Effect of Dietary Curcuma, Capsicum, and Lentinus on Enhancing Local Immunity against Eimeria acervulina Infection

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The protective effect of orally administered Curcuma longa (turmeric), Capsicum annuum and C. frutescens (hot pepper), and Lentinus edodes (shiitake mushroom) on avian coccidiosis was evaluated in young broilers. Broiler chickens were continuously fed with a standard diet or standard diet supplemented with Curcuma, Capsicum/Lentinus or Curcuma/Capsicum/Lentinus from hatch and body weight gains, fecal oocyst shedding, antibody titers, and pro-inflammatory cytokine gene expression were measured as parameters of protective immunity following challenge infection with E. acervulina. Chickens fed the Curcuma/Capsicum/Lentinus-supplemented diet showed significantly improved body weight gains compared with birds on the standard diet or birds given Capsicum/Lentinus-supplemented diet following challenge infection with E. acervulina. Chickens fed the Curcuma/Capsicum/Lentinus-supplemented diet shed significantly reduced fecal oocysts and produced higher serum antibody titers compared with the groups fed the standard diet or fed Curcuma or Capsicum/Lentinus. Finally, the levels of local cytokine transcripts of IL-1β, IL-6, IL-15, and IFN-γ were consistently greater in the Curcuma/Capsicum/Lentinus-fed group compared to the controls fed only the standard diet, Curcuma, or Capsicum/Lentinus groups. This study provides first immunological evidence that dietary supplementation of turmeric, hot pepper, and shiitake mixture significantly enhances local innate immunity and provides higher protective immunity against E. acervulina infection.

Key words: coccidiosis, cytokines, hot pepper, immunity, shiitake, turmeric

Introduction

Avian coccidiosis is an intestinal disease caused by several species of Eimeria protozoa and represents an economically important parasitic infection for the poultry industry worldwide (Lillehoj and Lillehoj, 2000). Due to increasing regulations with the use of prophylactic drugs, high cost of vaccines, and escalating consumers’ interest on naturally-raised chickens, much interest has been devoted toward the development of alternative strategies to control avian coccidiosis (Lillehoj and Lee, 2007a, b). It is now well accepted that many medicinal food and herbal products are highly effective promoting host defense mechanisms against microbial infections, tumors, and oxidative stress (Park et al., 2004; Lee et al., 2005, 2007a, b). For example, enhanced resistance against many infectious diseases including coccidiosis was demonstrated using dietary feeding of plant-derived phytonutrients (Banfield et al., 2002; Lee et al., 2007a, 2008c; Naidoo et al., 2008).

Hot pepper (Capsicum spp.) is a vegetable of importance in human nutrition and has many beneficial effects on human health. For example, C. frutescens is desired for its pungency, pigments, and its physiological and pharmaceutical uses, C. annuum exerts anti-oxidative effects in vitro (Conforti et al., 2007) and prevents Fe⁺⁺⁺-induced lipid peroxidation in brain (Oboh et al., 2007) and Capsicum oleoresin, prepared by organic extraction of pepper fruits, contains anti-bacterial activity and is effective in treating stomach illnesses (Spices board, 2008). Plants of the genus Curcuma, including C. longa (turmeric), have anti-oxidant and anti-inflammatory properties, and C. amada and C. caesi inhibit the growth of gram positive and negative bacteria as well as Candida albicans (Policegoudra et al., 2007; Sodsai et al., 2007; Mannangatti and Narayanasamy, 2008). Lentinus edodes (shiitake mush-
room) with its well known medicinal benefits, especially anti-tumor and anti-viral properties, have been effective in treating atopic diseases and arthritis (Park et al., 2004). These broad spectrums of biological properties suggest that these plant foods contain multiple beneficial phytonutrients operating through a variety of different mechanisms, and led us to hypothesize that they may protect against infectious diseases in poultry. Therefore, to evaluate their effects in enhancing innate immunity to avian coccidiosis, we compared seven parameters of protective immunity against coccidiosis (weight gain, fecal oocyst shedding, Eimeria antibody titers, and expression of IL-1β, IL-6, IL-15, and IFN-γ) in broiler chickens fed a diet containing Curcuma and/or Capsicum/Lentinus.

Materials and Methods

Experimental Animals
All experiments were approved by the Beltsville Agriculture Research Center-Small Animal Care and Use Committee. One day-old broiler chickens (Ross/Ross, Longenecker’s Hatchery, Elizabethtown, PA) were housed in Petersime starter brooder units and randomly assigned to 5 groups (20 birds/group). The birds were kept in brooder pens in an Eimeria-free facility for 2 weeks and transferred into large hanging cages (2 birds/cage) in a separate location where they were infected and kept until the end of experimental period.

Experimental Diets
All food extracts were obtained from Pancosma S.A. (Geneva, Switzerland). Crushed Capsicum annuum and C. frutescens were extracted with volatile solvents leading to an oleoresin and finally processed to produce a powder. And aqueous extract of Lentinus edodes was prepared at 95°C with pressure and spray-drying. Based on our preliminary results, Capsicum extract was always used in combination with Lentinus extract since this combination provided greater protection against experimental coccidiosis compared with either extract alone (unpublished data). Curcuma longa rhizomes were extracted with organic solvents. Chickens were continuously fed from hatch with a standard diet alone (control) or with diets supplemented with 35 mg/kg of C. longa alone (diet M), 35 mg/kg of C. annuum and C. frutescens plus 5 mg/kg of L. edodes (diet S), or Curcuma, Capsicum, and Lentinus at these concentrations (diet SM).

Eimeria Infection and Body Weight and Fecal Oocysts Measurements
Body weights of chickens were measured at days 1, 3, 14, 19, and 24 post-hatch to evaluate the effects of various diets on body weight changes. At day 14 post-hatch, chickens were either uninfected or orally infected with 2.0 × 10⁴ sporulated oocysts of E. acervulina as described by Lee et al. (2008a). Body weight gains were calculated over the period between days 0 and 10 post-infection. For determination of fecal oocyst shedding, birds were placed in oocyst collection cages (2 birds/cage) and fecal samples were collected from day 5 to day 10 post-infection. Oocyst numbers were determined as described (Lee et al., 2007a, b, 2008d) using a McMaster chamber according to the formula: Total oocysts/bird = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/2.

Serum Antibody Levels
Serum samples were obtained at day 10 post-infection (4 birds/group) and tested by ELISA to determine serum antibody levels against an apicomplexa antigen, EtMIC2, as described (Lee et al. 2007b, c). Briefly, 96-well microtiter plates were coated overnight with 10 μg/well of purified recombinant EtMIC2 protein. The plates were washed with PBS containing 0.05% Tween (PBS-T), and blocked with PBS containing 1% BSA. Diluted sera (1: 100) were added (100μL/well), incubated with agitation for 1 hr at room temperature, washed with PBS-T, and bound antibody detected with peroxidase-conjugated rabbit anti-chicken IgG (Sigma, St. Louis, MO) and peroxidase-reactive substrates made of phosphate citrate buffer (Sigma, St. Louis, MO), 3,3’,5,5’-Tetramethylbenzidine (Sigma, St. Louis, MO), and hydrogen peroxide (Sigma, St. Louis, MO). Optical density at 450 nm (OD₄₅₀) was determined with a microplate reader (Bio-Rad, Richmond, CA) and all experimental samples were analyzed in triplicates.

Quantification of Cytokine mRNA Levels
Intestinal duodenum tissues were obtained from uninfected chickens which were fed with various diets supplemented with plant extracts at day 14 post-hatch (4 birds/group), and local cytokine gene expression was determined using real time RT-PCR as previously described (Hong et al., 2006a, b). The intestinal duodenum was removed, cut longitudinally, and washed three times with ice-cold Hanks’ balanced salt solution (HBSS) containing 100 U/mL of penicillin and 100μg/mL of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel and the tissue was washed with HBSS. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Five micrograms of total RNA were treated with 1.0 U of DNase I and 1.0 μL of 10X reaction buffer (Sigma), incubated for 15 min at room temperature, 1.0 μL of stop solution was added to inactivate DNase I, and the mixture was heated at 70°C for 10 min. RNA was reverse-transcribed using the StrataScript first-strand synthesis system (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations. Briefly, 5.0μg of RNA was combined with 10X the first strand buffer, 1.0μL of oligo (dT) primer (5.0μg/μL), 0.8μL of dNTP mix (25 mM of each dNTP), and RNase-free water to a total volume of 19μL. The mixture was incubated at 65°C for 5 min, cooled to room temperature, 50 U of StrataScript reverse transcriptase was added, the mixture was incubated at 42°C for 1 hr, and the reaction was stopped by heating at 70°C for 5 min. Quantitative RT-PCR oligonucleotide primers for chicken cytokines and the GAPDH internal control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA using the Mx3000P system and
Brilliant SYBR Green qPCR master mix (Stratagene). Standard curves were generated using log10 diluted standard RNA. Levels of individual transcripts were then normalized to those of GAPDH analyzed by the Q-gene program (Muller et al., 2002). Each analysis was performed in triplicates. To normalize individual replicates, the logarithmic-scaled threshold cycle (C_T) values were transformed to linear units of normalized expression prior to calculating means and SEM for the references and individual targets, followed by the determination of mean normalized expression (MNE) using the Q-gene program (Lee et al., 2008a, c).

**Statistical Analyses**

Statistical analyses were performed using SPSS software (SPSS 15.0 K for Windows, Chicago, IL), and all data were expressed as means±SEM values. Comparisons of the mean values were performed by one-way analysis of variance, followed by the Duncan’s multiple range test and differences were considered statistically significant at P<0.05.

### Results

#### Body Weight Change

Dietary feeding of broiler chickens with diets containing the *Curcuma, Capsicum, and/or Lentinus* did not show any toxic effects on host based upon body weight changes and other physical appearance. Over the first 14 days post-hatch, birds given the supplemented diets did not exhibit any changes in body weights compared with those which were on standard diet alone (Table 2). Following *E. acervulina* infection, however, chickens fed the M or SM diet exhibited increased weight gains starting from day 0 to day 10 post-infection (days 14 and 24 post-hatch) compared with the infected controls given the non-supplemented diet (Fig. 1). Chickens fed the SM diet gained body weight by significantly greater extent than the birds given the S diet.

#### Oocyst Production

Fecal oocyst shedding was significantly reduced by 51% in chickens fed the SM diet (4.6 × 10^7) compared with the infected group given the non-supplemented diet (9.3 × 10^7). In contrast, neither the M nor the S diet alone

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### Table 1. Oligonucleotide primers used for quantitative RT-PCR of chicken cytokines

<table>
<thead>
<tr>
<th>RNA target</th>
<th>Primer sequences</th>
<th>PCR product size (bp)</th>
<th>Accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GGTGGTGCTAAGCGTGTTAT-3'</td>
<td>264</td>
<td>K01458</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-ACCTCTGTCATCTCTCCACA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TGGGCATCAAGGGGCTACA-3'</td>
<td>244</td>
<td>Y15006</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCGGGTGTTGGTGATG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CAAGGGTACAGGAGGGAC-3'</td>
<td>254</td>
<td>AJ309540</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TGCCAGGAGGGATTTCT-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-TCTGTCTTCTCTTCTGAGTGATG-3'</td>
<td>243</td>
<td>AF139097</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGTGATTTTGCTTCTGCTTTGGTA-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
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<td>259</td>
<td>Y07922</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GGCTTTTCCGTGGATTCC-3'</td>
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</tbody>
</table>

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### Table 2. Body weights of the birds fed plant-supplemented diets

<table>
<thead>
<tr>
<th>Groups/days post-hatch</th>
<th>1</th>
<th>3</th>
<th>14</th>
<th>19</th>
<th>24</th>
</tr>
</thead>
<tbody>
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<td><strong>Uninfected control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41.9±0.8NS</td>
<td>78.5±2.6NS</td>
<td>348.8±13.4NS</td>
<td>630.6±17.3ab</td>
<td>869.0±19.6ab</td>
</tr>
<tr>
<td><strong>Infected control</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>41.4±0.9</td>
<td>75.8±2.9</td>
<td>341.3±24.4</td>
<td>597.1±43.2b</td>
<td>835.6±46.7b</td>
</tr>
<tr>
<td><strong>S</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41.1±1.2</td>
<td>76.0±3.0</td>
<td>363.2±15.2</td>
<td>628.3±19.9ab</td>
<td>863.6±26.6ab</td>
</tr>
<tr>
<td><strong>M</strong></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>44.0±0.8</td>
<td>84.0±2.9</td>
<td>390.9±8.2</td>
<td>686.0±16.9a</td>
<td>943.1±19.1a</td>
</tr>
<tr>
<td><strong>SM</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>41.3±0.9</td>
<td>83.4±2.2</td>
<td>380.8±9.9</td>
<td>671.8±16.0ab</td>
<td>953.8±22.2a</td>
</tr>
</tbody>
</table>

*One-day-old broiler chickens were fed a standard diet or a standard diet supplemented with *C. annuum* and *C. frutescens* plus *L. edodes* (S), *C. longa* (M), or *C. annuum* and *C. frutescens*, *L. edodes*, and *C. longa* (SM), and their body weights were measured at various days post-hatch. Values indicate means±SEM. Within each group, values not sharing the same letters are significantly different (P<0.05) according to the Duncan’s multiple range test. NS=not significant.*
One-day-old broiler chickens were fed a standard diet alone (Control) or a standard diet supplemented with *C. annuum* and *C. frutescens* plus *L. edodes* (S), *C. longa* (M), or *C. annuum* and *C. frutescens*, *L. edodes*, and *C. longa* (SM). At 14 days post-hatch, the chickens were uninfected or infected with *E. acervulina*, and their body weight gains were measured starting from day 0 to day 10 post-infection. Each bar represents the mean ± SEM values. Bars not sharing the same letters are significantly different (*P* < 0.05) according to the Duncan’s multiple range test.

Reduced oocyst shedding compared to the control standard diet (Figure 2). No oocysts were detected from the uninfected control chickens (data not shown).

**Serum Antibody Responses**

The levels of serum antibodies reactive with the EtMIC2, an apicomplexa protein, were increased in *E. acervulina*-infected chickens fed the SM diet (0.34 ± 0.02), but not in birds given extracts of M diet (0.26 ± 0.01) or S diet (0.23 ± 0.04), when compared to the control birds on standard diet (0.21 ± 0.02) following *E. acervulina* infection (Figure 3).

**Local Cytokine Production**

As shown in Figure 4A, the level of transcripts encoding the pro-inflammatory cytokine IL-1β in the intestinal duodenum was significantly increased in uninfected chickens fed the SM diet at day 14 post-hatch compared with the control group (*P* < 0.05). Furthermore, the levels of transcripts encoding IL-6, IL-15, and IFN-γ were also increased in SM group compared with controls (Figures 4B, 4C, 4D). Of the 4 cytokines examined, IL-15 was significantly increased in all 3 groups with supplemented diets compared to the control group (Figure 4C).

**Discussion**

SM diet supplemented with *C. longa* and *C. annuum* and *frutescens* in combination with *L. edodes* provided significant body weight gains following infection with *E. acervulina* when compared to standard diet or diets containing *C. annuum* and *C. frutescens/L. edodes*. Furthermore, only the combination of *Curcuma/Capsicum/Lentinus* (SM diet) was efficient in reducing fecal oocyst shedding and increasing the titers of serum antibodies reactive with EtMIC2, an apical complex protein which plays an important role in host cell invasion of *Eimeria* parasites (Tomley et al., 1996; Lillehoj and Lillehoj, 2000). Taken together, these results provide clear evidence for an
One-day-old broiler chickens were fed a standard diet alone (Control) or a standard diet supplemented with and plus (S), (M), or and (SM). At days post-hatch, the intestinal duodenum was removed and the levels of transcripts for IL-1β (A), IL-6 (B), IL-15 (C), and IFN-γ (D) were quantified by real time RT-PCR. Each bar represents the mean ± SEM. Bars not sharing the same letters are significantly different (P<0.05) according to the Duncan’s multiple range test.

The effect of dietary plants on pro-inflammatory cytokine transcript levels of birds uninfected with *E. acervulina*. One-day-old broiler chickens were fed a standard diet alone (Control) or a standard diet supplemented with *C. annuum* and *C. frutescens* plus *L. edodes* (S), *C. longa* (M), or *C. annuum* and *C. frutescens*, *L. edodes*, and *C. longa* (SM). At 14 days post-hatch, the intestinal duodenum was removed and the levels of transcripts for IL-1β, IL-6, IL-15, and IFN-γ were quantified by real time RT-PCR. Each bar represents the mean ± SEM. Bars not sharing the same letters are significantly different (P<0.05) according to the Duncan’s multiple range test.

Effect of the different phytounit combinations in promoting local protective immunity against experimental avian coccidiosis. In addition, only the SM diet induced all four of the cytokines in the gut, further supporting the effective action of all 3 plants in stimulating local immunity. Alternatively stated, while all seven parameters of protective immunity to coccidiosis were augmented using the *Curcuma/Capsicum/Lentinus*-supplemented diet, *C. longa*-supplemented M diet also enhanced weight gain, IL-6, and IL-15 expression. Because IL-15 plays an important role in the development, survival, and function of NK cells (Waldmann and Tagaya, 1999) and acts as a memory-facilitating factor for helper T cells (Zhang et al., 1998; Kanegane and Tosato, 1996), future studies on the effect of feeding these plants in promoting prolonged vaccinal immune response will be valuable.

Historically, the severity of experimental *Eimeria* infection in chickens has been assessed by loss of body weight gain, excretion of fecal oocysts, and the presence of intestinal lesions (Idris et al., 1997). These disease parameters reflect host immunity status in avian coccidiosis (Lillehoj et al., 2007c). The challenge dose of *E. acervulina* that was used in this investigation is likely to be considerably higher than exposure levels that commercial flocks encounter (Wallach et al., 1995). Therefore, lower doses of the turmeric/hot pepper/shiitake mixture supplemented in standard poultry feed may still provide effective protection against coccidiosis in poultry raised under normal field conditions.

Given that markers of both humoral immunity (*Eimeria*-specific antibodies) and cell-mediated immunity (pro-inflammatory cytokines) were enhanced by the plant-supplemented diet, it will be important to investigate the cellular and molecular mechanisms responsible for immunoenhancing effects of these plants. Because cell-mediated immunity has been shown to play a major role in
protection against avian coccidiosis (Lillehoj and Ruff, 1987; Lillehoj and Trout, 1996; Lee et al., 2007b, c), it is possible that *Curcuma, Capsicum, and Lentinus*, when combined together, show increased effectiveness on stimulating local cell-mediated immunity protective against coccidiosis. In this study, *Curcuma/Capsicum/Lentinus* (SM diet) also enhanced serum antibody levels against a microneme protein 2 from *E. tenella* (EtMIC2). EtMIC2 has a putative function in parasite adhesion to the host cell and plays an important role in inhibiting sporozoite invasion of host cells (Sasai et al., 2008). *Curcuma/Capsicum/Lentinus*-treated birds showed higher antibody response to EtMIC2 protein and shed less oocysts. Therefore, increasing the level of serum antibodies which are directed against parasite antigens of survival importance will likely enhance local protection against coccidiosis.

Host immune response to *Eimeria* is accompanied by series of cell-mediated immune responses and several cytokines including IFN-γ, IL-1β, IL-6, and IL-15 which are involved in local inflammatory responses (Lillehoj et al., 2001, 2007c; Lee et al., 2008c; Hong et al., 2006a, b). IFN-γ is a common marker of cellular immunity and high levels are associated with protective immune responses to coccidiosis (Min et al., 2003; Lillehoj et al., 2004; Lee et al., 2008a). Administration of recombinant IFN-γ to chickens increased host protection against coccidiosis, significantly reduced the intracellular development of *Eimeria* parasites (Lillehoj and Trout, 1996), and showed adjuvant effect when given in a DNA vaccine (Min et al., 2001). IL-1β is a pro-inflammatory cytokine produced by macrophages, monocytes and dendritic cells, and is an important mediator of innate immunity. In mammals, IL-1β increases the expression of adhesion factors on endothelial cells to enable the transmigration of leukocytes to the sites of infection. IL-1β, when given simultaneously with a DNA vaccine, exerted an adjuvant effect by reducing fecal oocyst shedding following an oral infection with *Eimeria* (Min et al., 2001). IL-6 is produced by T-cells and macrophages and acts as both a pro-inflammatory and an anti-inflammatory cytokine whereas IL-15, primarily secreted by mononuclear phagocytes, enhances the activation of memory T cells (Waldmann and Tagaya, 1999; Kanegane and Tosato, 1996). In chickens, IL-15 promoted the survival of T-lymphocytes and NK cells (Lillehoj et al., 2001; Choi and Lillehoj, 2002) and enhanced protective immunity to coccidiosis when co-administered with a DNA vaccine (Lillehoj et al., 2001; Min et al., 2001). Enhanced production of these cytokines in birds which were continuously fed with the *Curcuma/Capsicum/Lentinus*-supplemented diet provides a new opportunity to utilize these dietary phytonutrients to increase local innate immunity and to reduce economic losses due to coccidiosis.

In conclusion, our results provide the first demonstration that a combination of *Curcuma, Capsicum, and Lentinus* effectively enhances the disease resistance of birds to *E. acervulina* infection. Although further studies are necessary to better understand the underlying immune mechanisms which are responsible for the dietary immune enhancement against avian coccidiosis using these plants, this study provides clear immunological evidence for their role in stimulating humoral and cell-mediated immunity in poultry. Furthermore, for a complex intestinal parasitic infection such as coccidiosis whose treatment has traditionally been relied upon prophylactic medication, dietary immune enhancement using food plants provides a safe alternative control method to reduce economic losses due to coccidiosis in poultry.

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