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Growth performance and immune responses in chickens after challenge with lipopolysaccharide and modulation by dietary different oils

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The study was conducted to investigate the effects of different oils on growth performance and immune responses of chickens after challenge with lipopolysaccharide (LPS). A total of 288 chickens were assigned in a 2 × 2 factorial design. Factors were dietary fat type (4.5% maize oil or 4.5% fish oil) and immunological challenge (LPS or saline). At 20 days and 27 days of age, chickens were injected intraperitoneally with either 1 mg/kg body weight of LPS or sterile saline. LPS decreased feed intake from 21 days to 28 days of age and body-weight gain from 21 days to 42 days of age. Fish oil improved feed-conversion efficiency of chickens after LPS challenge for the first time. Fish oil supplementation decreased lymphocyte proliferation (21 days: $P < 0.0001$*; 28 days:* $P < 0.0001$*) and the ratio of* $CD3^+CD4^+/CD3^+CD8^+$ *(21 days:* $P = 0.0479$*; 28 days:* $P = 0.0009$*) after LPS challenge. LPS challenge increased the levels of interleukin-1 (IL-1) (21 days:* $P < 0.0001$*; 28 days:* $P = 0.0030$*), IL-6 (21 days:* $P < 0.0001$*; 28 days:* $P = 0.0001$*) and tumor necrosis factor-α (TNF-α) (21 days:* $P = 0.0008$*; 28 days:* $P = 0.0018$*). And fish oil alleviated the elevations in the production of IL-6 (21 days:* $P = 0.0359$*; 28 days:* $P = 0.0302$*) and TNF-α (21 days:* $P = 0.0055$*; 28 days:* $P = 0.0391$*) induced by the LPS challenge. Fish oil alleviated the mRNA abundance elevation of nuclear factor kappa B (NFκB) (21 days:* $P = 0.0079$*; 28 days:* $P = 0.0017$*) after LPS challenge. These results showed that fish oil acts as an anti-inflammatory agent, which may be associated with down-regulation of the activated immune system. The results of pro-inflammatory cytokines and mRNA abundance results suggested that fish oil might alleviate the elevation of IL-6 and TNF-α induced by LPS through down-regulating NFκB expression.*

Keywords: chickens, fish oil, immune responses, maize oil, pro-inflammatory cytokines

Introduction

Immunological stress affected physiological and pathological processes of domestic animals. Stressful situation in which feed consumption, body-weight gain and feed-conversion efficiency of chickens are decreased (Benson *et al.*, 1993; Korver and Klasing, 1997). This change results in the partitioning of nutrients away from growth and toward processes associated with inflammatory immune responses in chickens (Korver and Klasing, 1997). Tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1) and IL-6 are among the most important pro-inflammatory cytokines produced by monocytes and macrophages. These cytokines activate neutrophils, monocytes and macrophages to initiate bacterial and tumour cell killing, stimulate T and B lymphocyte proliferation in inflammatory responses (Calder, 2001b). In addition, these cytokines mediate the systemic effects of inflammation such as fever, weight loss and acute-phase protein synthesis in the liver. Production of appropriate amounts of TNF-α, IL-1 and IL-6 is clearly beneficial in response to infection, but inappropriate or overproduction can be dangerous and these cytokines, especially TNFα, are implicated in causing some of the pathological responses that occur in inflammatory conditions (Calder, 2001a).

One of the goals for modulating the immune system of domestic animals by nutrition is to alleviate decreased performances following immune stimulation. Many animal studies investigating the effects of dietary oils on inflammation and immunity have now been published (Calder and Grimble, 2002; Sijben *et al.*, 2003). Lai *et al.* (2005a and 2005b) found that conjugated linoleic acid (CLA) was

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effective in preventing body-weight loss and suppressing the production and expression of IL-1, IL-6 and TNF-α in pigs following immune stimulation. Liu *et al.* (2003) showed that fish oil also improved performance and decreased indices of the inflammatory responses in pigs. Diets that have a relatively high ratio of n-3 polyunsaturated fatty acids (PUFA) have been demonstrated to offer protection against growth depression and inflammatory responses in chickens following LPS challenge (Korver and Klasing, 1997; Korver *et al.*, 1997 and 1998). Most of these studies indicate that, at the levels (5 g/kg, 10 g/kg, 20 g/kg, 40 g/kg, 60 g/kg) used in studies, fish oil is anti-inflammatory. However, not all studies agree with this generalisation (Revajová *et al.*, 2001). Reasons for contradictions might relate to the species of animal studied, the total fat content of the diets used, the amount of fish oil fed, the comparison being made and so on. A well-documented system for inducing sickness in laboratory animals is injection of lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria. Treatment of animals with LPS evokes a multitude of symptoms of sickness and elaboration of systemic inflammatory cytokines (Johnson and von Borel, 1994; Korver and Klasing, 1997; Warren *et al.*, 1997; Webel *et al.*, 1997; Balaji *et al.*, 2002). LPS is a potent inflammatory mediator and has been used to model bacterial infection experimentally in chickens (Korver and Klasing, 1997). The present study was designed to determine the effects of dietary fish oil and maize oil on growth performance and immune responses in chickens subjected to an inflammatory challenge, LPS.

Material and methods

Animals and experimental procedures

A total of 288 male Arbor Acres chicks on 1 day of age were used. Birds were fed with the starter diets from 1 day through 21 days of age, and the grower diets during 22 days to 42 days of age. The two kinds of isoenergetic and isonitrogenous maize-soya-bean meal-based diets were supplemented with maize oil 45 g/kg and fish oil 45 g/kg, respectively (Table 1). The fatty acids profiles of fish oil and maize oil were analysed by gas chromatography as shown in Table 2.

These 288 chickens were assigned in a 2×2 factorial arrangement. The main factors were dietary fat type (4.5% maize oil or 4.5% fish oil) and immunological challenge (LPS or saline). Birds were randomly assigned to four groups of 72 chicks each, with six replications of 12 chicks in a cage with constant lighting. At 20 days and 27 days of age, chickens were injected intraperitoneally with either 1 mg/kg live weight of *Escherichia coli* LPS (*E. coli* serotype 055: B5, Sigma, St Louis, MO, USA) or sterile saline. Body-weight gain, feed-conversion efficiency and feed intake were measured weekly throughout the experiment. This experiment was conducted according to protocols approved by the China Agricultural University Animal Care and Use Committee.

Table 1 *Ingredients and nutrient levels of trail diets*

Ingredients	4.5% maize oil (g/kg)		4.5% fish oil (g/kg)	
Composition (g/kg)	Weeks 0 to 3	Weeks 4 to 6	Week 0 to 3	Week 4 to 6
Maize	537.85	593.48	541.09	596.86
Soya-bean meal	374.51	286.43	373.84	285.80
Porphyries andesite	2.73	40.75	–	37.82
Maize gluten meal	–	–	–	–
Limestone	12.43	9.81	12.60	10.00
Dicalcium phosphate	17.88	15.24	17.86	15.23
Sodium chloride	3.50	3.50	3.50	3.50
Lysine-HCl	0.54	0.36	0.55	0.37
Methionine	1.67	0.83	1.67	0.83
Vitamins pre-mix[†]	0.20	0.20	0.20	0.20
Trace mineral pre-mix[‡]	2.00	2.00	2.00	2.00
50% choline chloride	1.00	1.00	1.00	1.00
Aureomycin	1.00	–	0.30	–
Antioxidants	0.40	0.40	0.40	0.40
Maize oil	45.00	45.00	–	–
Fish oil	–	–	45.00	45.00
Flavomycin		0.1		0.1
Metabolisable energy (Mcal/kg)	3.00	3.00	3.00	3.00
CP (g/kg)	210.00	190.00	210.00	190.00
Calcium (g/kg)	10.00	9.00	10.00	9.00
AP (g/kg)	4.50	4.00	4.50	4.00
Lysine (g/kg)	11.00	10.00	11.00	10.00
Methionine (g/kg)	5.00	3.80	5.00	3.80

[†]Supplied per kg of diet: vitamin A, 12 500 IU; vitamin D3, 2500 IU; vitamin K3, 2.65 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; vitamin B12, 0.025 mg; vitamin E, 30 IU; biotin, 0.0325 mg; folic acid, 1.25 mg; pantothenic acid 12 mg; niacin, 50 mg.
[‡]Supplied per kilogram of diet: Cu, 8 mg; Zn, 75 mg; Fe, 80 mg; Mn, 100 mg; Se, 0.15 mg; I, 0.35 mg.

Table 2 *Fatty acids composition of fish oil and maize oils*

Fatty acids	Composition	
	Fish oil	Maize oil
C14 : 0	8.2	0.03
C14 : 1	0.35	–
C16 : 0	15.42	12.36
C16 : 1	7.92	0.14
C18 : 0	4.21	1.84
C18 : 1	26.96	28.17
C18 : 2(n-6)	_	55.79
C18 : 3(n-3)	1.05	0.81
C20 : 1	13.88	0.33
C20 : 2	0.45	0.02
C20 : 3	0.68	–
C20 : 4(n-6)	0.16	–
C20 : 5(n-3)	10.02	–
C22 : 0	–	0.12
C22 : 6(n-3)	10.61	–
C24 : 0	–	0.40
n-3 PUFA/n-6 PUFA	136 : 1	1 : 100

Immune responses measurements

The ratio of $CD3^+CD4^+/CD3^+CD8^+$ of peripheral blood mononuclear cell (PBMC) isolation and culture of PBMC of birds were prepared by a modification of the method of Wallace *et al*. (2003) and Hayek *et al*. (1999). One day after the first and the second LPS or saline challenge, blood was aseptically sampled from the wing vein into 5-ml heparinised vacutainer tubes. Blood was layered onto two volume lymphocytes separation medium (Dingguo biotech Inc., Beijing, China), and centrifuged at 4800 r.p.m. for 30 min at 4°C, then viable mononuclear cells could be recovered and then washed in RPMI1640 (GIBCO; Invitrogen, Carlsbad, CA, USA). Viability of PBMC was determined by trypan blue exclusion, and cells were adjusted to 1×10^6 per ml. The fluorescence monoclonal antibodies were purchased from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA), including mouse anti-chicken CD3-FITC (cluster of differentiation, 3-fluorescein isothiocyanate, NO.8200-02), mouse anti-chicken CD4-PE (cluster of differentiation, 3-phycoerythrin, no. 8255-09) and mouse anti-chicken CD8-PE (no. 8220-09). In all, 5000 cells per sample were analysed using flow cytometer (BD, Franklin Lakes, NJ USA).

Cytokines of peripheral blood

Blood samples were obtained by puncturing the wing vein of chickens at 300 h postinjection of LPS. Blood was collected in 5-ml heparinised vacutainer tubes. The blood samples were centrifuged ($3000 \times \mathbf{g}$ for 15 min) to collect plasma. Plasma was harvested and stored at -30°C until analysis for IL-1, IL-6 and TNF-α. The levels of IL-1, IL-6 and TNF-α were measured with the commercial ELISA kits (Rapid & Biotech, Beijing, China) of IL-1, IL-6 and TNF-α, respectively, according to the manufacturer instructions. Briefly, This assay employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for cytokines has been pre-coated onto a microplate. Standards, controls and samples are pipetted into the wells and any cytokines present is bound by the immobilised antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for cytokines is added to the wells. Following a wash to remove any unbound antibody–enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the colour measured is proportionate to the amount of cytokines bound in the initial step. The sample values are then read off the standard curve.

Lymphocytes proliferation of peripheral blood

Isolation and culture of PBMC of birds were the same as above. Viability of PBMC was determined by trypan blue exclusion, and cells were adjusted to 1×10^7 per ml. The cells were cultured in 96-well microtitre plates and incubated for 48 h with 45 μg/ml of Concanavalin A (ConA) at 40°C, 5% CO_2 and optimum humidity. Subsequently, 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) solution (5 mg MTT/ml) was added to each well and the plates were incubated at 37°C for another 6 h. Following incubation, 100 μl of a 10% sodium dodecyl sulphate in 0.04 mol/l HCl solution was added to lyse the cells and solubilise the MTT crystals. Plates were read at 570 nm using an automated microplate reader (model 550; Bio-Rad, Hercules, CA, USA).

Serum IgG analysis

One day after the first and the second LPS or saline challenge, blood samples were obtained by puncturing the wing vein of chickens for the analysis of the serum immunoglobulin G (IgG) levels by the method of immunoturbidimetry. Serum samples were diluted with 0.01 mol/l phosphate-buffered saline (pH 7.2) at a dilution of 1 : 5. Briefly, the 10 μl diluted serum samples and 150 μl diluted (1 : 200) rabbit anti-chicken IgG (The Academy of Military Medical Sciences, China) were added to the 96-well microtitre plates. Then, a 10 μl of reactive buffer solution (0.01 mol/l phosphate-buffered saline including potyethytene glycol 53.0 g) was added to each well (Kastner and Jakse, 2003). After incubation for 30 min at room temperature, the plates were read at an absorbance of 260 nm using a SAFIRE 2 spectrophotometer (Tecan, Lausanne, Switzerland).

Real-time quantitative RT-PCR

Spleen was removed on 21 days and 28 days of age, respectively. Total splenic RNA was extracted using the TRIzol Reagent (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions. RNA integrity was verified electrophoretically by ethidium bromide staining. The RNA purity was determined using the Biophotometer (Eppendorf, Cambridge, UK) at OD260.

Total RNA was reverse transcribed according to the method described by Sijben *et al*. (2001) and Lai *et al*. (2005a). Briefly, 1.0 μl of each RNA sample was added to a 40 μl reaction system containing 1.0 μl of oligo-dT, 1.25 μl of dNTPs (Sigma), 0.5 μl of RNasin inhibitor (Promega, Madison, WI, USA), 1.0 μl of M-MLV transcriptase (Promega), 5.0 μl of M-MLV reverse transcriptase (RT) reaction buffer (Promega) and 15.25 μl of RNase-free water. Cycle parameters for the RT procedure were 1 cycle of 37°C, 1 h; 1 cycle of 70°C, 15 min; and 1 cycle of 4°C, 5 min. The RT products (cDNA) were stored at −20°C for relative quantification by PCR.

The reverse-transcribed products (cDNA) were for relative quantification by PCR. Nuclear factor kappa B (NFκB) amplification primers were designed using the Primer Premier 5.0 software program. The following sequences of PCR primer pairs were used: forward 5′-GTG TGA AGA AAC GGG AAC TG-3′, reverse 5′- GGC ACG GTT GTC ATA GAT GG -3′.

Real-time PCR for quantification of NFκB mRNA levels in chicken spleens were quantified using a method based on that of Sijben *et al*. (2003). Briefly, quantitative analysis of PCR was carried out according to optimised PCR protocols and SYBR Green PCR master Mix Kit (Applied Biosystems, Foster City, CA, USA). The volume of reaction system is 40 μl, including 20 μl SYBR Green PCR master Mix, 3.0 μl forward primer, 3.0 μl reverse primer, 2.0 μl template and 12 μl DNase-free water. For the PCR reaction, the following experimental run protocol was used: enzyme incubation (50°C for 2 min), denaturation program (95°C for 10 min), amplification and quantification program repeated 38 times (94°C for 20 s, different annealing temperature for different target genes for 20 s, 72°C for 20 s with a single fluorescence measurement), melting curve program (65°C to 95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and finally 72°C for 10 min. The annealing temperature for NFκB was 53°C. Quantification was based on the increased fluorescence detected by the DNA Engine Opticon 2 fluorescence detection system (MJ Research, Waltham, MA, USA).

To generate standard curves for the NFκB reaction, the NFκB products of RT-PCR were serially diluted from 10^{-3} to 10^{-7}. The housekeeping gene, β-actin, was used as an internal standard for the quantification. The threshold cycle value (Ct-value) was determined and was used to calculate the relative expression level compared with β-actin.

Statistical analysis

Statistical analysis of the chicken data were conducted using the GLM procedure of Statistical Analysis Systems Institute (2001) in a 2×2 factorial arrangement of treatments in a randomised complete block design with the two main effects of LPS challenge and dietary oil source. Meanwhile, the data were analysed by the procedure appropriated for a completely randomised design by the GLM procedure of SAS when the LPS challenge, dietary oil source main effects or the interaction between the two main effects is significant. Differences among each treatment group were tested by Duncan's multiple-range test. Differences due to diets treatment were considered significant if the P value for the effect was <0.05.

Results

Growth performance

Performance data are presented in Table 3. There were no significant effects of dietary oils on feed intake, body-weight gains and feed-conversion efficiency on 21 days of age in the chickens. During the first challenge period (from 21 days to 27 days of age), LPS challenge reduced feed intake ($P < 0.10$) of chickens. The interaction between dietary oils and LPS challenge was significant in feed-conversion efficiency during the first challenge. Feed-conversion efficiency was significantly higher in chickens fed the maize oil diet than in those fed the fish oil diet during the first challenge. After the second LPS challenge, chickens challenged with LPS had lower body-weight gains ($P < 0.05$) than that in those treated with saline during week 4 to week 6. Feed-conversion efficiency was significantly higher in chickens challenged with LPS than in those treated with saline. LPS challenge significantly reduced body-weight gains of chickens at week 4 to week 6. There was no significant interaction between dietary oils and LPS challenge during the second challenge and the whole experiment period.

Lymphocytes proliferation and IgG levels

Lymphocyte proliferation was measured in cultures of purified PBMCs after the first and the second LPS challenges, and the data are presented in Table 4. There was significant interaction between LPS challenge and dietary oils on lymphocyte proliferation in chickens *in vitro* during both the first and the second LPS challenge periods. Both the first and the second LPS challenges significantly increased lymphocyte proliferation of PBMCs incubated with ConA *in vitro*. Fish oil decreased lymphocyte proliferation of PBMCs in chickens challenged with LPS ($P < 0.05$). Serum IgG concentration in chickens after the first and the second LPS challenges was measured by the method of immunoturbidimetric quantification. LPS challenge significantly increased the levels of IgG in chickens. There was no significant interaction between LPS challenge and dietary oils on the serum IgG levels in chickens (Table 4).

The ratio of $CD3^+CD4^+/CD3^+CD8^+$ of peripheral blood mononuclear cells

The $CD3^+CD8^+$ and $CD3^+CD4^+$ proportion of T lymphocytes in the chickens is shown in Table 5. Dietary oils did not significantly affect the $CD3^+CD8^+$ and $CD3^+CD4^+$ proportion of T lymphocytes in the chickens. One day after the first and the second LPS challenges, the ratio of $CD3^+CD4^+/CD3^+CD8^+$ of PBMCs was significantly higher in chicken challenged with LPS than in those treated with saline. And there was significant interaction on the ratio of

Table 3 *Effects of dietary oils and lipopolysaccharide (LPS) challenge on growth performance of chickens*

	Saline		LPS			*P* value		
Week	45 g/kg CO	45 g/kg FO	45 g/kg CO	45 g/kg FO	s.e.	PUFA[†]	Stress	Interaction
Feed (kg)								
0–3	0.84	0.86	0.85	0.85	0.01	0.5290	–	–
4	0.73	0.74	0.76	0.76	0.01	0.8659	0.0714	0.5761
5	1.00	0.99	0.99	0.95	0.02	0.4249	0.4787	0.6335
4–6	2.40	2.26	2.38	2.35	0.03	0.1965	0.5935	0.4411
0–6	3.22	3.20	3.25	3.11	0.03	0.2442	0.6445	0.4483
Gain (kg)								
0–3	0.66	0.67	0.65	0.65	0.005	0.6449	–	–
4	0.39	0.37	0.37	0.40	0.01	0.7685	0.7764	0.0860
5	0.43	0.42	0.43	0.37	0.01	0.2246	0.4909	0.3468
4–6	1.09	1.09	0.98	1.00	0.02	0.7406	0.0108	0.8296
0–6	1.75	1.76	1.64	1.66	0.02	0.6385	0.0033	0.9407
FCR[†]								
0–3	1.27	1.28	1.30	1.30	0.01	0.8081	–	–
4	1.85	2.00	2.08	1.90	0.04	0.8006	0.3440	0.0184
5	2.36	2.37	2.34	2.62	0.06	0.2537	0.3425	0.2723
4–6	2.20	2.15	2.44	2.26	0.03	0.0431	0.0030	0.2114
0–6	1.84	1.81	1.98	1.88	0.02	0.0235	0.0011	0.2109

[†]FCR = feed conversion efficiency; PUFA = polyunsaturated fatty acids.

Table 4 *Effects of dietary oils and lipopolysaccharide challenge on lymphocytes proliferation isolated from peripheral blood and humoral responsesin chickens*[†]

	Lymphocytes proliferation		IgG	
	21 days	28 days	28 days	35 days
CO				
Saline	1.20	1.18	0.53	0.49
LPS	1.64	1.62	0.90	0.79
FO				
Saline	1.40	1.38	0.64	0.53
LPS	1.54	1.54	0.91	0.79
s.e.	0.03	0.04	0.04	0.03
P value				
Oils	0.0029	0.0058	0.1802	0.2581
Stress	<0.0001	<0.0001	<0.0001	<0.0001
Interaction	<0.0001	<0.0001	0.1976	0.3319

[†]Values are expressed as stimulation index calculated as OD_{570} absorbance of wells incubated with concanavalin A divided by $OD_{570\,nm}$ absorbance of wells incubated without concanavalin A. The values of IgG were expressed as absorbance at 490 nm when determined by a microplate reader.

$CD3^+CD4^+/CD3^+CD8^+$ of PBMCs in chickens between LPS challenge and dietary oils. Compared with maize oil, fish oil significantly decreased the ratio of $CD3^+CD4^+/CD3^+CD8^+$ in chickens at 1 day after the first and the second LPS challenges.

Pro-inflammatory cytokines

Table 6 shows the data for the pro-inflammatory cytokines levels in chickens at 3 h after the first and the second LPS challenges. LPS challenge significantly increased the release of IL-1, IL-6 and TNF-α in chickens compared with that in chickens treated with saline. There were no significant differences in the release of IL-1, IL-6 and TNF-α in chickens fed fish oil and those fed maize oil. However, the increased IL-6 and TNF-α production in chickens challenged with LPS were significantly attenuated by dietary fish oil.

Nuclear factor kappa B

The first and the second LPS challenges significantly increased ($P < 0.001$) the expression of NFκB of spleen in chickens at 1 day after challenge (Table 7). There was an interaction between LPS challenge and dietary oil ($P = 0.0079$ for the first challenge; $P = 0.0017$) for the mRNA abundance of NFκB. Among chickens challenged with LPS, those fed fish oil diets had significantly lower mRNA abundance of NFκB than those fed maize oil diets.

Discussion

Decreased feed consumption, body-weight gain and feed-conversion efficiency are the common results of an inflammatory response in different animals (Klasing and Korver, 1997; Korver and Klasing, 1997; Takahashi *et al.*, 2002; Gaines *et al.*, 2003). In this study, we found that LPS challenge decreased the performance in chickens (Table 3). When chickens were challenged with LPS firstly, the fish oil diets mitigated the decrease in feed-conversion efficiency. However, the beneficial effects were not observed after the second challenge. The present study indicated that fish oil would ameliorate the growth depression at acute inflammation early. The reason as to why an immediate effect of LPS challenge on immune response but a late

Table 5 *Effects of dietary oils and lipopolysaccharide (LPS) challenge on the distribution of $CD4^+$ and $CD8^+$ of lymphocytes isolated from peripheral blood in chickens*[†]

	$CD3^+CD4^+$		$CD3^+CD8^+$		$CD3^+CD4^+/CD3^+CD8^+$	
	21 days	28 days	21 days	28 days	21 days	28 days
CO						
Saline	0.58	0.71	0.51	0.47	1.08	1.49
LPS	0.68	0.75	0.37	0.34	2.00	2.42
FO						
Saline	0.58	0.74	0.39	0.42	1.04	1.58
LPS	0.63	0.73	0.40	0.43	1.30	1.60
s.e.	0.02	0.007	0.03	0.02	0.12	0.10
P value						
Oils	0.2858	0.6195	0.4335	0.5531	0.0296	0.0052
Stress	0.0337	0.3655	0.2669	0.1066	0.0015	0.0006
Interaction	0.8779	0.1042	0.2115	0.0560	0.0479	0.0009

[†]Data for $CD3^+CD4^+$ and $CD3^+CD8^+$ are the ratio of $CD3^+CD4^+$ cells and $CD3^+CD4^+$ cells divided by the total T cells of peripheral blood mononuclear cells in chickens.

Table 6 *Effects of dietary oils and lipopolysaccharide (LPS) challenge on cytokines production of chickens 3 h after LPS administration*

	IL-1 (pg/ml)		IL-6 (pg/ml)		TNF-α (pg/ml)	
Treatment	20 days	27 days	20 days	27 days	20 days	27 days
CO						
Saline	82.09	110.82	32.22	36.76	10.84	14.12
LPS	131.21	149.24	37.50	42.50	16.65	20.89
FO						
Saline	81.00	99.36	32.50	37.31	11.75	15.52
LPS	109.55	124.64	34.63	39.26	12.45	17.13
s.e.	5.69	6.01	0.55	0.60	0.60	0.76
P value						
Oils	0.1083	0.0665	0.0787	0.1146	0.0597	0.3235
Stress	$<$0.0001	0.0030	$<$0.0001	0.0001	0.0008	0.0018
Interaction	0.1438	0.4842	0.0359	0.0302	0.0055	0.0391

Table 7 *Effects of dietary oils and lipopolysaccharide challenge on NFκB mRNA of spleen in chickens*[†]

	NFκB	
	21 days	28 days
CO		
Saline	0.15	0.16
LPS	0.26	0.24
FO		
Saline	0.16	0.18
LPS	0.22	0.21
s.e.	0.01	0.01
P value		
Oils	0.2084	0.5363
Stress	$<$0.0001	$<$0.0001
Interaction	0.0079	0.0017

[†]Data of the NFκB expression are the ratio of the mRNA abundance of NFκB divided by corresponding housekeeping gene (β-actin) in spleen of chickens.

cumulated effect on performances (except for a significant interaction at week 4 for FCE) that fish oil diet did improved acute immune responses not stress situation over a long period of time. A previous study showed that a fish oil diet did initialise a reduced host natural resistance to infectious diseases (*Listeria monocytogenes*) in mice mainly because of the suppression of immune function generated by the fatty acids contained in this diet (Puertollano *et al*., 2004). Thus, this kind of experiment could be finally detrimental to the overall animal performance.

The acute-phase response is a hallmark of inflammation. Increased lymphocytes proliferation and humoral response are all associated with an inflammatory response (Liu *et al*., 2003). In our study, LPS challenge had similar dramatic effects on lymphocytes proliferation and humoral response in chickens (Table 4). The immune response led to the changes in the partitioning of nutrients away from growth and toward processes associated with the acute-phase response. It can be explained that LPS challenge decrease body-weight gains and feed-conversion efficiency in chickens. As has been shown with CLA, fish oil had no effects on antibody levels in chickens challenged with LPS (Cook *et al*., 1993). In our present study, fish oil diets did ameliorate the increased lymphocytes proliferation of PBMCs challenged with ConA (Table 4). The result is consistent with our previous results in laying hens (Guo *et al*., 2003 and 2004).

Generally, the generation of specific immune responses is believed to depends on the help of activated $CD4^+$ T cells. $CD4^+$ T cells can produce lymphokines that amplify the cytotoxic activity of $CD8^+$ T cells and other inflammatory cells. These findings imply that a high $CD4^+/CD8^+$ ratio may be a universal prognostic indicator in immune system activation (Sheu *et al*., 1999). In this study, inflammatory response of chickens challenged with LPS resulted in increased ratio of $CD4^+/CD8^+$ of lymphocytes of PBMCs in the chickens (Table 5). Similar to the present result, Lai *et al*. (2005b) reported that LPS challenge increased the ratio of $CD4^+/CD8^+$ of lymphocytes of PBMCs in pigs.

In our study, fish oil attenuated the increased ratio of $CD4^+$/$CD8^+$ of lymphocytes of PBMCs in chickens challenged with LPS (Table 5). It is believed that fish oil plays an important role in ameliorating inflammatory response. However, oral administration of seal oil with abundant n-3 PUFA to germ-free piglets resulted in a significant increase in the total value of $CD4^+$, $CD8^+$ lymphocytes in peripheral blood (Revajová *et al*., 2001). Differences between studies may be related to the animal species used, level and type of oils used and immune status of the animal under study.

The immunological challenge resulted in the poor performance of animals (Benson *et al*., 1993; Klasing and Korver, 1997; Halloy *et al*., 2004), which was attributed to the partitioning of nutrients away from growth and toward processes associated with inflammatory immune responses in chickens (Korver and Klasing, 1997). Some studies indicated that these changes are attributed to the release of pro-inflammatory cytokines (Johnson, 1997; Webel *et al*., 1997; Sijben *et al*., 2001), including TNF, IL-1 and IL-6. In this study, the first and the second LPS challenges significantly increased the pro-inflammatory cytokines. There was an interaction on IL-6 and TNF-α in chickens between LPS challenge and dietary oils (Table 6). This observation suggested that fish oil had beneficial effects on inflammation in chickens challenged with LPS. Dietary fish oil resulted in decreased release, relative to dietary maize oil, of IL-1 by peritoneal macrophages of inflammatory immune responses in chicks (Korver and Klasing, 1997), and improved performance of chickens challenged with LPS. During the challenge period, pigs treated with LPS had higher serum TNF-α and lower body-weight gain compared with pigs fed complex diets supplemented with maize oil as compared with pigs fed complex diets supplemented with menhaden fish (Gaines *et al*., 2003). It may be that in one way fish oil exerts its beneficial effects on chicken performance by lessening pro-inflammatory cytokines (IL-6 and TNF-α) of the inflammatory response in chickens, such as TNF-α, IL-6 and IL-1. Often presented as detrimental to the organism, inflammation is however required for culling a progressive infection (Puertollano *et al*., 2004). A fish oil diet did initialise a reduced host natural resistance to infectious diseases (*Listeria monocytogenes*) in mice mainly because of a suppression of immune function generated by the fatty acids contained in this diet (Puertollano *et al*., 2004). Thus, although presented here as a means to preserve good zootechnical parameters in acute stress period, this kind of experiment could possibly be finally detrimental to the overall animal health.

Several pro-inflammatory cytokines, such as TNF-α, IL-6 and IL-1, are encoded by target genes of NFκB activation pathway. The classical pathway of NFκB as a crucial transcriptional activator of pro-inflammatory cytokines is triggered by pro-inflammatory stimuli and genotoxic stress, including bacterial cell-wall components, such as LPS (Karin and Greten, 2005). In this study, LPS challenge in chickens resulted in increased abundance of NFκB mRNA (Table 7). Lo *et al*. (1998) reported that fish oil decreased macrophage TNFα gene transcription by altering the NFκB activity. It is believed that the NFκB plays an important role in the production of pro-inflammatory cytokines in chickens challenged with LPS. The present experiment showed that fish oil could ameliorate the increase of NFκB mRNA in chickens challenged with LPS (Table 7). Thus, the result of the present experiment suggested that fish oil decreased pro-inflammatory cytokines production in chickens challenged with LPS through ameliorating NFκB gene transcription.

In conclusion, fish oil decreases the release of pro-inflammatory cytokines possibly through ameliorating NFκB gene transcription, which would improve performance of chickens in an immunological challenge over a short time not over a long time.

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References

Balaji R, Wright KJ, Turner JL, Hill CM, Dritz SS, Fenwick B, Carroll JA, Zannelli ME, Beausang LA and Minton JE 2002. Circulating cortisol, tumor necrosis factor-α interleukin-1β, and interferon-γ in pigs infected with *Actinobacillus pleuropneumoniae*. Journal of Animal Science 80, 202–207.

Benson BN, Calvert CC, Roura E and Klasing KC 1993. Dietary energy source and density modulate the expression of immunologic stress in chicks. Journal of Nutrition 123, 1714–1723.

Calder PC 2001a. N-3 polyunsaturated fatty acids, inflammation and immunity: pouring oil on troubled waters or another fishy tale? Nutrition Research 21, 309–341.

Calder PC 2001b. The ratio of n-6 to n-3 fatty acids in the diet: impact on T lymphocyte function. European Journal of Lipid Science and Technology 103, 390–398.

Calder PC and Grimble RF 2002. Polyunsaturated fatty acids, inflammation and immunity. European Journal of Clinical Nutrition 56, s14–s19.

Cook ME, Miller CC, Park Y and Pariza M 1993. Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. Poultry Science 72, 1301–1305.

Gaines AM, Carroll JA, Yi GF, Allee GL and Zannelli ME 2003. Effect of menhaden fish oil supplementation and lipopolysaccharide exposure on nursery pigs: II. Effects on the immune axis when fed simple or complex diets containing no spray-dried plasma. Domestic Animal Endocrinology 24, 353–365.

Guo YM, Xia ZG, Chen SY and Yuan JM 2003. Effects of dietary polyunsaturated fatty acids on antibody production and lymphocyte proliferation of laying hens. Asian-Australasian Journal of Animal Sciences 16, 1320–1325.

Guo YM, Chen SY, Xia ZG and Yuan JM 2004. Effects of different types of polyunsaturated fatty acids on immune function and PGE2 synthesis by peripheral blood leukocytes of laying hens. Animal Feed Science and Technology 116, 249–257.

Halloy DJ, Bouhet S, Oswald IP, Goret-Nicaise M, Kobisch M, Mainil J and Gustin PG 2004. Pathophysiological changes occurring during *E. coli* endotoxin and *Pasteurella multocida* challenge in piglets: relationship with cough and temperature and predicitive value for intensity of lesions. Veterinary Research 35, 309–324.

Hayek MG, Han SN, Wu D, Watkins BA, Meydani M, Dorsey JL, Smith DE and Meydani SN 1999. Dietary conjugated linoleic acid influences the immune response of young and old C57BL/6NCrlBR mice. Journal of Nutrition 129, 32–38.

Johnson RW 1997. Inhibition of growth by pro-inflammatory cytokines: an integrated view. Journal of Animal Science 75, 1244–1255.

Johnson RW and von Borel E 1994. Lipopolysaccharide-induced sickness behavior in pigs is inhibited by pretreatment with indomethacin. Journal of Animal Science 72, 309–314.

Karin M and Greten FR 2005. NFκB: Linking inflammation and immunity to cancer development and progression. Nature Reviews Immunology 5, 749–759.

Kastner C and Jakse G 2003. Measurement of immunoglobulins in seminal fluid with modified nephelometry-an alternative diagnostic tool for chronic prostatitis. Prostate Cancer and Prostatic Diseases 6, 86–89.

Klasing KC and Korver DR 1997. Leukocytic cytokines regulate growth rate and composition following activation of the immune system. Journal of Animal Science 75, 58–67.

Korver DR and Klasing KC 1997. Dietary fish oil alters specific and inflammatory immune responses in chicks. Journal of Nutrition 127, 2039–2046.

Korver DR, Wakenell P and Klasing KC 1997. Effects of fish oil or lofrin, a 5-lipoxygenase inhibitor, decrease the growth-suppressing effects of coccidiosis in broiler chicks. Poultry Science 76, 1355–1363.

Korver DR, Roura E and Klasing KC 1998. Effects of dietary energy and oil source on broiler performance and response to an inflammatory challenge. Poultry Science 77, 1217–1227.

Lai CH, Yin JD, Li DF, Zhao LD, Qiao SY and Xing JJ 2005a. Conjugated linoleic acid attenuates the production and gene expression of proinflammatory cytokines in weaned pigs challenged with lipopolysaccharide. Journal of Nutrition 135, 239–244.

Lai CH, Yin JD, Li DF, Zhao LD, Qiao SY and Chen XJ 2005b. Effects of dietary conjugated linoleic acid supplementation on performance and immune function of weaned pigs. Archives of Animal Nutrition 59, 41–51.

Liu YL, Li DF, Gong LM, Yi GF, Gaines AM and Carroll JA 2003. Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotropic responses of weaned pigs after an *E. coli* lipopolysaccharide challenge. Journal of Animal Science 81, 2758–2765.

Lo CJ, Chiu KC, Fu M, Lo R and Helton S 1998. Fish oil decreases macrophage tumor necrosis factor gene transcription by altering the NF-κB activity. Journal of Surgical Research 82, 216–221.

Puertollano MA, Puertollano E, Ruiz-Bravo A, Jiménez-Valera M, De Pablo MA and De Cienfuegos GÁ 2004. Changes in the immune functions and susceptibility to *Listeria monocytogenes* infection in mice fed dietary lipids. Immunology and Cell Biology 82, 370–376.

Revajová V, Pistl J, Kaštel R, Bindas L', Magic SD, Levkut M, Bomba A and Šajbidor J 2001. Influencing the Immune parameters in germ-free piglets by administration of seal oil with increased content of ω-3 PUFA. Archiv für Tierernährung 54, 315–327.

Sheu BC, Hsu SM, Lin RH, Torng PL and Huang SC 1999. Reversed CD4/CD8 ratios of tumor-infiltrating lymphocytes are correlated with the progression of human cervical carcinoma. Cancer 86, 1537–1543.

Sijben JW, Schrama JW, Parmentier HK, van der Poel JJ and Klasing KC 2001. Effects of dietary polyunsaturated fatty acids on in vivo splenic cytokine mRNA expression in layer chicks immunized with Salmonella typhimurium lipopolysaccharide. Poultry Science 80, 1164–1170.

Sijben JWC, Klasing KC, Schrama JW, Parmentier HK, Van der Poel JJ, Savelkoul HFJ and Kaiser P 2003. Early in vivo cytokine genes expression in chickens after challenge with Salmonella typhimurium lipopolysaccharide and modulation by dietary n-3 polyunsaturated fatty acids. Developmental and Comparative Immunology 27, 611–619.

Statistical Analysis Systems Institute 2001. SAS software 8.02. SAS Inst. Inc., Cary, NC.

Takahashi K, Kawamata K, Akiba Y, Iwata T and Kasai M 2002. Influence of dietary conjugated linoleic acid isomers on early inflammatory responses in male broiler chickens. British Poultry Science 43, 47–53.

Wallace FA, Miles EA and Calder PC 2003. Comparison of the effects of linseed oil and different doses of fish oil on mononuclear cell function in healthy human subjects. British Journal of Nutrition 89, 679–689.

Warren EJ, Finck BN, Arkins S, Kelley KW, Scamurra RW, Murtaugh MP and Johnson RW 1997. Coincidental changes in behavior and plasma cortisol in unrestrained pigs after intracerebroventricular injection of tumor necrosis factor-α. Endocrinology 138, 2365–2371.

Webel DM, Finck BN, Baker DH and Johnson RW 1997. Time course of increased plasma cytokines, cortisol, and urea nitrogen in pigs following intraperitoneal injection of lipopolysaccharide. Journal of Animal Science 75, 1514–1520.