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Effects of *In Ovo* Injection of Conjugated Linoleic Acid on Hatchability, Growth Performance, Intestine Morphology and Avian B-Defensin Gene Expression in the Cecal Tonsils of Broiler Chicks

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Abstract

This experiment aimed to investigate the effects of in-ovo injection of conjugated linoleic acid (CLA) on growth performance and β-defensin (AvBD1 and AvBD2) genes expression in broilers. A total of 400 fertile eggs (Cobb 500) were randomly assigned to 4 treatments, each having five replicates of 20 eggs. CLA was injected into the air sack on 18 d of incubation (150 and 300 mg/egg; CLA150 and CLA300). Two groups of diluent injected and non-injected were also included as controls. Hatchlings were further evaluated in a 42-d rearing period. Data were analyzed using a completely randomized design. Results showed that the hatchability was not significantly affected by the treatments. The growth performance in the CLA300 group was improved ($P < 0.05$) compared to both controls. CLA300 increased ($P < 0.05$) the jejunal villus length on 42 d, leading to a significant increase in villus absorptive surface area ($P < 0.05$) compared to CLA150 or controls. Abdominal fat weight in the CLA300 group was significantly decreased on 42 d ($P < 0.05$). The expression of β-defensins was detectable in all groups on 21 and 42 d, irrespective of treatment and age. Differences in AvBD1 gene expression in chicks from different treatments were not significant on 21 d, but the expression level in CLA received groups was more than controls at 42 d ($P < 0.05$). AvBD2 gene expression in CLA-treated birds was increased compared to controls on 21 d, but only the CLA300 group showed a significant increase compared to both controls on 42 d ($P < 0.05$). *Research highlights: in ovo* feeding 300 mg CLA per egg into the air sac decreased abdominal fat pad, improved growth performance and villous absorptive surface area of the jejunum and increased AvBD1 and AvBD2 gene expression in broilers.

Introduction

Using technology similar to vaccination, developing embryos can be provided with certain nutrients and compounds in a process called *in ovo* feeding developed by Uni and Ferket (2003). Early provision of nutrients may improve hatchability and subsequent weight gain of hatchlings (Azadegan mehr *et al.*, 2014; Leão *et al.*, 2021). Linoleic acid (LA) is an unsaturated omega-6 fatty acid and conjugated linoleic acid (CLA) is a collective term for positional and geometric isomers of LA. CLA is found naturally in dairy products and meat of ruminants due to bacterial activity in the rumen (Wang *et al.*, 2017). One of the important influences of CLA feeding on performance of animals is the reduction of fat deposition in the body (Fu *et al.*, 2021) and promoting muscle growth (Wang *et al.*, 2017). CLA may affect key enzymes and mobilization and deposition of lipids (Choi, 2009). It has been reported that CLA significantly reduces fat storage and increases protein deposition (Fu *et al.*, 2020). Dietary supplementation with 1% CLA improved meat quality, antioxidant capability and fatty acids profile in broiler chicks, without remarkable influence on productive performance

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(Jiang *et al*., 2014). Both *in vivo* and *in vitro* studies have shown that CLA nutrition affects cell growth, utilization and deposition of nutrients, lipids metabolism, and energy partitioning from fat to skeletal muscle growth in animals (Kumari *et al*., 2017). Du and Ahn (2002) reported the body fat deposition was reduced in chicks fed with diets containing CLA in the levels of 20 and 30 g/kg during 3 to 6 weeks of age. Addition of CLA to the diet changes the abdominal fat profile in broiler chicks. This change is probably due to the inhibition of the Δ-9 desaturase, which is responsible for the desaturation of fats. Isomers of PUFA and CLA act as ligands for PPARs, and CLA nutrition decreases the abundance of PPARγ mRNA in the abdominal fat of broilers (Kumari *et al*., 2017).

The small intestine is the main site of digestive fluid secretion, feed digestion, and nutrient absorption, and the jejunum is the major absorptive site of the small intestine (Asare *et al*., 2022). Some reports have shown the positive effect of dietary CLA on jejunum morphology in broiler chicks (Alipour *et al*., 2010) resulting in improved performance.

Dietary supplementation with CLA improved the oxidative stability of broiler meat and reduced the amount of thiobarbituric acid reactive substances and the hexanal content of ground meat after aerobic storage (Du *et al*., 2001). Dietary supplementation with CLA at 2 or 4% doses in broiler chicks reduced the concentration of monounsaturated fatty acids in drumstick and breast meat (Sirri *et al*., 2003). Martin *et al*. (2008) showed that dietary supplementation of CLA improved the fatty acid profile of pork tissues by increasing the ratio of saturated fatty acids to unsaturated fatty acids. Jiang *et al*. (2014) concluded that dietary supplementation with 1% CLA improved the meat quality, antioxidant capacity, and fatty acid composition of broiler chicks while having no significant effect on growth performance.

β-defensins (AvBDs) are antimicrobial peptides in avian species that perform an important role in innate immunity. Generally, defensins are the body's first line of defense against pathogens and through their antimicrobial function, they break the bacterial cell wall and, therefore, play an important role (Sahl *et al*., 2005). Some defensins are probably chemoattractors for monocytes, lymphocytes, and dendritic cells that link innate and adaptive immune responses (Ganz, 2003). Then, the present study aimed to investigate the effects of *in ovo* injection of CLA on hatchability, growth performance, intestine morphology and avian β-defensins (AvBD1 and AvBD2) gene expression in the cecal tonsils of hatched broiler chicks.

Materials and Methods

Animal ethics

All experimental procedures were approved by the Animal Ethics Committee of the Animal Ethics Committee of the Ferdowsi University of Mashhad, Mashhad, Iran.

Birds, management and diets

A total of 500 fertile Cobb 500 broiler eggs with similar weight (62.0 ± 1 g) were obtained from a broiler breeder flock aged 40 wk. The eggs were incubated in a standard condition (relative humidity 65±2 percent and temperature 37.5 ± 0.2 °C). All eggs were candled on the 14th day of incubation and 400 embryonated eggs were selected and assigned to 4 treatments of 80 with five replications (20 eggs per replication). Treatments were included: 1) control (non-injected); 2) sham group injected with 0.5 mL/egg of a commercial vaccine carrier (as diluent); 3 and 4) Eggs injected with 150 and 300 mg of CLA dissolved in 0.5 mL of commercial diluent, respectively. The shell surface was disinfected by 70% ethanol at the egg's wide end prior to punching. The intra-egg infusion was implemented on d 18 of incubation into the air sac. After in-ovo injection, the injection sites were sealed with liquid Merck paraffin and returned to the incubator with non-injected control eggs (Fazli *et al*., 2015). The injection was done inside the setter room under standard incubation conditions (37.5 °C; RH=65%).

After hatching, all day-old chicks were tagged and transferred to the poultry house, and each ten chicks were assigned to a floor pen (four replicates per treatment) in a completely randomized design. The dimensions of each pen were (1.2 × 1.2 × 0.8 m; L × W × H). Bed materials of dried wood shavings with a depth of about 5 cm were used. A hanging feeder and a nipple drinker were provided in each pen. The birds were fed *ad libitum* and they had free access to drinking water and were exposed to 18 hours of light and 6 hours of darkness during the experiment. The temperature of the rearing house on the first day was set at 32 °C, which gradually decreased by 0.5 °C daily according to the usual commercial methods and remained constant at 21 °C thereafter. The birds were fed with mash diets formulated according to Cobb 500 broiler nutritional recommendations (Table 1).

Growth performance

During this experiment, average daily feed intake (FI), daily weight gain (WG) and feed conversion ratio (FCR) were measured. One bird per replicate (n=5) was selected on 21 and 42 d, slaughtered, and eviscerated. The carcass cuts and internal organs were removed, weighed, and expressed as a percentage of live weight (Kakhki *et al*., 2017).

Table 1. Ingredients and calculated analysis of experimental diets (*as fed basis*).

Ingredients (g/kg)	Starter (1–10 d)	Grower (11–22 d)	Finisher (23–42 d)
Corn	555.9	607.7	626.9
Soybean meal (44% CP)	367.4	310.4	283.1
Soybean oil	35.6	40.7	50.5
Common salt	3	3	3.5
Dicalcium phosphate	18.5	17.9	16.6
Limestone	12.5	12.2	11.5
DL–methionine	2.1	2.2	2.3
L–lysine HCl	0	0.9	0.6
Vitamin premix[1]	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5
Calculated composition (%)			
ME (kcal/kg)	2990	3058	3176
CP	21	19	18
Calcium	1.0	0.96	0.90
Available P	0.5	0.48	0.45
Lysine	1.20	1.10	1.05
TSAA	0.89	0.84	0.82
Threonine	0.95	0.89	0.83
Na	0.20	0.17	0.16

[1]Vitamin premix provided per kilogram of diet: vitamin A (retinyl acetate), 15,000 IU; vitamin D3, 5,000 IU; vitamin E (DL-α-tocopheryl acetate), 80 mg; vitamin K, 5 mg; thiamin, 3 mg; riboflavin,10mg; pyridoxine, 5 mg; vitamin B12, 0.02 mg; niacin, 70 mg; choline chloride, 1800 mg; folic acid, 2 mg; biotin, 0.4 mg; pantothenic acid, 20 mg.
[2]Mineral premix provided per kilogram of diet: Mn (manganese sulfate), 100 mg; Zn (zinc sulfate), 65 mg; Cu (copper sulfate), 5 mg; Se (Sodium Selenite), 0.22 mg; I (calcium iodate), 0.5 mg; and Co, 0.5 mg.

Intestinal morphology

At 21 d, one male chick from each replicate was selected and slaughtered. About 1 cm from the midpoint of the jejunum segment of the small intestine was removed, washed with 0.9% saline to clear the contents, and then fixed in 10% buffered formalin. The fixative solution of the tissues was replaced after 24 h and kept for subsequent histological processing. The samples were removed from the formalin solution, dehydrated in a graded ethanol series, cleared in xylene, and embedded in paraffin. Infiltrated tissues were placed in paraffin blocks. The tissues were cut using a rotary microtome with 5-6 µm thick sections. These sections were floated in distilled water at 40°C to be easily placed on a slide after smoothing out the wrinkles. The slides were placed on a hot plate at 45°C to melt excess paraffin during drying. The slides were stained with hematoxylin and eosin.

All chemical materials were purchased from Sigma-Aldrich (Sigma-Aldrich Chemical Co., St. Louis, MO). The prepared sections were photographed using an Olympus BX41 light microscope (Olympus Corporation, Tokyo, Japan) with a digital camera. Images were analyzed by image software. Measurements in ten healthy villi including villus height (VH) from villus tip to the crypt, villus width (VW; mean VW in one-third and two-thirds of the villus), crypt depth (CD) from villus base to the submucosa, and thickness of the muscular layer (MT) from the submucosa to the external layer of the intestine in each sample were recorded. The villous surface area (VSA) was calculated and shown in micrometers (Almamury *et al*., 2022).

Tissue collection, total RNA extraction and reverse transcription

At 42 d of age, one male chick from each replicate was selected, slaughtered, and eviscerated. Then, the cecal tonsils were removed and kept at -20 °C until processing for RNA extraction. Total RNA from cecal tonsil samples was extracted using Trizol reagent according to the manufacturer's instructions. For each 30 µL extracted RNA, 1 unit DNase I, 3µL 10×buffer, and 10-unit RNase inhibitor were added to each sample, then the samples were incubated at 37 °C for 30 min. After the incubation period, the volume of the samples increased to 200 µL using DEPC water, one volume of phenol: chloroform: isoamyl alcohol (25:24:1) to the samples, and then centrifuged at room temperature for 5 min at 16,000 × g. After centrifugation, the upper aqueous phase was removed, and the layer was transferred to a fresh tube. The tubes were kept at -20 °C overnight to precipitate the DNA from the sample. The samples were centrifuged at 4 °C for 15 min at 12,000 × g to pellet the cDNA. After centrifugation, the supernatant was carefully removed without disturbing the cDNA pellet and 200 µL of 70% ethanol. The samples were centrifuged at 4 °C for 5 min at 16,000 × g, the supernatant was carefully removed, and then 20 µL DEPC water was used to make the solution the pellet. The quality and integrity of extracted RNA were checked

spectrophotometrically on 1.2% agarose gel. RNA quality was also assessed spectrophotometrically. The common cause of an RNA sample losing integrity is contamination with RNase. Because RNase is a protein, ultraviolet (UV) spectrophotometry at 280 nm, based on the absorbance of the side chains of specific amino acids such as tryptophan and tyrosine, has been used as a measure to assess the contamination of RNA samples worldwide. This determination may be performed simultaneously as RNA quantification at a wavelength of 260 nm. Protein absorption peaks at 280 nm, and the full UV spectrum of protein absorption still indicates remarkable absorption at 260 nm wavelength. The opposite is true for RNA. Results showed the high quality of extracted RNA in our samples. Synthesis of the first cDNA was done by use of 5 µg of extracted RNA, 0.5 µg of oligo (dT) 12-18 primers and a reverse transcription kit (Fermentas Co) according to the manufacturer's instructions, then heated at 70 °C for 5 min and incubated on ice, prior to adding the rest of the reaction components, then incubated first-Strand cDNA synthesis reaction in a thermocycler. Then the gained cDNA was kept at -20°C until use. AvBD1 and AvBD2 gene sequences were provided from GenBank. These genes were chosen because their expressions were previously showed in caecal tonsils and chicks' intestine (Lynn *et al*., 2004). We used Primer Premier 5 as primer design software to design and analyze primer (Table 2). We used the ABI system using Cyber green Kit (Fermentage Co) and β-actin used for normalization control for real-time PCR. The obtained Ct was analyzed using the Livak method (2^-ΔΔCt) with Excel software and the Pffafl method with RESET software. After RESET analysis, the graphs were depicted using Excel software.

Table 2. The sequence of primers used for PCR analysis of AvBDs

Primer target	Orientation	Sequence	Product size (bp)	Temp (°C)
AvBD1	F	GGTTCTTACTGCCTTGCTGT	158	58
	R	TGACTTCCTTCCTAGAGCCT		
AvBD2	F	GGACTGCCTGCCACATACAT	239	58
	R	TTGCAGCAGGAACGGAAC		
β-Actin	F	CAACACAGTGCTGTCTGGTGG	205	64
	R	ATCGTACTCCTGCTTGCTGAT		

Statistical *analysis*

Data of the growth performance and carcass traits were tested for normality by UNIVARIATE before analysis and then subjected to ANOVA using the GLM procedure in a completely randomized design using the SAS software (Statistical Analyses System; 2012). Significant differences among the treatments were determined at $P < 0.05$. Mean values among treatment groups were compared by Tukey's HSD test. In addition, differences at $P < 0.05$ were considered significant for gene expression levels.

Results

Hatchability Characteristics

Effects of *in ovo* injection of CLA on hatchability (%) of fertile eggs are shown in Table 3. Results showed that the hatchability of the eggs was not significantly affected by the treatments.

Table 3. Effects of intra-air sac infusion of CLA on 18 d of incubation on hatchability and hatchlings' body weight

Treatments	Hatchability (%)	Hatchlings body weight (g)
Control (non-injected)	90.0	41.1
diluent Injected	89.0	40.9
150 mg/egg	88.8	40.6
300 mg/egg	88.5	40.5
SEM	1.66	0.650
P-value	0.719	0.700

Each mean represents five observations.

Growth performance

Effects of *in ovo* injection of different treatments on the average daily feed intake (FI) of the broiler chicks are shown in Table 4. Feed intake of the chicks receiving an intra-egg injection of CLA was significantly affected during the ages 1-10, 11-28, and 1-42 d ($P < 0.05$). Chicks hatched from the eggs injected with 300 mg CLA consumed more feed than both controls during the periods mentioned above. Feed consumption of chicks hatched from the eggs injected with 150 or 300 mg CLA was not significantly different during 1-42 d, and both groups consumed more feed than controls.

Table 4. Effects of *in ovo* injection of conjugated linoleic acid (CLA) on growth performance parameters of broiler chicks in different phases

Treatments	1–10 d	11–28 d	29–42 d	1–42 d
	Feed intake (g/bird/day)			
Control (Non-injected)	26.08^{b}	116.4^{bc}	181.5	116.59^{b}
Diluent-injected	26.96^{b}	110.4^{c}	180.1	113.77^{b}
150 mg CLA	27.86^{ab}	126.5^{ab}	182.3	121.61^{a}
300 mg CLA	28.57^{a}	134.6^{a}	186.7	126.72^{a}
SEM	0.78	3.95	5.20	2.41
P-value	0.036	0.048	0.775	0.045
	Weight gain (g/bird/day)			
Control (Non-injected)	16.96^{b}	59.71	81.80^{c}	56.89^{b}
Diluent-injected	17.63^{b}	57.43	83.22^{bc}	56.53^{b}
150 mg CLA	17.50^{b}	61.92	92.21^{b}	61.42^{ab}
300 mg CLA	19.20^{a}	61.65	108.80^{a}	67.24^{a}
SEM	0.62	1.67	4.13	1.31
P-value	0.021	0.259	0.016	0.027
	Feed conversion ratio (g/g)			
Control (Non-injected)	1.54	1.95^{b}	2.21^{a}	2.19^{a}
Diluent-injected	1.53	1.93^{b}	2.16^{ab}	2.14^{a}
150 mg CLA	1.59	2.04^{b}	1.98^{bc}	2.15^{a}
300 mg CLA	1.50	2.18^{a}	1.72^{c}	2.08^{b}
SEM	0.053	0.033	0.062	0.042
P-value	0.360	0.017	0.022	0.036

$^{a\text{-}b}$ Values in the same columns with no common superscript differ significantly ($P < 0.05$).
Each mean represents five observations.

Effects of *in ovo* injection of CLA on the average daily weight gain (WG) of the broiler chicks are shown in Table 4. WG of the chicks was significantly affected by intra-egg injection of CLA during the ages 1-10, 29-42, and 1-42 d ($P < 0.05$). Chicks hatched from the eggs injected with 300 mg CLA gained more than both controls during the periods mentioned above. The WG of chicks hatched from the eggs injected with 300 mg CLA was significantly more than controls and 150 mg CLA. There were no significant differences between the two control groups. Effects of *in ovo* injection of CLA on FCR of the broiler chicks are shown in Table 4. FCR of the chicks was significantly affected by intra-egg injection of CLA during the ages 11-28, 29-42, and 1-42 d ($P < 0.05$). Chicks hatched from the eggs injected with 300 mg CLA showed significantly lower FCR than 150 mg CLA and both controls during 11-28 d and 1-42 d. In general, 300 mg CLA improved FCR compared to both controls and 150 mg CLA. There were no significant differences in FCR between the two control groups.

Intestinal morphology

Morphological measurements of jejunum in chicks that received different treatments are shown in Table 5. In the present study, villi length in jejunum was increased ($P < 0.05$) with *in ovo* injection of 300 mg CLA as compared to 150 mg CLA or control groups at 42 d of age, which caused a significant increase in intestinal absorptive surface area ($P < 0.05$).

Table 5. Effects of *in ovo* injection of conjugated linoleic acid (CLA) on morphometric indices of jejunum in 21 d broiler chicks

Treatments	Villus length (µm)	Villus width (µm)	Crypt depth (µm)	Villus surface area ($\times 10^{-3}$, µm^2)	Villus length/ crypt depth
Control(Non-injected)	1124.7^{b}	187.2	162.0^{b}	661.2^{b}	6.94^{a}
Diluent-injected	1125.5^{b}	182.7	160.2^{b}	645.8^{b}	7.02^{a}
150 mg CLA	1116.6^{b}	185.3	158.3^{b}	649.8^{b}	7.05^{a}
300 mg CLA	1208.6^{a}	179.6	192.0^{a}	681.8^{a}	6.29^{b}
SEM	3.55	1.40	2.24	5.078	0.089
P-value	0.001	0.058	0.004	0.028	0.014

$^{a\text{-}b}$ Values in the same columns with no common superscript differ significantly ($P < 0.05$).
Each mean represents five observations.

Carcass characteristics

Effects of *in ovo* injection of CLA on carcass characteristics of broiler chicks are shown in Table 6. Results showed that carcass yield, breast, thighs, heart, liver, and gizzard relative weights were not affected by *in ovo* injection of CLA up to 300 mg at 21 or 42 d of age.

Table 6. Effects of *in ovo* injection of conjugated linoleic acid (CLA) on carcass characteristics[1] of broiler chicks (% of live weight)

Treatments	Carcass yield	Breast	Thighs	Heart	Liver	Gizzard	Pancreas	Abdominal fat
					21 d			
Control (Non-injected)	61.3	16.95	13.58	0.584	3.42	2.87	0.484	3.61
Diluent-injected	60.8	16.88	13.53	0.584	3.68	2.76	0.520	3.75
150 mg CLA	61.1	16.84	13.57	0.565	3.43	2.83	0.525	3.15
300 mg CLA	62.6	16.76	13.46	0.561	3.49	2.85	0.464	3.18
SEM	0.163	0.015	0.013	0.002	0.009	0.018	0.011	0.148
P-value	0.091	0.189	0.502	0.681	0.291	0.283	0.117	0.213
					42 d			
Control (Non-injected)	60.8	20.28	18.16	0.538	2.23	1.71	0.216	1.45[a]
Diluent-injected	62.3	19.85	18.73	0.567	2.35	1.69	0.260	1.37[a]
150 mg CLA	60.9	19.18	18.70	0.561	2.57	1.68	0.260	1.30[b]
300 mg CLA	61.2	19.7	18.04	0.551	2.52	1.67	0.267	1.26[b]
SEM	0.768	0.509	0.150	0.018	0.060	0.015	0.006	0.020
P-value	0.401	0.516	0.311	0.445	0.167	0.217	0.120	0.043

[a-b] Values in the same columns with no common superscript differ significantly ($P < 0.05$).
Each mean represents five observations.
[1] Carcasses were peeled before weighing.

Gene Expression of AvBD1 and AvBD2

The Effects of *in ovo* injection of CLA on the expressions of AvBD1 and AvBD2 are illustrated in Figures 1 to 4. Results showed that the expressions of AvBD1 and AvBD2 genes in the cecal tonsils of chicks were affected by CLA treatment. At 42 d of age, the expression levels of AvBD1 were significantly increased ($P < 0.05$) in the cecal tonsils of chicks *in ovo* fed with 150 or 300 mg CLA as compared to control groups (injected with diluent or non-injected). However, the expression levels of AvBD2 were only significantly increased ($P < 0.05$) in the cecal tonsils of chicks *in ovo* fed with 300 mg CLA compared to those received 150 mg and control groups.

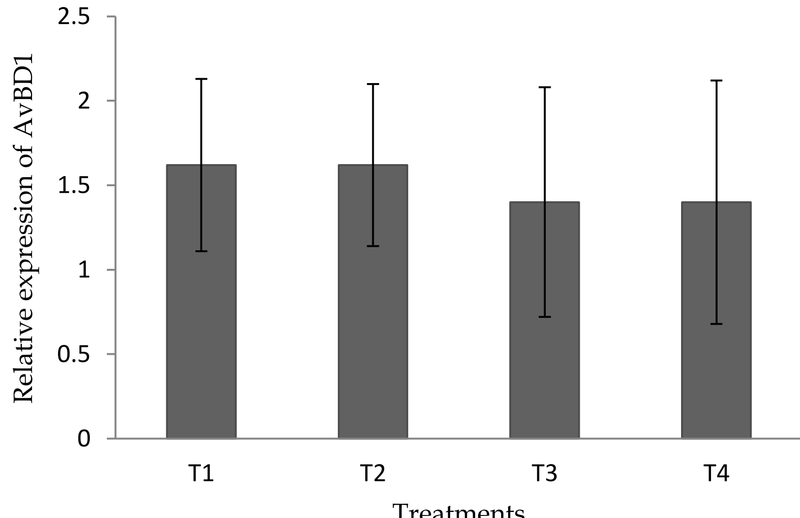

Figure 1. Relative expression levels of AvBD1 in the cecal tonsils of chicks at 21 d. The treatment groups of T1-T4 were as follows: T1) (Control) non-injected eggs T2) Eggs injected with 0.5 mL of commercial diluents (vaccine carrier) T3) Eggs with 150 mg of CLA dissolved in 0.5 mL of commercial diluent T4) Eggs injected with 300 mg of CLA dissolved in 0.5 mL of commercial diluent.
Error bars indicate the standard error of the mean.

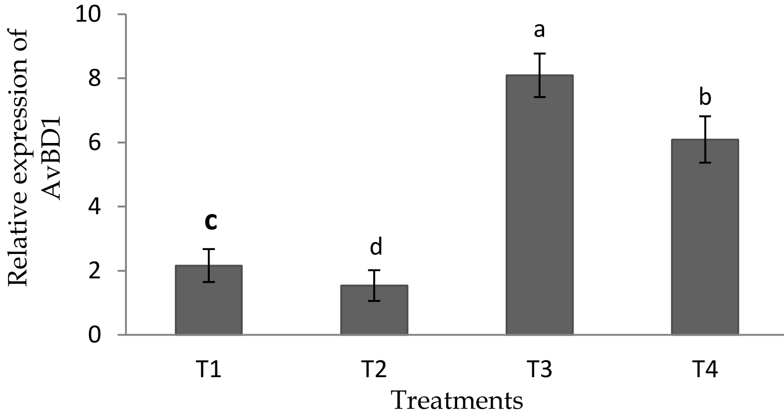

Figure 2. Relative expression levels of AvBD1 in the cecal tonsils of chicks at 42 d. The treatment groups of T1-T4 were as follows: T1) (Control) non-injected eggs T2) Eggs injected with 0.5 mL of commercial diluents (vaccine carrier) T3) Eggs with 150 mg of CLA dissolved in 0.5 mL of commercial diluent T4) Eggs injected with 300 mg of CLA dissolved in 0.5 mL of commercial diluent.
Error bars indicate the standard error of the mean.

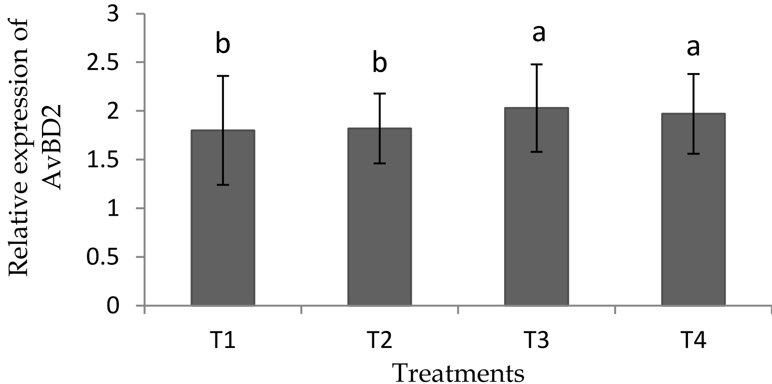

Figure 3. Relative expression levels of AvBD2 in the cecal tonsils of chicks at 21 d. The treatment groups of T1-T4 were as follows: T1) (Control) non-injected eggs T2) Eggs injected with 0.5 mL of commercial diluents (vaccine carrier) T3) Eggs with 150 mg of CLA dissolved in 0.5 mL of commercial diluent T4) Eggs injected with 300 mg of CLA dissolved in 0.5 mL of commercial diluent.
Error bars indicate the standard error of the mean.

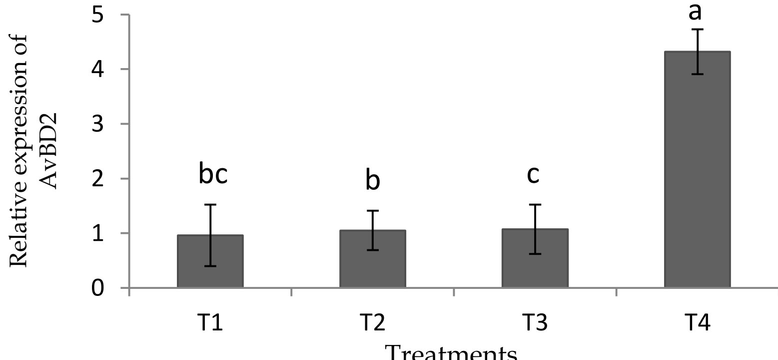

Figure 4. Relative expression levels of AvBD2 in the cecal tonsils of chicks at 42 d. The treatment groups of T1-T4 were as follows: T1) (Control) non-injected eggs T2) Eggs injected with 0.5 mL of commercial diluents (vaccine carrier) T3) Eggs with 150 mg of CLA dissolved in 0.5 mL of commercial diluent T4) Eggs injected with 300 mg of CLA dissolved in 0.5 mL of commercial diluent.
Error bars indicate the standard error of the mean.

Discussion

Hatchability characteristics

During the third week of incubation, the embryo increases the absorption of fatty acids, which may increase oxidative stress (Kumari *et al*., 2017). Another time of potential increased oxidative stress occurs as the chick begins piping when it switches to active pulmonary respiration (Moran, 2007). The CLA may benefit hatchability as a healthy fatty acid, but some authors have reported the opposite results. Results of this study showed that the hatchability of eggs was not affected by *in ovo* injection 150 or 300 mg CLA ($P > 0.05$). However, some authors showed reduced hatchability of fertile eggs when breeder hen diets were supplemented with CLA (Muma *et al*., 2006). The inconsistent results may be due to different feeding times, routes of administration, doses, and types of CLA.

Growth performance

Du and Ahn (2002) reported that dietary CLA up to 1% of the diet had no significant effect on the FI of broilers, although 2-3 % of dietary CLA slightly reduced FI. Badinga *et al*. (2003) showed that 5% CLA in the diet reduced FI of broiler chicks. However, in our experiment *in ovo* injection of 150 or 300 mg CLA could significantly increase FI ($P < 0.05$) at starter, grower, and whole rearing periods as compared to control groups (non-injected and diluent-injected).

Some reports showed that moderate dietary CLA concentrations could improve growth rate and FCR in growing rats (Tan *et al*., 2019) and pigs (Kumari *et al*., 2017), but some other reports showed no significant difference in growth rate with including dietary CLA (Du and Ahn, 2002). Stangl (2000) reported that the growth rate of rats feeding a diet supplemented with up to 5% CLA was not significantly influenced. One of the major effects of CLA on animal performance is muscle growth (Tan *et al*., 2019), so most authors showed improved WG and FCR in rats, mice, and pigs (Kumari *et al*., 2017) when feeding CLA at levels of 0.5 to 1.0 % in the diet. In this study, the WG of chicks hatched from *in ovo* administrated fertile eggs (150 or 300 mg CLA) was significantly ($P < 0.05$) higher than control chicks (diluent-injected or non-injected) during 1-42 d of age, but in 1-10 and 29-42 d of age, only chicks hatched from eggs received 300 mg of CLA gained more than control birds ($P < 0.05$). During the whole experimental period (1-42 d), the FCR of the chicks hatched from eggs that received 300 mg of CLA was significantly improved ($P < 0.05$) compared to chicks that received 150 mg CLA or control ones. The applied dose of CLA in this study was lower than CLA doses used in other trials, showing that feeding the CLA through *in ovo* at 18 d of incubation when the chick switches to active pulmonary respiration may be effective, and this procedure may help to better absorption during early post-hatch periods to support muscle growth.

Intestinal morphology

The jejunum is the main absorptive site in the small intestine, and the villi absorptive surface area in this section is vital for nutrient absorption. In a study, Alipour *et al*. (2010) showed that villi length and crypt depth in the jejunum of chicks fed with a diet supplemented with 3.5% CLA were increased compared to the control diet (no CLA supplemented). From a morphological point of view, it could have been expected that longer villi might result in an increased villous absorptive surface area (Amer *et al*., 2021), as shown in the jejunum section in the present study. This study indicated that the improved WG of the chicks hatched from *in ovo* injected eggs with 300 mg CLA may be partly due to improved absorption surface area in the jejunum.

Carcass characteristics

This result was in agreement with the findings of Suksombat *et al*. (2007), who did not observe significant effects of CLA on thighs and carcass yield in broiler chicks. Some authors showed that CLA has a major role in reducing fat accumulation by affecting key enzymes and processes involved in lipid mobilization and storage (Kumari *et al*., 2017). In the current study, the abdominal fat weight of the chicks was significantly decreased by *in ovo* injection of 300 mg CLA at 42 d, but not at 21 d. CLA may affect enzymes involved in post-hatch lipid storage, leading to decreased abdominal fat in the later periods. With CLA feeding, fatty acid synthase and acetyl-CoA carboxylase enzyme activity increase in the liver, the main enzymes controlling fatty acid synthesis. An increase in fatty acid synthase activity can elevate triglyceride levels in plasma. These results may indicate that CLA reduces lipogenesis in the liver and adipose tissues.

Gene expression of AvBD1 and AvBD2

The effects of CLA on chicken immune responses have been studied previously. Takahashi *et al*. (2002) reported that dietary CLA enhanced anti-SRBC antibody production in broilers. Azadegan mehr *et al*. (2014) showed that central lymphoid tissues such as the bursa of Fabricius and thymus relative weights were increased ($P < 0.05$) in chicks hatched from eggs treated with 300 mg CLA when compared to control hatchlings. IgG concentration was increased ($P < 0.05$) in chicks hatched from CLA300 compared to CLA150 and control ones at 42 days of age. Avian β-defensins (AvBDs) play essential roles in the immune function of poultry (Sahl *et al*., 2005). Pathogen recognition receptors such as Toll-like receptors stimulate innate immune responses. Many cellular pathways may be

activated upon stimulation of these receptors, leading to the expression of cytokines and antimicrobial peptides. Some defensins may act as chemoattractants for lymphocytes, monocytes, and dendritic cells, which is a link between immune responses of innate and adaptive (Ganz, 2003).

A total of 14 AvBD genes (AvBD1 to 14) have been recognized in different chicken tissues to date (Lynn *et al*., 2004, 2007). Expression of β-defensin 2 in human intestinal epithelial cells is regulated by some factors such as probiotic bacteria, like Lactobacillus (Schlee *et al*., 2008). Previous researchers showed that AvBDs are expressed in the mucosa of the gastrointestinal tract, such as the proventriculus in chickens. Probiotics reduced the expression of AvBDs which was increased by *Salmonella* infection of the caeca, where the colonization of bacteria is high (Rodríguez-Lecompte *et al*., 2012).

It has been reported in previous studies that the antimicrobial defensins family performs a significant role in the avian innate immune system and provides the first line of defense against pathogens (Sugiarto and Yu, 2004; Higgs *et al*., 2005). Avian beta-defensins, known as gallinacins, attack various microorganisms, such as gram-negative and gram-positive bacteria, yeasts, and fungi (Sugiarto and Yu, 2004). There are reports on the expression of avian β-defensins genes in various birds' organs (Guo *et al*., 2022). The beta-defensins are inducible genes, and bacterial infection or inflammation of the host tissues can increase their expression (Menendez and Finlay, 2007). Beta-defensin induction after infection of the bacteria or their components is also observed in the chickens. Inoculation of Salmonella enteritidis cell cultures and lipopolysaccharides to the laying hens has been reported to enhance AvBD1, AvBD2, and AvBD3 expressions (Yoshimura *et al*., 2006). In addition, the genes AvBD1, AvBD7, and AvBD12 are upregulated in ovarian follicles of hens injected with lipopolysaccharides (Subedi and Yoshimura, 2007). Milona *et al*. (2007) reported that *Salmonella enteritidis* and *Salmonella typhimurium* challenges remarkably increased expression of AvBD4 in the chicken's liver. Also, previous research has demonstrated the antimicrobial characteristics of beta-defensins in birds. For example, Higgs *et al*. (2005) Reported that AvBD13 reduced *Listeria monocytogenes* and *Salmonella typhimurium* at a concentration of 500 µg/mL. An in vitro study showed the antibacterial activity of AvBD6, AvBD4, and AvBD5 genes against *Salmonella* spp. (van Dijk *et al*., 2007). Another research showed that the expression of AvBD6 in the cecal tonsils of 42-d-old broiler chicks and its antimicrobial activity against gut pathogens was increased (Bommineni *et al*., 2007).

It was concluded that *in ovo* feeding of fertile eggs with 300 mg CLA/egg did not significantly affect the hatchability compared to the control groups (diluent injected or non-injected), but increased intestinal absorptive surface area in the jejunum, improved growth performance, and decreased abdominal fat in broiler hatchlings at 42 d of age.

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Disclosure statement
No potential conflict of interest was reported by the author(s).

Data Availability Statement
The data that support the findings of this investigation are available upon reasonable request from the corresponding author.

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