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RESEARCH ARTICLE

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# Effect of in ovo injection of conjugated linoleic acid on immune status and blood biochemical factors of broilers chickens

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## Abstract

This experiment evaluated the effects of an in ovo injection of conjugated linoleic acid (CLA) on immune status and blood metabolites of broiler chicks at 21 and 42 days of age. One hundred and sixty fertilized eggs were selected from a young broiler breeder flock and allocated to4 treatments with 4 replicates each. CLA was injected into the air sack of the selected eggs (150 and 300 mg, dissolved in 100 mL of commercial diluents, CLA150 and CLA300) on Day18 of incubation. Two control groups (injected with or without diluent) were also included. In ovo injection of 150 or 300 ppm CLA increased ($p < 0.05$) feed intake of chickens as compared to control groups up to 42 days of age. Weight gain and feed conversion ratio of chickens from CLA300, were improved ($p < 0.05$) as compared to chicks hatched from eggs injected diluent or control group during total period. Cutaneous basophil hypersensitivity response to injection of phytohaemagglutinin-P significantly ($p < 0.05$) increased in chickens from CLA300 comparing to chicks from CLA150 or control ones. In ovo injection of 300 ppm CLA, decreased blood serum cholesterol and low density lipoprotein (LDL) of chickens as compared to control chicks or those from CLA150 ($p < 0.05$). Central lymphoid tissues such as bursa of Fabricius and thymus relative weights were increased ($p < 0.05$) in chickens from CLA300 as compared to chicks injected diluent or control groups. It is concluded that feeding 300 ppm CLA through in ovo injection may be effective to subsequent growth rate, immune response and decrease blood serum cholesterol and LDL of broiler chickens during whole experimental period.

**Additional key words**: in ovo feeding; immune response; performance; serum fat parameters.

## Introduction

Modulation of the immune status of chickens may improve poultry health and production. Early provision of nutrients may help to immediate embryo survival and disease resistance. Conjugated linoleic acid (CLA) refers to the positional and geometric isomers of linoleic acid (cis-9, cis-12 18:2). It has double bonds that are separated by a single bond between two carbons (Pariza *et al*., 2001). Some researchers have shown the effects of CLA on stimulating the immune functions of broiler chickens (Zhang *et al*., 2005; Long *et al*., 2010). Takahashi *et al*. (2003) reported that dietary CLA enhanced anti-sheep red blood cells (anti-SRBC) antibody production in broilers. In addition to effects of CLA on immune status, effect of dietary CLA on growth rate and blood metabolites also has been studied. Downing *et al.* (2002) reported that use of 1% CLA in diet improved growth rate of broiler chickens. Also CLA has been shown to alter the lipid metabolism in chicken embryos derived from hens fed high levels of CLA (Latour *et al*., 2000). Nicolosi *et al*. (1997) showed that hamsters that were fed different levels of CLA had lower blood total cholesterol. Lee

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Abbreviations used: AI (avian influenza); ALT (alanine amino transferase); AST (aspartate amino transferase); BW (body weight); CBH (cutaneous basophil hypersensitivity response to phytohemagglutinin); CLA (conjugated linoleic acid); FCR (feed conversion ratio); FI (feed intake); HDL (high density lipoprotein); HI (haemaglutinin inhibition); LDL (low density lipoprotein); NDV (*Newcastle disease virus*); PBS (phosphate buffered saline); PHA-P (phytohaemagglutinin-P); SRBC (sheep red blood cell); WG (weight gain).

*et al*. (1994) reported that rabbits fed diets containing CLA showed lower low density lipoprotein (LDL) cholesterol and somewhat lower triglyceride concentrations. With in ovo injection of CLA in industrial hatcheries, we can improve subsequent performance of broiler chickens. Therefore, the aim of this experiment was to study the effects of in ovo injection of CLA at 18 days of incubation on growth performance, immune status and blood metabolites of broiler chicks at 21 and 42 days of age.

## Material and methods

This experiment was approved by the Animal Welfare Committee of the Ferdowsi University of Mashhad, Iran. One hundred and ninety two Cobb-500 broiler hatching eggs with similar weight (44 ± 2.5 g), were obtained from a broiler breeder flock at 30 weeks of age. The incubation conditions were controlled commercially during incubation period. On Day 18 of incubation, all of the eggs were injected 0.1 mL of treatments into air sack as the following experimental treatments: 1) control, non-injected eggs, 2) eggs injected with 100 mL of commercial diluent (vaccine carrier), 3) eggs injected with 150 mg of CLA dissolved in 100 mL of commercial diluent (CLA150), and 4) eggs injected with 300 mg of CLA dissolved in 100 mL of commercial diluent (CLA300).

The major CLA isomers in current experiment were cis-9, trans-11 and trans-10, cis-12 isomers. The eggs trays were randomly assigned to the 4 treatment groups giving 4 replicates with 12 eggs in each tray, so that treatment effects would not be influenced by their position within. After hatching, all chicks were tagged and after removing weak chicks from replicates, 160 chicks with average initial body weight (BW) of 45 g were selected and transferred to Ferdowsi University poultry house and allocated with four replicate floor pens (1.2 × 1.2 m) of 10 chicks each in a completely randomized design. Dried wood shavings were used as litter at a depth of about 5cm on floor pens. One hanging feeder and one water cup was provided for each pen. Birds had free access to feed and water and exposed to 23 h lighting: 1 h dark photoperiod throughout the experiment. Initial room temperature was set at 32°C and gradually reduced according to the usual commercial practices. The birds were fed mash diets formulated according to recommendations for Cobb-500 broiler chickens (Table 1).

**Table 1.** Composition of basal diet in starter, grower and finisher period

| | 0-14 d | 15-28 d | 29-42 d |
|---|---|---|---|
| *Ingredients (%)* | | | |
| Corn | 49.80 | 56.45 | 62.84 |
| Soybean meal | 41.50 | 36 | 30.76 |
| Soybean oil | 4.5 | 4 | 2.82 |
| Sodium chloride | 0.44 | 0.42 | 0.42 |
| Limestone | 1.36 | 1.10 | 1.10 |
| Dicalcium P | 1.66 | 1.33 | 1.35 |
| DL-methionine | 0.14 | 0.10 | 0.11 |
| L-lysine HCl | 0.10 | 0.10 | 0.10 |
| Vi t +Min premix[1] | 0.50 | 0.50 | 0.50 |
| *Calculated composition* | | | |
| ME (kcal kg$^{-1}$) | 2,950 | 3,000 | 3,050 |
| Crude protein (%) | 22.68 | 20.69 | 19.06 |
| Linoleic acid (%) | 1.22 | 1.14 | 0.95 |
| Crude fiber (%) | 3.36 | 3.33 | 3.23 |
| Calcium (%) | 1.02 | 0.86 | 0.81 |
| Nonphytate P (%) | 0.49 | 0.43 | 0.40 |
| Sodium (%) | 0.19 | 0.18 | 0.17 |
| Arginine (%) | 1.28 | 1.09 | 0.97 |
| Lysine (%) | 1.24 | 1.05 | 0.92 |
| Met (%) | 0.46 | 0.40 | 0.36 |
| Met + Cys (%) | 0.92 | 0.80 | 0.72 |
| Threonine (%) | 0.81 | 0.70 | 0.62 |
| Tryptophan (%) | 0.20 | 0.17 | 0.15 |

[1] Vitamin and mineral premixes supplied per kilogram of diet: vitamin A, 10,000 IU; vitamin D3, 9,790 IU; vitamin E, 121 IU; vitamin B12, 20 µg; riboflavin, 4.4 mg; calcium pantothenate, 40 mg; niacin, 22 mg; choline, 840 mg; biotin, 30 µg; thiamin, 4 mg; zinc sulfate, 60 mg; copper sulfate, 100 µg; selenium (sodium selenate), 0.2 mg; iodine, 1 mg; manganese oxide, 60 mg.

Corn-soybean based starter (0-14 days), grower (15-28 days) and finisher (29-42 days) diets were provided to all chickens.

### Production performance

During the experimental period, the average daily feed intake (FI), weight gain (WG) and feed conversion ratio (FCR) for each group of birds were calculated. One bird per replicate (n = 4) was selected to be killed and eviscerated on Days 21 and 42. The carcass cuts and internal organs such as breast, thighs, liver, heart, pancreas and abdominal fat pad were removed, weighed and expressed as percentage of live body weight.

### Blood metabolites

For measuring of blood biochemical factors, one chicken from each replicate close to pen average weight was selected; blood samples were taken from wing vein and kept at the room temperature for 30 min. Blood samples were centrifuged for 2,000 g for 15 min at room temperature to obtain serum. The samples were transferred to Eppendorf tubes and kept at –20°C until analyzed by an auto-analyzer system (Bio Systems, Barcelona, Spain) using commercially available kits (Bio Systems, Spain). The blood serum from each bird (1 mL) was used to determine aspartate aminotransferase (AST), alanine amino transferase (ALT), low density lipoprotein (LDL), high density lipoprotein (HDL), total cholesterol and triglyceride.

### Antibody titers against *Newcastle disease virus* and avian influenza

The chicks were vaccinated against *Newcastle disease virus* (NDV) and avian influenza (AI) on Day 8. On Days 14 and 20 (6 and 12 after vaccination), one chick per replicate was randomly selected (n = 4) and blood samples were taken from wing vein. The sera were separated by centrifugation for 2000 g for 15 min at room temperature and stored in –20°C in separate sterile vials prior to analysis. The antibody titers were measured using haemaglutinin inhibition (HI) test and antibody titer expressed as reciprocal log2 values for the highest dilution that displayed HI (Allan *et al.,* 1978).

### Antibody response to sheep red blood cell

One chicken per replicate (n = 4) was selected. They were intramuscularly injected with 1 mL 15% sheep red blood cells (SRBC) suspension in phosphate buffered saline (PBS) at Day 28. SRBC was used as an antigen to quantify the antibody response. Blood samples were collected at Days 35 and 42 (7 and 14 days after the injection). The serum from each sample was collected, heat inactivated at 56°C for 30 min and then analyzed for IgG (mercaptoethanol-resistant) and IgM (mercaptoethanol-sensitive) anti-SRBC antibodies as described by Cheema *et al*. (2003). The SRBCs used for inoculation and antibody titration were obtained from the same donor sheep.

### Cutaneous basophil hypersensitivity response to phytohemagglutinin

The cell mediated immune response was assessed using the cutaneous basophil hypersensitivity (CBH) response to phytohemagglutinin. At 35 days of age, a CBH test (Corrier & Deloach, 1990) was administered to 2 chickens per replicate (n = 8). Phytohaemagglutinin-P (PHA-P) was injected intradermally (100 µg of PHA-P suspended in 0.1 mL of PBS per bird) into the web between the third and fourth toes of the left foot and as a control, an equal volume of PBS was injected into the same toes web of the right foot. The thickness of the two toes webs was measured initially and 12 and 24 h afterwards with a pressure sensitive caliper. The swelling response was measured by subtracting the pre-injection measurement (initially) from the post-injection measurement (12 and 24 h afterwards) of the PHA-P-injected toe web.

### Immune organ weight

To assess the immune status, immune organ weights were evaluated. One bird per replicate (n = 4), with a weight similar to the pen average, was selected on Days 21 and 42. The immune organs such as bursa of Fabricius and thymus (all lobes on the left side of the neck) were removed, weighed and expressed as a percentage of live weight.

All data were analyzed by ANOVA using the procedure described by SAS (2008). All percentage data were subjected to arcsin transformation prior to analysis. All means were compared using Tukey's test at a 5% significance level. The antibody titers obtained in both serological tests were transformed in log10.

## Results

Hatchability results showed no significant differences between different treatments ($p > 0.05$). The hatchability (%) of non-injected eggs, eggs injected with commercial diluent, eggs injected with 150 ppm of CLA(CLA150) and eggs injected with 300 ppm of CLA (CLA300) were 89.8%, 90.2%, 90.2% and 90.5%, respectively. In ovo injection of 150 or 300 ppm CLA increased ($p < 0.05$) WG and FI of chickens as compared to control groups (injected with or without diluents, Table 2). Differences between FI and WG of chickens

**Table 2.** Effects of in ovo injection of different levels of conjugated linoleic acid (CLA) on production performance of broiler chickens (0-42 days)

| | Feed intake (g bird$^{-1}$ d$^{-1}$) | Weight gain (g bird$^{-1}$ d$^{-1}$) | Feed conversion ratio (g g$^{-1}$) |
|---|---|---|---|
| Control (without diluent) | 122.8$^{b}$ | 56.0$^{c}$ | 2.19$^{a}$ |
| Injected, diluent | 121.1$^{b}$ | 56.7$^{c}$ | 2.14$^{a}$ |
| 150 ppm CLA | 129.2$^{a}$ | 59.9$^{b}$ | 2.15$^{a}$ |
| 300 ppm CLA | 131.3$^{a}$ | 63.2$^{a}$ | 2.08$^{b}$ |
| Standard error of the mean | 2.41 | 1.31 | 0.042 |

$^{a,b}$ Values in the same column and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

from non-injected and injected diluents were not significant ($p > 0.05$), but FCR of chickens from CLA300, was improved ($p < 0.05$) as compared to chicks from CLA150 or control ones during total period.

The concentration of IgG was increased ($p < 0.05$) in chickens from CLA300as compared to CLA150 and control ones at 42 days of age but IgM, and Ig total production were not affected (Table 3). Results of antibody response to SRBC showed that antibody response to NDV and AI of broiler chickens (6 or 12 days after injection) were not influenced ($p > 0.05$) with in ovo injection of different levels of CLA (data not shown).

Effects of in ovo injection of different levels of CLA on CBH response to phytohemagglutinin are shown in Table 4. Results showed that cutaneous basophil hypersensitivity response to injection of PHA-P (after 12 or 24 h of injection) increased ($p < 0.05$) in chickens from CLA300 comparing to CLA150 or control ones.

Effects of in ovo injection of CLA on central lymphoid tissues such as bursa of Fabricius and thymus relative weights of broiler chicken are shown in Table 5. Results showed that the relative weight of bursa of Fabricius increased ($p < 0.05$) with the CLA treatments at Days21 and 42.

The amounts of AST and ALT in blood serum of broiler chicken are shown in Table 6. Results showed that AST levels were not changed in blood serum of chickens at 21 and 42 days of age with injection of 150 or 300 ppm CLA ($p > 0.05$) but ALT was decreased in

**Table 3.** Effects of in ovo injection of different levels of conjugated linoleic acid (CLA) on production of immunoglobulin G, immunoglobulin M and immunoglobulin total in broiler chickens

| | Anti-SRBC antibody titer | | |
|---|---|---|---|
| | Immunoglobulin G | Immunoglobulin M | Immunoglobulin total |
| *35 days* | | | |
| Control (without diluent) | 2.00$^{c}$ | 3.25 | 5.25 |
| Injected, diluents | 2.75$^{b}$ | 3.00 | 5.75 |
| 150 ppm CLA | 2.66$^{b}$ | 3.00 | 5.66 |
| 300 ppm CLA | 3.68$^{a}$ | 2.01 | 4.69 |
| Standard error of the mean | 0.389 | 0.410 | 0.648 |
| *42 days* | | | |
| Control (without diluent) | 2.00$^{b}$ | 3.00 | 5.00 |
| Injected, diluents | 2.00$^{b}$ | 2.50 | 4.50 |
| 150 ppm CLA | 2.33$^{b}$ | 3.33 | 5.66 |
| 300 ppm CLA | 3.66$^{a}$ | 2.00 | 5.00 |
| Standard error of the mean | 0.288 | 0.437 | 0.555 |

$^{a,b}$ Values in the same column and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

**Table 4.** Effects of in ovo injection of different levels of conjugated linoleic acid (CLA) on Cutaneous Basophil Hypersensitivity response to phytohemagglutinin (CBH) in broiler chickens

| | Thickness of the two toes webs (mm) | |
|---|---|---|
| | 12 h after injection | 24 h after injection |
| Control (without diluent) | 46.2[b] | 73.5[b] |
| Injected, diluents | 47.0[b] | 69.0[b] |
| 150 ppm CLA | 47.3[b] | 76.0[b] |
| 300 ppm CLA | 64.3[a] | 97.7[a] |
| Standard error of the mean | 1.911 | 2.709 |

[a,b] Values in the same columns and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

blood serum of chickens from CLA 300 at 21 days of age comparing to other groups.

Effects of in ovo injection of CLA on serum fat parameters of broiler chickens are shown in Table 7. Results showed that in ovo injection of 300 ppm CLA decreased blood serum cholesterol and LDL of chickens as compared to CLA150 or control ones ($p < 0.05$). Results of serum biochemical measurements of broiler chickens at 42 days of age showed that uric acid, glucose and albumin concentration in blood serum were not different ($p > 0.05$) among treatments (data not shown).

## Discussion

Badinga *et al*. (2003) reported that using 5% CLA in diet reduced feed consumption of broiler chickens, but similarly to our experiment, Downing *et al.* (2002) reported that use of 1-1.5% CLA in diet improved FI and weigh gain of broiler chickens during 0-42 days of age. In our experiment, CLA increased FI and consequently the weight gain of animals. One of the major causes of increased weight in our study might be due to increased feed consumption of chickens from CLA300.The used dose of CLA in this study might be different from CLA doses used in other trials, but this study showed that in ovo injection of CLA at 18 days of incubation when chicks switch to active pulmonary

**Table 5.** Effects of in ovo injection of conjugated linoleic acid (CLA) on bursa of Fabricius and thymus relative weight of broiler chickens

| | Bursa of Fabricius | | Thymus | |
|---|---|---|---|---|
| | 21 d | 42 d | 21 d | 42 d |
| | (% of live weight) | | | |
| Control (without diluent) | 0.219[b] | 0.116[b] | 0.282[c] | 0.328[c] |
| Injected, diluents | 0.247[b] | 0.125[b] | 0.325[c] | 0.365[c] |
| 150 ppm CLA | 0.325[a] | 0.205[a] | 0.560[b] | 0.446[b] |
| 300 ppm CLA | 0.354[a] | 0.188[a] | 0.671[a] | 0.466[a] |
| Standard error of the mean | 0.016 | 0.008 | 0.022 | 0.005 |

[a,b] Values in the same column and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

**Table 6.** Effects of in ovo injection of different levels of conjugated linoleic acid (CLA) on liver enzymes (IU $L^{-1}$) in blood serum of broiler chickens

| | Alanine amino transferase | | Aspartate amino transferase | |
|---|---|---|---|---|
| | 21 d | 42 d | 21 d | 42 d |
| Control (without diluent) | 10.5[ab] | 10.5 | 224.6 | 257.4 |
| Injected, diluents | 11.2[a] | 9.9 | 250.4 | 245.1 |
| 150 ppm CLA | 11.3[a] | 9.2 | 233.8 | 237.4 |
| 300 ppm CLA | 9.5[b] | 9.5 | 234.1 | 239.4 |
| Standard error of the mean | 0.42 | 0.448 | 10.91 | 6.01 |

[a,b] Values in the same column and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

**Table 7.** Effects of in ovo injection of different levels of conjugated linoleic acid (CLA) on serum fat relating parameters of broiler chickens at 42 days of age

| | Triglycerides (mg dL$^{-1}$) | Total cholesterol (mg dL$^{-1}$) | HDL (mg dL$^{-1}$) | LDL (mg dL$^{-1}$) | LDL/HDL |
|---|---|---|---|---|---|
| Control (without diluent) | 84.2 | 140.0$^{a}$ | 59.8 | 83.9$^{a}$ | 1.40$^{c}$ |
| Injected, diluents | 86.6 | 145.5$^{a}$ | 59.7 | 84.9$^{a}$ | 1.42$^{c}$ |
| 150 ppm CLA | 88.8 | 141.3$^{a}$ | 61.2 | 79.8$^{b}$ | 1.30$^{b}$ |
| 300 ppm CLA | 84.5 | 132.2$^{b}$ | 65.9 | 65.3$^{c}$ | 0.99$^{a}$ |
| Standard error of the mean | 3.09 | 5.40 | 2.38 | 0.39 | 0.085 |

$^{a,b}$ Values in the same column and variable with no common superscript differ significantly ($p < 0.05$). Values are means for 4 replicates with one bird per replicate.

respiration may support subsequent growth of chickens. Chin *et al*. (1994) showed that moderate doses of dietary CLA (≤0.5%) could improve growth rate and FCR of growing rats. Due to significant increase in weight gain of chickens from CLA300, the FCR was improved as compared to other groups during whole experimental period. Due to weak environmental conditions in our state such as high altitude and lower relative humidity, it looks that productive results of birds were lower than Cobb-500 standards, but 300 ppm CLA at 18 days of incubation could help the hatched chicks to reach productive results closer to the Cobb standards. The effects of CLA on mammalian immune responses have been extensively studied but little is known about the effects of dietary CLA on chicken immunity. Yamasaki *et al*. (2000) and Corino *et al*. (2009) have shown that dietary CLA enhances immunoglobulin production in mammals. Cook *et al*. (1993) showed that antibody production in chicks against SRBC was not affected by feeding CLA, while Takahashi *et al*. (2003) reported that dietary CLA enhanced anti-SRBC antibody production in broilers. Limited information is available on other measures of immune response in birds. In this study, antibody response to NDV and AI of broiler chickens were not influenced ($p > 0.05$) with in ovo injection of different levels of CLA. The cell mediated immune response in the chickens was assessed using the CBH response to phytohemagglutinin. In ovo injection of 300 ppm CLA could enhance ($p < 0.05$) this type of immune response in broiler chicks as compared to control chicks or chicks from CLA150. It is well known that the lymphoid tissue plays an important role in the defense against microorganisms in birds. The chicken has central (thymus and bursa of Fabricius) and peripheral (spleen and all mucosa associated lymphoid tissue) lymphoid tissues (Getty, 1975). The thymus-dependent component is represented by the smaller lymphocytes and is responsible for cell mediated immunity (CMI), including immunosurveillance, whereas the bursa-dependent component is represented by larger lymphocytes which transform into plasma cells in the tissue and play an important role in humoral immunity (HI), remaining both of them active over lifetime (Sakhare *et al*., 2007). Concerning this immunological point of view, the histology of the lymphoid tissues of the chicken is very important. The relative weight of bursa of Fabricius, which plays an important role in humoral immunity of chicks, were significantly increased ($p < 0.05$) in chicks from CLA150 and CLA300 at 21 and 42 days of age. The relative weight of thymus, which plays an important role in cell mediated immunity of chicks, were significantly increased ($p < 0.05$) in chicks at 21 and 42 days of age, indicating that 150 or 300 ppm CLA could also influence cell-mediated immunity of chicks. Increasing the amount of AST or ALT, which are released from chicken liver to blood stream, is an indicator of liver damage (Chatila & West, 1996). The AST levels were not changed in blood serum of chickens at 21 and 42 days of age with injection of 150 or 300 ppm CLA ($p > 0.05$) but ALT, the other liver enzyme found mainly in liver and in smaller amounts in kidneys, heart, muscles, and pancreas, was decreased in blood serum of chickens from CLA300 at 21 days of age. Nicolosi *et al*. (1997) also reported that hamsters that were fed different levels of CLA showed lower total circulating cholesterol concentrations. Similarly, Munday *et al*. (1999) reported that mice fed CLA showed lower serum triglyceride concentrations. The decreased level of serum LDL in chicks from CLA300 was similar to Lee *et al*. (1994) results, which reported that 0.5 g CLA day$^{-1}$ in rabbits lowered blood LDL concentrations. The beneficial effects of CLA might be related to the fact that CLA de-

creases the activity of lipoprotein lipase (Park *et al*., 1997). Szymczyk *et al*. (2001) reported that CLA increased total cholesterol and HDL, but reduced the ratio of HDL: Total cholesterol in the plasma of broilers. Increased serum activities of ALT may decrease serum concentrations of albumin, total protein and uric acid, but results of this study showed that uric acid, glucose and albumen levels in blood serum were not different ($p > 0.05$) among the treatments. It is concluded that in ovo injection of 300 ppm CLA could increase subsequent growth rate and immune response and decrease blood serum cholesterol and LDL of broiler chickens during the whole experimental period.

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