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ABSTRACT There is interest in the enrichment of poultry meat with long-chain n-3 polyunsaturated fatty acids in order to increase the consumption of these fatty acids by humans. However, there is concern that high levels of n-3 polyunsaturated fatty acids may have detrimental effects on immune function in chickens. The effect of feeding increasing levels of fish oil (FO) on immune function was investigated in broiler chickens. Three-week-old broilers were fed 1 of 4 wheat-soybean basal diets that contained 0, 30, 50, or 60 g/kg of FO until slaughter. At slaughter, samples of blood, bursa of Fabricius, spleen, and thymus were collected from each bird. A range of immune parameters, including immune tissue weight, immuno-phenotyping, phagocytosis, and cell proliferation, were assessed. The pattern of fatty acid incorporation reflected the fatty acid composition of the diet. The FO did not affect the weight of the spleen, but it did increase thymus weight when fed at 50 g/kg ($P < 0.001$). Fish oil also lowered bursal weights when fed at 50 or 60 g/kg ($P < 0.001$). There was no significant effect of FO on immune cell phenotypes in the spleen, thymus, bursa, or blood. Feeding 60 g/kg of FO significantly decreased the percentage of monocytes engaged in phagocytosis, but it increased their mean fluorescence intensity relative to that of broilers fed 50 g/kg of FO. Lymphocyte proliferation was significantly decreased after feeding broiler chickens diets rich in FO when expressed as division index or proliferation index, although there was no significant effect of FO on the percentage of divided cells. In conclusion, dietary n-3 polyunsaturated fatty acids decrease phagocytosis and lymphocyte proliferation in broiler chickens, highlighting the need for the poultry industry to consider the health status of poultry when poultry meat is being enriched with FO.

Key words: fish oil, n-3 fatty acid, phagocytosis, cell proliferation

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INTRODUCTION

In recent years, polyunsaturated fatty acids (**PUFA**, particularly those of the n-3 family) have received considerable attention in both human and animal nutrition. Dietary supplementation with fish oil (**FO**), which is rich in n-3 PUFA, is reported to have nutritional benefits (Schmidt, 1997; Leaf and Kang, 2001; Mayser et al., 2002; Calder, 2006; Schwalfenberg, 2006). However, consumption of n-3 PUFA by humans is low, particularly the long-chain (>18 carbon atoms) PUFA. As a means of addressing the low consumption of the long-chain n-3 PUFA by humans, there has been some interest in the enrichment of poultry meat with these fatty acids.

Modulation of fatty acid profiles as a result of n-3 PUFA supplementation or dietary modification is well-documented in many species, including mice (Lokesh et al., 1988; Fritsche and Johnston, 1990; Fujikawa et al., 1992), rats (Gudbjarnason and Oskarsdottir, 1977; Nassar et al., 1986; Yaqoob et al., 1995a; Jeffery et al., 1996; Peterson et al., 1998a; Yang and O'Shea, 2009), chickens (Ratnayake et al., 1986; Fritsche et al., 1991b; López-Ferrer et al., 1999; López-Ferrer et al., 2001a; Wang et al., 2002; Bou et al., 2004; Puthpongsiriporn and Scheideler, 2005; Rymer and Givens, 2006; Svedova et al., 2008; Zelenka et al., 2008; Kartikasari et al., 2010), and humans (Meydani et al., 1991; Kew et al., 2004; Miles et al., 2004; Surette et al., 2004; Reis and Hibbeln, 2006). In general, studies demonstrate that the fatty acid profile of plasma and immune tissues reflects the fatty acid composition of the diet.

Several studies in the literature demonstrate that chickens fed marine oils accumulate significant amounts of n-3 PUFA in their eggs and meat. For example, Bou et al. (2004) reported that supplementing broiler diets with 25 g/kg of FO doubled the amount of eicosapentaenoic (**EPA**) and docosahexaenoic (**DHA**) acids in

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their meat compared with that from chickens fed diets supplied with 12.5 g/kg of FO (0.06 vs. 1.00 and 0.09 vs. 1.38 for EPA and DHA, respectively). Supplementing broiler chickens with 4% FO decreased the proportions of saturated (from 43.77 to 39.84% of total fatty acids) and monounsaturated fatty acids (**MUFA**; from 41.26 to 37.60% of total fatty acids) and increased the proportion of n-3 PUFA (from 2.09 to 8.14% of total fatty acids) in thigh samples (López-Ferrer et al., 2001b). López-Ferrer et al. (1999) substituted 82 g/kg of FO in a supplemented broiler diet with the same amount of linseed and rapeseed and concluded that the total amount of n-3 PUFA in the chicken meat decreased when FO was removed from the diet, whereas the proportions of n-6 PUFA and MUFA (in the form of oleic acid) increased.

Recently, there has been some concern that diets enriched with n-3 PUFA may have detrimental effects on chicken immunity and impair resistance to infection. However, it is not clear whether this concern is justified, given that some studies show no effect (Puthpongsiriporn and Scheideler, 2005), some show a detrimental effect (Fritsche et al., 1991a, Babu et al., 2005), and some show an improvement (Phipps et al., 1991; Korver and Klasing, 1997; Parmentier et al., 1997; Puthpongsiriporn Sijben et al., 2000; and Scheideler, 2005; Yang and Guo, 2006).

The main immune organs in poultry are the thymus, spleen, and bursa of Fabricius. During an immune response, mature lymphocytes and other immune cells interact with antigens in these tissues. Consequently, immune tissue mass can in some cases indicate immune status (Moller and Erritzoe, 2000; Grasman, 2002; Smith and Hunt, 2004). Wang et al. (2000) observed that feeding laying chickens diets rich in n-3 PUFA promoted the growth of the thymus, spleen, and bursa up to 4 wk of age. However, from the age of 4 wk onward, immune tissue weights began to decline, and the bursa degenerated between 4 and 8 wk of age. Nevertheless, the authors suggested that changes in the weights of the thymus and spleen did not correlate with the immune function. Interestingly, the same phenomenon was observed in the thymus and spleen of mice fed n-3 PUFA diets (Ellis et al., 1986; Huang et al., 1992).

Studies in chickens report inconsistent effects of dietary n-3 PUFA on subsets of immune cells. One study showed that 5% FO increased spleen IgM^+ B-lymphocytes, but had no effect on $CD4^+$ cells (Wang et al., 2000); one study showed decreased proportions of $CD8^+$ and increased proportions of $CD4^+$ and $CD3^+$ T cells (Yang and Guo, 2006); one study showed no effect on any cell subset (Selvaraj and Cherian, 2004); and one showed a decrease in the ratio of $CD4^+$ to $CD8^+$ (Yang et al., 2008).

The reported effects of n-3 PUFA on phagocytosis in animal models are inconsistent and a matter of debate. There are no published studies investigating the effect of dietary n-3 PUFA on phagocytosis in chickens.

There is evidence of suppressive effects of dietary n-3 PUFA on lymphocyte proliferation in mice (Wallace et al., 2001; Pompos and Fritsche, 2002; Switzer et al., 2003; Barber et al., 2005; Kim et al., 2008), rats (Sanderson et al., 1995; Yaqoob et al., 1995a; Jeffery et al., 1996; Peterson et al., 1998a;1998b), chickens (Cassity et al., 1990; Fritsche et al., 1991a; Wang et al., 2000; ZhaoGang et al., 2004; Yang et al., 2008), and humans (Meydani et al., 1991; Thies et al., 2001; Kew et al., 2004).

In light of the above, poultry diets enriched with n-3 PUFA may have the potential to modulate the avian immune response, and thus, affect the bird's ability to resist invading pathogens. Therefore, the aim of this study was to investigate the effects of n-3 PUFA in the form of increasing levels of FO on the immune functions in broiler chickens, including immune tissue weight, immune cell phenotypes, phagocytosis, and cell proliferation.

MATERIALS AND METHODS

Birds and Diets

One-day-old male Ross 308 broiler chicks (PD Hook Hatcheries, Bampton, Oxfordshire, UK), vaccinated against infectious bronchitis, were used in this study. Water and feed were provided ad libitum. In total, 48 birds were randomly housed in 16 cages (106 × 106 × 108 cm), 3 chicks per cage. Upon hatching, all chicks were given the same basal diet for 21 d. Following this, the 3-wk-old broilers were fed the experimental diets until being killed after 21 to 26 d; that is, animals were killed on 2 staggered days. Body weight gain, immune tissue weight, immune cell subsets, and phagocytosis were studied at 47 d of age. Cell proliferation and fatty acid composition of immune tissues were studied at 42 d of age. The experimental diets were wheat- and soybean meal-based diets that contained 30, 50, or 60 g/kg of FO. Every 4 cages (i.e., 12 birds) received one of the experimental diets. The control birds received no FO. The diets were formulated according to Ross 308 guidelines for broiler chickens (Ross Broilers, 2007). Due to time limitation, BW gain and immune cell subsets were studied using 30 and 60 g/kg of FO, and immune cell proliferation was studied using 50 g/kg of FO, whereas immune tissue weight, phagocytosis, and fatty acid composition of immune cells were studied using 30, 50, or 60 g/kg of FO. Table 1 shows the fatty acid composition of the FO mixture used. The FO was purchased from United Fish Industries UK Ltd., Gilbey Road, Grimsby, Lincolnshire, UK. The temperature for the broilers was kept at 30°C for 14 d and then gradually reduced to 21°C by 21 d.

Sample Collection

Birds were killed by stunning and bleeding. Blood was collected in heparinized tubes. The thymus, spleen,

Table 1. Fatty acid composition of the fish oil mixture used

Fatty acid	Weight (%)
14:0	5.50
16:0	17.21
16:1 n-7	6.45
17:1	0.81
18:0	3.36
18:1 n-9	17.55
18:2 n-6	4.15
18:3 n-3	1.50
20:1 n-9	2.82
20:3 n-6	8.14
20:5 n-3	9.87
22:5 n-3	3.35
22:6 n-3	12.92

and bursa were aseptically removed and weighed. The thymus and spleen were placed in cell culture medium (**CCM**) on ice. The CCM was composed of RMPI-1640 (Sigma-Aldrich, Gillingham, UK) supplemented with glutamine and antibiotics. The bursae were frozen for fatty acid analysis. All fat and adherent tissue was removed from the thymus and spleen.

Preparation of Leukocytes from Immune Tissues

The heparinized blood was layered onto an equal volume of Lympholyte-H (Cedarlane Laboratories Ltd., Burlington, Canada) and centrifuged at 800 × *g*, and the interface containing leukocytes was collected and suspended in CCM. The cell suspension was then layered again on Lympholyte, and the previous step was repeated to further remove red blood cells. The cells were washed and suspended in CCM. Lymphocytes from freshly harvested spleens and thymi were prepared by dispersing their tissues through a stainless steel wire-mesh strainer. Debris was removed by filtering the cell suspension through lens tissue. Cells were collected by centrifugation at 250 × *g* and were resuspended in CCM. Separation of leukocytes from the thymus and spleen was performed as for the blood.

Fatty Acid Analysis by Gas Chromatography

Fatty acid methyl esters were analyzed using a Hewlett Packard 6890 series gas chromatography system (Hewlett Packard, Basingstoke, UK). Approximately 5 to 9 × 10^7 cells were centrifuged at 250 × *g* and suspended in 400 μL of 0.9% (wt/vol) NaCl. For lipid extraction, 5 mL of chloroform:methanol [2:1 (vol/vol), 50 mg/L of butylated hydroxytoluene] and 1 mL of 1 *M* NaCl were added to the sample in a 10-mL glass tube. Tubes were vortexed and centrifuged at 930 × *g* for 10 min at 25°C. The bottom layer was transferred to a glass tube and evaporated to dryness under nitrogen. For transmethylation, 400 μL of toluene (+butylated hydroxytoluene 50 mg/L) and 800 μL of 1.5% sulphuric acid in methanol were added to the dried samples. The tubes were vortexed and heated in a water bath at 70°C for 1 h. After cooling, 2 mL of neutralizing agent (0.1 *M* K_2CO_3, 0.1 *M* $KHCO_3$) and 2 mL of hexane were added, and the suspension was mixed and centrifuged at 1,162 × *g* for 10 min at 25°C. The upper layer was transferred to a glass tube and evaporated to dryness under nitrogen. The extract was suspended in 100 μL of hexane, vortexed, and transferred to a gas chromatography vial. Samples were run against a known analytical standard solution (47885-U Supelco 37 component FAME Mix, 10 mg/mL in methylene chloride; Sigma-Aldrich).

Flow Cytometric Analysis of Immune Cell Phenotypes

Cells (10^6) were stained with anti-CD3, anti-CD4, anti-CD8, or BU-1A (B-cell marker), purchased from Serotec, Oxfordshire, UK, for 30 min at 4°C. A negative sample was processed without antibody staining. Samples were treated with lysis buffer for 20 min at room temperature to get rid of the red blood cells and then washed twice and fixed with 500 μL of Cell Fix (AbD Serotec, Oxford, UK; diluted 1:10 with dH_2O). Proportions of immune cells were determined using the FACSCalibur (Becton Dickson, Franklin Lakes, NJ) flow cytometer. Dead cells and debris were determined using forward and side scatter and were excluded from phenotype analysis by gating of the desired viable populations.

Phagocytic Activity of Peripheral Blood Leukocytes in Whole Blood

Phagocytic activity in whole blood was performed using phagotest commercial kits (ORPEGEN-Pharma, Heidelberg, Germany). Briefly, 100 μL of heparinized blood was cooled on ice for 10 min and incubated for 60 min at 41°C with 20 μL of *Escherichia coli* bacterial suspension, opsonized, and conjugated with a fluorochrome substrate (fluorescein isothiocyanate). Control samples were prepared and kept on ice. Samples were washed, lysed, and fixed according to the kit instructions. Leukocyte DNA was then stained with 200 μL of DNA staining solution. Samples were kept in the dark on ice until analysis with an FACSCalibur flow cytometer. Data were collected from 50,000 events. Discrete populations of polymorphonuclear heterophils and monocytes were gated in the software program, based on identification by forward and side scatter. Phagocytic activity was expressed as the percentage of cells participating in phagocytosis. Mean fluorescence intensity (**MFI**) was also recorded; this is the relative degree or extent of phagocytosis, reflected by the mean number of ingested *E. coli* bacteria per phagocyte.

Mitogenic Responses of Lymphocytes

Carboxyfluoroscein succinimidyl ester stain was diluted by approximately 1:100 in spleen and thymus cell

Table 2. Body weight gain (g) of birds fed different dietary treatments during the period from 19 to 47 d of age[1]

	Fish oil inclusion in diet (g/kg)				
Age (d)	0	30	60	SEM	*P*-value
19–26	427	383.1	381.8	42.49	0.699
26–33	639	643.2	603.3	86.91	0.939
33–40	435.2	405.8	386.8	112.89	0.955
40–47	421.7	327.3	564.2	98.72	0.263

[1]Differences between the treatment groups are statistically different at $P \leq 0.05$; n = 6 per treatment.

suspensions. The solution (cell suspension + stain) was incubated at 41°C for 10 min in an atmosphere containing 5% CO_2. Cells were washed twice, adjusted to 1×10^6 cells/mL, and cultured in 48-well microtiter plates in the presence or absence of concanavalin-A (**ConA**, 25 μg/mL), phytohaemagglutinin (**PHA**, 10 μg/mL for splenocytes and 50 μg/mL for thymocytes) or Pansorbin cells (*Staphylococcus aureus*, which stimulate B cells in spleen preparations; used at 80×10^5/well), and 10% autologous plasma. The optimum concentration of each mitogen was determined in preliminary studies (data not shown). The cultures were incubated for 72 h at 41°C in an atmosphere containing 5% CO_2. After this time, cells were transferred to FACS tubes and kept on ice until analysis using the FACSCalibur flow cytometer. FlowJo (Flow Cytometry Analysis Software, Tree Star Inc., Ashland, OR) was used to model cell proliferation data obtained. Lymphocyte proliferation was assessed as a division index (average number of divisions; for example, if half of the cells in the starting population divided and the average number of divisions was 4, the division index would be 2), proliferation index (average number of divisions that those cells that divided underwent, ignoring undivided cells), and the percentage of divided cells (proportion of the starting cell population that participated in the division event).

Statistical Analysis

The overall differences between dietary treatments were analyzed using one-way ANOVA, and the GLM procedure of Minitab (Minitab Inc., State College, PA) was applied. Differences between the treatment groups were considered statistically different at $P \leq 0.05$. When significant differences occurred, treatment mean differences were identified by pairwise comparison using the Bonferroni test. Some data were arcsine transformed to achieve normality. In case of nonparametric cases, medians were used and the Kruskal-Wallis test was applied.

RESULTS

BW Gain

The average weekly BW gains of the broilers fed 0, 30, and 60 g/kg of FO during the period from 19 to 47 d of age are shown in Table 2. There were no significant differences after feeding FO.

Immune Tissue Weight

The effect of increasing levels of FO on immune tissue weight (as a percentage of final BW) in broiler chickens is shown in Table 3. Results show that FO did not affect the weights of the spleens of broiler chickens. Chickens fed diets containing 50 g/kg of FO had significantly greater thymus weights compared with chickens fed 0, 30, or 60 g/kg of FO ($P < 0.001$). Chickens fed a diet containing 50 and 60 g/kg of FO had significantly lower bursa weights ($P < 0.001$) than those of chickens fed diets containing no FO or 30 g/kg of FO. In addition, the bursae were thinner in appearance compared with control bursae and those from chickens fed diets containing less FO (not shown).

Plasma Fatty Acid Profile

The n-3 PUFA-enriched diets (30, 50, and 60 g/kg of FO) significantly decreased the proportion of arachidonic acid (**AA**, C20:4 n-6). There was a substantial increase in the level of EPA (C20:5 n-3) and DHA (C22:6 n-3) after feeding FO (30, 50, and 60 g/kg of FO). Docosapentaenoic acid (C22:5 n-3) was significantly increased after feeding diets containing 50 and 60 g/kg of FO but not after feeding the diet containing 30 g/kg of FO (Table 4). Feeding chickens a diet containing FO (30, 50, and 60 g/kg) significantly decreased

Table 3. Effect of fish oil on immune tissue weights in broiler chickens[1]

	Tissue (% of BW)		
Diet (g/kg of fish oil)	Spleen	Thymus	Bursa
0	0.12	0.15^{B}	0.30^{A}
30	0.15	0.17^{B}	0.28^{A}
50	0.11	0.33^{A}	0.14^{B}
60	0.12	0.12^{B}	0.14^{B}
SEM	0.01	0.02	0.02
P-value	0.394	<0.001	<0.001

[A,B]For each tissue weight percentage in columns, values with different superscripts are significantly different.

[1]Differences between the treatment groups are statistically different at $P \leq 0.05$; n = 6 per treatment.

Table 4. Plasma fatty acid composition of 6-wk-old broilers fed increasing levels of fish oil (0, 30, 50, and 60 g/kg)[1]

Fatty acid (% of total weight)	Fish oil (g/kg)				SEM	*P*-value
	0	30	50	60		
C14:0	21.06	10.27	10.18	10.33	3.105	0.051
C16:0	9.76^{B}	19.77^{A}	21.90^{A}	20.31^{A}	2.124	0.006
C16:1	0.38^{C}	0.58^{BC}	1.51^{A}	0.91^{B}	0.096	<0.001
C18:0	13.34	13.10	14.24	11.82	0.185	0.563
C18:1 n-9	13.76^{A}	12.73^{A}	14.61^{A}	8.48^{B}	0.872	0.002
C18:2 n-6 *trans*	1.50	1.55	1.80	1.69	0.099	0.176
C18:2 n-6 *cis*	21.34^{a}	16.11^{ab}	16.91^{ab}	14.71^{b}	1.440	0.035
C18:3 n-6[2]	0.13	0.09	0.09	0.07	—	0.104
C18:3 n-3	1.80	1.46	1.89	1.22	0.156	0.051
C20:4 n-6	8.73^{A}	3.00^{B}	2.11^{B}	2.54^{B}	0.924	0.001
C20:5 n-3	1.16^{C}	4.68^{B}	8.85^{A}	9.16^{A}	0.391	<0.001
C22:5 n-3	1.00^{B}	1.54^{B}	2.57^{A}	2.68^{A}	0.138	<0.001
C22:6 n-3	2.52^{C}	6.85^{B}	9.23^{A}	11.09^{A}	0.578	<0.001
ΣSAT[3]	44.17	43.14	37.13	42.46	2.475	0.238
ΣMUFA[4]	14.15^{A}	13.32^{A}	16.12^{A}	9.39^{B}	0.906	0.002
ΣPUFA[5]	38.19	35.28	43.45	43.16	2.781	0.161
Σn-6[6]	31.71^{A}	20.76^{B}	20.91^{B}	19.01^{B}	2.129	0.005
Σn-3[7]	6.48^{C}	14.52^{B}	22.54^{A}	24.16^{A}	0.950	<0.001
Σn-6:Σn-3	4.95^{A}	1.43^{B}	0.93^{B}	0.79^{B}	0.162	<0.001

$^{A–C}$Means within rows are significantly different at $P \leq 0.01$.

^{a,b}Means within rows are significantly different at $P \leq 0.05$; one-way ANOVA and the GLM procedure of Minitab were applied. Treatment mean differences were identified by Bonferroni tests. Some data were arcsine transformed to achieve normality.

[1]n = 4 for each dietary treatment, pooled SEM.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

[3]ΣSAT = sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[4]ΣMUFA = sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1 n-9 *trans*, C18:1 n-9 *cis*, C22:1 n-9).

[5]ΣPUFA = sum percentage of polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C22:5 n-3, C22:6 n-3).

[6]Σn-6 = sum percentage of n-6 polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C20:4 n-6, C20:2 n-6, C20:3 n-6).

[7]Σn-3 = sum percentage of n-3 polyunsaturated fatty acids (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

the percentage of total n-6 PUFA, increased the percentage of total n-3 PUFA, and decreased the ratio of n-6:n-3 fatty acids (Table 4).

Fatty Acid Profile of Immune Tissues

The effect of feeding broiler chickens increasing levels of FO (0, 30, 50, and 60 g/kg) on bursal, splenic, thymic, and blood leukocytes is shown in Tables 5 to 8. There was no significant effect of FO-enriched diets on proportions of α-linolenic acid in the bursa. Feeding diets containing FO significantly lowered the total amount of n-6 PUFA, AA (C20:4 n-6), and the n-6:n-3 ratio in the case of bursa (Table 5), spleen (Table 6), thymus (Table 7), and blood leukocytes (Table 8) compared with those of the control diet. Conversely, the proportions of n-3 PUFA (EPA, docosapentaenoic acid, and DHA) in the bursa, spleen, thymus, and blood leukocytes were significantly increased compared with those of the control diet when FO was fed.

Immune Cell Phenotypes

The effect of FO on leukocyte subsets in the peripheral blood (Table 9), spleen, and thymus was investigated after feeding 30 and 60 g/kg of FO (Table10 and Table 11). Statistically, there was no significant effect of FO on the percentage positive or MFI of the leukocyte subsets under investigation. However, there was a trend toward a decrease in the MFI of $CD8^{+}$ subsets in the spleen, which was close to statistical significance ($P = 0.054$).

Phagocytic Activity of Monocytes and Heterophils

The percentage of monocytes engaged in phagocytosis was significantly lower after feeding 60 g/kg of FO than after feeding 50 g/kg of FO. On the contrary, feeding 60 g/kg of FO significantly increased MFI of monocytes relative to feeding 50 g/kg of FO (Table 12). There was no significant effect of FO on phagocytosis by heterophils.

Mitogen-Stimulated Proliferation of T and B Lymphocytes

Concanavalin A-, PHA-, and Pansorbin-mediated thymocyte and splenocyte proliferation, expressed as a division index, proliferation index, and percentage of

Table 5. Bursa fatty acid composition of 6-wk-old broilers fed increasing levels of fish oil (0, 30, 50, and 60 g/kg)[1]

Fatty acid (% of total weight)	Fish oil (g/kg)				SEM	*P*-value
	0	30	50	60		
C14:0[2]	1.07	1.11	1.8	1.2	—	0.206
C16:0	23.43	22.46	20.99	25.21	0.751	0.051
C16:1	3.66	3.55	3.84	4.47	0.309	0.205
C18:0	11.03	10.69	10.78	9.50	1.511	0.892
C18:1 n-9	33.72	36.06	27.90	32.27	2.055	0.086
C18:2 n-6	14.42^{A}	11.39^{A}	6.07^{B}	5.20^{B}	1.310	0.001
C18:3 n-6	0.61^{a}	0.23^{b}	0.17^{b}	0.26^{b}	0.087	0.015
C18:3 n-3	1.13	1.10	1.86	1.63	0.422	0.523
C20:3 n-6	1.18^{A}	0.71^{B}	0.43^{B}	0.66^{B}	0.071	<0.001
C20:4 n-6	5.97^{A}	2.39^{B}	1.70^{B}	2.56^{B}	0.281	<0.001
C20:5 n-3	0.64^{C}	2.43^{B}	3.97^{A}	4.21^{A}	0.310	<0.001
C22:5 n-3	2.59^{B}	3.67^{AB}	4.28^{AB}	5.25^{A}	0.450	0.009
C22:6 n-3	1.22^{C}	3.36^{B}	3.75^{AB}	5.04^{A}	0.335	<0.001
ΣSAT[3]	35.56	34.28	33.42	36.03	1.025	0.304
ΣMUFA[4]	37.38	39.62	31.74	36.74	2.250	0.143
ΣPUFA[5]	27.77	25.29	22.24	24.82	1.461	0.118
Σn-6[6]	34.13^{A}	19.52^{B}	11.79^{C}	13.81^{C}	1.320	<0.001
Σn-3[7]	5.59^{C}	10.56^{B}	13.86^{AB}	16.13^{A}	0.962	<0.001
Σn-6:Σn-3	6.12^{A}	1.88^{B}	0.87^{C}	0.87^{C}	0.143	<0.001

$^{A–C}$Means within rows are significantly different at $P \leq 0.01$.

^{a,b}Means within rows are significantly different at $P \leq 0.05$; one-way ANOVA and the GLM procedure of Minitab were applied. Treatment mean differences were identified by Bonferroni tests. Some data were arcsine transformed to achieve normality.

[1]Values are expressed as means (n = 4 for each dietary treatment), pooled SEM.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

[3]ΣSAT = sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[4]ΣMUFA = sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1 n-9 *trans*, C18:1 n-9 *cis*, C22:1 n-9).

[5]ΣPUFA = sum percentage of polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C22:5 n-3, C22:6 n-3).

[6]Σn-6 = sum percentage of n-6 polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C20:4 n-6, C20:2 n-6, C20:3 n-6).

[7]Σn-3 = sum percentage of n-3 polyunsaturated fatty acids (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

divided cells, was analyzed. Collectively, there was an inhibition on the order of 50 to 70% after feeding 50 g/kg of FO.

Dietary FO significantly decreased thymocyte proliferation, expressed as the division index in response to 25 μg/mL of ConA ($P = 0.001$); see Figure 1a. There was a significant effect of dietary treatment on the proliferation index of thymocytes stimulated with 25 μg/mL of ConA ($P = 0.018$). As with the division index, feeding FO significantly decreased the proliferation index of thymocytes compared with that of chickens fed the control diet (Figure 1b). There was no effect of FO on percentage of divided thymocytes ($P = 0.173$; Figure 1c). There was a significant effect of dietary treatment on the proliferation index of thymocytes stimulated with 50 μg/mL of PHA (T-cell mitogen). Feeding FO significantly decreased the proliferation index of thymocytes compared with that of chickens fed the control diet ($P = 0.010$; Figure 1a). Feeding 50 g/kg of FO did not affect the division index ($P = 0.200$) or the percentage of divided cells ($P = 0.836$) of PHA-stimulated thymocytes; see Figure 1a,c.

The division indices for 25 μg/mL of ConA-stimulated splenocytes were significantly decreased after feeding FO compared with those of chickens fed the control diet ($P = 0.023$; Figure 2a). Similarly, the proliferation index of ConA-stimulated splenocytes was significantly decreased after feeding FO compared with that of chickens fed the control diet ($P = 0.007$; Figure 2b). Feeding FO did not affect the percentage of divided splenocytes stimulated with ConA; see Figure 2c ($P = 0.157$).

Feeding 50 g/kg of FO did not affect the division index ($P = 0.351$), the proliferation index ($P = 0.159$), or the percentage of divided splenocytes ($P = 0.417$) stimulated with PHA; see Figure 2a,b,c, respectively.

The division and the proliferation indices of splenocytes to Pansorbin stimulation (B-cell mitogen) were significantly decreased after feeding FO compared with those of chickens fed the control diet (Figure 2a,b; division index, $P = 0.017$; and proliferation index, $P = 0.005$). Feeding FO did not affect the percentage of divided splenocytes to Pansorbin ($P = 0.111$; Figure 2a).

DISCUSSION

The objective of this study was to investigate the effects of different dietary levels of FO on immune function in broiler chickens. The immune parameters studied were immune tissue weight, immune phenotypes,

Table 6. Spleen leukocytes fatty acid composition of 6-wk-old broilers fed increasing levels of fish oil (0, 30, 50, and 60 g/kg)[1]

Fatty acid (% of total weight)	Fish oil (g/kg)				SEM	*P*-value
	0	30	50	60		
C14:0	0.38	0.33	0.43	0.64	0.075	0.058
C16:0	20.57^{B}	22.50^{AB}	19.01^{C}	24.40^{A}	0.663	0.001
C16:1	0.47^{B}	0.49^{B}	1.48^{A}	1.06^{AB}	0.201	0.010
C17:1[2]	0.11	0.07	2.50	0.06	—	0.039
C18:0	18.26^{AB}	17.28^{B}	15.39^{C}	18.89^{A}	0.251	<0.001
C18:1 n-9	22.23^{A}	17.87^{B}	13.38^{C}	14.24^{C}	0.354	<0.001
C18:2 n-6	12.74^{A}	12.68^{A}	8.15^{C}	10.56^{B}	0.266	<0.001
C18:3 n-6[2]	0.40	0.20	0.15	0.20	—	0.119
C18:3 n-3[2]	0.65	0.46	1.22	0.24	—	0.004
C20:3 n-6[2]	1.19	1.30	0.14	1.15	—	0.013
C20:4 n-6	13.51^{A}	9.72^{B}	5.90^{C}	7.32^{C}	0.390	<0.001
C20:5 n-3	0.50^{B}	3.60^{B}	6.21^{A}	6.29^{A}	0.325	<0.001
C22:5 n-3	2.10^{B}	4.54^{A}	5.29^{A}	5.25^{A}	0.190	<0.001
C22:6 n-3	1.65^{C}	5.23^{B}	5.29^{B}	6.50^{A}	0.272	<0.001
ΣSAT[3]	39.20^{B}	40.11^{B}	34.83^{C}	43.93^{A}	0.686	<0.001
ΣMUFA[4]	22.81^{A}	19.17^{B}	17.38^{B}	15.37^{C}	0.634	<0.001
ΣPUFA[5]	39.10^{C}	58.43^{AB}	54.01^{B}	63.33^{A}	1.540	<0.001
Σn-6[6]	27.83^{A}	23.89^{A}	14.74^{C}	19.23^{B}	0.474	<0.001
Σn-3[7]	5.08^{B}	13.81^{B}	18.43^{A}	18.29^{A}	0.591	<0.001
Σn-6:Σn-3	5.53^{A}	1.73^{B}	0.81^{C}	1.06^{B}	0.176	<0.001

$^{A–C}$Means within rows are significantly different at $P \leq 0.05$; one-way ANOVA and the GLM procedure of Minitab were applied. Treatment mean differences were identified by Bonferroni tests. Some data were arcsine transformed to achieve normality.

[1]Values are expressed as means (n = 4 for each dietary treatment), pooled SEM.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

[3]ΣSAT = sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[4]ΣMUFA = sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1 n-9 *trans*, C18:1 n-9 *cis*, C22:1 n-9).

[5]ΣPUFA = sum percentage of polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C22:5 n-3, C22:6 n-3).

[6]Σn-6 = sum percentage of n-6 polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C20:4 n-6, C20:2 n-6, C20:3 n-6).

[7]Σn-3 = sum percentage of n-3 polyunsaturated fatty acids (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

phagocytosis, and lymphocyte proliferation. The effect of feeding broiler chickens diets containing increasing levels of FO on the fatty acid composition of plasma and immune cells was also investigated in this study. The doses used in this work were 30, 50, and 60 g of FO/kg. This range was used to include the dose that is used commercially (30 g/kg) and to cover doses (50 and 60 g/kg) that would significantly increase the enrichment of n-3 PUFA in chicken meat.

Immune tissue development can in some cases reflect immune system response and functionality. In the current study, FO did not affect the growth of the spleen. However, some studies do suggest that feeding PUFA to chickens (Wang et al., 2000) and mice (Ellis et al., 1986) results in increased spleen weights. In the study of Wang et al. (2000), the author used single-comb White Leghorn layers fed sunflower oil, animal oil, linseed oil, or fish oil at 5% (wt/wt). The results demonstrated that chicks fed the 3 PUFA-rich diets (sunflower, linseed, or fish oils) had significantly higher weights of the thymus, spleen, and bursa compared with those of chicks fed the diet with animal oil. In the current study, chickens fed diets containing 50 g/kg of FO had significantly greater thymus weights compared with chickens fed 0, 30, and 60 g/kg. It is not clear why the increased thymus weight was not maintained after feeding chickens on diets with 60 g/kg of FO. The increased thymus weight at 50 g/kg of FO is in agreement with the study by Wang et al. (2000). The increased thymus weight in the case of chickens fed 50 g/kg of FO in the current study did not affect the proportions of T cells in the blood, thymus, or spleen. Chickens fed a diet containing 50 and 60 g/kg of FO had significantly lower bursa weights than those of chickens fed the control diet or 30 g/kg of FO. In addition, the bursae were thinner in appearance compared with bursae from chickens fed diets containing less FO.

In the current study, fatty acid data are reported for plasma and immune cells isolated from the blood, spleen, thymus, and bursa in chickens fed n-3 PUFA. Results indicated that, as expected, the fatty acid composition of the plasma and immune tissues reflected that of the diet, especially for the key fatty acids that characterize the dietary treatment. The effect of the FO diet was most evident for the long-chain n-3 PUFA. In general, chickens fed an FO-enriched diet had significantly higher EPA, docosapentaenoic acid, DHA, and total n-3 fatty acids in their plasma, bursae, spleens, thyme, and blood leukocytes compared with those of chickens fed the control diet. The AA, total n-6 fat-

Table 7. Thymus leukocytes fatty acid composition of 6-wk-old broilers fed increasing levels of fish oil (0, 30, 50, and 60 g/kg)[1]

Fatty acid (% of total weight)	Fish oil (g/kg)				SEM	*P*-value
	0	30	50	60		
C16:0	21.66^{B}	24.86^{AB}	22.01^{B}	28.39^{A}	1.045	0.002
C16:1	1.19	1.54	1.38	1.15	0.185	0.443
C18:0	14.73	14.71	14.42	16.46	0.515	0.060
C18:1 n-9	21.54^{A}	21.57^{A}	16.24^{AB}	14.26^{B}	1.482	0.008
C18:2 n-6	12.05^{A}	12.44^{A}	7.07^{B}	8.00^{B}	0.801	0.001
C18:3 n-6[2]	0.60	0.42	0.27	0.31	—	0.026
C18:3 n-3	0.25^{b}	0.89^{b}	0.98^{b}	1.36^{a}	0.193	0.011
C20:2 n-6	0.96^{A}	0.62^{B}	0.26^{C}	0.45^{C}	0.072	<0.001
C20:4 n-6	7.40^{a}	4.64^{ab}	4.14^{ab}	3.02^{b}	0.936	0.036
C20:5 n-3	0.73^{C}	4.27^{B}	7.04^{A}	8.64^{A}	0.494	<0.001
C22:5 n-3	1.54^{C}	3.37^{B}	4.86^{A}	5.56^{A}	0.275	<0.001
C22:6 n-3	1.41^{C}	4.46^{B}	5.51^{B}	7.40^{A}	0.341	<0.001
ΣSAT[3]	36.39^{B}	39.57^{AB}	36.43^{B}	44.85^{A}	1.502	0.006
ΣMUFA[4]	22.73^{a}	23.12^{a}	17.62^{ab}	15.41^{b}	1.636	0.014
ΣPUFA[5]	26.11^{b}	31.11^{ab}	29.35^{ab}	34.76^{a}	1.522	0.012
Σn-6[6]	21.44^{A}	18.11^{A}	10.57^{B}	12.91^{B}	1.165	<0.001
Σn-3[7]	4.66^{C}	12.99^{B}	18.78^{A}	21.85^{A}	0.814	<0.001
Σn-6:Σn-3	4.77^{A}	1.40^{B}	0.58^{B}	0.59^{B}	0.199	<0.001

$^{A–C}$Means within rows are significantly different at $P \leq 0.01$.

^{a,b}Means within rows are significantly different at $P \leq 0.05$; one-way ANOVA and the GLM procedure of Minitab were applied. Treatment mean differences were identified by Bonferroni tests.

[1]Values are expressed as means (n = 4 for each dietary treatment), pooled SEM.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

[3]ΣSAT = sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[4]ΣMUFA = sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1 n-9 *trans*, C18:1 n-9 *cis*, C22:1 n-9).

[5]ΣPUFA = sum percentage of polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C22:5 n-3, C22:6 n-3).

[6]Σn-6 = sum percentage of n-6 polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C20:4 n-6, C20:2 n-6, C20:3 n-6).

[7]Σn-3 = sum percentage of n-3 polyunsaturated fatty acids (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

ty acids, and n-6:n-3 ratios were significantly lower in plasma and immune tissues of FO-fed chickens. There was a tendency for EPA and DHA incorporation into the plasma and immune tissues to be associated with a significant decrease in the proportion of AA by 50 to 67%. This trend was also observed in all human and rodent studies in the field of n-3 PUFA enrichment (Fritsche, 2007). It was reported that n-3 PUFA, particularly EPA, competes with AA for incorporation into the phospholipid bilayer of cell membranes of all body cells (Simopoulos, 1994; Surai and Sparks, 2000). Both AA and EPA are precursors for eicosanoids, which play a crucial role in inflammatory responses in both humans and animals, including poultry. It was suggested earlier by Edwards and Marion (1963) and Miller and Robisch (1969) that the reduced level of AA after feeding FO might be because of the inhibitory effect of n-3 PUFA on the synthesis of AA from linoleic acid. It was noted from the current results that the spleen contained more AA, EPA, and DHA than the thymus, bursa, or plasma (in most cases). This observation may be related to the fact that the spleen is a major immune organ with a high capacity for eicosanoid production, which is predominated by AA. This confirms the findings of Fritsche et al. (1991b), who investigated the effect of dietary fat source on the relative amount of eicosanoid precursors present in the spleen, thymus, and bursa of chickens. Authors of that study reported that the highest proportion of AA was present in the splenocytes. Feeding broiler chickens n-3 PUFA in the current study (30, 50, and 60 g/kg of FO) did not significantly alter the proportions of total saturated fatty acids in the plasma and bursae, which is in agreement with other studies (Fritsche et al., 1991b; Friedman and Sklan, 1995). Contrary to those observations, feeding FO significantly increased the total amount of saturated fatty acids in the spleens and thymi. Similarly, the total amount of PUFA was not affected by FO feeding in the plasma and bursae, but was significantly increased in the spleens and thymi of chickens fed diets containing 60 g/kg of FO.

Immunophenotyping represents a potentially important measure of immune status. Results in the current study showed that there was no effect of FO on subsets of $CD3^+$, $CD4^+$, $CD8^+$, and B-lymphocytes in the peripheral blood or spleen. Similarly, there was no effect of FO on subsets of $CD3^+$, $CD4^+$, and $CD8^+$ in the thymus. However, there was a trend toward a decrease in the MFI of $CD3^+$ subsets ($P = 0.088$) and of $CD8^+$ subsets ($P = 0.054$) in the spleen after feeding 60 g/kg of FO. The ratio between $CD4^+$ and $CD8^+$ T-lymphocytes after vaccination is thought to be a good reflection of the adaptive immune response, and any changes in this ratio may reflect changes in the functional immune

Table 8. Blood leukocytes fatty acid composition of 6-wk-old broilers fed increasing levels of fish oil (0, 30, 50, and 60 g/kg)[1]

Fatty acid (% of total weight)	Fish oil (g/kg)				SEM	*P*-value
	0	30	50	60		
C14:0[2]	0.38	0.30	2.63	0.31	—	0.177
C16:0	15.86	11.89	14.02	13.86	1.262	0.553
C16:1	0.17	0.64	1.13	0.71	0.154	0.124
C18:0	21.29^{A}	13.73^{B}	13.95^{B}	19.89^{A}	0.574	0.004
C18:1 n-9	19.53^{B}	33.37^{A}	15.28^{B}	14.90^{B}	0.626	<0.001
C18:2 n-6	11.75^{ab}	14.19^{a}	7.41^{b}	9.80^{ab}	0.913	0.039
C18:3 n-6	0.29	0.51	0.45	0.31	0.109	0.660
C18:3 n-3	0.31	2.03	2.15	0.08	0.543	0.181
C20:2 n-6	0.74	0.69	0.45	0.54	0.124	0.584
C20:3 n-6	1.98	0.90	0.60	1.97	0.232	0.053
C20:4 n-6	21.93^{A}	7.05^{C}	6.04^{C}	11.66^{B}	0.235	<0.001
C20:5 n-3	0.34^{C}	1.69^{C}	3.91^{B}	5.16^{A}	0.177	0.001
C22:5 n-3	1.33^{b}	1.55^{b}	3.97^{ab}	4.63^{a}	0.372	0.018
C22:6 n-3	0.83^{C}	2.40^{B}	4.23^{AB}	6.02^{A}	0.310	0.004
ΣSAT[3]	37.31^{A}	25.92^{D}	32.31^{C}	34.06^{B}	0.263	<0.001
ΣMUFA[4]	19.70^{B}	34.01^{A}	16.40^{B}	15.61^{B}	0.492	<0.001
ΣPUFA[5]	39.49^{A}	31.00^{B}	29.20^{B}	40.16^{A}	0.956	0.005
Σn-6[6]	36.68^{A}	23.34^{B}	14.95^{C}	24.27^{B}	0.777	0.001
Σn-3[7]	2.81^{C}	7.65^{B}	14.25^{A}	15.89^{A}	0.389	<0.001
Σn-6:Σn-3	13.05^{A}	3.05^{B}	1.05^{D}	1.53^{C}	0.057	<0.001

$^{A–D}$Means within rows are significantly different at $P \leq 0.01$.

^{a,b}Means within rows are significantly different at $P \leq 0.05$; one-way ANOVA and the general linear model procedure of Minitab were applied. Treatment mean differences were identified by Bonferroni tests.

[1]Values are expressed as means (n = 4 for each dietary treatment), pooled SEM.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

[3]ΣSAT = sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[4]ΣMUFA = sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1 n-9 *trans*, C18:1 n-9 *cis*, C22:1 n-9).

[5]ΣPUFA = sum percentage of polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C18:3 n-3, C20:2 n-6, C20:3 n-6, C20:4 n-6, C20:5 n-3, C22:2, C22:5 n-3, C22:6 n-3).

[6]Σn-6 = sum percentage of n-6 polyunsaturated fatty acids (C18:2 n-6 *trans*, C18:2 n-6 *cis*, C18:3 n-6, C20:4 n-6, C20:2 n-6, C20:3 n-6).

[7]Σn-3 = sum percentage of n-3 polyunsaturated fatty acids (C18:3 n-3, C20:5 n-3, C22:5 n-3, C22:6 n-3).

response in chickens (Parmentier et al., 1995; Erf et al., 1998; Li et al., 2008). For example, Erf et al. (1998) reported that this ratio in the spleen of 7-wk-old broilers reared on litter was less (0.12) than values for layers of a similar age reared in laboratory conditions (0.5–0.2). Authors suggested that this may reflect enhanced cytotoxic activity within the splenic T-cell compartment of broilers grown on litter compared with layers raised in laboratory conditions. Moreover, it was suggested that the low splenic CD4:CD8 ratio in broilers may be the result of genetic selection for growth performance, and suggests a negative effect of this selection on the broiler's immune system (Parmentier et al., 1995). In the current study, the ratio between $CD4^+$ and $CD8^+$ T cells in the thymus was lower than the range reported for broiler chickens (1.2–2.3; Erf et al., 1998). Lower $CD4^+$:$CD8^+$ ratios indicate enhanced cytotoxic activity exhibited by immune cells. The ratio between $CD4^+$ and $CD8^+$ T cells in the spleens of chickens in the current study was within the range of those reported for broiler chickens (0.5–0.2; Chen et al., 1991; Parmentier et al., 1995; Ewald et al., 1996). The ratio between $CD4^+$ and $CD8^+$ was lower in the spleen than in the thymus and blood after feeding all of the dietary treatments. This indicated that more cytotoxic activity is exhibited in the spleen than in the thymus and the

Table 9. Effect of fish oil on leukocyte subsets in peripheral blood[1]

Fish oil (g/kg)	Percentage of positive cells					Mean fluorescence intensity			
	CD3	CD4[2]	CD8[2]	BU-1A	CD4:CD8	CD3	CD4[2]	CD8[2]	BU-1A
0	87.09	29.00	21.13	7.69	1.55	365.54	153.8	274.70	191.00
30	79.76	26.70	21.73	6.89	1.38	357.07	153.4	296.10	145.10
60	89.19	29.29	22.46	11.75	1.38	412.87	160.4	284.80	215.30
SEM	2.97	—	—	—	0.19	65.99	—	—	—
P-value	0.094	0.587	0.932	0.271	0.779	0.815	0.796	0.755	0.810

[1]Differences between the treatment groups are statistically different at $P \leq 0.05$; n = 6 per treatment.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

Table 10. Effect of fish oil on leukocyte subsets in spleen[1]

Fish oil (g/kg)	Percentage of positive cells					Mean fluorescence intensity			
	CD3	CD4	CD8	BU-1A	CD4:CD8	CD3	CD4[2]	CD8	BU-1A
0	92.71	23.33	54.26	11.47	0.45	430.52	121.30	210.62	125.94
30	90.52	22.11	54.27	16.95	0.41	374.52	121.60	205.74	158.51
60	91.76	23.91	50.14	17.68	0.48	272.16	100.10	140.84	137.70
SEM	0.93	1.31	2.52	2.09	0.04	47.36	—	20.59	28.67
P-value	0.278	0.621	0.43	0.104	0.554	0.088	0.143	0.054	0.723

[1]Differences between the treatment groups are statistically different at $P \leq 0.05$; n = 6 per treatment.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

blood. A higher $CD4^+$:$CD8^+$ ratio in the blood compared with the spleen and thymus was also observed by Erf (1997).

The phagocytic activity of polymorphonuclear heterophils and monocytes was expressed as the percentage of cells participating in phagocytosis as well as MFI. To our knowledge, this is the first study investigating the effect of FO feeding on phagocytosis in broilers using flow cytometry. Results showed that the percentage of monocytes engaged in phagocytosis was significantly lower after feeding 60 g/kg of FO than after feeding 50 g/kg of FO. On the contrary, feeding 60 g/kg of FO significantly increased MFI, relative to feeding 50 g/kg of FO. There was no significant effect of FO on phagocytosis by heterophils. Studies in the literature reporting effects of n-3 PUFA on phagocytosis are inconsistent, with some studies showing enhanced phagocytosis, some showing a decrease, and some showing no effect. The enhanced phagocytic activity upon supplementation with n-3 PUFA in rodent and human studies was explained on the basis that the fatty acid composition of cell membranes affects their fluidity as well as changes in membrane-bound enzymes and receptor functions (Brenner, 1984; Stubbs and Smith, 1984; Calder and Newsholme, 1993). It was further suggested by Calder et al. (1990) and Calder (1998b) that, on the basis of in vitro experiments, membrane fluidity is important in determining macrophage adhesion and phagocytic activity upon enrichment with FO-derived fatty acids. On the other hand, some studies report that n-3 PUFA incorporation into cell membranes following dietary supplementation does not affect membrane fluidity (Yaqoob, 1993; Grimble and Tappia, 1995; Yaqoob et al., 1995b). Calder (1996) explained these contradictory results by suggesting that incorporation of dietary n-3 PUFA into membrane phospholipids may induce only a minimal effect on fluidity, that cholesterol contents may also be changed at the same time, and that undetectable partial changes of fluidity may occur in particular regions of the cell membrane. Furthermore, the composition and fluidity of immune cell membranes can be substantially altered in in vitro studies, whereas dietary studies achieve only a limited alteration of cell membrane composition and fluidity. Nevertheless, Kew et al. (2003) demonstrated that phagocytic activity and membrane fluidity of neutrophils and monocytes was negatively correlated with the palmitic acid content and with the ratio of saturated to PUFA. In the same study, a positive correlation between the activity of phagocytes and total PUFA, total n-6 PUFA, and total n-3 PUFA was also observed. The apparent immunosuppressive evidence of n-3 PUFA on the phagocytic activity of monocytes in the current study is of potential importance with respect to the concept of immunomodulation in poultry. Recruitment and activation of circulating phagocytes with rapid migration to the site of pathogen invasion is an effective host defense mechanism against infections, such as salmonellosis (Kogut et al., 1998, 2005). An important aspect of this concept is that the activated phagocytes act nonspecifically, thus providing protection against a multitude of invasive agents. When pathogens are phagocytosed, monocytes and heterophils present antigens of the pathogens to T-lymphocytes to stimulate the immune response, so to some extent, phagocytosis can reflect disease resistance of chickens.

The most profound effects of FO reported in this paper are for lymphocyte proliferation. Using several stimulants and carboxyfluoroscein succinimidyl ester staining, lymphocyte proliferation was assessed as a di-

Table 11. Effect of fish oil on leukocyte subsets in thymus[1]

Fish oil (g/kg)	Percentage of positive cells				Mean fluorescence intensity		
	CD3	CD4	CD8	CD4:CD8	CD3	CD4	CD8[2]
0	97.99	80.01	89.19	0.90	319.13	70.92	257.50
30	98.05	81.66	89.72	0.91	322.94	68.91	225.00
60	98.28	76.08	86.18	0.88	322.29	63.46	211.80
SEM	3.30	2.42	1.53	0.03	32.71	5.61	—
P-value	0.945	0.277	0.24	0.801	0.996	0.632	0.264

[1]Differences between the treatment groups are statistically different at $P \leq 0.05$; n = 6 per treatment.

[2]A nonparametric case in which medians were used and Kruskal-Wallis test was applied.

Table 12. Effect of fish oil on phagocytosis by monocytes and heterophils in broiler chickens fed 0, 30, 50, and 60 g/kg of fish oil[1]

	Monocyte		Heterophil	
Fish oil (g/kg)	% of positive cells	MFI[2]	% of positive cells	MFI
0	46.78^{ab}	2,148.00^{AB}	69.77	1,302.20
30	48.76^{ab}	2,325.60^{A}	81.04	3,775.10
50	55.82^{a}	224.5^{B}	73.14	314.70
60	35.16^{b}	2,379.4^{A}	72.85	1,850.30
SEM	4.840	513.8	5.107	—
P-value	0.050	0.024	0.465	0.008

^{A,B}Means within columns are significantly different at $P \leq 0.01$.

^{a,b}Means within columns are statistically different at $P \leq 0.05$; n = 6 per treatment.

[2]MFI = mean fluorescence intensity.

vision index, proliferation index, and the percentage of divided cells. Results from the current study indicate that feeding 50 g/kg of FO significantly decreased proliferation in response to ConA in both thymocytes and splenocytes, in most cases to such an extent that proliferation was almost entirely abolished. The proliferative response to PHA was much more variable than that to ConA, and as a result, there was no significant effect of FO when cells were stimulated with PHA, except for the case of proliferation index of thymocytes. However, FO significantly inhibited the proliferative response to Pansorbin, particularly when assessed as division and proliferation indices. It was generally observed that ConA exerted a greater stimulatory effect on lymphocyte proliferation than PHA or *Staph. aureus* bacteria. The poor response to bacteria could be related to the relative insensitivity in vivo to the effect of bacterial endotoxins (Adler and DaMassa, 1979; Fritsche et al., 1991a). Lower responses of spleen and thymus lymphocytes to PHA were also reported in other studies (Leca et al., 1986; Chilson and Kelly-Chilson 1989; Wang et al., 2000). Differences between ConA and PHA-mediated proliferative responses in this experiment may be explained by differences in signal transduction pathways and differences in the subsets of T-cells being stimulated. The lower response of lymphocytes to PHA

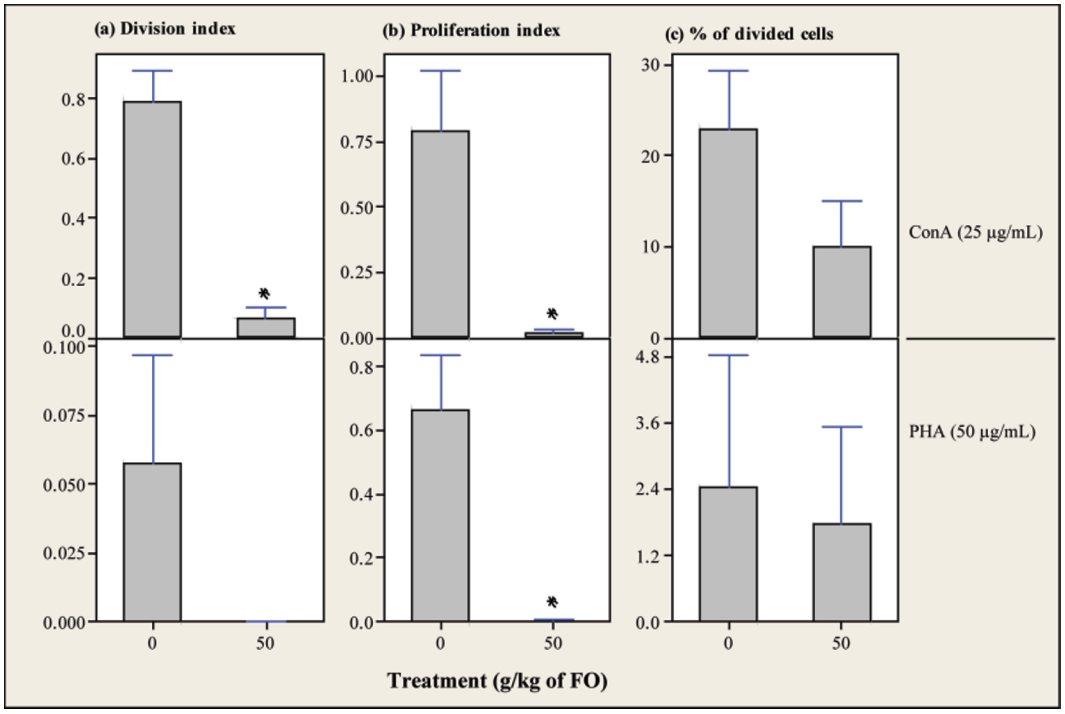

Figure 1. Effect of dietary fish oil (0–50 g/kg) on ConA- and PHA-mediated thymocyte proliferation of broiler chickens, expressed as (a) division index, (b) proliferation index, and (c) percentage of divided cells. Thymocytes were labeled with carboxyfluoroscein succinimidyl ester and then incubated with optimal concentrations of either ConA (25 µg/mL) on the upper part of the graph or PHA (50 µg/mL) on the lower part of the graph for 72 h in culture medium. Cells were then analyzed by flow cytometry. Each bar represents mean ± SEM (n = 4 per treatment) relative to the control (unstimulated sample). *Means are statistically different at $P \leq 0.05$. ConA = concanavalin A (µg/mL); PHA = phytohaemaglutinin (µg/mL); and FO = fish oil. Color version available in the online PDF.

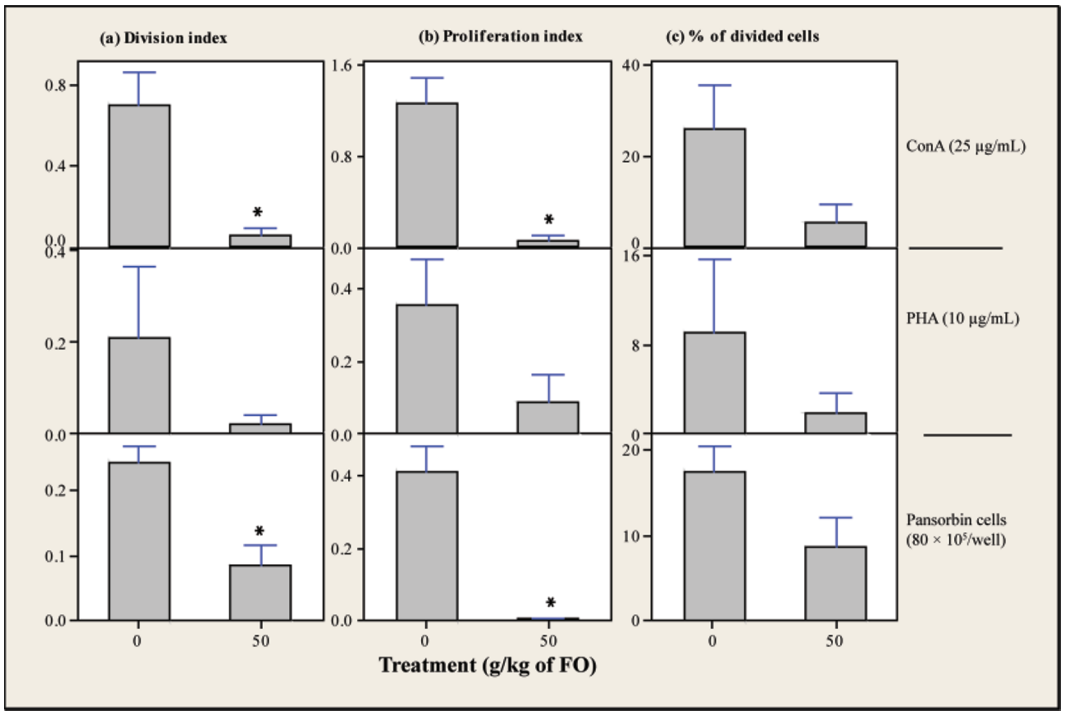

Figure 2. Effect of dietary fish oil (0–50 g/kg) on ConA-, PHA-, and Pansorbin-mediated splenocyte proliferation of broiler chickens, expressed as (a) division index, (b) proliferation index, and (c) percentage of divided cells. Splenocytes were labeled with carboxyfluoroscein succinimidyl ester and then incubated with optimal concentrations of ConA (25 µg/mL) on the upper part of the graph; PHA (10 µg/mL) on the middle part of the graph; or Pansorbin cells (80×10^5/well) on the lower part of the graph for 72 h in culture medium. Cells were then analyzed by flow cytometry. Each bar represents mean ± SEM (n = 4 per treatment) relative to the control (unstimulated sample). *Means are statistically different at $P \leq 0.05$. ConA = concanavalin A (µg/mL), PHA = phytohaemaglutinin (µg/mL), Pansorbin cells = *Staphylococcus aureus*, and FO = fish oil. Color version available in the online PDF.

compared with ConA may explain why there was little effect of FO on PHA-mediated T-cell proliferation compared with that of ConA in the current study. Taken together, there was an immunosuppressive effect of feeding broiler chickens diets containing 50 g/kg of FO on the proliferative response of splenocytes and thymocytes. Results of the current study are in agreement with 5 other studies in chickens (Cassity et al., 1990; Fritsche et al., 1991a; Wang et al., 2000; ZhaoGang et al., 2004; Yang et al., 2008). On the other hand, there are 3 studies reporting increased lymphocyte proliferation after feeding chickens diets rich in FO (Sijben et al., 2000, 2001; Babu et al., 2005) and 2 studies reporting no effect (Korver and Klasing, 1997; Puthpongsiriporn and Scheideler, 2005). The contradictory results might be because of different species and strains, age of birds, basal diet, level and type of oils used, type of stimulant, immune status of the animal under study, cell type, ratio of n-6 to n-3 PUFA, and different fatty acids of n-3 PUFA used for various studies. For example, the levels of FO used in the studies of Babu et al. (2005) and Klasing and Korver (1997) were moderate (30 and 20 g/kg of FO, respectively) compared with the levels used in the current study. In addition, the chicken strain and the techniques used were different among the 3 studies. Yaqoob (2010) also attributed the inconsistent results in human studies to the doses of fatty acids used, levels of EPA and DHA in different preparations of FO, differences in methodological approaches used, and the different range of immune function parameters under investigation.

The mechanism by which n-3 PUFA modulated the immune response was not investigated in the current study. However, it was suggested that eicosanoids regulate the production of cytokines (Miles and Calder, 1998). If n-3 PUFA are present, EPA compete with AA, inhibit arachidonic acid's oxygenation and direct the metabolic pathway toward leukotrienes, hydroperoxyeicosatetraenoic acid, hydroxyeicosatetraenoic acids, and lipoxins by the action of 5-, 12-, or 15-lipoxygenase (Yaqoob and Calder, 1995; Calder, 1997, 1999, 2005, 2006, 2008; Calder et al., 2002; Yaqoob, 2010). Some of the immunomodulatory effects of n-3 PUFA may result from effects on intracellular signaling pathways and transcription factor activity (Calder, 1998a, 2002, 2005; Calder and Burdge, 2004) and on lipid rafts (Schroeder et al., 1998; Cheng et al., 1999; Katagiri et al., 2001; Stulnig et al., 2001; Heerklotz, 2002), and suggested that the immunomodulatory effect of n-3 PUFA may be mediated by their effect on lipid raft structure and composition.

An ideal measurement of immunocompetence depends on vaccine- or pathogen-specific mounting of an immune response. The ex vivo immune markers used in this study (i.e., phagocytosis, immunophenotyping, immune tissue weights, natural killer cell activity, and

cell proliferation) have been considered to be suitable as biomarkers of immunity, especially if a combination of immune markers is investigated together (Albers et al., 2005). But, further work is required in animal models of pathogen exposure to evaluate this in a relevant pathological setting.

In summary, the current study suggests that feeding broiler chickens diets rich in n-3 PUFA suppresses some aspects of the immune response that are considered to be important lines of defense against tumor, viral, bacterial, and other infections. It remains to be determined whether this has an effect on risk of infection, which could have important implications for the poultry industry.

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