Effects of dietary conjugated linoleic acid on the intestinal mucosal immunity of broiler chickens

Yong Xiang Liu, Jian Ping Yang, Guo Pan Tang and Dong Feng Jiang

College of Animal Science and Technology, Henan University of Animal Husbandry and Economy, Zhengzhou, China

ABSTRACT

The effects of dietary conjugated linoleic acid (CLA) on the intestinal mucosal immunity of broiler chickens were investigated. A total of 96 1-day-old Arbor Acres male broiler chickens were assigned to two dietary treatments (0 and 1% CLA) with eight replicates each of six chicks. The intestinal mucosal immune status of broiler chickens was evaluated at 21-day old. The jejunal mucosa SIgA level increased by 43.58% \((p < .01)\) while its mRNA expression increased by 22.95% \((p < .01)\). The broilers receiving the CLA diet exhibited higher gene expression of jejunal peroxisome proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) \((p < .01)\) while the mRNA expression level of transforming growth factor \(\beta\) 4 (TGF\(\beta\)4) was not different between two dietary treatments group \((p > .05)\). After dietary CLA supplementation, the percent of Peyer’s node CD8\(^+\) T lymphocytes increased \((p < .01)\) and lymphocytes proliferation of Peyer’s node increased \((p < .05)\). In conclusion, the CLA diet enhanced jejunal SIgA expression, and increased lymphocyte transformation rate and the percent of CD8\(^+\) T lymphocyte in Peyer’s node of broiler chickens.

INTRODUCTION

Conjugated linoleic acid (CLA) refers to a class of spatial and geometric isomers of linoleic acid containing conjugated double bond, belonging to the polyunsaturated fatty acids family members. CLA has many important properties that regulate physiological and metabolic responses, for example, immune modulation, anti-cancer effects, prevention of cardiovascular disease and diabetes and weight control. Studies on the effects of CLA on immune function have thus far been conducted in the normal physiological status or inflammatory challenge status (O'Shea et al. 2004; Lai et al. 2005a; Renner et al. 2012).

In poultry science, enrichment of chicken meat and egg with CLA by the addition of CLA in the diet has been successfully demonstrated by several researchers (Kawahara et al. 2009; Herzallah 2013; Shinn et al. 2015, 2016). CLA has positively influences carcass traits, meat quality, antioxidant capacity, and fatty acid (FA) composition of poultry (Jiang et al. 2014; Fesler & Peterson 2013). In addition, little attention has been drawn to the beneficial immunomodulatory properties of CLA in broiler chickens (Takahashi et al. 2003; Zhang et al. 2005; He et al. 2007; Moraes et al. 2016).

Intestinal mucosal immune system is the largest and the most complicated immune system of animal organism. Pérez-Cano et al. (2009) reported that dietary CLA increased intestinal secretory IgA (SIgA) level of young rats. Bontempo et al. (2004) found dietary CLA improved intestinal SIgA level and cellular immune function in late pregnancy and lactation period of sows and growth and fattening period of offsprings. When piglets were infected with \(B.\) hyodysenteriae, dietary CLA effectively enhanced the expression of colonal proliferator-activated receptor \(\gamma\) (PPAR\(\gamma\)) and alleviated the intestinal mucosal inflammatory injury (Hontecillas et al. 2002). These researches hinted a close relationship between dietary CLA and intestinal mucosal immune response. So it was possible that CLA affects the intestinal mucosal immunity of broiler chickens. Although it was accepted that conjugated linoleic acid (CLA) has immunomodulatory properties in broiler chickens, no studies were available on the roles of CLA in intestinal mucosal immunity of poultry. The purpose of this study was to determine the effects of dietary CLA on the intestinal mucosal immunity of broiler chickens fed with 1.0% CLA diet.
Materials and methods

Experimental design

The animal management protocol for this research was approved by Animal Care and Use Committee of the Henan University of Animal Husbandry and Economy. A total of 96, one-day-old Arbor Acres male broiler chickens were purchased from Lvy Poultry Huabandry Co., Ltd. and were randomly distributed into two dietary treatments, fed either on the control or 1% CLA-supplemented diet. Each treatment group had eight replicates of six chickens each.

A maize soybean meal diet was used, and energy was adjusted using soybean oil. In CLA diet, 1% soybean oil was replaced by 1% CLA oil to keep both the CLA-supplemented diet and the control diet isonergic. The 1% adding amount of CLA was used in the experiment as described in the previous studies (Zhang et al. 2005; Jiang et al. 2014). The compositions of the control diet are presented in Table 1. The experimental diets were formulated to meet poultry requirements according to the National Research Council (1994) requirements. CLA was provided by the Qingdao Aohai Biology Technology Co., Ltd (Qingdao, China), 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.

Feeding experiment was conducted in the scholastic experiment pasture. The broilers were housed in wire cages and maintained on a 16L:8D light pro-

Growth performance

The body weights and feed consumption of broiler chickens were registered at the end of the third week. Average daily gain (ADG), average daily feed intake (ADFI) and feed conversion ratio (FCR) were calculated for this interval.

Sample collection and jejunal mucosa SIgA

On day 21, six birds (one per replication) were randomly chosen from each treatment and killed by cervical dislocation, manual exsanguination. The body cavity was opened and jejunum was removed. The jejunum was flushed with ice-cold KCl (1.5%, w/v). About a 2 cm portion of the jejunum was snap-frozen in liquid nitrogen for RNA isolation. The remaining jejunum was opened length-wide on an ice-cooled surface. The mucosa was gently scraped off using a blade and snap-frozen in liquid nitrogen for determining SIgA. The jejunum and mucosa were stored at −80 °C until analysis.

After thawing, the mucosa was homogenised (10%, w/v) at 15,000 rpm for 2 min at 4 °C in 0.88% sodium chloride water solution. Homogenate was centrifuged at 6000 g for 5 min at 4 °C. The supernatant was collected, sub-packed in 0.5 ml tubes and stored at −80 °C until further analysis. The level of jejunal mucosa SIgA was determined with a double antibody sandwich ELISA method according to the manufacturer’s instructions (chicken IgA ELISA quantitation kit from Shanghai SHireike Biotechnology Co., Ltd.).

RNA isolation and real-time polymerase chain reaction analysis

Total RNA of jejunum samples was extracted using TRIZOL reagent according to the manufacturer’s instruction (15596026, Introvgen Company, CA, USA). The quantity and quality were assessed by OD260:280. About 5 μl purified RNA was used as template for cDNA synthesis in the presence of 1.25 μl M-MLV RT reverse transcriptase (200 U) (28025-013, Introvgen Company, CA, USA), 1 μl of Oligo dT8 (30 mM), 3 μl of RNase-free water, 1.25 μl of 10 mmol dNTP Mix, 1 μl of RNasin ribonuclease inhibitor (50 U/μl) (Vigorous Biotechnology Co., Ltd. Beijing, China). After incubation for 60 min at 37 °C, the reverse transcriptase was inactivated at 70 °C for 15 min.

Gene expressions of SIgA, PPARγ, transforming growth factor β4 (TGFβ4) were detected by real-time
PCR with β-actin as reference. The following primer pairs were used: SlgA (Gene accession number: S4060), F (Forward) 5′-GGGGCAACAATTGCACCTGA-3′ and R (Reverse) 5′-GGTTAGGGGTAAAGTGTCG-3′; PPARγ (Gene accession number: AF470456.1), F 5′-CCCTAATACACGTCAC-3′ and R 5′-GGGTCGAGCAGTGAGC-3′; TGFβ4 (Gene accession number: M360), F 5′-CAACCCGGTACTAGGCGC-3′ and R 5′-GGGAGGCCTCCGTCG-3′; β-actin (Gene accession number: AF173602), F 5′-TATGCTCGTGCAATTGCACTG-3′ and R 5′-GGATTAGCGGGTTACCTGTG-3′. cDNA was amplified by real-time PCR using the Applied Biosystem ABI-PRISM 7900 HT sequence detection system (ABI Company, MA, USA). Each PCR was on 1 µl of cDNA mixed with 0.25 µmol of each primer, 5.3 µl of RNAse-free water, 7.5 µl of SYBR Green PCR master Mix (ABI Company, MA, USA) for a final volume of 15 µl. Amplifications were performed as follows: 2 min at 55 °C and 10 min at 94 °C followed by 40 cycles of 5 s denaturation at 94 °C, 30 s annealing at 53.5–63 °C, and 30 s primer extension at 72 °C. Each sample had three replicates in a real-time PCR run.

**Lymphocytes separation, lymphocyte transformation rate and lymphocyte subsets in Peyer’s node**

Six birds (one per replication) were randomly chosen from each treatment on day 21 and killed by cervical dislocation, manual exsanguination. Separation of Peyer’s node was conducted according to the method of Vaughan et al. (2006). Briefly, the intestinal segment between the proximal of McColl’s diverticulum and the distal of the ileocecal junction was excised, rinsed thoroughly with distilled water, ligated with tweezers near the junction of jejunum and ileum, perfused with 10–20 ml low concentration eosin solution, after holding for 1 min, and with 10–20 ml crystal violet solution. Peyer’s node was removed, then stored in cold RPMI-1640 solution (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). As shown in Table 2, the ADG, ADFI and FCR were not different between the 2 groups (p > .05).

For detection of T lymphocyte subpopulations, the lymphocytes were incubated with an optimal concentration of fluorescence-labeled anti-CD monoclonal antibody for 30 min at 4 °C in the dark, and later with a fluorescein isothiocyanate-conjugated goat anti-mouse IgG. Cells were washed and re-suspended in HBSS buffer, and analysed with a Beckman–Counter flow cytometer. A minimum of 10⁴ viable cells from each experiment was analysed. PE-labeled rat anti-chicken CD4⁺ and PE-labeled mouse anti-chicken CD8⁺ antibody were obtained from Southern Biotechnology Associates, Inc (Birmingham, AL).

**Statistical analysis**

Data were expressed as the means ± SE. The means were compared by student’s t-test.

### Results

As shown in Table 2, the ADG, ADFI and FCR were not different between the 2 groups (p > .05).

| Items       | Control diet | CLA diet | Significance |
|-------------|--------------|----------|--------------|
| ADG, g      | 31.14 ± 1.19 | 30.95 ± 1.18 | ns           |
| ADFI, g     | 48.02 ± 2.35 | 47.65 ± 2.58 | ns           |
| FCR         | 1.54 ± 0.08  | 1.54 ± 0.07  | ns           |

CLA: conjugated linoleic acid; ADG: daily gain; ns: non-significant; ADFI: average daily feed intake; FCR: feed conversion ratio.
CLA: conjugated linoleic acid; SIgA: secretory IgA; R: relative value.
a,bDifferent superscripts within a line mean extremely significant difference (p < .01).

Table 4. Effects of dietary CLA supplementation on PPARγ and TGFβ4 gene expression in jejunum.

| Items                          | Control diet | CLA diet    |
|-------------------------------|--------------|-------------|
| PPARγ transcription level, R  | 0.64 ± 0.16  | 0.97 ± 0.15 |
| TGFβ4 transcription level, R  | 0.78 ± 0.06  | 0.76 ± 0.08 |

CLA: conjugated linoleic acid; PPARγ: Peroxisome proliferator-activated receptor γ; R: relative value; TGFβ4: transforming growth factor β4.
a,bDifferent superscripts within a line mean extremely significant difference (p < .01).

CLA supplementation increased the percent of CD8+ T lymphocytes in Peyer’s node by 7.0% (p < .05). After dietary CLA supplementation, lymphocytes transformation rate of Peyer’s node increased by 7.0% (p < .05).

Discussion

We demonstrated that the CLA diet increased the jejunal mucosa SIgA level and the percent of Peyer’s node CD8+ T lymphocytes in broiler chickens. This is the first in vivo report, to our knowledge, showing an increase of intestinal mucosal immune responses in broiler chickens after feeding CLA.

Effects of dietary CLA on the jejunal SIgA levels of broiler chickens

SIgA is the principal immunoglobulin on the surface of the mucosa and particularly important in preventing bacterial or viral infection, and acts as a potentiator of the immune response in intestinal tissue by uptaking antigen to dendritic cells. The present experiment found that 1% dietary CLA increased the expression of jejunal SIgA both at protein and gene level. This result is basically in line with Pérez-Cano et al. (2009) who reported that dietary CLA supplementation in the maternal diet increased SIgA levels and its mRNA expression in the intestine and colon of newborn mouse. It has already been demonstrated that long-term feeding of diet supplemented with CLA increased the specific OVA-IgA production in mesenteric lymph node of mouse (Ramírez-Santana et al. 2009). Moreover, the better humoral enhancing effects were observed after feeding CLA to young rodents, which reportedly increased concentrations of spleen IgG, IgM, and IgA (Yamasaki et al. 2000, 2003), although specific adaptive responses were not addressed in such studies.

The specific mechanism by which CLA enhances IgA levels at mucosal sites remains unknown. It was well accepted that a great deal of CLA’s metabolic effects supplementation. However, literature is inconsistent because several studies found that dietary supplementation with CLA at levels greater than 10 g/kg decreased the growth rate of broilers (Szymczyk et al. 2001; Suksumt et al. 2007). Moraes et al. (2016) found that CLA exhibited negative effects on performance that could be observed not only at 2% CLA inclusion, but also at 1% CLA inclusion. This effect could be explained with the negative effect of trans 10 cis12-CLA supplementation on the body weight and growth because CLA used in the present study is a blend of isomers where trans 10 cis 12-CLA representing 50% of the contained isomers. Therefore, the use of CLA in broiler diets has been kept low to avoid adverse effects on growth performance.

Effects of dietary CLA on the jejunal SIgA levels of broiler chickens

CLA: conjugated linoleic acid.
a,bDifferent capital superscripts within a line mean extremely significant difference (p < .01).
a,bDifferent lowercase superscripts within a line mean significant difference (p < .05).

Table 3. Effects of dietary CLA on jejunal mucosa SIgA levels and its mRNA expression of broiler chickens at 21-day old.

| Items                          | Control diet | CLA diet    |
|-------------------------------|--------------|-------------|
| SIgA level, μg/mL             | 2.96 ± 0.33  | 4.25 ± 0.45 |
| SIgA transcription level, R   | 0.82 ± 0.11  | 2.56 ± 0.23 |

CLA: conjugated linoleic acid; SIgA: secretory IgA; R: relative value.
a,bDifferent superscripts within a line mean extremely significant difference (p < .01).

Table 5. Effects of dietary CLA on transformation rate and subsets of lymphocytes in Peyer’s node.

| Items                          | Control diet | CLA diet    |
|-------------------------------|--------------|-------------|
| CD8+, %                       | 27.5 ± 1.46  | 29.8 ± 2.87 |
| CD8−, %                       | 37.6 ± 1.80  | 44.5 ± 2.34 |
| Stimulation index             | 1.27 ± 0.04  | 1.36 ± 0.05 |

CLA: conjugated linoleic acid.
a,bDifferent capital superscripts within a line mean extremely significant difference (p < .01).
a,bDifferent lowercase superscripts within a line mean significant difference (p < .05).
are mediated through alterations in gene expression, either directly or indirectly via PPARs. PPARs are novel members of the nuclear receptor superfamily with several isoforms (a, b and γ), of which PPARγ is the predominant isoform in immune cells and enterocytes. PPARs were expressed by monocytes, macrophages, T cells, dendritic cells, and gastrointestinal epithelial cells in gut mucosa (Tontonoz et al. 1998; Thompson 2007).

Transforming growth factor β (TGFβ)-mediated signalling has a pivotal role in the stimulation of IgA responses at mucosal sites (Borsutzky et al. 2004). TGFβ attracts lymphocytes move to the intestinal epithelial layer, and plays important role in the course of the differentiation and proliferation of IgA+ B cells and then maturation into IgA-phlogocytes (Ehrhardt et al. 1992). We quantified TGFβ and PPARγ mRNA expression as possible mediators of CLA’s immunomodulatory effects on the intestinal mucosal immune response of broiler chickens. Here, we have shown that after dietary CLA supplementation, jejunal PPARγ mRNA expression was enhanced, and simultaneously SIgA expression level at both transcription and protein level increased. Taken together, these results indicated that the increase of PPARγ expression may promote the SIgA expression. No difference in TGFβ gene expression was observed between two diet groups in the present experiment. Thus, the increase of SIgA expression at transcription and protein level could not be attributed to TGFβ stimulation.

**Effects of dietary CLA on lymphocytes subsets and lymphocytes transformation rate of Peyer’s node**

Peyer’s node, known as the second lymphoid organ, is an important component of the intestinal mucosal immune system, characterised by a mediatior and amplifier role in the process of intestinal immune response to external antigen. The present study found that proliferative responses of Peyer’s node lymphocytes to ConA were also increased by dietary CLA supplementation. The result is consistent with previous experiments which found dietary CLA enhanced the proliferation activity of peripheral blood and spleen lymphocytes in animals such as rats and pigs (Chew et al. 1997; Bassaganya-Riera et al. 2001; Lai et al. 2005b). In addition, following hepatitis B vaccination, specific lymphocyte proliferation was higher in humans fed CLA 50:50 than in the control group (Yaqoob 2004). However, Zhang et al. (2006) reported dietary CLA significantly increased peripheral blood lymphocyte proliferation response to Con A in 42-day-old broiler chicks, but not on the 21-day-old broiler chickens. This might be due to different proliferation response to ConA between peripheral blood lymphocytes and Peyer’s node lymphocytes. Alternatively, intestine is in direct contact with CLA, thus responses to CLA may be faster than peripheral blood lymphocytes.

Dietary CLA increased the percent of Peyer’s node CD8+ T lymphocytes in the present study. In the absence of exogenous stimulus, several studies in rat, pig and poultry have shown that dietary CLA increased CD8+ T lymphocyte percentage in such tissues as peripheral blood, thymus, spleen (Chew et al. 1997; Yamasaki et al. 2003; Zhang et al. 2006). Moreover, the present data agrees with previous study that reported a higher specific proliferative response of CD8+ T lymphocytes from pigs fed a CLA diet (50:50 isomers mix) (Bassaganya-Riera et al. 2002, 2003).

CD8+ T lymphocytes are the effectors of cellular immune, directly destroy infected cells and play an important role in the body’s immune resistance to the virus infection. Therefore, the increase of Peyer’s node CD8+ T lymphocytes resulted from dietary CLA on may imply enhance of antiviral (bacteria) capacity in birds. Although this needs further challenge test to verify, this effect has been confirmed in the trials of swine enteritis induced by intestinal pathogenic bacteria *B. hyodysenteriae* in which dietary CLA was conducive to the maintenance of normal lymphocyte subsets (CD4+ and CD8+) and reduced the intestinal mucosal damage (Hontecillas et al. 2002).

It is unclear why dietary CLA increased the percent of Peyer’s node CD8+ T lymphocytes. Maturation and lineage choice of T cells takes place in the thymus. Therefore, if the observed changes in the profile of Peyer’s node CD8+ T lymphocytes originated in the thymus, phenotypic shifts in this organ were expected. Bassaganya-Riera et al. (2001) found that an increase in CD8xβ+ cells was detected earlier in the thymus than in peripheral blood, and that the increase in percentages of CD8xβ+ thymocytes could be correlated with greater numbers of thymic lymphoid progenitor cells (CD4−CD8− or CD4+CD8+ thymocytes) in pigs fed CLA-supplemented diets. We previously found that dietary CLA significantly increased the percentages of CD4+CD8− double-positive and CD8+ single-positive T lymphocytes in the thymus of piglets in the thymus after CsA injection (Liu et al. 2016). Thus, dietary CLA appears to initially modulate the cellular profiles within primary lymphoid pool (i.e. thymus).

**Conclusions**

Under the conditions of the experiment, 1% dietary CLA enhanced jejunal SIgA expression, and increased
lymphocyte transformation rate and the percent of CD8⁺ T lymphocyte in Peyer’s node of broiler chickens. The results confirmed the previous findings that dietary CLA changed intestinal mucosal immune response in animal and human. In poultry production, this means that CLA might be used as a nutrient to improve the intestinal mucosal immunity of broiler chickens.

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Disclosure statement

None of the authors have any conflicts of interest that could inappropriately influence their work.

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