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Comparison of different dietary sources of n-3 polyunsaturated fatty acids on immune response in broiler chickens

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ABSTRACT

The study aims to research the effects of varied dietary sources of n-3 polyunsaturated fatty acids (PUFA) on the immune response in broiler chickens with stress on natural killer (NK) cell activity. Diets supplemented with one of the four sources of n-3 PUFA: linseed oil-, echium oil-, fish oil (FO) or algal biomass-enriched diets at levels of 18, 18, 50 and 15 g/kg fresh weight, were provided for one-d-old male Ross 308 broilers, totaling 340 in number, until they were slaughtered. The analyses included total lipid profile using gas chromatography (GC) for plasma, spleen, thymus, and blood. Additionally, NK cell activity and cell proliferation were investigated for thymocytes and splenocytes. The results indicated that the source of n-3 PUFA had a strong influence on fatty acid composition across all tissues. NK activity was highest in splenocytes and PBMCs from broilers fed linseed oil, followed by those fed algal biomass or echium oil, and lowest for those from broilers fed FO. The proliferative response of lymphocytes from algal biomass-fed chickens tended to be the highest, followed by those fed linseed oil in most cases. Lymphocytes from chickens fed fish oil showed the lowest proliferative response. These results could mean that a docosahexaenoic acid (DHA)-rich algal product might enrich chicken meat with n-3 PUFA without significant damaging effects on chicken immunity.

1. Introduction

Production of added-value poultry meat and eggs has received a considerable attention in recent years to diversify poultry products and to satisfy the needs of humans seeking healthy life styles. This can be approaches by using effective and functional ingredients in the poultry feed such as medicinal herbs, probiotics, prebiotics, natural growth promotors, and n-3 polyunsaturated fatty acids (PUFA) (Al-Khalaifa et al., 2019; Al-Khalaifah and Al-Nasser, 2018; Al-Khalaifah, 2018; Al-Khalifa, 2009; Al-Khalifa and Al-Naser, 2014; Al-Khalifa and Al-Nasser, 2012; Al-Khalifa and Ragheb, 2013; Al-Khalifa et al., 2013; Al-Nasser et al., 2015; Attia et al., 2017).

In the last decade or so, there has been some concern that diets enriched with PUFA may be harmful to chickens, and may negatively have an effect on their immunity and resistance to infections. However, this concern cannot be grounded as studies have shown varied results, with some studies showing no effect (Puthpongsiriporn and Scheideler, 2005), some showing the damaging effects of these PUFAs (Attia et al., 2019; Al-Khalifa et al., 2012, 2016; Babu et al., 2005; Fritsche et al., 1991), and yet others showing only marginal improvement (Parmentier et al., 1997; Puthpongsiriporn and Scheideler, 2005; Sijben et al., 2000; Yang and Yuming, 2006).

Previous research has indicated the immunosuppressive effect of n-3 PUFA on natural killer (NK) cell cytotoxicity in mice (Fritsche and Johnston, 1989; Meydani et al., 1988a), rats (Jeffery et al., 1996, 1997; Yaqoob et al., 1994), and humans (Almallah et al., 2000; Almallah et al., 1998; Yamashita et al., 1991). Research on animals has suggested that both, eicosapentaenoic acid (EPA) and DHA have immunomodulatory effects. A diet containing a total of 4.4 g/100 g total fatty acids of α-linolenic (ALA), γ-linolenic, arachidonic (ARA), EPA, and DHA were fed to rats. When ALA was replaced with EPA, it led to a decrease in ex vivo lymphocyte proliferation and NK cell activity (Peterson et al., 1998). The study by Thies et al. (2001b) on the effects of supplementation with fish oil, highly purified DHA, or a placebo on lymphocyte proliferation in healthy human subjects, showed that fish oil suppresses lymphocyte proliferation whereas DHA has no effect. This could mean either that EPA is responsible for the repressive effect or that both, EPA and DHA are required. It was also determined that fish oil, and not DHA, decreased NK

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cell activity (Thies et al., 2001a). Kelley et al. (1999) studied the results obtained on replacing 20% of dietary linoleic acid with 6 g DHA/d, there was no effect of DHA on lymphocyte proliferation, production of IL-2, antibody production, or delayed type hypersensitivity. In contrast, DHA appeared to decrease NK cell activity. Also, Yamashita et al. (1991) showed that peripheral blood mononuclear cells (PBMCs) of healthy human subjects exhibited lower NK activity after intake of EPA-enriched triacylglycerol. Thus, these observations suggested that EPA is chiefly responsible for the immunomodulatory effects of fish oil (FO). However, this is not unequivocal, either in rodents (Jenski et al., 1998; Sasaki et al., 1999) or in humans (Kew et al., 2004; Soyland et al., 1993).

A decrease in the proliferative capacity of splenocytes by 30–50% to Concanavalin A (Con A) in comparison to control, due to FO and linseed oil was observed in chickens (Fritsche et al., 1991; Wang et al., 2000; ZhaoGang et al., 2004). On the other hand, Sijben et al. (2001, 2001) fed laying hens different levels of α-linolenic acid (ALA). The result showed that the high level of ALA (1.8, and 2.7% of total diet) enhanced the proliferation of peripheral blood leukocytes with con A in *M. butyricum* immunized birds. Also, Puthpongsiriporn and Scheideler (2005) fed laying hens on diets with different ratios of linoleic acid and ALA. There was no effect of diet on proliferation of lymphocytes.

Based on the aforementioned studies it may be safe to make the assumption that the effect of n-3 PUFA on immune response in animals and humans is contradictory. Moreover, literature concerning the relative influence of EPA and/or DHA on immune function in chickens is limited. Studies have also shown that a dietary imbalance of n-6: n-3 PUFA ratios affect human health due to the presence of these high ratios in modern diets. This in turn results in an enlarged production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1 and interleukin-6 and thus excessively augments inflammation. Typically, formulated broiler diets are deficient in n-3 PUFA due to widening n-6: n-3 PUFA ratios. A study was conducted to assess the effect of modifying dietary n-6: n-3 PUFA ratio from plant and animal sources on performance, behavior, cytokine mRNA expression, antioxidative status and meat fatty acid profile of broilers. Birds were fed 7 diets enriched with different dietary oil sources such as sunflower oil, fish oil and linseed oil. The results showed that narrowing down the n-6:n-3 ratios by the addition of FO or linseed oil improved performance and immune response of broilers and therefore, resulted in healthy chicken meat enriched with n-3 PUFA (Ibrahim et al., 2018). The objective of this study was to compare the effect of feeding normal broiler chickens four different sources of n-3 PUFA containing linseed oil, echium oil, FO or algal biomass at levels of 18, 18, 50 and 15 g/kg fresh weight, respectively, to match for n-3 PUFA content, on NK cell activity and proliferative response in broiler chickens.

2. Materials and methods

2.1. Animals and diets

For this study, a total of 340 one-day-old male Ross 308 broiler chicks were arranged from the poultry farm (PD Hook Hatcheries, Oxfordshire, UK). These chicks were vaccinated for the avian Infectious Bronchitis (IB). They were also supplied with water and feed as and when required. The ambient temperature was maintained at 30 °C without any fluctuations for duration of 14 d, and then gradually lowered to 21 °C till day 21. For the next 21 days, the chicks consumed a basal diet, post hatching. Four n-3 PUFA enriched sources were fed to the 3-wk-old broilers. They were distributed into four batteries, 85 birds per battery. Each battery consisted of five levels and the space of each level was 0.85 m^2. There were 17 birds in each level, providing a space of 0.05 m^2 for each bird. A random distribution of the four dietary supplements was followed for the four batteries. Twenty birds were slaughtered from each dietary treatment and were randomly used for analyses (n = 20). Samples derived from each bird were analyzed in quintuplicate. The procurement of the various n-3 PUFA sources used were fish oil from United Fish Industries Ltd, (Gilbey Road, Grimsby, UK), algal biomass (DHA Gold) from Martek Biosciences Corporation (Maryland, USA), echium oil from Croda (Leek, UK) and linseed oil from Dairy Direct (Bury St Edmunds, UK). Diets were formulated to consist of uniform amounts of n-3 PUFA. Formulation of broiler diets was done as per the Ross 308 broiler guidelines (Ross Broilers, 2007). Table 1 shows the composition of the four finisher experimental diets used. The fatty acid composition of the various oils employed for the experimental diets is shown in Table 2. The fatty acid profile of the four experimental diets is shown in Table 3.

2.2. Sample collection

All the bleeding procedures were approved by the Poultry Production Department at Kuwait Institute for Scientific Research and by the University of Reading, UK. This procedure follows the official decrees of the Ministry of Agriculture in Egypt relevant to animal welfare No. 27 (1967). These procedures are humane, respect animal rights and welfare and do not cause any suffering to animals. The practice of stunning and bleeding were used to slaughter the birds. To further elaborate, the bird was restrained and electrically stunned, it was then bled through a cut made in the neck. This procedure is aimed at minimizing the pain felt by the bird and the distress that could occur during bleeding out. It also immobilizes the bird to allow easy and accurate neck cutting (Gregory, 1995; Guarnieri et al., 2004). Approximately 15 ml of blood was collected in heparinized tubes into which additional heparin was added to prevent clotting. The thymus and spleen were separated carefully to avoid any contamination, all extra fat and tissues were removed, it was then weighed and placed in cell culture medium (CCM) on ice. Composition of CCM included RMPI-1640 supplemented with 10% fetal calf serum, 2 mmol/1 glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin, Boehringer Mannheim, Germany).

2.3. Preparation of leukocytes from lymphoid tissues

Lympholyte-H (Cedarlane Laboratories Ltd) was used to layer the collected heparinized blood by 1:1 volume. After centrifugation, the leukocytes in the interface layer were collected and suspended in CCM. The layering and centrifugation step was repeated with the interface layer to eliminate the red blood cells. The leukocytes were washed and suspended again in CCM. The freshly harvested spleen and thymus were dispersed across a wire mesh strainer and filtered to eliminate the excess debris. The solution containing the lymphocytes was then layered, centrifuged, and re-suspended in CCM as described earlier for the blood.

2.4. Fatty acid (FA) analysis by gas chromatography

Fatty acid methyl esters were analyzed using a Hewlett Packard (HP) 6890 series gas chromatography (GC) system (HP, Basingstoke, UK). Approximately 5–9x10^7 cells were centrifuged and suspended in 400 μl 0.9% w/v NaCl. For lipid extraction, 5 ml chloroform: methanol (2:1 v/v, 50 mg/l butylated hydroxytoluene, BHT) and 1ml 1M NaCl were added to the sample in a 10ml glass tube. Tubes were vortexed and centrifuged at 930 x g for 10 min. The bottom layer was transferred to a glass tube and evaporated to dryness under nitrogen. For transmethylation, 400 μl toluene (+BHT 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 neutralizing agent (0.1M K_2CO_3, 0.1M $KHCO_3$) and 2 ml hexane were added and the suspension was mixed and centrifuged at 1162 x g for 10 min. The upper layer was transferred to a glass tube and evaporated to dryness under nitrogen. The extract was suspended in 100 μl hexane, vortexed and transferred to a GC vial. Samples were run against a known fatty acid methyl esters standard solution (47885-U Supelco® 37 component FAME Mix, 10 mg/mL in methylene chloride).

Table 1. Composition of finisher experimental diets[1] used.

Feed	Linseed	Echium	FO	Algal biomass
Wheat	583	583	583	581
Soyabean meal	285	285	285	276
$CaCO_3$	13	13	13	13
Dicalcium phosphate	10	10	10	10
Algae	0	0	0	18
Echium oil	0	18	0	0
Linseed oil	15	0	0	0
Fish oil	0	0	50	0
Soya oil	35	32	0	40
Salt	3.5	3.5	3.5	3.5
Vitamin/mineral supplement	50	50	50	50
DL Methionine	2	2	2	2
NaHCO3	2	2	2	5
Lysine	1.5	1.5	1.5	1.5
Vitamin E (i.u./kg)	100	100	100	100
Nutrient composition	Crude Protein (5)	Metabolisable Energy (kcal/kg)	Fat (g/kg)	
	21.03	3105.30	6.002	

[1] On as-fed basis.

2.5. NK cell activity assay

The SOP12 of the School of Medicine at the University of Southampton was followed for NK cell activity assay, with some modifications as described by Al-Khalaifa (2013), Al-Khalifa (2016), Kushima et al. (2003), Betts et al. (2003). Dr. Peter Kaiser from Roslin Institute, provided the LSCC-RP9 cell line.

2.6. Mitogenic responses of lymphocytes

The mitogenic responses of lymphocyte assay were determined in accordance with the SOP14 from the School of Medicine in the University of Southampton. From the stock of the CFSE stain, 10 μl was diluted by 990 μl spleen and thymus cell suspensions. The solution containing the cell suspension along with the stain was incubated at 41 °C for 10 min in an atmosphere containing 5% CO_2. This was followed by washing the cells twice and then adjusting it to 1×10^6/ml. A 48-well micro titre plates was used to culture the cells in the presence or absence of Con A (25 μg/ml), PHA (75 μg/ml) or Pansorbin cells (*Staphylococcus aureus*, which stimulate B-cells in spleen preparations; used at 2×10^5/well for splenocytes only) in CCM and 10% autologous plasma. The most favourable concentration of each mitogen was previously determined (data not shown). To each of the control wells, CCM and 10% autologous plasma were added. The cell cultures were transferred to a 5% CO_2 charged atmosphere and were incubated for 72 h at 41 °C. Post incubation period, the cells were then transferred to FACS tubes and kept on ice until analysis using a FACSCalibur™ flow cytometer. To analyse the data, a dot plot of the control profile, with no added mitogen should be displayed so that cells were grouped by size (FCS) and granularity (SSC). The small and rested lymphocytes were then gated (R1). A FITC-CFSE staining histogram against cell count depicted the gated lymphocytes. The control cells produced fluorescence that ranged between 102 and 103. A marker was set on the control sample, to include at least 98.5% of the unstimulated cells (M1). The fluorescence of stimulated cells moved out of M1 and into M2. The total percentage of cells that proliferated (M2) was reported. FlowJo (Flow Cytometry Analysis Software, Tree Star, Inc.) was used to model the cell proliferation data. It gave the information about proportions of cells in each generation of the subset.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) and the general linear model in Minitab software were used to analyze the overall differences between dietary treatments. Statistical difference was considered at $P \leq 0.05$. Significantly different means were identified using post-hoc Bonferroni comparison tests. Normal distribution of data was achieved by arcsine transformation.

3. Results

3.1. Lymphoid tissue weight

The effect of feeding four different sources of n-3 PUFA on lymphoid tissue weight (as a percentage of final body weight) was investigated. There were no significant differences between diets with respect to lymphoid tissue weights in broiler chickens.

3.2. Plasma fatty acid profile

The effect of feeding 6-wk-old broilers various n-3 PUFA sources on the fatty acid profiles of plasma, splenocytes, thymocytes and blood leukocytes is shown in Table 4. The highest concentration of plasma EPA was observed in broilers fed FO, followed by those fed the algal biomass, and then those fed linseed or echium oils (Table 4). EPA in the plasma of chickens fed the echium oil-enriched diet was significantly lower than in those fed FO or the algal biomass-enriched diet, but not the linseed oil-enriched diet (Table 4). DHA was highest in the chickens fed the algal biomass diet, followed by those fed the FO diet; chickens fed linseed and echium oil diets showed similar levels of plasma DHA, which were lower than the other two diets (Table 4). The highest proportion of plasma α-linoleic acid was observed in broilers fed linseed, followed by those fed algal biomass, echium and FO (Table 4). The highest proportion of ALA was observed in plasma of broilers fed echium oil, followed by those fed linseed and algal biomass, and then by those fed FO (Table 3). Broilers fed echium and linseed oils showed the highest proportions of plasma total n-6 PUFA, followed by those fed algal biomass, and then those fed FO (Table 4). The lowest ratios of n-6:n-3 were observed in broilers fed FO diet and algal biomass, followed by those fed linseed and echium oils which showed similar ratios (Table 4).

3.3. Fatty acid composition of spleen, thymus and blood leukocytes

The effect of feeding 6-wk-old broilers various n-3 fat sources on the fatty acid profile of spleens, thymus and blood leukocytes is shown in (Table 4). The greatest enrichment of EPA in splenocytes was observed in the broilers fed FO, followed by those fed algal biomass, while broilers fed linseed or echium oil had low levels of EPA in their splenocytes; this closely reflects the fatty acid profile of the plasma (Table 4). Proportions of DHA were highest in splenocytes from broilers fed algal biomass, followed by FO, while those from broilers fed echium or linseed oils had low levels of DHA (Table 4). ALA was higher in broilers fed linseed and echium oils than those fed FO and algal biomass. Proportions of α-linolenic acid were similar in broilers fed echium and FO. There was no significant difference in proportions of stearidonic acid (SDA) between broilers fed different dietary treatments. The total amounts of n-3 PUFA were highest in broilers fed FO, followed by those fed algal biomass, and then by those fed linseed and echium oils which showed similar proportions of total n-3 PUFA (Table 4). The proportion of ALA in spleens from broilers fed linseed and echium oils was higher than that in spleens from broilers fed FO and algal biomass. Spleens of broilers fed algal biomass, echium, and linseed oils showed similar total amounts of n-6 PUFA, which were higher than in those fed FO. The lowest ratio of n-6: n-3 was observed in broilers fed FO and algal biomass, followed by those fed linseed and echium oils (Table 4). The impact of the four diets on the

Table 2. Fatty acid composition of the oils used in experiment.

Fatty acids (wt %)				
	Linseed	Echium	FO	Algal Biomass
C12:0	0.00	0.00	0.13	0.00
C14:0	0.04	0.66	8.67	0.79
C14:1	0.00	0.00	0.16	0.00
C15:0	0.00	0.00	0.49	0.00
C16:0	5.08	20.65	17.15	22.40
C16:1n7	0.06	2.26	12.66	0.00
C17:0	0.06	0.00	0.49	0.00
C17:1	0.04	0.00	1.35	0.00
C18:0	3.42	12.84	3.59	13.00
C18:1n9t	0.00	0.08	2.55	32.27
C18:1n9c	18.95	0.00	7.01	0.00
C18:2n6t	0.56	12.96	3.51	5.66
C18:2n6c	16.50	0.00	1.12	1.04
C20:0	0.13	0.00	0.88	0.08
C18:3n6	0.20	0.62	0.43	0.28[a]
C18:3n3	54.44	25.90	0.69	3.29
C18:4n3	0.00	10.89	0.00	0.00
C22:0	0.03	1.59	3.18	0.23
C20:3n6	0.12	0.65	0.17	0.80
C22:1n9	0.00	1.69	1.39	0.29
C23:0	0.00	0.93	2.25	0.00
C22:2	0.00	1.74	0.60	0.00
C24:0	0.00	3.40	0.18	0.00
C20:5n3	0.00	0.00	16.48	10.12
C24:1n9	0.08	0.00	0.14	0.00
C22:6n3	0.02	1.26	9.42	4.98
∑SAT[1]	8.75	34.15	33.82	36.36
∑MONO[2]	19.12	32.74	25.25	36.55
∑PUFA[3]	71.85	31.15	35.60	25.66
∑n-6[4]	17.39	14.23	5.23	10.33
∑n-6:∑n-3[6]	0.32	0.50	0.20	0.67

[5] ∑n-3 = Sum percentage of n-3 polyunsaturated fatty acids.
Means within rows are significantly different at $p \leq 0.05$, mean in the same row with different superscripts are significantly different.
[1] ∑SAT = Sum percentage of saturated fatty acids.
[2] ∑MONO = Sum percentage of monounsaturated fatty acids.
[3] ∑PUFA = Sum percentage of polyunsaturated fatty acids.
[4] ∑n-6 = Sum percentage of n-6 polyunsaturated fatty acids.
[6] ∑n-6:∑n-3 = ratio of ∑n-6 to ∑n-3.

fatty acid profiles of thymocytes and blood leukocytes were almost identical to that on the splenocytes (Table 4).

3.4. NK cell activity of splenocytes

NK cell activity of splenocytes of broilers fed the linseed oil diet was the highest and for those fed the FO was the lowest at all ratios of effector: target cells tested (Table 5). At a ratio of 100:1, NK cell activity of splenocytes from broilers fed the linseed oil diet was significantly higher ($P < 0.001$) than that of broilers fed the echium oil and FO diets (Table 5). NK activity of splenocytes from the algal biomass-fed broilers was higher than that of broilers fed fish oil, but no different from that of broilers fed linseed or echium oil (Table 5). Broilers fed the echium oil diet had significantly lower NK activity than those fed linseed oil, but there were no significant differences compared with those fed algal biomass or fish oil (Table 5). At lower ratios of effector to target cells, the pattern was similar. Overall, NK activity of splenocytes tended to be highest in the linseed oil group, followed by the algal biomass and echium oil groups, and the lowest activity was observed in the FO group (Table 5).

3.5. NK activity of PBMCs

NK activity of blood leukocytes was much lower than that of splenocytes, but the impact of the four diets was generally very similar in that NK activity tended to be highest in the linseed oil group, followed by the algal biomass and echium oil group, with the FO group demonstrating the lowest NK activity (Table 7).

3.6. Mitogen-stimulated proliferation of splenocytes and lymphocytes

Con A-, PHA- and Pansorbin-mediated cell proliferation, expressed as division index was analyzed. There was no significant effect of dietary treatment on the division indices of splenocytes in response to 75 μg/ml PHA, 25 μg/ml Con A, and *Staphylococcus aureus* (i.e. pansorbin) among the four dietary groups, although there was a strong trend for division index to be lowest following fish oil feeding.

There was no significant effect of dietary treatment on the division indices of thymocytes in response to 75 μg/ml PHA. The division index of algal biomass fed group was numerically the highest among the other dietary groups, but failed to reach statistical significance ($P = 0.067$). There was no significant effect of dietary treatment on the division indices of thymocytes stimulated with 25 μg/ml Con A. Once again, however, there remained a trend for the fish oil diet to produce the lowest index of division.

4. Discussion

The objective of this study was to investigate the effect of feeding various n-3 PUFA sources on the FA composition of plasma and lymphoid tissues and on immune function in broiler chickens. The objective was to determine whether enrichment of the broiler diet with n-3 PUFA could be implemented without undesirable effects on immune function. The n-3 PUFA sources included linseed oil, echium oil, fish oil and algal biomass. The linseed oil diet served as a control because it is well known that conversion of the abundant α-linolenic acid in this oil to long chain (LC) n-3 PUFA is very limited (Al-Khalifa, 2015; Bioriginal, 2010; Brenna, 2002; Burdge and Calder, 2006; James et al., 2003; Rymer and Givens, 2005; Williams and Burdge, 2006). Echium oil was selected because it is rich in SDA, and there is evidence to suggest a reasonable conversion of this precursor to EPA in animals (Yamazaki et al., 1992; Yang and O'Shea, 2009) and humans (Al-Khalifa, 2015; Guil-Guerrero, 2007; Harris et al., 2008; James et al., 2003; Miles et al., 2004; Whelan, 2009). There are some data in chickens suggesting the conversion efficiency of SDA to EPA is limited, but there is no information with respect to immune function.

Changes in the fatty acid compositions of plasma and immune cells were as expected, with FO producing the most dramatic increases in EPA. This was followed by algal biomass, which resulted in an EPA content which was between 30-50% of that in the FO-fed broilers. This increase in EPA following feeding of high levels of DHA is a common feature which results from retroconversion of DHA (Al-Khalifa, 2015; Al-Khalifa et al., 2012; Conquer and Holub, 1996; Gronn et al., 1991; Kelley et al., 2008; Stark and Holub, 2004; Su et al., 2001). Levels of EPA and DHA following feeding of linseed or echium oils were low and not significantly different, suggesting that echium oil is no better than linseed oil in terms of increasing plasma or tissue levels of EPA in broilers, confirming the observation of Rymer et al. (2011) that the conversion of SDA to EPA is no more efficient (in the chicken) than the conversion of ALA to EPA. Echium oil also contains a considerable amount of ALA, which explains why the proportions of this fatty acid in plasma, meat and lymphoid tissues of chickens fed this diet were increased. This is in agreement with other studies of echium oil fatty acid modulation in rats (Yang and O'Shea, 2009) and humans (Surette et al., 2004). It is noteworthy that negligible traces of SDA were present in the samples, which is in agreement with other studies (James et al., 2003; Miles et al., 2004; Miller et al., 2007) although this may be because of the relatively low concentration of SDA even in echium oil.

Table 3. Fatty acid composition of the diet mixtures used.

Fatty acids (wt %)				
	Linseed	Echium	FO	Algal biomass
C14:0	3.71	0.16	0.21	4.07
C16:0	15.64	11.16	11.08	19.30
C16:1	4.53	0.20	0.21	0.29
C18:0	3.18	3.81	4.10	2.91
C18:1n9	20.63	18.94	19.78	13.56
C18:2n6	16.00	42.74	42.82	33.38
C18:3n-6	0.48	3.26	0.40	0.44
C18:3n3	3.01	13.05	17.45	4.78
C20:1n-9	4.61	0.64	0.63	0.06
C18:4 n-3	0.96	3.45	0.17	0.24
C20:4n6	6.37	0.25	0.28	0.86
C23:0	1.09	0.05	0.07	0.33
C20:5n3	6.49	0.10	0.10	0.49
C22:5n3	2.65	0.10	0.12	0.20
C22:6n3	7.23	0.24	0.24	17.24
∑SAT[1]	23.62	15.17	15.45	26.61
∑MUFA[2]	29.78	19.78	20.62	13.91
∑PUFA[3]	43.70	63.27	61.70	57.72
∑n-6[4]	23.35	46.33	43.62	34.77
∑n-3[5]	20.35	16.94	18.08	22.95
∑n-6:∑n-3[6]	1.15	2.74	2.41	1.53

[1] ∑SAT = Sum percentage of saturated fatty acids (C14:0, C15:0, C16:0, C18:0, C20:0, C21:0, C22:0, C23:0).

[2] ∑MUFA = Sum percentage of monounsaturated fatty acids (C14:1, C16:1, C17:1, C18:1n-9t C18:1n-9c, C22:1 n-9).

[3] ∑PUFA = Sum percentage of PUFA (C18:2n-6t, C18:2n-6c, C18:3n-6, C18:3n-3, C20:2n-6, C20:3n-6, C20:4n-6, C20:5n-3, C22:2, C22:5n-3, C22:6n-3).

[4] ∑n-6 = Sum percentage of n-6 PUFA (C18:2n-6t, C18:2n-6c, C18:3n-6, C20:4n-6, C20:2n-6, C20:3n-6).

[5] ∑n-3 = Sum percentage of n-3 PUFA (C18:3n-3, C20:5n-3, C22:5n-3, C22:6n-3).

[6] ∑n-6:∑n-3 = ratio of ∑n-6 to ∑n-3.

The concentration of docosapentaenoic acid (DPA) (C22:5 n-3) was highest in the birds fed FO (which contained both EPA and DHA), and intermediate in birds fed echium or linseed oil (in which EPA would have been formed as a precursor to the DPA). In birds fed algal biomass (which contained only small amounts of EPA), the concentration of DPA in the plasma was much lower. The concentration of DPA in all diets was low, but this accumulation of LC n-3 PUFA as DPA has been observed before. The results from this study would suggest that the accumulation of DPA comes from the metabolism of EPA to DPA rather than from retro-conversion of DHA.

Levels of DHA were highest in broilers fed the algal biomass, followed by FO; in most cases, the algal biomass resulted in significantly higher levels of DHA than FO, but the relative difference was not great, demonstrating that small amounts of algal biomass can result in greater incorporation of DHA and about a third of the EPA as a large amount of FO containing an equivalent amount of total n-3 PUFA. Recently, Rymer et al. (2010) investigated the suitability of algal biomass as an alternative to FO in enrichment of poultry meat with the LC n-3 PUFA and reported that the breast content of DHA was linearly increased as the algal biomass dose increased. Also, feeding 3.6 g of algal biomass DHA per bird for 49 days resulted in a 6-fold increase in the DHA content of breast meat and a 2–3-fold increase in the DHA content of dark meat (Abril and Barclay, 1998). Results of these studies, as well as the current study, confirm that algal biomass enrichment of broiler meat can be used as efficiently as FO in enrichment of poultry meat with n-3 PUFA, especially DHA.

To our knowledge, this is the first study comparing the effects of feeding different sources of n-3 PUFA on NK activity of splenocytes or PBMCs of broiler chickens. In the current study, splenocytes exhibited higher NK activity than PBMCs, which is consistent with earlier studies (Kushima et al., 2003; Sharma and Coulson, 1979; Sharma and Lee, 1983; Sharma and Okazaki, 1981). The current study also demonstrated that for both splenocytes and PBMCs, NK activity in the FO group was significantly lower than that in the linseed oil group, with the algal biomass and echium oil groups in between and not significantly different from each other. The effects were particularly dramatic in splenocytes, largely because NK activity of PBMCs was considerably lower overall. This result is generally in agreement with other studies in animals and humans. In mice studies, Meydani et al. (1988b) reported that young mice fed FO had moderately lower NK activity than those fed corn oil. Older mice did not exhibit the reduced NK activity after FO feeding, which the authors attributed to slower fatty acid turnover rate and/or larger fatty acid pool size in old mice compared with young mice. In rats, Yaqoob et al. (1994) demonstrated that several high fat diets (coconut oil, olive oil, safflower oil and fish oil) decreased NK cell activity of rat spleen lymphocytes compared with a low fat diet, and that the lowest NK activity was exhibited by splenocytes from rats fed the FO-enriched diet. There are no studies in the literature examining the effects of dietary fat on NK cell activity in chickens. However, Fritsche and Cassity (1992) fed female broiler chickens on a corn and soybean-based diet containing 7% lard, corn oil, linseed oil, or FO to investigate the effect of these dietary treatments on Antibody Dependent Cell Cytotoxicity (ADCC). Results of this study showed that the ADCC of splenocytes was decreased by 50% when broiler chickens were fed the FO-enriched diet compared to the other diets. In human studies, injection of EPA and DHA emulsions significantly decreased NK cell activity by up to 65% in healthy humans (Hamazaki et al., 1987). In addition, intravenous infusion of EPA emulsion (3 g) significantly decreased NK cell activity by up to 35% in healthy humans. Kelley et al. (1999) demonstrated that feeding DHA as triacylglycerol significantly decreased NK activity of human PBMCs relative to the control diet feeding. Supplementing patients with active proctocolitis with 15 ml FO per day decreased NK cell activity by 30% after 3 months (Almallah et al., 2000). FO also decreased NK cell activity in healthy elderly humans by 48% (Thies et al., 2001a). Also, reported that supplementing the human diet with EPA and DHA capsules decreased the proportions of NK cells in the circulation, but the authors did not assess NK cell activity in this study.

It has been suggested that the inhibitory effect of FO on NK activity in rats (Peterson et al., 1998), and humans (Kelley et al., 1997; Thies et al., 2001a; Yamashita et al., 1991) is due to EPA rather than DHA, although this is controversial. Accordingly, it was predicted that the lowest NK activity would be exhibited by splenocytes with the highest contents of EPA. However, this was not entirely the case. While FO feeding resulted in the lowest NK activity, algal biomass did not decrease NK activity as much as predicted (based on EPA enrichment of immune cells), and the second most inhibitory treatment was in fact echium oil. This suggests that although algal biomass enriches tissues with both EPA and DHA, it is not associated with the dramatic impairment of NK activity that is observed following FO feeding. However, it should be noted that the FO diet in the current study was fed at 50 g/kg, whereas algal biomass was fed at 18 g/kg. The purpose of this was to provide equivalent amounts of total n-3 PUFA, but it is possible that the inhibition of NK activity by the FO diet is at least partly due to a higher level of fat, since high fat diets have been shown to impair NK activity relative to low fat diets in rats (Yaqoob et al., 1994). Nevertheless, the aim of this study was to ascertain whether different means of enriching chicken tissues with n-3 PUFA are associated with undesirable effects on immunity, and this study demonstrates that feeding small amounts of algal biomass results in a reasonable level of enrichment of n-3 PUFA without the dramatic immunosuppression that is associated with feeding large amounts of fish oil.

The correlation analysis supported the suggestion that EPA is responsible for the inhibitory effects of FO on NK activity, since EPA, but not DHA, was consistently negatively correlated with NK activity, albeit

Table 4. Fatty acid composition of plasma, spleen, thymus and blood leukocytes of 6-wk-old broilers fed various n-3 fat sources.

% of total weight						
	Linseed	Echium	FO	Algal biomass	SEM	P-value
Plasma						
C18:3n6	0.27^{b}	0.71^{a}	0.36^{b}	0.33^{b}	0.071	<0.001
C18:3n3	3.29^{a}	1.82^{b}	0.76^{b}	0.81^{b}	0.251	<0.001
C18:4n3	0.05	0.11	0.20	0.35	0.080	0.098
C20:4n6	7.93^{a}	11.22^{b}	3.45^{c}	6.00 ^{ac}	0.577	<0.001
C20:5n3	1.46^{c}	1.92 ^{bc}	9.37^{a}	3.32^{b}	0.348	<0.001
C22:5n3	1.84 ^{bc}	2.33^{b}	3.78^{a}	1.45^{c}	0.189	<0.001
C22:6n3	3.42^{c}	3.55^{c}	12.35^{b}	16.37^{a}	0.514	<0.001
∑n-6[1]	39.59^{a}	39.69^{a}	16.32^{c}	28.43^{b}	1.855	<0.001
∑n-3[2]	10.16^{b}	9.62^{b}	26.26^{a}	21.95^{c}	0.868	<0.001
∑n-6:∑n-3[3]	3.92^{a}	4.26^{a}	0.631^{b}	1.31^{b}	0.220	<0.001
Spleen						
C18:3n6	0.37	1.85	0.55	0.97	0.541	0.187
C18:3n3	1.05^{a}	0.72 ^{ab}	0.38 ^{bc}	0.32^{c}	0.085	<0.001
C18:4n3	0.14	0.11	0.49	0.12	0.186	0.431
C20:4n6	14.05^{a}	13.90^{a}	7.15^{b}	11.79 ^{ab}	1.024	0.002
C20:5n3	0.45^{c}	0.95^{c}	5.39^{a}	1.78^{b}	0.299	<0.001
C22:5n3	3.39^{c}	2.79 ^{bc}	5.84^{a}	1.90^{b}	0.280	<0.001
C22:6n3	1.76^{b}	2.62^{b}	6.27^{c}	9.68^{a}	0.222	<0.001
∑n-6[1]	33.95^{a}	33.69^{a}	20.13^{b}	29.95^{a}	1.263	<0.001
∑n-3[2]	7.20^{b}	7.03^{b}	17.93^{a}	13.68^{c}	0.548	<0.001
∑n-6:∑n-3[3]	4.72^{a}	4.79^{a}	1.12^{b}	2.19^{b}	0.266	<0.001
Thymus						
C18:3n-6(γ-Linolenic acid)	0.78^{a}	0.74^{a}	0.31^{b}	0.21^{b}	0.136	0.016
C18:3n3(α-Linolenic acid)	2.27^{a}	1.18^{b}	0.44 ^{bc}	0.26^{c}	0.188	0.002
C18:4n3 (Stearidonic acid)	0.48	0.22	0.32	0.06	0.168	0.401
C20:4n6	9.33^{a}	9.51^{a}	3.86^{c}	8.52^{b}	0.180	<0.001
C20:5n3 (Eicosapentaenoic acid)	1.01^{b}	1.91^{b}	7.11^{a}	3.87^{c}	0.415	<0.001
C22:5n3 (Docosapentaenoic acid)	3.15^{b}	3.71^{b}	5.18^{a}	2.80^{b}	0.340	<0.001
C22:6n3 (Docosahexaenoic acid)	1.63^{c}	2.48^{c}	6.94^{b}	8.07^{a}	0.238	<0.001
∑n-6[1]	28.41^{a}	28.25^{a}	14.47^{c}	22.29^{b}	0.978	<0.001
∑n-3[2]	8.06^{b}	9.32^{b}	19.67^{a}	14.82^{a}	0.780	<0.001
∑n-6:∑n-3[3]	3.25^{a}	3.03^{a}	0.76^{b}	1.50^{b}	0.424	<0.001
% of total weight						
Blood						
C18:3n-6	0.26^{b}	0.82^{a}	0.40 ^{ab}	0.46 ^{ab}	0.395	0.037
C18:3n3	2.19^{b}	0.73^{b}	0.22^{a}	0.28^{a}	0.331	0.014
C18:4n3	0.15	0.15	0.13	0.12	0.034	0.322
C20:4n6	16.30^{a}	9.58^{a}	8.57^{b}	16.57^{a}	2.557	<0.001
C20:5n3	0.57^{b}	0.59^{b}	4.28^{a}	1.17^{c}	0.353	<0.001
C22:5n3	1.93 ^{bc}	2.06^{b}	6.05^{a}	1.30^{c}	0.161	<0.001
C22:6n3	1.02^{b}	1.09^{b}	6.13^{a}	6.54^{a}	0.110	<0.001
∑n-6[1]	37.70^{a}	27.06^{b}	20.35^{b}	34.51^{a}	2.574	<0.001
∑n-3[2]	5.71^{b}	4.47^{b}	16.68^{a}	9.30^{c}	0.329	<0.001
∑n-6:∑n-3[3]	6.62^{a}	6.27^{a}	1.22^{b}	3.71^{b}	0.713	<0.001

Means within rows are significantly different at $p \leq 0.05$, mean in the same row with different superscripts are significantly different, one-way analysis of variance (ANOVA) and the general linear model procedure of Minitab was applied. Treatment mean differences were identified by Bonferroni tests.
Values are expressed as means (n = 20 per treatment, each sample was analysed in quintuplicate), pooled standard error of means (SEM).
[1] ∑n-6 = Sum percentage of n-6 PUFA (C18:3n-6, C20:4n6).
[2] ∑n-3 = Sum percentage of n-3 PUFA (C18:3n3, C18:4n3, C20:5n3, C22:5n3, C22:6n3).
[3] ∑n-6:∑n-3 = ratio of ∑n-6 to ∑n-3.

weakly in some cases. Interestingly, n-6 PUFA tended to be positively correlated with NK activity (Tables 6, 8, and 9).

To our knowledge, this is the first study comparing the effect of feeding different sources of n-3 PUFA on the proliferative response of immune cells in chickens. Also, there are limited data investigating the relative effects of individual fatty acids, particularly EPA and DHA on immune function in chickens. Overall, data from this study showed that the highest division indices of mitogen-stimulated splenocytes and thymocytes were observed in the algal biomass-fed chickens, followed by those fed linseed-, and echium oils, while FO tended to result in the lowest level of proliferation.

There are no poultry studies evaluating the separate effects of EPA and DHA on immune function. However, unlike the NK cell activity results, algal biomass and echium oil tended to produce the highest division

Table 5. Effect of feeding various n-3 fat PUFA sources on NK cell activity of splenocytes from 6-week old broilers against the LSCC-RP9 cell line.

	Treatment			Algal Biomass	SEM	P-value
	Linseed	Echium	FO			
E:T		% cytotoxicity of effector cells				
100:1	41.15^{a}	25.09^{bc}	11.53^{c}	34.62^{ab}	3.685	<0.001
50:1	29.74^{a}	13.47 ^{ab}	3.28^{b}	18.16 ^{ab}	4.211	0.006
25:1	22.11^{a}	9.37 ^{ab}	5.40^{b}	15.25 ^{ab}	3.624	0.032
12.5:1	13.00^{a}	3.35^{b}	1.07 $_{b}$	6.87 ^{ab}	1.605	0.001

Means within rows with no common superscripts are significantly different ($p \leq 0.05$).
Chicks were fed linseed oil, echium oil, FO or algal biomass enriched diets.
Values are expressed as means (n = 20 per treatment, each sample was analysed in quintuplicate), pooled standard error of means (SEM).
FO, fish oil, E:T, effector cell/target cell ratio.

Table 6. Correlation coefficients for the relationships between percentage of individual fatty acids in the splenocytes and their NK-activity.

Fatty acid	NK cell activity at L:T			
	100		50	
	r	*p*	*r*	*p*
C14:00	-0.096	0.724	0.032	0.906
C16:00	0.011	0.967	0.208	0.439
C16:1n-7	-0.358	0.173	-0.150	0.580
C18:00	0.190	0.482	0.153	0.571
C18:1n-9	-0.154	0.569	0.070	0.797
C18:2n-6	0.609	0.012	0.616	0.011
C18:3n-6	-0.330	0.212	-0.398	0.127
C18:3n-3	0.333	0.208	0.396	0.129
C20:2	0.623	0.010	0.523	0.038
C20:3n6	0.054	0.841	0.034	0.901
C18:4 n-3	-0.003	0.991	-0.280	0.294
C20:4n-6	0.527	0.036	0.507	0.045
C20:5n-3	-0.406	0.118	-0.462	0.071
C22:5n-3	-0.475	0.063	-0.401	0.124
C22:6n-3	0.093	0.733	-0.066	0.807
∑SAT	0.135	0.618	0.246	0.359
∑MUFA	-0.173	0.523	0.055	0.840
∑PUFA	0.434	0.093	0.415	0.110
∑n-6	0.619	0.011	0.587	0.017
∑n-3	-0.484	0.057	-0.457	0.075
∑n-6:∑n-3	0.369	0.160	0.384	0.142

Correlation coefficient (*r*) is spearman linear rank when data is not normally distributed or pearson when data is normally distributed.

Table 7. Effect of feeding various n-3 PUFA sources on NK cell activity of PBMCs from 6-week old broilers against the LSCC-RP9 cell line.

Treatment						
E:T	Linseed	Echium	FO	Algal biomass	SEM	P-value
	% cytotoxicity of effector cells					
100:1	14.91^{a}	6.55 ^{ab}	2.97^{b}	8.87 ^{ab}	2.471	0.047
50:1	9.18	9.93	4.62	3.91	3.309	0.494
25:1	7.70	5.49	5.78	0.83	1.961	0.164
12.5:1	4.27	2.33	0.82	1.97	1.490	0.470

Means within rows with no common superscripts are significantly different ($p \leq 0.05$).
Values are expressed as means (n = 20 per treatment, each sample was analysed in quintuplicate), pooled standard error of means (SEM).
Chicks were fed algae oil-, echium oil-, FO- or linseed-oil enriched diets.
FO, fish oil, E:T, effector cell/target cell ratio.

indices. Unlike NK activity, division index was not negatively correlated with EPA, but there was evidence of a positive correlation with DHA. It is also pertinent to note that algal biomass is not pure oil and that other ingredients may have had an effect on immune function. For example, algal cells and nucleotides are reported to act as immunomodulators in mice (Adjei et al., 1999), rats (Ameho et al., 1997), chickens (Deng et al., 2005; Frankic et al., 2006) and humans (Carver et al., 1991; Hawkes et al., 2006). In the study of Adjei et al. (1999), authors fed mice on diets supplemented with a 0.5% nucleoside–nucleotide mixture before and after inoculation with *Cryptosporidium parvum*. Spleens from mice receiving the supplemented diet had higher Con A- and antigen-specific induced cell proliferation compared with the controls. Also, these spleen cells produced significantly more IL-2 and IFN-γ than cells from the control mice. In rats, Ameho et al. (1997) investigated the effect of either 2% or 4% glutamine on the inflammatory cytokines interleukin-8 and tumor necrosis factor-α in induced colitis. Results demonstrated that both cytokines in inflamed colonic tissues were lower in the glutamine groups than in the control group (no glutamine). In the chicken study of Deng et al. (2005), authors supplemented leghorn-type chickens with 5 or 10 g yeast RNA/kg for 4 weeks and then all birds were given a commercial

Table 8. Correlation coefficients for the relationships between percentage of individual fatty acids in the PBMCs and their NK-activity.

NK cell activity at L:T				
Fatty acid	100		50	
	r	*p*	*r*	*p*
C16:00	-0.570	0.053	-0.168	0.601
C16:1n-7	-0.676	0.016	-0.197	0.539
C18:00	0.423	0.171	0.126	0.697
C18:1n-9	-0.613	0.034	-0.261	0.413
C18:2n-6	0.725	0.008	0.193	0.547
C18:3n-6	-0.486	0.109	0.133	0.680
C18:3n-3	0.641	0.025	0.183	0.569
C20:3n-6	0.162	0.615	0.113	0.727
C18:4 n-3	-0.224	0.484	0.063	0.846
C20:4n-6	0.458	0.135	-0.149	0.645
C20:5n-3	-0.754	0.005	-0.585	0.046
C22:5n-3	-0.549	0.064	0.190	0.554
C22:6n-3	-0.134	0.678	-0.317	0.316
∑SAT	0.007	0.983	0.000	0.998
∑MUFA	-0.690	0.013	-0.162	0.615
∑PUFA	0.592	0.043	-0.276	0.385
∑n-6	0.268	0.400	0.070	0.828
∑n-3	-0.451	0.141	-0.387	0.214
∑n-6:∑n-3	0.599	0.040	0.252	0.430

Correlation coefficient (*r*) is Spearman linear rank when data is not normally distributed or Pearson when data is normally distributed.

Table 9. Correlation coefficients for the relationships between percentage of individual fatty acids of splenocytes and thymocytes and their proliferative response, expressed as division index.

Fatty acid	Division index			
	Splenocytes		Thymocytes	
	r	*p*	*r*	*p*
C14:00	-0.097	0.310	-0.326	0.008
C16:00	0.206	0.029	0.061	0.633
C16:1n-7	-0.246	0.009	-0.251	0.045
C18:00	0.162	0.088	0.222	0.078
C18:1n-9	-0.132	0.164	-0.223	0.076
C18:2n-6	0.194	0.041	0.025	0.847
C18:3n-6	-0.122	0.200	-0.108	0.397
C18:3n-3	-0.060	0.528	-0.127	0.319
C20:2 n-6	0.227	0.016	0.095	0.454
C20:3n-6	0.009	0.925	-0.074	0.562
C20:4n-6	0.095	0.319	0.113	0.375
C20:5n-3	-0.109	0.251	0.042	0.739
C22:5n-3	-0.156	0.102	-0.163	0.199
C22:6n-3	0.097	0.310	0.261	0.037
∑SAT	0.240	0.011	0.151	0.233
∑MUFA	-0.143	0.131	-0.214	0.089
∑PUFA	0.225	0.017	0.312	0.012
∑n-6	0.155	0.102	0.013	0.921
∑n-3	-0.033	0.732	0.141	0.268
∑n-6:∑n-3	0.025	0.793	-0.106	0.404

r = Pearson Correlation coefficient.

pullet grower feed for another 8 weeks. The toe-web PHA response was significantly higher at week 8 in control birds (no yeast RNA) than in the supplemented birds. Also, yeast RNA stimulated the development of the spleen in young birds. In the study of Frankic et al. (2006), male broiler chickens were exposed to 10 mg/kg feed of either T-2 toxin or deoxynivalenol with or without addition of dietary nucleotides. Results demonstrated that dietary nucleotides reduced the extent of DNA damage induced by the action of toxins in splenocytes.

In conclusion, the current work suggests that the immunosuppressive effects of FO are largely dependent on EPA enrichment of lymphoid tissues, and that a DHA-rich algal product may enrich chicken meat with n-3 PUFA without significant detrimental effects on chicken immunity.

Declarations

Author contribution statement

H. Al-Khalaifah, A. Al-Nasser, D. I. Givens, C. Rymer, P. Yaqoob: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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The authors declare no conflict of interest.

Additional information

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