The effect of dietary flaxseed meal on liver and egg yolk fatty acid profiles, immune response and antioxidant status of laying hens

Tarek M. Shafey, Hamad A. Al-Batshan, Ahmed M.S. Farhan
Department of Animal Production, King Saud University, Riyadh, Saudi Arabia

Abstract

The effects of supplementing laying hen diets with 0, 50, or 100 g flaxseed meal (FSM)/kg over a 12-week period on liver and egg yolk fatty acids (FA) composition, liver and serum lipid peroxidation [thiobarbituric acid reactant substances (TBARS), activities of superoxide dismutase (SOD) and glutathione peroxidase (GPx)], serum lipids (triglycerides, total cholesterol, high density lipoprotein cholesterol), proteins (total protein, globulin and albumin), and immune response [serum antibody titres to sheep red blood cells (SRBC) and white blood cell count (WBC) and differential (heterophils (H), lymphocytes (L), monocytes (M), eosinophils (E) and basophils (B))] of laying hens were studied. The FSM diets increased total polyunsaturated FA (PUFA) and omega-3 FA of α-linolenic acid (C18:3n-3), docosapentaenoic (C22:5n-3) and docosahexaenoic (C22:6n-3), and they reduced total monounsaturated FA (MUFA) and total omega-3/total omega-6 FA (2n-6:2n-3) ratio in the liver and egg yolk. Hens fed the FSM diets had a higher serum anti-SRBC and a lower blood total WBC. It was concluded that the addition of FSM to the diet of laying hens occurred leading to the development of potentially toxic compounds (Cortinas et al., 2005). Lipid peroxidation has been implicated in the pathogenesis of diseases, including heart disease, cancer and atherosclerosis (Rose, 1997; Yashodhara et al., 2009; Chang and Cho, 2009). Since ALA is highly susceptible to oxidation, then the addition of FSM to the diet may result in a significantly higher lipid peroxidation and influence immune response of laying hens. There is a lack of information on the effects of FSM on the immune system and oxidative stress in laying hens. Therefore, this study was designed to assess the effects of FSM addition to the diet of laying hens on liver and egg yolk fatty acids (FA) composition, immune response and oxidative stress in laying hens.

Materials and methods

A total of 48 Hi-Line birds at 22 weeks of age were used in this study. Birds were housed individually in cages 50x60x56 cm (length x width x depth) under a controlled environment with a temperature of 24.65°C±0.20 at the poultry house (Department of Animal Production, College of Food and Agriculture Sciences, King Saud University). Each cage was fitted with water nipples and a galvanized...
feed trough. The nipple-type drinkers (2 nipples/cage) were positioned in the rear side of each cage. Birds were housed 2 weeks before the beginning of the feeding trial. Each bird was treated as an experimental unit. Sixteen replicates were randomly assigned to either one of three experimental diets. The experimental diets included a control diet with 0% FSM and two diets with 50 and 100 g FSM/kg. The experimental diets were formulated to be isonitrogenous and isocaloric. The composition of the experimental diets is shown in Table 1. The addition of FSM to the basal diet increased the ALA content as a percentage of FA from 2.85 to 16.42 and 25.86 for the 50 and 100 g FSM/kg diets, respectively. The experiment lasted 12 weeks. A photoperiod of 16 h was maintained throughout the trial. Feed and water were provided ad libitum throughout the experiment.

At the end of the experiment, seven eggs from each treatment were collected for lipid extraction and FA analysis. Blood samples for haematological and serum analysis were collected using sterilized syringes and needles from the wing vein of seven randomly selected birds from each treatment. The blood samples were dispensed into tubes containing Ethylene Diamine Tetra Acetic Acid (EDTA) as anticoagulant and another set into tube without anticoagulant for serum analysis. Serum samples were separated by centrifuging blood samples at 1,500×g for 15 min. Serum samples were maintained at -20°C until biochemical analysis. Blood sera were analyzed for triglycerides (TG), serum total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), total protein (TP) and albumin (ALB), using an automatic blood chemical analyzer with Roche testing kits (Roche COBAS MIRA PLUS). The serum globulin (GLB) fraction was determined by subtracting the albumin from the total protein. EDTA anticoagulated blood samples were used to prepare blood films for cell count. Blood films were air dried then stained with Giemsa-Wright’ stain. Differential white blood cell counts (WBC) were performed using the standard avian guidelines of Ritchie et al. (1994), and the hemoglobin to lymphocyte ratio (HL ratio) was calculated. WBC was determined by a manual method using a haemacytometer.

After blood collection, birds were sacrificed by decapitation. Livers were rapidly removed and washed free of blood with 0.9% NaCl solution and distilled water. They were perfused with a 50 mM (sodium phosphate buffer saline (100 mM Na2HPO4/NaH2PO4, PH 7.4) in an ice-containing medium, containing 0.1 mM EDTA to remove any red blood cells and clots.

Livers were then cut into two pieces, and one portion was frozen and stored at -0°C for FA analysis, while the other half was homogenized in 5-10 mL cold buffer per gram tissue and centrifuged at 3000 x g for 30 min. The resulting supernatant was transferred into Eppendorf tubes, and preserved at -80°C until used for various biochemical assays.

Lipid peroxidation in the blood plasma and liver was measured in the form of thiobarbituric acid reactive substance (TBARS) by biochemical kit (Cat. No. MD 2529, Biodiagnostic Company, Egypt) according to the method of Ohkawa et al. (1979). Activity of the antioxidative enzyme glutathione peroxidase (GPX) was determined using a kit from biodiagnostic (Cat. No. GR 2524, Biodiagnostic Company, Egypt) according to the method of Table 1. Composition of the experimental diets.

|                  | Flaxseed meal, g/kg |
|------------------|---------------------|
|                  | 0                   | 50                  | 100                 |
| Feed ingredients, g/kg |                     |                     |                     |
| Corn             | 600.3               | 562.2               | 548.1               |
| Soybean meal     | 250.0               | 241.1               | 234.7               |
| Wheat bran       | 30.0                | 20.0                | 0.0                 |
| Flaxseed meal    | 0.0                 | 50.0                | 100.0               |
| Corn oil         | 9.7                 | 14.8                | 13.0                |
| Limestone        | 81.5                | 81.2                | 81.1                |
| Dicalcium phosphate | 15.5            | 15.2                | 15.1                |
| Potassium bicarbonate | 5.1              | 3.7                 | 3.2                 |
| Salt             | 4.1                 | 4.0                 | 4.0                 |
| DL-methionine    | 1.2                 | 1.2                 | 1.2                 |
| Premix*          | 2.0                 | 2.0                 | 2.0                 |
| L-lysine         | 0.6                 | 0.7                 | 0.8                 |
| ME, kcal/g       | 2.80                | 2.82                | 2.85                |
| CP, %N x 6.25    | 17.9                | 18.0                | 18.2                |
| EE               | 43.6                | 59.2                | 69.8                |
| Calcium, g/kg    | 35.0                | 35.0                | 35.0                |
| AP, g/kg         | 5.0                 | 5.0                 | 5.0                 |
| Electrolyte balance, mEq/kg | 321.0        | 315.0               | 315.0               |
| Lysine, g/kg     | 10.0                | 10.0                | 10.0                |
| Met + cy%, g/kg  | 7.0                 | 7.0                 | 7.0                 |
| Fatty acid, %    |                     |                     |                     |
| MA (C18:0)       | 0.20                | 0.12                | 0.16                |
| PA (C16:0)       | 11.09               | 10.85               | 10.74               |
| SA (C18:2)       | 1.81                | 1.76                | 1.82                |
| PO (C16:1)       | 0.11                | 0.14                | 0.13                |
| OA (C18:1)       | 24.73               | 23.30               | 22.84               |
| LA (C18:2n-6)    | 59.21               | 47.41               | 38.45               |
| ALA (C18:3n-3)   | 2.85                | 16.42               | 25.86               |
| ΣSFA             | 13.10               | 12.73               | 12.72               |
| ΣMUFA            | 24.84               | 23.44               | 22.97               |
| ΣPUFA            | 62.06               | 63.83               | 64.31               |
| Σn-3 FA          | 2.85                | 16.42               | 25.86               |
| Σn-6 FA          | 59.21               | 47.41               | 38.45               |
| Σn-6+2n-3 FA     | 20.77               | 2.89                | 1.49                |

ME, metabolizable energy; CP, crude protein; EE, ether extract; AP, available phosphorus (calculated on the basis of 30% availability of phosphorus in plant products); Electrolyte balance, Na+ + K+ + Cl-; Met + cy% = methionine + cysteine; MA, myristic acid; PA, palmitic acid; SA, stearic acid; PO, palmitoleic; OA, oleic acid; LA, linoleic acid; ALA, α-linolenic acid; Σ SFA, sum of saturated fatty acid; Σ MUFA, sum of monounsaturated fatty acid; Σ PUFA, sum of polyunsaturated fatty acid; Σ n-3 FA, sum of (n-3) fatty acids; Σ n-6 FA, sum of (n-6) fatty acids. "The composition of vitamins and minerals in the premix (per kg of diet): Vitamin A, 6000 U; vitamin E, 10 U; menadione, 2.5 mg; Vitamin D, 2000 U; riboflavin, 25 mg; Ca pantothenate, 10 mg; nicotinic acid, 12 mg; choline chloride, 500 mg; vitamin B6, 4 g; vitamin B12, 5 mg; thiamine, 3 mg; folic acid, 0.5 mg; biotin, 0.2 mg; Zn, 40 mg; Fe, 40 mg; Cu, 4 mg; Se, 0.10 mg; carrier (polysorbate) made up to 2 g. Calculated values from National Research Council (1994).
feeding dietary FSM at the level of 50 and 100 g/kg diet did not influence body weight, feed intake, feed conversion ratio (feed/kg egg) and egg production. Results on the performance were not shown.

Results and discussion

The FA profiles of liver and egg yolk of hens fed the FSM diets are shown in Table 2. The lipids found in the egg yolk are synthesized in the liver of the laying hen and transported in the blood stream by lipoproteins to the developing yolk (Chapman, 1980). The addition of FSM to the diet significantly (P<0.01) increased liver contents of ALA and DHA (100>50>0 g FSM/kg diet); DPA and Σ3 FA (100>50>0 g FSM/kg diet); and AA and (P<0.05) ΣPUFA (100>0 g FSM/kg diet), and reduced (P<0.01) OA (100<50<0 g FSM/kg diet); PA

Table 2. Fatty acid composition of liver and yolk of laying hens fed different dietary levels of flaxseed meal.

|                  | Flaxseed meal, g/kg | SEM | P       |
|------------------|---------------------|-----|---------|
|                  | 0                   | 50  | 100     |
| Liver fatty acid |                     |     |         |
| MA (C16:0), %    | 0.18                | 0.14| 0.13    |
| PA (C16:1), %    | 18.63               | 19.28|17.11   | 0.32 ** |
| SA (C18:0), %    | 15.14               | 14.81|15.87   | 0.37 ns |
| PO (C16:0), %    | 3.91                | 3.38| 4.11    | 0.29 ns |
| OA (C18:2), %    | 41.01               | 39.89|38.48   | 0.34 ** |
| LA (C18:3), %    | 17.80               | 16.82|16.38   | 0.16 ** |
| AA (C20:4), %    | 1.52                | 1.90| 2.61    | 0.26 ** |
| ALA (C18:3), %   | 0.21                | 0.12| 0.17    | 0.03 ns |
| SDA (C22:4), %   | 0.30                | 0.31| 0.28    | 0.12 ns |
| EPA (C20:5), %   | 0.13                | 0.17| 0.12    | 0.03 ns |
| DPA (C22:6), %   | 0.03                | 0.22| 0.34    | 0.06 ** |
| DHA (C22:6), %   | 0.17                | 0.22| 0.21    | 0.22 ** |
| ΣSFA, %          | 33.95               | 34.23|33.11   | 0.78 ns |
| ΣMUFA, %         | 44.92               | 43.27|42.60   | 0.67 *  |
| ΣPUFA, %         | 20.16               | 21.88|23.62   | 0.98 *  |
| Σn-3 FA, %       | 0.84                | 3.16| 4.63    | 0.55 ** |
| Σn-6 FA, %       | 19.32               | 18.72|18.99   | 0.36 ns |
| Liver total lipid, mg/g | 118.9 | 93.3 | 89.2 | 5.1 ** |
| Σn-6: n-3 FA, %  | 23.0                | 5.92| 4.10    | 0.62 ** |
| Egg yolk fatty acid, % |               |     |         |
| MA (C14:0), %    | 0.34                | 0.26| 0.25    | 0.024 * |
| PA (C16:1), %    | 26.79               | 26.22|24.15   | 0.212 ** |
| ALA (C18:3), %   | 11.61               | 10.97|11.37   | 0.243 ns |
| PO (C16:0), %    | 2.95                | 2.15| 1.58    | 0.281 ** |
| OA (C18:2), %    | 37.73               | 36.86|36.18   | 0.561 ns |
| LA (C18:3), %    | 14.80               | 15.82|17.33   | 0.472 ** |
| AA (C20:4), %    | 2.52                | 1.90| 1.37    | 0.124 ** |
| ALA (C18:3), %   | 1.26                | 2.92| 4.86    | 0.218 ** |
| SDA (C22:4), %   | 0.26                | 0.22| 0.19    | 0.012 ** |
| EPA (C20:5), %   | 0.03                | 0.07| 0.13    | 0.011 ** |
| DPA (C22:6), %   | 0.08                | 0.29| 0.32    | 0.027 ** |
| DHA (C22:6), %   | 0.66                | 1.62| 1.86    | 0.134 ** |
| ΣSFA, %          | 38.74               | 37.45|35.77   | 0.429 ** |
| ΣMUFA, %         | 40.66               | 39.01|37.76   | 0.418 ** |
| ΣPUFA, %         | 19.61               | 22.84|26.06   | 0.782 ** |
| Σn-3 FA, %       | 2.29                | 5.12| 7.30    | 0.369 ns |
| Σn-6 FA, %       | 17.32               | 17.72|18.70   | 0.544 ns |

**MA, myristic acid; PA, palmitic acid; SA, stearic acid; PO, palmitoleic; OA, oleic acid; LA, linoleic acid; AA, arachidonic; ALA, α-linolenic acid; SDA, stearidonic; EPA, eicosapentaenoic; DPA, docosapentaenoic; DHA, docosahexaenoic; Σn-3 FA, sum of saturated fatty acid; Σn-6 FA, sum of (α-6) fatty acids. Values reported as percentage of total fatty acids for n=5. *Means with different superscripts within a row differ significantly (P<0.05); ns, not significant.
Dietary level of FSM meal, g/kg

Flaxseed in laying hen diets

Table 3. Differential count (mean±SE) of white blood cells of laying hens fed different dietary levels of flaxseed meal.

| Dietary level of FSM meal, g/kg | H: L | M | E | B | H:L ratio |
|-------------------------------|------|---|---|---|----------|
| 0                             | 57.1±0.80<sup>a</sup> | 38.1±0.86<sup>b</sup> | 3.3±0.87<sup>a</sup> | 1.1±0.34 | 0.9±0.26 | 1.50±0.05<sup>a</sup> |
| 50                            | 38.4±5.44<sup>a</sup> | 53.1±5.95<sup>b</sup> | 5.1±1.08<sup>a</sup> | 1.4±0.48 | 0.7±0.18 | 0.86±0.21<sup>b</sup> |
| 100                           | 44.8±3.80<sup>a</sup> | 50.3±3.61<sup>b</sup> | 3.4±0.43<sup>a</sup> | 0.9±0.34 | 0.6±0.20 | 0.96±0.15<sup>b</sup> |
| SEM                           |     |   |   |   | **      |       |

Table 4. Serum triglycerides, total cholesterol, high-density lipoprotein cholesterol, total protein, albumin and globulin of laying hens (mean ± SE) fed different dietary levels of flaxseed meal.

| Dietary level of FSM meal, g/kg | TG, mg/dL | TCHL, mg/dL | HDL-C, mg/dL | HDL-C:TCHL, mg/dL | TP, g/dL | ALB, g/dL | GLB, g/dL | ALB:GLB ratio |
|--------------------------------|-----------|-------------|--------------|-------------------|---------|----------|----------|--------------|
| 0                              | 1236.4±175.2 | 126.6±9.6  | 68.8±7.75    | 0.64±0.05        | 5.58±0.26 | 3.64±0.21 | 0.54±0.02 |
| 50                             | 1514.8±53.0  | 130.3±4.6  | 95.7±7.23    | 0.73±0.04        | 5.45±0.30 | 3.51±0.25 | 0.56±0.03 |
| 100                            | 1471.4±147.0 | 130.8±9.9  | 83.9±7.23    | 0.61±0.05        | 4.91±0.15 | 3.73±0.09 | 0.55±0.05 |
| SEM                            | 145.2      | 7.46       | 7.77         | 0.04             | 0.26     | 0.09     | 0.21     | 0.03         |

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*P<0.05; **P<0.01; ns, not significant (P>0.05).
SRBC from birds fed diets with up to 15% FS or canola seed (ALA enriched-diets) were higher than those of birds fed the control diets, albeit non-significantly. A similar non-significant effect of ALA-enriched diets on antibody production was reported by different authors (Fritsche et al., 1992; Phetteplace and Watkins, 1992; Wang et al., 2000). Differences in results on the effects of dietary n-3FA on the humoral immunity of birds are more likely related to differences in dietary levels and sources of n-3 FA used in these studies.

Results from the percentages of H and L differential counts indicated that dietary FSM reduced the H:L ratio. This finding was in agreement with Wang et al. (2011) who recorded a reduction in H:L ratio in geese due to feeding lower dietary n-6:n-3 PUFA ratios. Similarly, Hill et al. (2007) found that diets containing higher n-3:n-6 PUFA ratios could decrease H:L ratios. H:L ratio has been used as an index of stress in chicken (Gross and Siegel, 1983; Zulkifli et al., 2000). These results suggested that the addition of FSM to the diet modified immune response of birds. This suggestion is supported by Arrington et al. (2001) who reported that immune responses of birds could be modified by the ratio and structure of PUFA in diet.

The addition of FSM to the diet did not alter serum contents of TG, TCHL, HDL-C, HDL-C:TCHL ratio, TP, ALB, GLB and ALB:GLB ratio of laying hens (Table 4). However, the findings from most of the previous trials in animals and humans on the effects of FSM on blood parameters were inconsistent. Results from this study were partly in agreement with Stuglin and Prasad (2005) who reported no effect of supplementation of FSM in diets of humans on serum TCHL, HDL-C, TP, ALB, but serum TG levels were increased. Whereas in rats, Bhathena et al. (2003) found that dietary FSM...
supplementation decreased plasma TCHL and TG. Similar disagreement has been reported in response to FS diets in men. FS diet increased TG levels in the blood (Cunnane et al., 1995), or decreased (Djousse et al., 2005; Singer et al., 1986) or no effect on circulating TG levels (Rallidis et al., 2003; Paschos et al., 2007). Dietary FS did not influence the level of HDL in men (Cunnane et al., 1995; Arjmandi et al., 1998; Clark et al., 2001; Lemay et al., 2002; Lucas et al., 2002). However, Bloedon et al. (2008) reported a reduction of 16% in HDL. In quails, Parmentier et al. (1997) found that fish oil increased increase GLB when compared with those fed the same amount of chicken fat or soybean oil. The discrepancies in these studies could be attributed to diversity of the FA used in the experimental diets (FS, FSM, fish oil), experimental design and duration, sample size and/or species differences.

The effects of dietary level of FSM on levels of TBARS, SOD in the liver and serum and GPX in the liver are shown in Figures 2 to 4, respectively. The addition of 100 g FSKM/kg diet significantly (P<0.05) increased the level of TBARS in the liver when compared with those of the control or 50 g FSKM/kg (Figure 2). Whilst TBARS level in the serum was not influenced by the addition of FSKM to the diet. The increase in TBARS content in the liver of birds fed the highest FSKM diet (100 g FSKM diet vs. 50 g FSM or 0 g FSKM diet) is more likely related to the presence of higher concentration of long chain PUFA that are highly peroxidized. This diet may suggest that high dietary level of FSKM increased the formation of lipid peroxidation product (Goyens et al., 2006). It appears that the amount of dietary polyunsaturated FA plays a key role in whether FSKM increases tissue susceptibility to oxidation. The non-significant effect of dietary FSKM on serum level of TBARS may suggest that serum TBARS was maintained in normal levels by some protective mechanisms such as hepatic microsomes which have the ability of generating and degenerating TBARS (Venkatraman et al., 1998), or lipid peroxides are produced in different organs or tissues (Kotelnikova et al., 2008), transported via blood circulation and accumulated in fat tissues, or lipid peroxides are produced in subcutaneous fat tissue itself and products of TBARS are increased because serum antioxidant activity is decreased (Gotoh et al., 1997). These findings may suggest that TBARS levels in the liver is a more sensitive indicator of the peroxidative tendency because of the abundance of substrate for TBARS generation in the liver and/or the accumulation of TBARS generated in other transported via circulating blood. The levels of SOD in the serum and liver, and GPX in the serum were not influenced by dietary level of FSKM (Figures 3 and 4, respectively). The non-significant effect of dietary FSKM on GPX and SOD suggested that the addition of dietary FSKM for up to 100 g/kg did not influence the cellular antioxidative defense system and consequently the oxidative stress of the birds. This finding was in agreement with Romieu et al. (2008) and Grim et al. (2011) who reported that diets supplemented with fish oils do not increase cellular oxidative damage, but may even exert an antioxidant effect.

It was concluded that the addition of FSKM to the diet of laying hens enhanced their immune response, increased omega-3 FA and PUFA and reduced MUFA and Σ-6:Σ-3 ratio in the liver and egg yolk, and that of 100 g FSKM/kg diet increased lipid peroxidation in the liver. TBARS measurement in the liver is more sensitive than that of the serum.

Conclusions

The addition of 50 and 100 g/kg FSKM to the diet of laying hens from 24 to 36 weeks of age increased the Σ(3) FA and PUFA in the liver and egg yolk, serum anti-SRBC and reduced blood H:L ratio without altering count of monocytes, eosinophils and basophils or total WBC. The liver TBARS level was increased by the addition of 100 g FSKM/kg to the diet. However, FSKM did not influence many of the oxidative stress parameters (TBARS in the serum, SOD in liver and serum, GPX in liver), nor on lipid and protein levels (total protein, globulin and albumin) in the serum. These findings suggest that the addition of FSKM to the diet of laying hens improved the health status of eggs for human consumption and immune response of birds and that of 100 g FSKM/kg diet increased hepatic lipid peroxidation.

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