The Degree of Fat Unsaturation in the Tissue and Potential Immune Response of Broiler Fed *Chlorella sp.*

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Abstract: This study was carried out to investigate the effect of *Chlorella sp*. administered in the diet of broilers on the degree of fat unsaturation, ratio of EPA to AA in the tissue and potential immune response of broiler. A total of 90 heads of one-day-old Ross chicks were assigned in completely randomized design by 3 dietary treatments with 6 repetitions and 5 chicks in each pen. The diets were T1: control (basal diet without enrichment with *Chlorella sp*.); T2: basal diet enriched with 5-g of *Chlorella sp*./kg feed; T3: basal diet enriched with 10-g of *Chlorella sp*./kg feed. Skinless breast meat was sampled for FA determination at d-36. Chickens were vaccinated at d-6 and d-17 with live vaccine against ND to activate antibodies production, and then 2 ml of blood was collected at d-24 for IgG and IgM quantification. Administration of *Chlorella sp*. in broiler's diet had no significant effect on the degree of fat unsaturation, the ratio between EPA and AA contained in the breast muscle and the concentration of IgG and IgM of broiler. In conclusion administration of *Chlorella sp*. from tropical marine origin in the diet of broiler has no significant effect on the degree of fat unsaturation, ratio of EPA to AA in the tissue, and potential immune response of broiler. The culture temperature in which the *Chlorella sp*. was cultivated may affect the FA composition of *Chlorella sp*.

Abbreviations: AA: arachidonic acid, ALA: α-linolenic acid, EPA: eicosapentaenoic acid, FA: fatty acids, LA: linoleic acid, PGE_2: Prostaglandin E_2, PUFA: polyunsaturated fatty acids, SFA: saturated fatty acid

Key Words: antibody, broiler, *Chlorella sp*., fatty acid, PUFA.

Introduction

Chicken meat is an important component of a modern human diet. As a staple diet, chicken meat should have a higher proportion of unsaturated than saturated FA. Increasing the level of unsaturation especially polyunsaturated fatty acid (PUFA) is recommended by human nutritionist to prevent cardiovascular diseases (Galli and Calder, 2009). This fact therefore encourages the broiler producer to include different PUFA sources in the broiler's diet to produce high and low content of PUFA and SFA, respectively, in the meat. Instead of a the ratio between PUFA and SFA, the ratio among PUFA composition, primarily between EPA and AA should also be considered. Enhanced tissue level of EPA is needed for optimal health (Ratyanake and Galli, 2009), whereas enhanced AA leads to enhanced PGE_2 possessing immunosuppressive effects (Calder et al., 2006). The contrasting effects of EPA and of AA on various cell functions, particularly in cells involved in immunomodulation, has therefore raised the issue whether the ratio of n-3 and n-6 FA in the body should be controlled through nutritional strategies.

Human and mice studies have shown that *Chlorella sp*. is able to improve immune response (Liang et al., 2004; Spolaore et al., 2006). It is most likely due to the high content of n-3 FA in this alga (Bergé and Barnathan, 2005). However, beside rich in n-3 FA, *Chlorella sp*. especially that of tropical marine origin contains high amount of n-6 FA as well (Seto et al., 1984; Khotimchenko, 2002). Therefore, it becomes considerably interesting to include this type of alga in the diet of broiler in order to investigate its effect on the degree of fat unsaturation, ratio of EPA to AA in the tissue and potential immune response of broiler.

Materials and Methods

Animals and Diets

Ninety mixed-sexes of one day old Ross chicks were used in the experiment conducted with completely randomized design of 3 dietary treatments with 6 repetitions (experimental units). Each experimental unit consisted of 5 chicks that were housed in one pen, therefore, there were 18 pens in this trial. The chicks were sexed, weighed and marked (using cable ties) before being allotted into 18 wire floors pens (app. 2 male and 3 female chicks each pen) situated a semi closed house system. The diets were formulated by adding ('on top') *Chlorella sp.* to the basal diet (equivalent to commercial chick starter crumbles) as the last ingredient in the mixing process and fed (ad libitum) to the chickens from d-0 to d-35. The treatment diets were chemically iso-calory and iso-nitrogen (data not shown). The diets were T_1: control (basal diet without enrichment wiwth *Chlorella sp*.); T_2: basal diet enriched with 5-g of *Chlorella sp*./kg feed; T_3: basal diet enriched with 10-g of *Chlorella sp*./kg feed.

FA composition of the diets was described in Table 1. The *Chlorella sp.* used in this experiment was in the form of meal, obtained from Brackishwater Aquaculture Development Centre Situbondo, East Java province, Indonesia.

Table 1. Determined FA composition (mg/100g)

Fatty acids	T_1	T_2	T_3
ALA	137.14	141.49	174.20
EPA	0.52	0.68	0.67
LA	1837.20	1953.01	2340.24
AA	1.18	1.71	1.77
Ratio ALA/LA	0.07	0.07	0.07
Ratio EPA/AA	0.44	0.40	0.38
Ratio PUFA/SFA	1.02	1.04	1.03

AA: arachidonic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; FA: fatty acids; LA: linoleic acid; PUFA: polyunsaturated fatty acids; SFA: saturated fatty acid,

T_1 :control (basal diet without enrichment with *Chlorella sp*.)

T_2 :basal diet enriched with 5-g of *Chlorella sp*./kg feed

T_3: basal diet enriched with 10-g of *Chlorella sp*./kg feed

FA Profile and Iodine Value

Skinless breast meat was sampled from 1 selected male bird from each pen at d-36 after being killed and used for FA determination. The FA composition was determined based on methodology described by Bligh and Dyer (1959) with some modifications. It was performed with an HP-6890 gas chromatograph equipped with an autosampler, FID, and fused-silica capillary column (30 m x 0.25 mm x 0.2 µm film thickness). The sample (1 µl) was injected with helium as a carrier gas onto the column programmed for ramped oven temperatures (initial temperature was 110°C held for 1.0 min, then ramped at 15°C/min to 190°C and held for 5 min, then ramped at 5°C/min to 230°C and held for 5 min). Inlet and temperature detector were held at 220°C. Peak areas and percentages were calculated using Hewlett-Packard ChemStation software. FA methyl esters were identified by comparison with retention times of authentic standard. FA values were reported as weight percentages and iodine value was calculated from FA profile obtained from gas chromatograph.

Serum IgG and IgM Concentration

All chickens were vaccinated at d-6 and d-17 with live vaccine against ND to activate the production of IgG and IgM. At d-24, 2 ml blood was collected from wing vein and serum was prepared thereafter. Quantitative ELISA (Sandwich) immunoassay kits for measurement of chicken IgG and IgM from Bethyl Laboratories (Montgomey, TX, USA) were used to measure those immunoglobulins. The ELISA assay was, with few exceptions, performed according to the kit manual. Serum used for IgG assay was diluted 25000 times and 1500 times for IgM assay. Conjugate diluents pH 8.0 (0.05 M Tris, 0.15 M NaCl, 1% BSA, 0.05% Tween 20) were used for diluting all of those samples. In brief, microtitre plates (96-wells) were coated with 100µl of the capture antibody (anti-chicken IgG and IgM) followed by incubation at room temperature (20-25^{0}C) for 2 h. After incubation, the solution was removed and the plate was washed 3 times with wash solution pH 8.0 (0.05 M Tris, 0.15 M NaCl, 0.05% Tween 20). Then 200µl blocking solution was added to each well and incubated at room temperature for 30 min, the solution was removed and washed 3 times hereafter. Standards and samples (100 µl) were added to each well followed by room temperature incubation for 1 h and washed 3 times. The IgG and IgM concentrations were detected by incubation

with HRP-conjugated goat anti-chicken IgG and IgM and then incubated for 1h and washed 3 times hereafter. TMB (3,3′,5,5′-tetramethyl benzidine) was used as chromagen and 1M H_2SO_4 as stop solution. The result was monitored as OD at 450 nm. The IgG and IgM concentrations were calculated from a standard curve as suggested by the company. The final result was accounted by multiplying with the dilution factor for each sample.

Statistical analysis

All data were presented as the mean ± the standard error of the mean. The iodine values, FA composition in the breast muscle and antibodies concentration were analyzed using a one-way ANOVA procedure. Effect of inclusion of *Chlorella sp*. in the diet was analyzed. All analyses were performed by SPSS 15.0 for Windows. A p-value of less than 0.05 was considered statistically significant.

Results and Discussion

The degree of fat unsaturation can be measured by iodine value (Gunstone, 1996). However, the iodine value is somehow less informative compared to FA profile obtained by gas chromatograph. Our study showed that administration of *Chlorella sp*. in the broiler's diet had not significant effect (P>0.05) on the degree of fat unsaturation contained in the breast muscle of broiler (Table 2).

Deposition of PUFA in the tissue depends on the dietary supplementation. FA composition of the tissues is more or less similar to the FA composition of the diets (Barroeta, 2004). Since the contents of total PUFA and SFA (data not shown) as well as the ratio between those FAs in the diets (Table 1) were rather similar, the insignificant degree of fat unsaturation reflected from iodine value and the ratio of PUFA to SFA could be expected. Increased level of *Chlorella sp*. in the diet was not only accompanied by the increased of PUFA concentration but also SFA concentration as *Chlorella sp*. used in this experiment was of tropical marine origin. Exposure to high temperature in tropical regions causes algae to increase their relative amount of FA saturation (Guschina and Harwood, 2009) and may also elicit the high content of n-6 FA, especially LA in *Chlorella sp*. (Seto et al., 1984; Goss and Wihelm, 2009).

The ratio of n-3 to n-6 FA may influence a potential immune competence of broiler (Guo et al., 2004; Puthpongsiriporn and Scheideler, 2005). We expected previously that the content of n-3 FA in *Chlorella sp*. might help to improve the health status of broiler. However, high content of n-6 FA in this alga perhaps attenuated its potential role, since the metabolisms of both FAs are intertwined (Sijben et al., 2001). Puthpongsiriporn and Scheideler (2005) demonstrated that increasing the dietary ratio of ALA to LA increased tissue levels of EPA and decreased AA. The increase and decrease of EPA and AA, respectively, will imply to the increase of antibody production since it reduces synthesis of PGE_2 (Guo et al., 2004; Calder et al., 2006).

Table 2. Iodine value and ratio between total PUFA to total SFA in the breast muscle of broiler

Fatty acids	T_1	T_2	T_3	P value
Iodine value (g/100gFA)	102.20±2.13	105.50±2.23	105.67±1.99	0.474
Ratio PUFA/SFA	0.93±0.03	0.97±0.03	0.96±0.03	0.744

T_1 :control (basal diet without enrichment with *Chlorella sp.*); T_2 : basal diet enriched with 5-g of *Chlorella sp.*/kg feed;
T_3: basal diet enriched with 10-g of *Chlorella sp.*/kg feed.

Table 3. Ratio of EPA to AA in the breast muscle of broiler

Fatty acids	T_1	T_2	T_3	P value
EPA (mg/100gDM)	2.29±0.16	2.24±0.07	1.92±0.15	0.132
AA (mg/100gDM)	31.20±1.65	32.14±2.75	28.90±1.28	0.513
Ratio EPA/AA	0.07±0.00	0.07±0.01	0.07±0.01	0.818

T1 :control (basal diet without enrichment with *Chlorella sp.*); T2 : basal diet enriched with 5-g of *Chlorella sp.*/kg feed;
T3: basal diet enriched with 10-g of *Chlorella sp.*/kg feed.

Table 4. Serum antibodies concentration ((µg/ml)

Antibodies	T_1	T_2	T_3	P value
IgG	3434.59 ± 455.86	2185.11 ± 380.21	2892.48 ± 336.72	0.129
IgM	167.43± 17.77	142.26 ± 32.70	223.59 ± 32.30	0.152

T_1 :control (basal diet without enrichment with *Chlorella sp*.); T_2 : basal diet enriched with 5-g of *Chlorella sp*./kg feed;
T_3: basal diet enriched with 10-g of *Chlorella sp*./kg feed.

In our study, the similar ratio between ALA and LA in the diets (Table 1) resulted in similar ratio of EPA and AA in the tissue (breast muscle) of broiler (Table 3), which led to the insignificant differences (P>0.05) of serum antibody concentrations.

Conclusions

In conclusion administration of *Chlorella sp*. from tropical marine origin in the diet of broiler has no significant effect on the degree of fat unsaturation, ratio of EPA to AA in the tissue, and potential immune response of broiler. The culture temperature in which the *Chlorella sp*. was cultivated may affect the FA composition of *Chlorella sp*.

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