Omega-3 fatty acid composition of chicken plasma fed diets varying in LA to ALA ratio

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Abstract. The impact of dietary supplementation of a vegetable source of n-3 fat in the form of alpha-linolenic acid (ALA, 18:3n-3) on the accumulation of n-3 fatty acids in plasma chicken was investigated. Sixty unsexed one-day-old broiler chickens were randomly allocated to six diets (n=10 birds/diet) for 4 weeks. The birds were fed a proprietary starter commercial feed for the first two days after hatch. Six dietary treatments ranging in fat content from 5 to 10% were prepared by supplementing the basal diet with vegetable oils. The ALA levels varied from 1 to 8% energy (%en). The n-6 fatty acid linoleic acid (LA, 18:2n-6) level was limited to less than 5%en. Results showed that the metabolites of dietary ALA, EPA, DPA, and DHA in plasma tissues increased in a curvilinear manner as dietary ALA increased, achieving 3- to 13-fold compared to the levels in the control birds (P<0.001). The total PUFA content of plasma samples increased (P<0.001) due to increasing the diets' ALA content, achieving 41.20% of the total fatty acids when the LA to ALA ratio of diets was at the lowest level. The increase in total PUFA was mainly due to an increase in total n-3 PUFA.

1. Introduction
The health benefits associated with the consumption of omega-3 long-chain polyunsaturated fatty acids (n-3 LCPUFA) have been well documented, so that consumption of omega-3 fatty acids is recommended to be increased. Foods that are the main sources of n-3 LCPUFAs are fish and fish products [1], besides that it also includes foods fortified with fish oil [2]. However, some people do not like food sourced from marine products, in addition, some researchers report that food enriched with fish oil or chicken products produced from chickens fed diets supplemented with fish oil [3] caused a negative effect on the sensory quality of meat [3,4], and heavy metal contamination that negatively impacts human health [5].

Plant sources containing high n-3 PUFA (ALA) can be an alternative to marine sources (fish meal and fish oil). Some plants, such as chia seed, flaxseed, and canola, contain high n-3 PUFA to be supplemented in the chicken diet to enhance the content of omega-3 fatty acids in animal products such as egg and meat. ALA in plants or plant oils is a precursor to n-3 LCPUFA and can be metabolized into EPA, DPA, and DHA. In this process, the availability of desaturation and elongation enzymes plays an
important role in the metabolism of ALA to EPA, DPA, and DHA (n-3 LCPUFA). However, the conversion of n-6 PUFA (LA) to arachidonic acid (AA, 20:4n-6) also requires these enzymes, and this causes competition between n-3 PUFA (ALA) and n-6 PUFA, linoleic acid. (LA, 18:2n-6) in the use of these enzymes. This suggests that the decreased production of EPA and DHA and increased AA levels may be caused by the high consumption of LA [6]. Thus, the LA to ALA ratio in the diet is an important determinant for optimal ALA conversion. Diets enriched with n-3 PUFA were able to increase the n-3 PUFA content in chicken tissues [7,8] but have not succeeded in showing a relatively high increase in n-3 LCPUFA in meat. The lack of response to the accumulation of tissue n-3 LCPUFA in these studies was probably due to the relatively high LA to ALA ratio of the diet given. The results of Kartikasari et al. [9] found that increasing dietary ALA while keeping LA levels low in the diet resulted in a 5- and 4-fold enhance in n-3 LCPUFA production in chicken breast, respectively.

It is interesting to investigate whether there was a positive correlation between breast EPA and DHA and plasma phospholipid (PL) EPA and DHA. The results of blood phospholipid EPA and DHA can be good markers of the n-3 LCPUFA (EPA and DHA) levels in chicken meat. This study aims to examine the impact of increasing dietary alpha-linolenic acid (ALA, 18:3n-3) from plant sources rich in n-3 fat on the accumulation of n-3 fatty acids in plasma PL chickens.

2. Materials and methods

2.1. Birds, rearing, and management
Cobb 500 broilers (n=70) were housed in rearing cages (1.2 × 0.9m² each pen) for 28 days (PPPI, SARDI, Roseworthy Campus, the University of Adelaide) and fed and watered ad libitum. The procedures for rearing chickens followed Kartikasari et al. [9]. During the growing period, the chickens used a 24-hour light program, and the cages were also heated from infrared lamps (175 watts) for 21 days. Chickens are observed regularly during the first few days to ensure that all have access to adequate feed and water and are comfortable with environmental conditions. During the 28-day dietary intervention, feed consumption and body weight were recorded weekly. Feed efficiency (g feed: weight gain g) and body weight gain were calculated.

2.2. Diets
The design used in this research was a one-way classification, and the variable factor is the level of increased levels of ALA in food while maintaining constant LA levels. Dietary treatments were based on the LA to ALA ratio in the dietary treatments. The feed provided consisted of a control diet and six dietary treatments (n = 10 chickens for each diet), which varied on the ALA content of the feed. Six experimental feeds were made from a basal diet supplemented with pure vegetable oils or mixtures obtained by varying the proportions of several vegetable oils. The fat content of the dietary treatment ranged from 5 to 10%, and all diets met or exceeded the requirements (NRC) [10] for broilers. For the first two days after hatching, the broiler chickens were fed an exclusive starter commercial diet. The ALA level and the ratio of LA to ALA of the diets (A1-A5) are presented in Table 1. During the 28-day growth period, each diet was administered ad libitum.

2.3. Fatty acid analysis of chicken plasma phospholipids
To separate erythrocytes and plasma, whole blood was spun (1559g for 10 min). Next, plasma was placed into labeled tubes and stored (-20°C) for fatty acid analysis. Next, total plasma lipid was extracted (methanol/chloroform; 1:2, v/v). The sample was mixed thoroughly with methanol (2mL) and chloroform (4mL), and the glass tube was shaken vigorously. Then, the tube was left at room temperature for 5 minutes. The next step was separating the organic phases and aqueous by centrifuging the tube at 1559g for 10 minutes (Megfuge 1.0, Heraeus Sepatech, Hanau, Germany). The next step was to remove or place the chloroform layer into a labeled scintillation bottle (20 mL), and using a vacuum concentrator, and the chloroform was evaporated to dryness. The total lipid extract obtained was added with 150µL of chloroform/methanol (9:1, v/v) and spotted on a thin layer chromatography (TLC) plate.
The lipid components were separated and developed in petroleum spirit/acetone (3:1, v/v). Then, the plates were analyzed under UV light to identify the phospholipids. Then, together with silica, the phospholipid band was scraped into a 5mL glass bottle containing 2mL of 1% H2SO4 in methanol and sealed.

The phospholipid samples were left to methylate for 3 hours at 70°C. The methyl ester obtained was then extracted into 0.5mL heptane and 250µL distilled water and then stored at -20°C for further analysis. Fatty acid analysis by gas chromatography followed Kartikasari et al. [9].

2.4. Data analysis
The data collected were analyzed using a one-way analysis of variance (SPSS version 15.0) to evaluate the impact of increased levels of ALA in feed on omega-3 fatty acid content in broiler plasma phospholipids. If there were significant differences between experimental diets, the analysis was followed by the Tukey-b test with a significance level of P<0.05.

Table 1. Omega-3 and omega-6 fatty acid levels of the experimental diets

| Dietary treatments | LA (% en) | A1 | A2 | A3 | A4 | A5 |
|--------------------|-----------|----|----|----|----|----|
| Control            | 2.72      | 3.96 | 3.77 | 3.67 | 4.06 | 4.59 |
| ALA (% en)         | 0.26      | 1.10 | 1.61 | 3.19 | 5.82 | 7.97 |
| LA:ALA ratio       | 10.46:1   | 3.60:1 | 2.34:1 | 1.15:1 | 0.70:1 | 0.58:1 |
| n6:n-3 ratio       | 9.34:1    | 3.58:1 | 2.35:1 | 1.15:1 | 0.70:1 | 0.58:1 |
| Fat Content (%)    | 5.29      | 5.37 | 5.13 | 5.38 | 7.26 | 9.41 |

3. Results and discussion
The plasma fatty acid composition is shown in Table 2. As the dietary LA to ALA ratio reduced, the proportion of ALA in plasma phospholipids significantly increased (P<0.001). Increased availability of food ALA will increase the metabolites of ALA, n-3 LCPUFA (P<0.001), including EPA, DPA, and DHA. It indicates that the response to an increase in EPA, DPA, and DHA in the blood sample is the same either by reducing the LA to ALA ratio or improving the ALA content of the diet. There appears to be a direct correlation between the level of ALA of the dietary treatments and the plasma phospholipid n-3 level of broiler chickens, especially as n-3 LCPUFA.

Treatment diets containing 8% energy ALA (lowest LA to ALA ratio) resulted in increased plasma accumulation of EPA, DPA, and DHA between 3- and 13-fold. When compared with DHA accumulation, greater EPA deposition in plasma phospholipids was achieved. This is probably due to the metabolic pathway of ALA to EPA and DPA requiring only one Δ6-desaturase pathway, which means it follows simple zero-order kinetics [11]. In contrast, the conversion from DPA to DHA is more complex [12] because it requires a second pathway after being extended to 24:5n-3. This resulted in competition between 24:5n-3 and LA in the use of Δ6-desaturase. The next step is the process of beta-oxidation of 24:6n-3 to DHA in peroxisomes, which provides another potential regulatory point. The observations obtained are in accordance with previous studies [9, 13, 14].

This suggests that taking blood samples is non-lethal and an easy way to check alters in fatty acid levels in meat caused by dietary treatments. Plasma phospholipid EPA and DHA of chickens can be a good marker of n-3 LCPUFA levels in breast and thigh meat. It seems that the ability to incorporate n-3 LCPUFA fatty acids for each tissue reflects its specificity. For example, the content of EPA was low in breast and thigh phospholipids; however, EPA accumulation was found to be high (4.7%) in plasma phospholipids [9]. This may be due to EPA having a limited ability to deposit in breast and thigh tissues. In addition, the major n-3 LCPUFA in the breast and thigh meat was DPA, but in plasma phospholipid, DPA was found to be only moderate (Figure 1). This may reflect that DPA can accumulate more easily with less conversion to DHA in breast and thigh tissues.

While the level of total n-6 fatty acids in plasma phospholipids reduced slightly, the total n-3 fatty acid levels increased due to the increase in ALA levels of the diets (P<0.001). The lowest total plasma phospholipids n-6 PUFA were obtained from the feed with the highest ALA content (the lowest LA to
ALA ratio). Increasing the ALA content in the diets did not change the plasma phospholipid LA level. As expected, there was a significant reduction in the AA content (P<0.01) when EPA accumulation increased in the plasma samples. Plasma phospholipid AA levels in chickens decreased by 37.78% with a high ALA diet (LA to ALA ratio of 0.6:1) compared to those fed a 3.6:1 LA to ALA ratio. These findings are consistent with other investigators, who indicated that the content of LA and AA in meat samples decreased with improving the levels of flaxseed oil in the diets [9,14]. The biosynthetic competition between ALA and LA precursors may cause the decrease in the AA content of the tissue samples, but this may also be due to the competition between n-3 LCPUFA and AA for inclusion in plasma membrane phospholipids.

**Table 2.** Fatty acid profiles of plasma phospholipids from broiler chickens fed dietary treatments varying in the ratio of LA to ALA

| ALA (%)en | Control | A1 | A2 | A3 | A4 | A5 |
|-----------|---------|----|----|----|----|----|
|           |         | 1.10 | 1.61 | 3.19 | 5.82 | 7.97 |
| Fatty acid | (% of total fatty acids) | | | | | | P Value | Significance |
| 16:0 | 20.47c | 22.24a | 21.95bc | 24.04a | 22.01b | 21.16bc | 0.000 | *** |
| 18:0 | 24.82bc | 23.73c | 24.67bc | 23.42a | 26.07ab | 27.42a | 0.000 | *** |
| SFA | 46.74d | 47.29d | 47.74c | 48.64c | 49.34a | 49.76a | 0.000 | *** |
| 18:1n-9 | 16.13a | 13.10ab | 12.59b | 11.59b | 7.76c | 6.65c | 0.000 | *** |
| 18:1n-7 | 2.64a | 1.90b | 1.95b | 1.72b | 1.11c | 0.88c | 0.000 | *** |
| MUFA | 21.35a | 16.77b | 16.30b | 15.02b | 9.96c | 8.48c | 0.000 | *** |
| Total n-9 | 18.31a | 14.96b | 14.33b | 12.94c | 8.70c | 7.40c | 0.000 | *** |
| 18:2n-6 (LA) | 20.83 | 22.44 | 21.32 | 20.19 | 21.41 | 21.17 | 0.131 | NS |
| 20:3n-6 | 1.34a | 1.12ab | 1.18ab | 0.97bc | 0.78c | 0.72c | 0.000 | *** |
| 20:4n-6 | 4.77ab | 5.61a | 4.89ab | 3.90ab | 3.55ab | 3.49b | 0.026 | * |
| Total n-6 | 28.10ab | 30.41a | 28.48ab | 26.09b | 26.49b | 25.99b | 0.001 | ** |
| 18:3n-3 (AA) | 0.30c | 0.74a | 1.02d | 1.84a | 3.42b | 3.91a | 0.000 | *** |
| 20:5n-3 (DPA) | 0.35c | 0.84a | 1.49d | 2.60a | 3.69b | 4.70a | 0.000 | *** |
| 22:5n-3 (DPA) | 0.35d | 0.84d | 1.01c | 1.76a | 2.50a | 2.97a | 0.000 | *** |
| 22:6n-3 (DHA) | 1.00d | 1.56a | 2.51c | 2.84bc | 3.69a | 3.39ab | 0.000 | *** |
| n-3 LCPUFA | 1.71f | 3.25a | 5.01d | 7.21c | 9.89b | 11.05b | 0.000 | *** |
| Total n-3 FA | 2.11c | 4.10f | 6.18d | 9.28a | 13.68b | 15.39a | 0.000 | *** |
| Total PUFA | 30.21c | 34.51b | 34.66b | 35.36b | 40.17b | 41.38b | 0.000 | *** |
| LA to ALA | 68.98a | 30.27b | 21.59c | 10.96d | 6.27ab | 5.48c | 0.000 | *** |
| n-6 to n-3 | 13.40d | 7.51b | 4.65a | 2.82d | 1.94c | 1.69c | 0.000 | *** |

NS, not significant; *P<0.05; **P<0.01; ***P<0.001.

**Figure 1.** Increasing levels of dietary ALA significantly enhance (P<0.01) phospholipid EPA, DPA, and DHA of chicken breast (A) and thigh [9].
Observations showed that the level of plasma phospholipid MUFA decreased in response to an increase in the level of ALA of the diets (P<0.001). Broiler chickens fed with the lowest LA to ALA ratio (0.6 to 1) produced the lowest MUFA level, 2.5 times lower than broilers fed the control diet. In contrast, the total PUFA level of plasma phospholipids enhanced (P<0.001) due to a decrease in the LA to ALA ratio (Table 2).

4. Conclusions
Increased levels of dietary ALA improved the accumulation of n-3 LCPUFA (EPA, DPA, and DHA) in chicken plasma phospholipids. Experimental diets with the lowest LA to ALA ratio increased the incorporation of EPA and DHA into chickens plasma to levels of 3- and 13-fold, respectively, compared to chickens fed the diets with the highest LA to ALA ratio. In contrast, as the ratio of LA to ALA reduced, the levels of n-6 LCPUFA, arachidonic acid decreased. Further research is needed to test whether there is a strong correlation between n-3 LCPUFA in chicken plasma and the levels of n-3 LCPUFA in meat.

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