Immunomodulatory properties of dietary plum on coccidiosis

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Abstract

The current study was conducted to evaluate the effect of dietary supplementation with a lyophilized powder made from plums (P) on host protective immune responses against avian coccidiosis, the most economically important parasitic disease of poultry. One-day-old White Leghorn chickens were fed from the time of hatch with a standard diet either without P (control and P 0 groups) or supplemented with P at 0.5\% (P 0.5) or 1.0\% (P 1.0) of the diet. Animals in the P 0, P 0.5, and P 1.0 groups were orally challenged with 5000 sporulated oocysts of \textit{Eimeria acervulina} at day 12 post-hatch, while control animals were uninfected. Dietary supplementation of P increased body weight gain, reduced fecal oocyst shedding, and increased the levels of mRNAs for interferon-\gamma and interleukin-15 in the P 1.0 group at 10 days post-infection compared with the P 0 group. Furthermore, chickens fed either the P 0.5 or P 1.0 diets exhibited significantly greater spleen cell proliferation compared with the non-plum P 0 group. These results indicate that plum possesses immune enhancing...
properties, and that feeding chickens a plum-supplemented diet augments protective immunity against coccidiosis.

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1. Introduction

Avian coccidiosis is economically the most important parasitic disease of the poultry industry and is caused by intestinal infection with *Eimeria* spp. Although drugs have been used as the main method for coccidiosis control, there is increasing interest in the development of alternative strategies of disease prevention due to the escalating incidence of drug resistant coccidia strains in the field [1]. Our laboratory has investigated the ability of dietary natural products to enhance protective immunity against *Eimeria* infections. For example, feeding broilers with a lectin derived from the mushroom *Fomitella fraxinea* significantly improved protective immunity against coccidiosis [2]. Moreover, broilers fed *Pediococcus* and *Saccharomyces*-based probiotic MitoMax® showed enhanced humoral immunity against *E. acervulina* [3].

These promising results provide a rational basis for further investigation of the effects of other types of dietary products on the immune response to avian coccidiosis. In humans, consumption of fruit juices has been associated with stimulated immune cell functions [4], and phenolic and flavonoid compounds from
mulberry, strawberry, and red onion stimulated splenocyte proliferation in mice [5]. Park et al. [6] reported that extracts of *Lentinus edodes* (shiitake mushroom) inhibited tumor growth in a human papilloma virus 16 oncogene-transformed animal tumor model via an apoptotic mechanism. Increasing evidence that particular dietary supplementations have the ability to influence host inflammatory responses and enhance protective immunity against various infectious microorganisms prompted us to investigate the role of traditional medicinal plants on avian coccidiosis.

Oriental plum (*Prunus salicina*) is a long-established medicinal fruit, which has been extensively used in Korea and other Asian countries to enhance immunity and treat tumors in humans. Several prior studies have documented the medicinal effects of plum phenolic compounds [5,7], its antioxidant effect [8], and its capacity to decrease blood cholesterol [9]. However, the underlying mechanisms of its immunoenhancing effects have not been well characterized. Thus, the current investigation was conducted to examine the effect of dietary plum supplementation on protective immunity against experimental coccidiosis in chickens.

### 2. Materials and methods

#### 2.1. Experimental animals, diets, and parasite infection

Fertilized eggs of specific pathogen-free White Leghorn chickens were obtained from SPAFAS (Charles River Laboratories, Preston, CT) and hatched at the Animal and Natural Resources Institute, USDA (Beltsville, MD). One-day-old chickens were randomly assigned to four pens (*N* = 10/pen) of an electrically heated battery and fed *ad libitum* a standard chicken diet either without plum (20 birds; 10 uninfected controls and 10 infected controls [P 0]) or with plum supplemented at 0.5% (P 0.5) or 1.0% (P 1.0) of the diet (10 birds each). Plum diets were prepared by mixing the standard chicken diet with a freeze-dried powder of oriental plum (*P. salicina*) supplied by the National Rural Resources Development Institute (Suwon, South Korea). All diets were formulated to meet the nutrient requirements for chickens as recommended by the National Research Council [10]. Thirty birds (P 0, P 0.5, and P 1.0 groups) were orally inoculated with 5000 sporulated oocysts of *E. acervulina* at day 12 post-hatch as described [11]. All experiments were performed according to the guidelines established by the Beltsville Area Institutional Animal Care and Use Committee.

#### 2.2. Body weight measurements and oocyst shedding

Body weights were measured at 0 and 10 days post-inoculation (dpi) as described [12]. Oocyst shedding was determined between days 6 and 10 dpi [12]. Briefly, fecal material collected over the 5-day period was pooled, resuspended, two 35 ml samples were taken, diluted, and the number of oocysts was counted microscopically using a McMaster chamber. The total number of oocysts was calculated using the
formula: total oocysts/bird = oocyst count × dilution factor × (fecal sample volume/counting chamber volume)/# of birds per cage.

2.3. *Spleen lymphocyte proliferation*

Spleens were removed at 10 dpi and placed in a Petri dish with 10 ml of Hank’s balanced salt solution (HBSS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, St. Louis, MO). Single-cell suspensions of spleen lymphocytes were prepared[13] and lymphocyte proliferation was carried out as described[14]. In brief, splenocytes were adjusted to 1 × 10^7 cells/ml in RPMI-1640 medium without phenol red (Sigma) supplemented with 10% fetal bovine serum (FBS) (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin (Sigma). Splenocytes (100 μl/well) were incubated in 96-well flat bottom plates at 41 °C in a humidified incubator (Forma, Marietta, OH) with 5% CO₂ and 95% air for 48 h. Cell proliferation was determined with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8, Cell-Counting Kit-8®, Dojindo Molecular Technologies, Gaithersburg, MD) as described[15]. Optical density (OD) was measured at 450 nm using a microplate spectrophotometer (BioRad, Richmond, CA).

2.4. *Flow cytometric analysis*

Spleens were removed at 10 dpi and single-cell suspensions were resuspended in 1.0 ml of HBSS containing 3% FBS and 0.01% sodium azide (flow buffer). One hundred microliters aliquots (~10^6 cells) were incubated on ice for 40 min with 100 μl of appropriately diluted monoclonal antibodies against chicken CD4, CD8, αβ-TCR, or γδ-TCR. After washing twice with 2.0 ml of flow buffer, the cells were incubated with fluorescein isothiocyanate-conjugated anti-mouse IgG antibody (Sigma) for 30 min on ice, washed twice, resuspended in 2.0 ml, and analyzed with an Epics model XL flow cytometer (Coulter, Miami, FL). Data were obtained from a total of 10^6 viable cells.

2.5. *Interferon-γ and Interleukin-15 mRNA levels in intestinal intraepithelial lymphocytes (IELs)*

2.5.1. *Isolation of intestinal IELs*

At 10 dpi, the intestinal duodenum was removed from three chickens, cut longitudinally, and washed three times with ice-cold HBSS containing 100 U/ml of penicillin and 100 μg/ml of streptomycin. The mucosal layer was carefully scraped away using a surgical scalpel, the tissue was washed twice with HBSS, and IELs were isolated and pooled as described[16].

2.5.2. *cDNA synthesis*

Total RNA was extracted from intestinal IELs using TRIzol (Invitrogen, Carlsbad, CA) as described[17]. Five micrograms of RNA were treated with 1.0
I of DNase I and 1.0 μl of 10 × 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, 5.0 μg of RNA were combined with 10 × first-strand buffer, 1.0 μl of oligo(dT) primer (5.0 μg/μl), 0.8 μl of dNTP mix (25 mM each), and RNase-free water to a total volume of 19 μl. 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 h, and the reaction was stopped by heating at 70 °C for 5 min.

2.5.3. Quantitative RT–PCR

Quantitative RT–PCR oligonucleotide primers for chicken interferon-γ (IFN-γ), interleukin-15 (IL-15), and GAPDH control are listed in Table 1. Amplification and detection were carried out using equivalent amounts of total RNA from intestinal IELs using the Mx3000P system and Brilliant SYBR Green QPCR master mix (Stratagene). Standard curves were generated using log_{10} diluted standard RNA and levels of individual transcripts were normalized to those of GAPDH analyzed by the Q-gene program [18]. Each analysis was performed in triplicate. To normalize RNA levels between samples within an experiment, the mean threshold cycle (C_t) values for the amplification products were calculated by pooling values from all samples in that experiment.

2.6. Statistical analyses

Data analyses were performed using InStat® software (Graphpad, San Diego, CA). The ANOVA test was used to test for differences between the groups. The data were expressed as mean ± SEM. The Tukey–Kramer multiple range test was used to determine the significance of differences between the mean values of the treatment groups at the level of P<0.05.

Table 1
Oligonucleotide primers used for quantitative RT–PCR of chicken IFN-γ, IL-15, and GAPDH

<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>5'-GGTGGTGCTAAGCGTGTATT-3' 5'-ACCTCTGTACATCTCTCCACA-3'</td>
<td>264</td>
<td>K01458</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>5'-AGCTGACGGTGGACCTATTATT-3' 5'-GGCTTTGCGGTGGATTC-3'</td>
<td>259</td>
<td>Y07922</td>
</tr>
<tr>
<td>IL-15</td>
<td>5'-TCTGTTCTCTGTCTCTGAGTGATG-3' 5'-AGTGATTGGTCTCTGGTATGTA-3'</td>
<td>243</td>
<td>AF139097</td>
</tr>
</tbody>
</table>
3. Results

3.1. Body weight gain

Previous studies have established that two key parameters providing an accurate measure of protective immunity during the course of experimental coccidiosis are body weight gain and fecal oocyst shedding [19]. Therefore, both of these factors were evaluated in order to assess the effects of dietary supplementation with plum on the host immune response to coccidia infection. Mean body weight gains of uninfected and *E. acervulina*-infected birds were calculated over the 10-day infection period (0–10 dpi). As shown in Fig. 1, body weight gains were significantly reduced by more than 20% in the *E. acervulina*-infected birds of the P 0 and P 0.5 groups compared with the uninfected control group (*P* < 0.05). By contrast, the body weight gain in the P 1.0 group increased by more than 14.5% compared with the P 0 and P 0.5 groups and, indeed, was not significantly different from the uninfected control group.

3.2. Oocyst shedding

Oocyst shedding in the P 0.5 and P 1.0 groups decreased by 16% (from $1.05 \times 10^8$ to $0.88 \times 10^8$) and by 46% (from $1.05 \times 10^8$ to $0.56 \times 10^8$), respectively, compared with the P 0 group (Fig. 2). Oocysts shedding was significantly reduced in the P 1.0 group compared with the P 0 group (*P* < 0.05). As expected, no fecal oocysts were detected in uninfected chickens (data not shown).

3.3. Spleen lymphocyte proliferation

As shown in Fig. 3, splenocytes from *E. acervulina*-infected chickens fed the 0.5% plum-supplemented diet (P 0.5) displayed significantly greater spontaneous proliferation compared with infected animals on a normal diet (P 0) or with the uninfected controls. Interestingly, however, while the P 1.0 group of chickens also

![Fig. 1. Body weight gains of chickens fed plum-supplemented diets. Chickens were uninfected, or infected with 5000 *E. acervulina* oocysts at day 12 post-hatch, fed diets supplemented with 0% (control, P 0), 0.5% (P 0.5), or 1.0% (P 1.0) plum, and body weights were measured at 0 and 10 dpi. Each value represents the mean ± SEM values from 10 chickens. Bars not sharing the indicated letters are significantly different (*P* < 0.05) according to the Tukey-Kramer multiple range test.](image-url)
displayed greater lymphocyte proliferation compared with the P 0 group, their proliferation was less than that of the P 0.5 group and not significantly different compared with the uninfected controls.

### 3.4. Spleen T-cell subpopulations

Prior studies from our laboratory showed that the percentage of T lymphocytes expressing the γδ-T cell receptor (γδ-TCR) cell surface marker was significantly increased during experimental coccidiosis, and that the increased level of γδ-TCR+ cells was positively correlated with protective immunity against the parasite [20]. Thus, it was of interest to determine the effects of plum-supplemented diets on T-cell subpopulations during *E. acervulina* infection. As shown in Table 2, the percentages of CD4+, CD8+, and αβ-TCR+ cells at 10 dpi were not significantly different between uninfected control birds and those infected with *E. acervulina*, irrespective of whether or not the latter group had received the plum diets. Furthermore, while
the fraction of $\gamma$$\delta$-TCR$^+$ cells increased following *E. acervulina* infection, there was no effect of plum diets on this change. We concluded that dietary plum supplementation does not alter the distribution of spleen T-cell subpopulations during coccidiosis.

3.5. *Ifn-γ* and *il-15* transcript levels

Two key chicken cytokines involved in the local protective immune response against experimental *Eimeria* infection are IFN-γ and IL-15 [21]. Therefore, we next determined the effect of plum-supplemented diets on IFN-γ and IL-15 gene expression in intestinal IELs during coccidiosis. As shown in Fig. 4, the levels of IFN-γ and IL-15 mRNAs were significantly increased in chickens on the P 1.0 diet. In these animals, the IFN-γ transcript level increased 2.81-fold compared with the uninfected control group and 2.40-fold compared with the P 0 group, and the level of the IL-15 mRNA increased by 11.20- and 8.32-fold compared with the uninfected control and P 0 groups, respectively.

4. Discussion

Epidemiological and experimental studies have revealed a negative correlation between human consumption of diets rich in fruits and vegetables and the risk of developing a variety of acute and chronic diseases [22–26]. These health-promoting properties of fruits and vegetables have been explained, in part, by their abundant content of phenolic compounds, including flavonoids and anthocyanins, and carotenoids [27–30]. Plum, a fruit rich in phenolics, is a traditional medicinal fruit that has been used for centuries in Korea and other Asian cultures to enhance human disease resistance [5]. Therefore, the present study was undertaken to examine the effects of dietary plum on the immune responses of chickens during infection by *E. acervulina*, one of the most prevalent *Eimeria* species in chickens.

### Table 2

<table>
<thead>
<tr>
<th><em>E. acervulina</em></th>
<th>Plum (%)</th>
<th>CD4</th>
<th>CD8</th>
<th>$\alpha$$\beta$-TCR</th>
<th>$\gamma$$\delta$-TCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected</td>
<td>0</td>
<td>46.6±2.7</td>
<td>47.2±2.7</td>
<td>67.3±3.9</td>
<td>7.5±0.4$^b$</td>
</tr>
<tr>
<td>Infected</td>
<td>0</td>
<td>51.1±2.9</td>
<td>46.4±2.6</td>
<td>65.7±3.8</td>
<td>11.6±0.7$^a$</td>
</tr>
<tr>
<td>Infected</td>
<td>0.5</td>
<td>50.3±2.9</td>
<td>45.3±2.6</td>
<td>68.1±3.9</td>
<td>10.4±0.6$^a$</td>
</tr>
<tr>
<td>Infected</td>
<td>1.0</td>
<td>52.6±3.1</td>
<td>43.4±2.5</td>
<td>63.4±3.6</td>
<td>11.3±0.6$^a$</td>
</tr>
</tbody>
</table>

Chickens were uninfected or infected with 5000 *E. acervulina* oocysts at day 12 post-hatch and fed diets supplemented with 0% (control, P 0), 0.5% (P 0.5), or 1.0% (P 1.0) plum. At 10 dpi, spleen cells were examined by flow cytometry for expression of the indicated surface markers. Each value represents the mean±SEM value from three chickens. Values in the same column with different superscripts are significantly different ($P<0.05$).
[31,32], and to determine the potential protection that plum might provide against the pathogen.

*E. acervulina* infects the intestinal duodenum causing diarrhea, ranging from mucoid and watery to hemorrhagic, weight loss and morbidity, with death
sometimes seen in young birds [33,34]. The severity of *Eimeria* infection is commonly assessed by reduced body weight gain and fecal excretion of oocysts [19]. Although it is desirable to see the combined positive effects of increased weight gain and reduced oocyst shedding as indicators of host resistance to coccidiosis, direct correlation between these two parameters has not always been observed [3,12,35]. Nevertheless, in this report, we demonstrated both increased weight gain and lowered parasite excretion in infected chickens fed the 1.0% plum diet. Based on these initial physiological experiments, we hypothesized that the effects of dietary plum were mediated through its ability to stimulate protective immunity. Therefore, we next conducted a series of studies to examine the ability of a plum-supplemented diet to influence three particular parameters of chicken inflammation previously documented to correlate with protective immunity during experimental coccidiosis, i.e., spleen lymphocyte proliferation, spleen T-cell subsets, and gut IEL cytokine gene expression.

Chickens fed plum diets showed significantly enhanced spleen cell proliferation compared with those fed a standard diet without plum. A number of prior studies have demonstrated that the immunoenhancing effects of natural food and herbal products on host defense against microbes and tumors are directly correlated with their ability to influence lymphocyte proliferation [36–38]. In the case of coccidiosis, medicinal foods and probiotics have been suggested to afford protection against parasite infection by enhancing anti-*Eimeria* cellular immunity and/or inducing parasite-specific antibodies [39–41]. Because both of these factors are related to increased splenocyte proliferation, we speculate that constituents of the plum fruit, most likely phenolic compounds [5], stimulate lymphocyte mitosis leading to elevated cellular and humoral protective immunities against *E. acervulina*.

Alterations of intestinal lymphocyte subpopulations during plum supplementation were investigated to better understand the characteristics of protective immunity during coccidiosis. CD4⁺ and CD8⁺ cells are involved in different phases of host immunity [21], and the CD4⁺/CD8⁺ cell ratio in SC strain chickens was reported to be related to disease resistance [42]. αβ-TCR⁺ and γδ-TCR⁺ cells contribute differently to the host immune competence. γδ-TCR⁺ cells elaborate immunoregulatory cytokines, as well as other soluble factors that influence epithelial cell growth and repair [43]. γδ-TCR⁺ cells also mediate specific cellular immune functions without the requirement for antigen processing and can directly recognize invading pathogens or damaged cells [44,45]. In the case of coccidiosis, γδ-TCR⁺ cells are involved in cytotoxic effector mechanisms known to be important for coccidia control [16,41]. However, our results indicated that the percentages of CD4⁺, CD8⁺, and αβ-TCR⁺ cells were not significantly altered between uninfected and infected chickens. Moreover, whereas γδ-TCR⁺ cells were increased following *E. acervulina* infection, corroborating our previous report [20], dietary plum supplementation did not modify this effect. It should be noted, however, that the percentages of CD8⁺, αβ-TCR⁺, and γδ-TCR⁺ cells seen in *Eimeria*-infected birds were lower at 10 dpi (the time point used in this study) compared with those evaluated at 6–8 days after infection in the previous study [20].
possible that the effect of dietary plum on lymphocyte subpopulations in *Eimeria*-infected chickens may be detected at an earlier time point following coccidian infection. It may also be instructive to examine subsets of IELs in the duodenum of infected animals.

Various experimental strategies to enhance chicken immunity to pathogens using IFN-γ and IL-15 have been reported [41,46]. Early cellular immune responses characterized by IFN-γ production are critical for the host response against coccidiosis [41]. Chicken IFN-γ regulates acquired immunity by activating lymphocytes and enhancing the expression of MHC class II antigens [47]. Further, IFN-γ is a common marker of cellular immunity and higher levels have been correlated with protective immune responses to coccidial infections [48–51]. Chicken IFN-γ production during coccidiosis has been examined by quantitative RT–PCR [50–52] and more recently by gene expression profiling [17]. Additionally, administration of exogenous recombinant IFN-γ to chickens significantly hindered the intracellular development of *Eimeria* parasites and reduced body weight loss [53]. Thus, we hypothesize that reduced body weight loss, decreased oocyst shedding, and activation of lymphocyte proliferation seen in animals fed a plum-supplemented diet is the consequence, at least in part, of increased IFN-γ gene expression.

The IL-15 is a Th1 cytokine produced by mononuclear phagocytes and other cell types in response to viral or protozoan infections [54,55]. During avian coccidiosis, IL-15 acts by stimulating the proliferation of antigen-specific T lymphocytes and NK cells [55,56]. In our previous report, the efficacy of an experimental *Eimeria* subunit vaccine against coccidiosis was markedly enhanced when coadministered with recombinant IL-15 [46]. Thus, increased IL-15 expression in intestinal IELs of chickens fed the 1.0% plum diet, particularly in unison with augmented IFN-γ expression, may have stimulated T-cell proliferation thereby modulating the physiological parameters mentioned above. Further studies are ongoing in our laboratory to elucidate the role of additional chicken cytokines and chemokines whose intestinal expression may be enhanced by plum.

In summary, the results of this study demonstrate that plum promotes protective immunity against coccidiosis as assessed by reduced body weight loss, decreased oocyst shedding, enhanced splenocyte proliferation, and elevated expression of transcripts encoding IFN-γ and IL-15. These immunoprotective effects were most evident by the inclusion of 1.0% plum in the standard chicken diet. Supplementation of chicken diets with plum should be studied further as an alternative to prophylactic coccidiostats as well as chemotherapy of other poultry diseases.

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References


