Effects of dietary conjugated linoleic acid on the duodenal mucosal immunity response and redox status of broiler chicks infected with Eimeria acervulina

Y.X. Liu¹, Y.L. Liu¹, J.P. Yang², W.T. Li²

¹College of Animal Science and Technology, Henan University of Animal Husbandry and Economy, Zhengzhou, P.R. China
²Henan Engineering Technology Research Center of Animal Nutrition and Feed, Zhengzhou, P.R. China

ABSTRACT: The present experiment investigated the effects of conjugated linoleic acid (CLA) on the growth performance, oocyst shedding, duodenal mucosal immune response, and redox status of broiler chicks infected with Eimeria acervulina. Totally 144 birds were fed either the basal diet or 1.5% CLA diet for 21 days, and then half of them from each dietary treatment were inoculated with 10 000 Eimeria acervulina sporulated oocysts. Compared with the basal diet, the 1.5% CLA diet partially ameliorated the growth inhibition ($P < 0.01$) and decreased the oocyst shedding of broiler chicks at ca. 6–9 days post-inoculation ($P < 0.01$). Additionally the dietary CLA significantly increased the percentage of CD8+ T lymphocytes in the duodenal epithelium ($P < 0.05$), and showed no obvious effect on the interferon γ expression in duodenal mucosa ($P > 0.05$). The dietary CLA also notably suppressed the increase of malondialdehyde ($P < 0.05$) and the decrease of glutathione concentration ($P < 0.01$) in duodenal mucosa of the infected birds, without changing the activities of total superoxide dismutase and catalase ($P > 0.05$). These results demonstrate the protective effect of dietary CLA on the broiler chicks infected with Eimeria acervulina, and anticoccidial effects of dietary CLA are most likely related to the increased proportions of CD8+ T lymphocytes in duodenal epithelium and the inhibition on the decrease of duodenal mucosal glutathione after Eimeria acervulina infection.

Keywords: CLA; intestinal mucosal immunity; oxidative stress; broiler chick; coccidiosis

INTRODUCTION

Coccidiosis is one of the major diseases causing severe economic losses to the poultry industry. Currently, the control of coccidiosis mainly relies on anticoccidial drugs and vaccines. However, due to increasing concerns about the prolonged drug usage and the high cost of vaccines, alternative strategies are being sought.

Conjugated linoleic acid (CLA) is a mixture of geometrical and positional isomers of linoleic acid in which the two double bonds are conjugated. In the test of piglets with B. hyodysenteriae induced enteritis, dietary CLA was found to alleviate intestinal mucosal injury from inflammation by maintaining the intestinal cytokine profiles (i.e. interferon γ (INFγ) and interleukin-10 (IL-10)) and T lymphocyte subset distributions (Hontecillas et al. 2002). Dietary CLA reduced the proportion of macrophages in the mouse mesenteric lymph, inhibited the expression of colonic TNFα, and prevented the colon cancer development induced by inflammation (Evans et al. 2010). Moreover, some studies have revealed the antioxidant properties of CLA (van Den Berg et al. 1995; Yu 2001). For instance, in rat and chicken, CLA could alleviate...
oxidative damages induced by inflammatory agents such as the lipopolysaccharide, gliadin, and sodium arsenite (Zhang et al. 2008; Bergamo et al. 2011). Palacios et al. (2003) considered that the antioxidant capacity of CLA far exceeded that of vitamin A.

Coccidiosis is a disease caused by *Eimeria* parasi-tizing in the intestinal mucosa epithelial cells, and the main immune barrier against coccidiosis *in vivo* is the intestinal mucosal immune system. Cell immunity mediated by T lymphocytes in intestinal epithelium and mucosal lamina propria plays an important role in resistance to coccidial infection (Lillehoj et al. 2004). Coccidial infection is known to induce oxidative stress by weakening the antioxidant capacity of body and reducing the activity of superoxide dismutase (SOD) and the concentrations of blood vitamins A, E, and C to induce oxidative stress (Koinarski et al. 2005).

From the aforementioned studies, it may be concluded that: (1) dietary CLA has regulating effects on the intestinal mucosal immunity and oxidative stress status in animals such as pig, mouse, and chicken; (2) the intestinal mucosal immunity and oxidative stress status are closely related to the antico-cidial capacity of broiler chicks. Therefore, we hypothesized that coccidiosis infection of broiler chicks may be ameliorated by dietary CLA supplementation. The objective of the present experiment was to test this hypothesis by investigating the effects of dietary CLA on the growth performance, oocyst shedding, intestinal mucosal immune response, and antioxidant capacity of broiler chicks infected with *Eimeria acervulina*.

**MATERIAL AND METHODS**

**Experimental design.** The present experiment was conducted according to the protocols approved by the Animal Care and Use Committee of the Henan University of Animal Husbandry and Economy. A total of 144 one-day-old Arbor Acres male broiler chicks were randomly assigned to two dietary treatments: the basal diet and the CLA diet. Each dietary treatment consisted of 12 replications (cages) with 6 broiler chicks each. Diets were formulated on the basis of the NRC (1994) recommendation without anticoccidial drugs. Composition and nutrient levels of the basal diet are shown in Table 1. In the basal diets, 1.5% CLA was replaced by 1.5% soybean oil to keep both the CLA-supplemented and basal diets isoenergetics. CLA was provided by the Qingdao Aohai Biology Technology Co., Ltd. and contained 80.80% conjugated dienes with 39.24% 9c, 11t-CLA and 38.93% 10t, 12c-CLA representing 96.74% of the contained isomers. The 1.5% adding amount

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>1–3 weeks (g/kg)</th>
<th>4–6 weeks (g/kg)</th>
<th>Nutrition levels</th>
<th>1–3 weeks</th>
<th>4–6 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maize</td>
<td>600.0</td>
<td>657.6</td>
<td>metabolizable energy (MJ/kg)</td>
<td>12.20</td>
<td>12.42</td>
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<tr>
<td>Soybean meal</td>
<td>342.1</td>
<td>285.0</td>
<td>crude protein (g/kg)</td>
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<td>179.8</td>
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<td>Soya oil</td>
<td>20.0</td>
<td>20.0</td>
<td>calcium (g/kg)</td>
<td>9.2</td>
<td>9.0</td>
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<tr>
<td>Limestone</td>
<td>13.0</td>
<td>13.0</td>
<td>available P (g/kg)</td>
<td>4.2</td>
<td>4.1</td>
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<tr>
<td>CaHPO₃</td>
<td>16.0</td>
<td>16.0</td>
<td>lysine (g/kg)</td>
<td>10.5</td>
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<tr>
<td>dl-Methionine</td>
<td>2.0</td>
<td>2.0</td>
<td>methionine (g/kg)</td>
<td>5.0</td>
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</tr>
<tr>
<td>Sodium chloride</td>
<td>3.5</td>
<td>3.5</td>
<td>methionine + cysteine (g/kg)</td>
<td>8.5</td>
<td>8.0</td>
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<tr>
<td>Choline chloride (50%)</td>
<td>1.0</td>
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<tr>
<td>Multivitamin premix¹</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Trace-minerals premix²</td>
<td>2.0</td>
<td>2.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BHT (33%)</td>
<td>0.2</td>
<td>0.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1000.0</td>
<td>1000.0</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BHT = 2,6-di-tert-butyl-4-methylphenol

¹multivitamin premix provided per kg of diet (in mg): retinyl acetate 4.3, cholecalciferol 0.0625, α-tocopherol 18.75, me-nadione sodium bisulphite 2.65, thiamine-HCl 2.0, pyridoxine-HCl 6.0, cyanocobalamin 0.025, niacin 50, d-pantothenic acid 12, folic acid 1.25

²trace-minerals premix provided per kg of diet (in mg): Cu 8, Fe 80, Mn 100, Zn 75, Se 0.15, I 0.35
of CLA was used in the experiment as described in a previous study (Narciso-Gaytan et al. 2011). The conventional broiler chicks were purchased from a local hatchery and were raised in layered galvanized cages. The temperature was controlled throughout the experiment: 31~33°C for the first 5 days, and then a decrease of 3°C weekly to 22°C. The broiler chicks were vaccinated according to the routine immunization program for broiler chicks, and had free access to feed and water. The coccidiosis vaccine was not included in the vaccination program.

On day 21 of the study, half of the cages in each dietary treatment were experimentally infected by oral dosing with 10,000 *Eimeria acervulina* sporulated oocysts provided by the veterinary laboratory of the Henan University of Animal Husbandry and Economy. The study automatically became a 2 × 2 factorial arrangement of treatments, i.e. two levels of CLA (0 or 1.5% in diets) and with or without *Eimeria acervulina* challenge.

**Average daily growth (ADG) and oocysts shedding.** The broiler chicks were weighed between days 0 and 10 post-infection on the basis of cage, and daily gain was calculated at this interval. The fecal droppings of broiler chicks were collected between days 6 and 9 post-infection. *Eimeria acervulina* counting was performed according to Mike McMaster’s method. Briefly, each fecal material was suspended in 3 l of water, and the oocyst numbers were determined in two 0.15 ml samples using the McMaster counting chamber according to the following formula:

\[
\text{total oocysts/bird} = \frac{\text{oocyst count} \times \text{dilution factor} \times (\text{fecal sample volume/counting chamber volume})}{2}
\]

**Separation of duodenal intraepithelial lymphocytes (IEL) and detection of T lymphocyte subpopulations.** The IEL were separated by Lillehoj’s method (Lillehoj 1989). One bird from each cage was slaughtered at day 10 post-infection. Segments (two birds pooled) of approximately 3–4 cm in length from middle duodenum were dissected, opened by longitudinal cutting, and washed with HBSS. About a 30–50 mg sample of mucosa was collected, snap frozen in liquid nitrogen, and stored at −80°C.

Total mRNA was extracted using TRIzol reagent according to the manufacturer’s instruction (Invitrogen, Grand Island, USA). About 5 ml of purified RNA was used as template for cDNA synthesis in the presence of 1.25 ml of M-MLV RT reverse transcriptase (200 U) (Invitrogen), 1 ml of Oligo dT18 (30mM), 13 ml of RNAse-free water, 1.25 ml of 10mM dNTP Mix, and 1 ml of RNasin ribonuclease inhibitor (50 U/ml). After incubation for 60 min at 37°C, the reverse transcriptase was inactivated at 70°C for 15 min.

The relative IFNγ expression was measured by real-time fluorescent PCR using the ABI-PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, USA) with β-actin as reference. The following primer pairs were used: *IFNγ*: 5’-AGCTGACGGTGAGCAGACCTATATT-3’, 5’-GGCTTTGCGCTGGATTC-3’; *β-actin*: 5’-TCTGGTGTTGACCACTACATGTACCCT-3’, 5’-CCAGTAATTTGGTACCGGCTCCTC-3’. The
primer sequences for IFNγ and β-actin were designed according to the sequences published by GenBank (Accession Nos. FJ977575.1 and NM 205518, respectively). The PCR amplifications were performed as follows: 2 min at 55°C and 10 min at 94°C followed by 40 cycles of 15 s denaturation at 94°C, 30 s annealing at 53.5°C, and 30 s primer extension at 72°C. Each sample had 3 replicates in a real-time PCR run.

Preparation of intestinal mucosa homogenate and measurement of malondialdehyde (MDA) content, antioxidant enzyme activity, and glutathione (GSH) content. Duodenum samples were obtained from 24 birds (one bird per cage). The lumen of duodenum was washed with cold KCl (1.5%, w/v). Mucosa was scraped off using a blade, and a 10% homogenate (w/v) was made in PBS buffer (pH 7.4, 50mM). After 8000 g centrifugation for 15 min at 4°C, the supernatant was collected and frozen at –30°C. Total SOD activity was assayed according to the methods of Spitz and Oberly (1989). Units of SOD activity were defined by the amount of enzyme required to inhibit the rate of formazan dye formation by 50% under defined conditions. CAT activity was estimated by the decomposition of H2O2 to yield H2O and O2, and changes in absorbance at 240 nm were monitored for 2 min (Cohen et al. 1970). MDA, the marker of lipid peroxidation, was quantified by measuring the thiobarbituric acid reactive substances with a spectrophotometer at 535 nm (Wills 1966). GSH was measured by 2-nitrobenzoic acid and thiol compound reaction method (Beutler 1975). Commercial kits were purchased from the Nanjing Jiancheng Biological Engineering Research Institute.

Statistical analysis. Data were expressed as mean ± SD. Data were analyzed using the GLM model of SPSS software (Version 11.5, 2002) with diet and coccidial infection as main effects along with their interaction. A P < 0.05 was considered to be statistically significant. Multiple comparisons were carried out using least significant difference tests. The oocyst output was compared by Student’s t test.

RESULTS

As shown in Table 2, Eimeria acervulina infection significantly decreased the average daily gain of broiler chicks between days 5 and 9 post-infection (P < 0.01) while dietary CLA did not change it (P > 0.05). There was a significant interaction between dietary CLA and Eimeria acervulina infection on the average daily gain (P < 0.05). Dietary CLA partly ameliorated the decrease of the average daily gain by Eimeria acervulina infection (P < 0.05). Compared with the infected basal diet group, the oocyst output in the infected CLA-fed group decreased by 24.27% between days 6 and 9 post-infection (P < 0.01).

As shown in Table 3, Eimeria acervulina infection increased the percentages of CD4+ and CD8+ T lymphocytes in the duodenal epithelium of broiler chicks (P < 0.05), and significantly enhanced IFNγ gene expression (P < 0.01). Dietary CLA significantly increased the percentage of CD8+ T lymphocytes in the duodenal epithelium (P < 0.05), but showed no obvious effect on the IFNγ expression in the duodenal epithelium (P > 0.05).

As shown in Table 4, Eimeria acervulina infection significantly increased the content of MDA, the activity of CAT (P < 0.01), and the content of GSH (P < 0.01) in duodenal mucosa (P < 0.05), and significantly decreased the activity of total superoxide dismutase (TSOD) (P < 0.05). Dietary CLA significantly inhibited the increase of MDA content (P < 0.05) and the decrease of GSH content (P < 0.05) in duodenal mucosa, but had no effect on the activities of TSOD and CAT (P > 0.05). There was no interaction between dietary

Table 2. Effects of dietary CLA supplementation on average daily gain and oocyst shedding of broiler chicks after Eimeria acervulina infection

<table>
<thead>
<tr>
<th>Variable</th>
<th>Uninfected</th>
<th>Infected</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control diet</td>
<td>CLA diet</td>
<td>control diet</td>
</tr>
<tr>
<td>Average daily gain (g)</td>
<td>40.85 ± 2.12^a</td>
<td>41.63 ± 2.25^a</td>
<td>32.57 ± 2.09^c</td>
</tr>
<tr>
<td>Oocyst production (10^8/bird)</td>
<td>–</td>
<td>–</td>
<td>4.45 ± 0.68^a</td>
</tr>
</tbody>
</table>

CLA = conjugated linoleic acid

^a,b^values with different superscripts within a row significantly differ (P < 0.05)
DISCUSSION

In the present experiment, 1.5% dietary CLA significantly reduced the oocyst output of broiler chicks infected with Eimeria acervulina, and partly alleviated their growth inhibition caused by Eimeria acervulina infection. These results demonstrated that the dietary CLA had protective effect on the broiler chicks infected with Eimeria acervulina.

The present experiment revealed that the proportions of CD4+ and CD8+ T lymphocytes in duodenal epithelium significantly increased after broiler chicks infection with Eimeria acervulina, which is consistent with previous experiments (Lillehoj 1994; Bessay et al. 1996). The collective findings confirmed that the invasion of Eimeria acervulina into duodenal epithelium activated CD4+ and CD8+ T lymphocytes (Lillehoj and Chung 1992). In the present experiment, with or without Eimeria acervulina infection, dietary CLA increased the percentage of CD8+ T lymphocytes in duodenal epithelium of broiler chicks (P < 0.05). Bassaganya-Riera et al. (2002, 2003) have reported that dietary CLA was conducive to maintain the numbers of CD8+ T lymphocytes in swine infected with pseudorabies virus and porcine circovirus type 2. Moreover, in the absence of exogenous stimulus as viral infection and lipopolysaccharide stimulation, dietary CLA increased the percentages of CD8+ T lymphocytes in the peripheral blood, thymus, and spleen of animals such as rat, pig, and poultry (Bassaganya-Riera et al. 2001; Yamasaki et al. 2003; Zhang et al. 2008). These results suggest that the regulating property of dietary CLA on CD8+ T lymphocytes is present in a variety of animal tissues.

The immune response of chickens to Eimeria tenella infection is very complex, which involves the systemic immunity and the intestinal local mucosal immunity. Cell-mediated immunity, especially IEL and lamina propria lymphocytes, play important roles in the response to Eimeria tenella infection (Lillehoj et al. 2004). Trout and Lillehoj (1995) found that Eimeria acervulina sporophyte significantly increased the duodenal epithelium lymphocytes of broiler chicks in 24 h after infection with Eimeria acervulina, indicating the involvement of CD8+ T lymphocytes in the immune process of coccidian infection. Zigterman et al. (1993) reported that CD8+ T lymphocytes prevented the sporozoite migration and inhibited Eimeria tenella (P < 0.05).
growth in the early stage of infection, and directly lysed and destroyed target cells in the later stage of *Eimeria tenella* infection. In the present study, after the infection of broiler chicks with *Eimeria acervulina*, dietary CLA increased the percentage of CD8⁺ T lymphocytes in duodenum epithelium, and simultaneously decreased the oocyst output, leading to partial recovery of the daily gain of the birds. Taken together, these results indicated that the increased percentage of CD8⁺ T lymphocytes may facilitate the inhibition or killing of *Eimeria acervulina*.

In this experiment, *Eimeria acervulina* infection markedly enhanced the gene expression of duodenal IFNγ, which was similar to the result observed in the chickens infected with *Eimeria tenella* (Hong et al. 2008). IFNγ plays an important role against coccidial infection. Recombinant IFNγ reduced oocyst output and relieved the growth inhibition of broiler chicks infected with *Eimeria acervulina* (Lillehoj and Choi 1998). In the present experiment, the enhanced gene expression of duodenal IFNγ indicated that *Eimeria acervulina* infection activated the duodenal immune response to increase the self-protection of broiler chicks, however, no difference was found here in IFNγ gene expression between the CLA diet and the basal diet, indicating that the beneficial effects of CLA on the reduction of oocyst output and alleviation of growth inhibition cannot be attributed to the enhanced IFNγ gene expression.

During the *Eimeria tenella* infection, the depletion of CD4⁺ T lymphocytes in the cecal tonsil of broiler chicks suppressed the IFNγ expression, while the depletion of CD8⁺ T lymphocytes enhanced the IFNγ expression, which is why Yun et al. (2000) considered that IFNγ was derived from CD4⁺ T lymphocytes. In this experiment, dietary CLA did not change the percentage of duodenal CD4⁺ T lymphocytes but had no effect on IFNγ expression, which is consistent with the viewpoint of Yun et al. (2000). However, Lauret et al. (2001) found that the IFNγ from CD8⁺ T lymphocytes was directly involved in the cellular immune response against coccidiosis in the test of broiler chicks infected with *Eimeria tenella* and *Eimeria maxima*. In addition, in piglets infected with circovirus type 2, dietary CLA inhibited the IFNγ expression in CD4⁺ T lymphocytes, but increased the IFNγ expression in CD8⁺ T lymphocytes (Bassaganya-Riera et al. 2003). The above two studies suggested the possibility that dietary CLA stimulated the IFNγ production by CD8⁺ T lymphocytes. However, in this experiment, CLA increased the percentage of CD8⁺ T lymphocytes subsets without affecting the IFNγ expression. To clarify this, further studies are necessary.

In the present experiment, *Eimeria acervulina* infection increased the content of MDA and decreased the activity of TSOD in duodenal mucosa of broiler chicks (*P* < 0.01), which was in line with previous experiments (Koinarski et al. 2005). The collective findings indicated that *Eimeria acervulina* infection weakened the antioxidant capacity of cells and induced oxidative stress. Cells have evolved several antioxidant strategies aimed at the detoxification of free radicals. One of them is the enzymatic antioxidant defense system, consisting of the enzymes including superoxide dismutase, CAT, and glutathione peroxidase. In our experiment, after *Eimeria acervulina* infection, the activity of SOD decreased and CAT activity increased in duodenal mucosa. SOD plays an important role in eliminating oxygen free radicals and protecting against chromosome damage and cell death from oxidative stress (McCord et al. 1983). The decreased activity of SOD would result in an accumulation of more free radicals and induce the expression of CAT through a negative feedback mechanism. Therefore, the increased activity of CAT perhaps was a compensation for oxidative damage. Similar phenomena have also been observed in the test of broiler chicks experimentally infected with *Eimeria tenella* (Georgieva et al. 2006).

In the present experiment, dietary CLA decreased the content of MDA, the lipid peroxidation marker, in the duodenal mucosa of broiler chicks infected with *Eimeria acervulina*, which demonstrated that dietary CLA alleviated the oxidative stress resulting from *Eimeria acervulina*. The previous studies found that the enhanced antioxidant capacity may help improve the anticoccidial capacity in the chickens. For example, vitamin A effectively suppressed the oxidative damage induced by *Eimeria acervulina* to reduce the shedding of sporulated oocysts in chickens (Sklan et al. 1994). Therefore, the enhanced resistance to *Eimeria acervulina* by dietary CLA was likely associated with its effect on alleviating oxidative stress induced by *Eimeria acervulina* infection.

GSH is an important non enzymatic antioxidant and free radical scavenger in *vivo*. Dietary CLA did not change the activities of TSOD and CAT.
in duodenal mucosa, but partially suppressed the decrease of the duodenal mucosal GSH content after *Eimeria acervulina* infection, suggesting that dietary CLA probably alleviated the oxidative stress caused by *Eimeria acervulina* infection through increasing the GSH content. The rate limiting enzyme in the synthesis of GSH is γ-glutamylcysteine synthase (γGCS), the main regulator of which are peroxisome proliferator activated receptors (PPARs). As the ligands of PPARs, CLA, directly or indirectly, influenced the expression and activity of PPARs (Houseknecht et al. 1998), thereby providing a possible mechanism for CLA to influence GSH.

**CONCLUSION**

Dietary CLA reduced the oocyst output and partly alleviated growth inhibition of broiler chicks infected with *Eimeria acervulina*. The anticoccidial effects of dietary CLA are probably related to the increased proportions of CD8⁺ T lymphocytes in duodenal epithelium and its inhibitory effect on the decrease of duodenal mucosal GSH after *Eimeria acervulina* infection.

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