Effects of flaxseed supplementation on omega-6 to omega-3 fatty acid balance, lipid mediator profile, proinflammatory cytokines and stress indices in laying hens

Sang-Mok Lee\textsuperscript{a,1}, Hee Kyum Kim\textsuperscript{a}, Ho-Bin.Lee\textsuperscript{b}, Oh-Dae Kwon\textsuperscript{b}, Eun-Bi.Lee\textsuperscript{b}, Jin-Duck.Bok\textsuperscript{b}, Chong-Su Cho\textsuperscript{a,c}, Yun-Jaie Choi\textsuperscript{a,c}, Sang-Kee Kang\textsuperscript{b,*}

\textsuperscript{a} Department of Agricultural Biotechnology, Seoul National University, Seoul 08826, Republic of Korea

\textsuperscript{b} Institute of Green-Bio Science & Technology, Seoul National University, Pyeongchang, Gangwon-do 25354, Republic of Korea

\textsuperscript{c} Research Institute of Agriculture and Life Sciences, Seoul National University, Seoul 08826, Republic of Korea

\textsuperscript{*} Corresponding author. Tel: 82-33-339-5731; Fax: 82-33-339-5763; EM: kangsk01@snu.ac.kr

\textsuperscript{1} This author is the first author
Abstract

**Background:** Although polyunsaturated fatty acids are in the spotlight due to their physiological effects on inflammation and stress of livestock animals, the biological roles of their derivatives, termed lipid mediators, have been little reported in laying hens.

**Results:** In this study, two hundred 33-week-old laying hens were fed 0, 0.9, 1.8, or 3.6% (w/w) dietary flaxseed (Lintex170) with a commercial basal diet for 4 weeks to determine the physiological effects of dietary flaxseed, an omega-3-rich ingredient, on host inflammation or stress states regarding lipid mediator profiles, and also its impact on laying performance. The physiological changes in the omega-6 to omega-3 ratio, lipid mediator profiles, serum proinflammatory cytokine (tumor necrosis factor-alpha, interleukin-1beta, interleukin-6) levels, serum corticosterone levels, and the ratio of heterophils to lymphocytes were monitored. Supplementing dietary flaxseed greatly reduced the omega-6 to omega-3 fatty acid ratio from 25.85 to 4.16 in eggs and from 19.23 to 4.08 in serum samples between groups fed with 0% and 3.6% dietary flaxseed after the experimental period. In addition, the lipid mediator profiles of laying hens were modulated by supplementation with flaxseed, mainly resulting in enrichment of omega-3 fatty acid-derived lipid mediators. Furthermore, the level of proinflammatory cytokines TNF-alpha decreased when fed 3.6% (w/w) dietary flaxseed. Two stress indices, corticosterone in the serum and the heterophil to lymphocyte ratio showed significant reductions in laying hens fed 3.6% (w/w) dietary flaxseed. Additionally, overall laying performance indices were significantly improved by supplementary flaxseed.

**Conclusion:** Taken together, these findings suggest that the decreased omega-6 to omega-3 ratio and enrichment of omega-3-derived lipid mediators induced by dietary flaxseed may contribute to reducing the stress state in laying hens, improving laying performance. These findings broaden the understanding of lipid mediator profiles in laying hens, and the results
will be applied to develop antiinflammatory and antistress feed additives for the poultry industry.

**Keywords:** omega-6 to omega-3 fatty acid balance, flaxseed, inflammation, lipid mediator, stress
Introduction

Polyunsaturated fatty acids (PUFAs) have been reported to broadly regulate homeostatic and inflammatory processes, either directly or through transformations to bioactive metabolic compounds such as lipid mediators [1]. PUFAs can be classified into two principal families, omega-3 and omega-6 fatty acids, depending on the position of the first double bond from the methyl group. Among them, linoleic acid (LA), an omega-6 fatty acid and alpha-linolenic acid (ALA), an omega-3 fatty acid are essential fatty acids, which must be obtained from the diet, and omega-3 fatty acids cannot be converted to omega-6 fatty acids and vice versa in mammals and poultry. When they enter the body, they undergo bioconversion by common elongases and desaturases, which result in the conversion of LA to arachidonic acid (AA) and ALA to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These PUFAs are incorporated into the cell membrane and, used as resources to produce and release various derivatives termed lipid mediators, which are converted by cell membrane-incorporated enzymes such as lipoxygenases (LOXs), cyclooxygenases (COXs) and cytochrome P450 (CYP450) [2]. Lipid mediators have known roles in the onset and resolution of inflammation and their systemic profiles can be nutritionally controllable depending on the quantity of ingested precursors such as omega-6 and omega-3 fatty acids [3]. In general, omega-6-derived lipid mediators exert proinflammatory actions, whereas omega-3-derived lipid mediators exert anti-inflammatory actions [4]. Meanwhile, cytokines are regulators of host responses to various states, including inflammation. Some cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1), and interleukin-6 (IL-6), act as promoters of inflammation and are therefore called proinflammatory cytokines [5]. They play key roles in the acute phase response. However, when they persist in their actions so that inflammation is not resolved, chronic inflammation occurs and results in various problems in the body such as tissue damage. Levels of circulating proinflammatory cytokines are elevated in several
inflammatory diseases, such as Crohn’s disease [6-8] and obesity [9]. Recently, the contribution of the network between lipid mediators and cytokines in immune homeostasis has been investigated. In general, it has been reported that omega-6 derived lipid mediators increase proinflammatory cytokines, while omega-3-derived lipid mediators raise the level of anti-inflammatory cytokines and suppress that of proinflammatory cytokines [10]. For these reasons, balanced intake of omega-6 and omega-3 fatty acids is essential for homeostasis associated with inflammation control.

However, modern human diets and animal feeds commonly contain excessive levels of omega-6 fatty acids but low levels of omega-3 fatty acids due to the high dependence of omega-6-rich resources, such as corn and soybean, to manufacture cooking oils and animal feeds. It was reported that the ratio of omega-6 to omega-3 in modern diets has increased to approximately 20:1 from 1:1 in the past [11]. Since the fatty acid composition in the inner body has also been influenced by this omega-6 and omega-3 fatty acid imbalance in the diet, the incidence of chronic inflammatory diseases could be increased [12]. Various studies have found that omega-3 fatty acids counter the effects of chronic inflammatory diseases associated with cardiovascular diseases [13-15], central nervous system diseases [16], diabetes [17], obesity [18] and even several cancers [19-21]. Similarly, the omega-6 to omega-3 fatty acid ratio of animal feed is also obviously distorted. One of the rational inferences would be that livestock animals might suffer from problems such as chronic inflammation during feeding, which increase stress and adverse effects on their performance. Therefore, restoration of the fatty acid composition through supplementation with omega-3 fatty acids in diets and feeds could be an applicable strategy to improve both human and animal health by recovering immune homeostasis.

Flaxseed has been widely used as a dietary supplement of omega-3 fatty acids for humans and animals because it contains abundant amounts of ALA, representing approximately 53%
of total fatty acids [22]. However, whole flaxseed has been known to contain antinutritional factors such as mucilage, cyanogenic glycosides, phytic acid and trypsin inhibitors [23], which could induce adverse effects on digestion and absorption and even cause diarrhea [24]. One of the common treatments available to reduce these side effects of flaxseed is extrusion [25].

Currently, there are various reports about omega-3 fatty acid-fortified livestock products through the supplementation of omega-3 fatty acid-rich resources such as flaxseed [26-28]. However, it is difficult to find any report on the physiological effects of feeding omega-3 fatty acid-rich resources to livestock animals. In this study, the effect of flaxseed supplementation in laying hens not only to improve the omega-6 to omega-3 fatty acid ratio in eggs, but also its effects on animal physiology and health, mainly by profiling lipid mediators after feeding was investigated. Associated with the regulation of inflammation by lipid mediators and cytokines, it was hypothesized that supplementing omega-3 fatty acids to laying hens could improve their performance through the alleviation of inflammation and stress during the laying period. To examine this hypothesis, the profile of lipid mediators and representative proinflammatory cytokines in serum, corticosterone levels and the heterophil to lymphocyte ratio (H/L) for stress indices, and the performance of laying hens were monitored and analyzed after supplementation with dietary flaxseed.
Methods

Materials

The basal diet used in this study was commercial laying hen feed product provided by a domestic feed manufacturing company. Dietary flaxseed was obtained from a commercial product termed Lintex170 (HANYOU BNF Co., Ltd, Seoul, Republic of Korea) which was developed by Seoul National University and HANYOU BNF Co., Ltd and manufactured by HANYOU BNF Co., Ltd. The product consists of 85% extruded flaxseed and 15% starch source so that 17% of the product is ALA. The lipid mediator standards were as follows: 14, 15-epoxyeicosatrienoic acid (EET)-d11, 5(S)hydroxyeicosatetraenoic acid (HETE)-d8, leukotriene B4 (LTB4)-d4, prostaglandin D2 (PGD2)-d4 and arachidonic acid (AA)-d8 (Cayman Chemical, Ann Arbor, MI, USA). The fatty acid methyl ester standard (Supelco® 37 Component FAME Mix, FAME 37), internal standard (tridecanoic acid, C13:0), potassium hydroxide and sulfuric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade water, acetonitrile, isopropyl alcohol and hexane were purchased from J.T. Baker (Avantor Performance Material, INC., Center Valley, PA, USA). A Strata-x-33-μm polymerized solid reverse-phase extraction column (Cat no.8B-S100-UBJ) was purchased from Phenomenex (Torrance, CA, USA).

Animals, Treatments, and Management

The experiment involving laying hens (n = 200, 33 weeks old, Lohmann Brown-Lite) was conducted at the Seoul National University animal farm (Pyeongchang, Republic of Korea). The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-180219-1). The hens were divided randomly into 4 groups with 5 hens per cage (48 cm x 45 cm x 45 cm, width x depth x height) and 10 cages per group. Fifty hens were assigned to each of the following diet
treatments for 4 weeks: 1) commercial basal diet (C); 2) basal diet with 0.9% (w/w) Lintex170 (T1); 3) basal diet with 1.8% (w/w) Lintex170 (T2); and 4) basal diet with 3.6% (w/w) Lintex170 (T3). The ratio of omega-6 to omega-3 of each feed was 21.8 (C), 8.1 (T1), 5.2 (T2), and 2.9 (T3), calculated by comparison with the FAME 37 peak pattern (Table 2). Feed was offered 600 g per cage daily and fresh water was offered ad libitum during the experimental period.

Sample and Data Collection

Performance was assessed for each cage. The number of eggs and their weights were recorded daily. Abnormal eggs, such as broken, cracked or shell-less eggs, were also noted daily. Feed intake was measured every week, and then the weekly feed conversion ratio [feed consumed (g)/egg mass (g)] was calculated. Egg samples [10 eggs/(group × week)] were randomly selected and analyzed for albumen height, Haugh unit, eggshell thickness and egg fatty acid profile. Albumen height was measured with a micrometer. The Haugh unit was calculated with egg weight using the following formula: Haugh unit = 100 × log (H + 7.57 – 1.7 × W\(^{0.37}\)), where H = albumen height (mm) and W = egg weight (g) [29]. The value of eggshell thickness was determined by averaging measurements taken at 3 different locations on the egg (air cell, equator, and sharp end) by using a dial pipe gage. After these procedures, 1 g of egg yolk was separated and collected in a glass tube to measure the egg fatty acid profile.

Blood samples were collected from 10 randomly selected hens per group at week 0, week 2 and week 4. They were collected in 2 tubes: BD Vacutainer SST\(^{TM}\) II Advance Tubes (Becton Dickinson, Le Pont de Claix, France) for sampling serum and V-Tube\(^{TM}\) EDTA K3 Tubes (AB MEDICAL, Republic of Korea) for measuring complete blood cell count. Blood samples were centrifuged at 6,000 rpm for 10 min, and supernatant serum was transferred to 1.5 mL plastic
tubes and stored at -20°C until use. Blood samples for complete blood cell count were stored at 4°C until use.

Gas Chromatography for Fatty Acid Profile

The fatty acid profiles were analyzed using feed, egg, and serum samples (feed, 1 g × 3 replicates; egg, 1 g × 5 replicates; serum, 500 μL × 5 replicates). Fatty acids were extracted and methylated in one tube using the direct methylation method [30] with some modifications. Briefly, 0.5 mg of tridecanoic acid was added to samples in 15 mL glass tubes, followed by the addition of 5.3 mL of methanol and 700 μL of 10 N KOH. Tubes were incubated in a water bath at 55°C for an hour and a half with brief vortexing every 20 min. Then, tubes were chilled at RT, and 580 μL of 24 N H₂SO₄ was added. Incubation and chilling steps were repeated as before. Finally, 3 mL of hexane was added, and the sample was vortexed for 5 min and centrifuged at 3,000 rpm for 5 min. The upper phase was transferred to a GC vial (Agilent, Santa Clara, CA, USA) and analyzed with a gas chromatography-flame ionization detector (GC-FID, Agilent 7890B, Santa Clara, CA, USA) with SP-2560 (100 m × 0.25 mm, L × I.D; 0.2 μm, df, Sigma-Aldrich, St. Louis, MO, USA). FAME 37 was used as a reference for peak identification. The running condition of GC-FID followed the FAME 37 manual (oven temperature: 140°C, 5 min; ramp: 240°C at 4°C/min and hold for 28 min; injector and detector temperature: 260°C; split ratio: 1:30; injection volume: 1 μL).

Ultra-performance Liquid Chromatography for Serum Lipid Mediator Profile

Lipid mediators in serum were separated by the solid-phase extraction method [31] using a Strata-x-33-μm polymerized solid reverse-phase extraction column. Briefly, the columns were activated with 3.5 mL of methanol followed by the same amount of water for equilibration. Samples were prepared by adding 10 ng of each deuterated internal standard to 1 mL of serum
with 200 μL of methanol and 800 μL of distilled water. Samples were then loaded on columns and washed with 3.5 mL of 10% methanol in distilled water. Finally, samples were eluted with 1mL of methanol. These samples were concentrated using a Speed-Vac concentrator (Labconco, Kansas City, MO, USA), resuspended in 90 μL of solvent A (70:30:0.02, water:acetonitrile:acetic acid, v:v:v) and stored at -80°C until use.

The UPLC analyses and MS/MS analyses followed previously reported methods [32]. The Skyline software package (MacCoss Laboratory, Seattle, WA, USA) was used to determine the peak area of each lipid from raw data. The extracted peak areas were normalized by internal standard. Partial least squares discriminant analysis (PLS-DA) plots of quantified lipid mediators and variable importance in projection (VIP) plots of 15 lipid mediators (VIP scores Top 15) were performed on the MetaboAnalyst website [33].

**Enzyme-linked Immunosorbent Assay for Serum Proinflammatory Cytokine and Corticosterone Levels**

The levels of serum TNF-alpha, IL-1beta, IL-6 and corticosterone were determined with Week 0 and Week 4 serum samples (5 replicates per group) with ELISA kits specific for chicken TNF-alpha, IL-1beta, IL-6, and corticosterone (CUSABIO, Wuhan, China) following the provider’s method.

**Complete Blood Cell Count for Heterophils to Lymphocytes (H/L) Ratio**

To assess the stress state of hens, the ratios of H/L were measured using the complete blood cell count method. First, 10 μL blood samples in EDTA K3 tubes were collected to make blood smears on slide glasses. Then, the smears were stained with Wright’s stain. White blood cells, such as heterophils, lymphocytes, eosinophils, and monocytes were identified with a compound microscope (Axio Scope.A1, Carl Zeiss, Germany) and the ratio of H/L was calculated.
**Statistical Analysis**

Using SAS 9.3, significant differences among 4 groups were determined by one-way ANOVA with post hoc Tukey HSD tests. To compare before and after flaxseed supplementation in each group, paired two-sided Student’s t-tests were conducted with data from Week 0 and Week 4. The fixed effect (Trt) in this model was a flaxseed (Lintex 170) treatment. Orthogonal polynomial contrasts were also used to validate the linear (Lin) and quadratic (Quad) effects when the level of Lintex 170 supplementation increased (*p-value<0.05; **p-value<0.01; ***p-value<0.001). Variability in the data were shown as the pooled SEM.
Results

Flaxseed Improved Ratio of Omega-6 to Omega-3

Flaxseed supplementation improved omega-6 to omega-3 ratios in both egg and serum samples (Table 3). In both samples, the omega-6 to omega-3 ratio decreased with increasing flaxseed concentration. The average ratios in eggs at week 4 were 9.81 (T1), 6.87 (T2) and 4.16 (T3) compared to 25.85 (C). Likewise, the average ratios in serum samples were 8.33 (T1), 5.66 (T2), and 4.08 (T3) compared to 19.23 (C). The content of each PUFA component was also similar between egg and serum samples (Fig. S1).

Flaxseed Altered Serum Lipid Mediator Profile

Exploiting the multiple-reaction monitoring (MRM) method based on UPLC-MS/MS, the profiles of lipid mediator profiles in serum samples of laying hens at week 4 were investigated. As a result, a total of 91 lipid mediators were identified in serum samples (Table S1).

Partial least square discriminant analysis (PLS-DA) was performed to observe the dissimilarities of the lipid mediators profile among groups (Fig. 1A). PLS-DA plot analysis revealed clustering among groups according to flaxseed concentration in the corresponding PLS-DA score plot including Component 1 (19.4%) and Component 2 (11.7%). Fig. 1B is the variable importance in projection (VIP) plot of 15 lipids (VIP scores of Top 15) that were differentially regulated among the 4 groups, which shows that individual lipid mediator species such as hydroxyoctadecatrienoic acid (HOTrE), prostaglandin F3 alpha (PGF3α), and hydroxydocosahexaenoic acid (HDoHE), mainly contributed to the clustering among groups.

Next, each concentration of serum PUFAs and lipid mediators among groups was analyzed by one-way ANOVA and orthogonal polynomial contrasts to determine flaxseed effects on the host lipid profile. In the case of PUFAs, arachidonic acid (AA), an omega-6 PUFA was lower in the flaxseed-fed groups (T1, T2 and T3) than in the C group (Fig. 2A), while EPA and DHA
were higher in the T3 group (Fig. 2B, 2C). In addition, the concentrations of several lipid mediators were changed by flaxseed. Lipid mediators derived from the omega-3 fatty acids ALA (9-HOTrE, p-value: 0.004 for Trt, <0.001 for Lin) (Fig. 3A), EPA (PGF-3α, p-value: 0.038 for Trt) (Fig. 3B) and DHA (4-HDoHE, p-value: 0.022 for Trt, 0.006 for Lin) (Fig. 3C) were linearly increased by flaxseed addition. Additionally, one ALA-derived lipid mediator 13-HOTrE (p-value: 0.014 for Quad) (Fig. S2), two EPA-derived lipid mediators 5-HEPE (p-value: 0.045 for Lin) (Fig. S3A) and 9-HEPE (p-value: 0.023 for Lin) (Fig. S3B) and three DHA-derived lipid mediators 8-HDoHE (p-value: 0.013 for Trt, 0.017 for Lin, 0.022 for Quad) (Fig. S4A), 14-HDoHE (p-value: 0.026 for Lin) (Fig. S4B), and 16-HDoHE (p-value: 0.018 for Lin) (Fig. S4C) were also increased by dietary supplementation with flaxseed.

Flaxseed Decreased Proinflammatory Cytokine Levels in Serum

To evaluate the effect of dietary flaxseed-induced changes in lipid mediator profiles on inflammatory processes, cytokine levels were examined in serum samples from laying hens (Table 4). No significant differences among groups were observed at any levels of TNF-alpha, IL-1beta, or IL-6 in serum samples at week 4. However, when comparing values of week 0 and week 4 per group, the levels of TNF-alpha (p-value: 0.043) were significantly decreased in the T3 group.

Flaxseed Suppressed Serum Corticosterone Levels and Lowered the H/L Ratio

To investigate the effect of flaxseed supplementation on stress state, two stress indices, serum corticosterone level (Table 5) and H/L ratio (Table 6) were examined. During the experiment, the T3 group showed a significant reduction in serum corticosterone levels compared to Week 0 (p-value: 0.046) (Table 5). Similarly, the T2 (p-value: 0.015) and T3 (p-value<0.001) groups
also showed significantly decreased H/L ratios compared to Week 0 (Table 6). The results clearly showed that flaxseed supplementation decreased both stress indices.

**Performance and Egg Quality**

By supplementing flaxseed, all treatment groups (T1, T2, and T3) significantly improved both hen-day egg production (week 1 to 2 p-value: 0.003 for Trt, 0.003 for Quad; week 2 to 3 p-value: 0.029 for Trt, 0.005 for Lin; week 3 to 4 p-value: <0.001 for Trt, <0.001 for Lin, 0.002 for Quad) and egg mass production (week 1 to 2 p-value: 0.001 for Trt, <0.001 for Quad; week 2 to 3 p-value: 0.021 for Trt, 0.014 for Lin, 0.048 for Quad; week 3 to 4 p-value: <0.001 for Trt, <0.001 for Lin) in the second, third and fourth weeks compared to the C group. Additionally, average egg weight was elevated in all treatment groups during the overall period (week 0 to 1 p-value: 0.005 for Trt, 0.015 for Lin, 0.004 for Quad; week 1 to 2 p-value: <0.001 for Trt, 0.001 for Quad; week 2 to 3 p-value: 0.015 for Quad; week 3 to 4 p-value: 0.014 for Trt, 0.003 for Quad). Although there was no significant difference among the group in terms of feed intake during the experiment, the feed conversion ratio improved over the overall period (week 0 to 1 p-value: 0.034 for Trt; Week 1 to 2 p-value: <0.001 for Trt, <0.001 for Quad; Week 2 to 3 p-value: 0.029 for Quad; Week 3 to 4 p-value: 0.026 for Trt, 0.006 for Quad).

Collectively, dietary supplementation with flaxseed appeared to have positive effects on the performance of the laying hens. In particular, hen-day egg production and egg mass production linearly increased with the level of flaxseed addition. The average egg weight and feed conversion ratio were greatest in the T2 group, which showed a quadratic effect of flaxseed supplementation.

Meanwhile, the indices of egg quality (albumen height, Haugh unit, and eggshell thickness) showed no notable improvement with flaxseed supplementation despite albumen height (p-
value: 0.026 for Trt, 0.003 for Lin) and Haugh unit (p-value: 0.027 for Trt, 0.003 for Lin) being significantly greater in week 2 (Table 8).
Discussion

As PUFAs have been studied for their important roles in controlling inflammation, the imbalance in the omega-6 to omega-3 PUFA ratio in the diet due to excessive omega-6 fatty acids has been suspected to be the main cause of disease involving chronic inflammation [34]. Thus, restoration of the balance is a relevant issue, and sufficient intake of omega-3 fatty acid-rich diets is often recommended as a solution for health.

Meanwhile, in the livestock industry field, corn, an ingredient containing high contents of omega-6 fatty acids, is mainly used as an energy source in animal feed. Thus, the ratio of livestock products such as meat, eggs, and milk is generally unbalanced, which can adversely affect the health of consumers. Hence, a variety of studies have tried to produce omega-3 fatty acids-fortified livestock products, and dietary supplementation with omega-3 fatty acid-rich feed ingredients, such as flaxseed, has resulted in the successful production of meats, eggs, and milks with an improved omega-6 to omega-3 fatty acid ratio [35-37]. However, contrary to improving the quality of livestock products for consumers, few studies have investigated physiological changes in livestock when their ratio of omega-6 to omega-3 ratios is balanced. In particular, lipid mediator profiles of livestock are rarely studied despite their strong biological impact on chronic inflammation. In the case of laying hens, owing to their high stocking density, their breeding environment is poor, which makes them chronically exposed to inflammatory stimuli.

The present study aims to validate the hypothesis that dietary supplementation with flaxseed, an omega-3 fatty acid (ALA)-enriched feed ingredient, could not only affect the production of omega-3 fatty acid-enriched eggs but also improve laying performance by alleviating inflammation and the stress state in laying hens by modulating the lipid mediator profile.

Similar to a previous study in which flaxseed oil was supplemented with laying hens [38], the supplementation of flaxseed in the present study showed a decrease in the omega-6 and omega-
3 fatty acid ratios in eggs as the omega-3 fatty acid content increased. Additionally, the serum PUFA profile showed similar patterns with eggs in groups of flaxseed-fed hens. Moreover, from a total of 91 lipid mediators identified by UPLC-MS/MS, 9 lipid mediators (9-HOTrE, 13-HOTrE, PGF3α, 5-HEPE, 9-HEPE, 4-HDoHE, 8-HDoHE, 14-HDoHE, 16-HDoHE) showed significant alterations among the groups by both one-way ANOVA and contrast analysis. Interestingly, these lipid mediators all originated from omega-3 fatty acids. Thus, it can be inferred that dietary flaxseed enriched the lipid concentration of not only omega-3 fatty acids but also omega-3 derived lipid mediators.

It has already reported that omega-3 fatty acids and their derivatives exert anti-inflammatory effects [39]. For example, the ALA-derived lipid mediators, 9-HOTrE and 13-HOTrE which were increased by flaxseed supplementation in this study, are known to exert anti-inflammatory effects by inactivating the nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome [40-42]. Likewise, all EPA-derived lipid mediators (PGF3α, 5-HEPE, 9-HEPE) and DHA-derived lipid mediators (4-HDoHE, 8-HdoHE, 14-HDoHE and 16-HDoHE) have been identified as anti-inflammatory lipid metabolites by countering proinflammatory signals in other animals such as mice, rats and monkeys [43-47]. In particular, 5-HEPE is recognized as a potent inducer of regulatory T cells that play a pivotal role in regulating excessive immune responses [45]. Collectively, dietary flaxseed can enrich the lipid mediators alleviating inflammation in laying hens.

Meanwhile, independent of the results for the lipid mediators, serum levels of several representative proinflammatory cytokines and stress indices were also investigated to determine whether dietary flaxseed actually affects host inflammation and stress state, as many studies have demonstrated the crosstalk between inflammation and stress such as stress-stimulated production of proinflammatory cytokines, through the activation of the nuclear factor kappa B (NF-κB) signaling pathway [48].
As a result, although the serum levels of proinflammatory cytokines such as IL-1beta and IL-6 showed only ambiguous tendencies among the groups, the serum level of TNF-alpha and two stress indices (corticosterone, H/L ratio) in laying hens [49] were significantly reduced by dietary flaxseed supplementation, which indicates its potential to alleviate the effects of inflammation and the stress state of laying hens.

With the increased levels of omega-3 derived anti-inflammatory lipid mediators and reduced levels of stress indices, the groups of flaxseed-fed hens also showed improvement in overall laying performance. Although increases in metabolizable energy and fat content due to flaxseed addition (Table 1) might positively affect laying performance, several studies have reported no effect on laying performance after extra caloric supplementation by adding lipid resources [50-52]. Therefore, dietary flaxseed is likely to improve laying hen performance by enriching omega-3-derived lipid mediators and alleviating inflammation and stress states.
Conclusions

In conclusion, the results in this study imply that dietary supplementation of flaxseed, as an omega-3-rich feed ingredient, to laying hens could alleviate inflammation and stress states by adjusting the lipid mediator profile strengthen anti-inflammatory lipid mediators originating from omega-3 fatty acids, which consequently improved overall laying performance. Therefore, it is inferred that the balance of omega-6 and omega-3 ratio can improve laying performance by downregulating inflammation and stress state.

Meanwhile, this is the first report demonstrating changes in lipid mediator profiles in laying hens. Moreover, this study suggests that flaxseed might be used as a new category of functional feed additives with antiinflammatory and antistress effects in the livestock industry. In addition, differentially regulated lipid mediators depending on dietary supplementation of lipid resources would be an interesting research subject to expand understanding of the mechanism underlying the regulation of immune homeostasis in livestock animals.

Abbreviations

PUFA, polyunsaturated fatty acid; LA, linoleic acid; ALA, alpha-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid, LOX, lipoxygenase; COX, cyclooxygenase; CYP450, cytochrome P450; TNF, tumor necrosis factor; IL, interleukin; H/L, heterophil to lymphocyte ratio; PLS-DA, partial least squares discriminant analysis; VIP, variable importance in projection; MRM, multiple-reaction monitoring; HOTrE, hydroxyoctadecatrienoic acid; PGF, prostaglandin F; HDoHE, hydroxydocosahexaenoic acid; EpETE, epoxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; HETE, hydroxyeicosatetraenoic acid; LTB, leukotriene B; PGD, prostaglandin D; NLRP3, nod-like receptor family, pyrin domain containing 3; NF-κB, nuclear factor kappa B
**Declarations**

**Ethics approval and consent to participate**

This study was approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-180219-1).

**Consent for publication**

Not applicable.

**Availability of data and material**

All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary information. Additional data related to this paper may be available from the corresponding authors.

**Competing interests**

The authors declare that they have no competing interests.

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**Authors’ contributions**
SML designed and conducted the whole experiment, and wrote the paper. HKK, HBL, ODK, EBL performed the animal feeding experiment. JDB and CSC discussed the results and edited the paper, YJC and SKK supervised the experiment.

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| Items (g/kg)          | Commercial basal diet<sup>a</sup> | Lintex170<sup>c</sup> (Flaxseed) |
|----------------------|-----------------------------------|----------------------------------|
| Crude protein        | 165.0                             | 205.9                            |
| Fat (ether extract)  | 25.0                              | 344.1                            |
| Crude fiber          | 80.0                              | 352.7                            |
| Crude ash            | 200.0                             | 53.8                             |
| Calcium<sup>b</sup>  | 40.6                              | -                                |
| Phosphorous<sup>b</sup> | 2.0                              | -                                |
| Methionine + Cysteine| 6.0                               | -                                |
| Metabolizable energy, Mcal/kg | 2.7                           | 5.3                             |

<sup>a</sup>The commercial basal diet was a commercial laying hen feed product termed ‘Laying hen mid laying period No.4’, which was manufactured by a domestic feed manufacturing company.

<sup>b</sup>The contents of calcium and phosphorus were included in the crude ash content.

<sup>c</sup>The content of ALA in Lintex170 is 17% (w/w) of the total product.
Table 2. Omega-6 to omega-3 fatty acid ratio in experimental diet

| Items                                      | Group                          | p-value² | SEM  |
|--------------------------------------------|--------------------------------|----------|------|
| Feed composition                           | C + 0.9% Lintex170 (w/w)      | Trt      | Lin  | Quad |
| Commercial basal diet (C)                   | T1                             |          |      |      |
| Omega-6 to omega-3 fatty acid ratio        | 21.88d                         | <0.00    |      |      |
|                                             | T2                             | <0.001   |      |      |
|                                             | T3                             | <0.01    |      |      |
|                                             | SEM                            | 0.524    |      |      |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.

1 Each value represents the least squares means of 3 replicates.

2 Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01, ***p-value<0.001).

a-d Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Table 3. Changes in omega-6 to omega-3 fatty acid ratios of egg and sera samples during the experiment\(^1\)

| Items                          | Group          | p-value\(^2\) |
|-------------------------------|----------------|---------------|
|                               | C  | T1  | T2  | T3  | SEM | Trt | Lin | Quad |
| Feed composition              |    |     |     |     |     |     |     |      |
| Commercial basal diet (C)     |    |     |     |     |     |     |     |      |
| Lintex1 70                    |    |     |     |     |     |     |     |      |
| Comm + 0.9% (w/w) Lintex1 70  |    |     |     |     |     |     |     |      |
| Comm + 1.8% (w/w) Lintex1 70  |    |     |     |     |     |     |     |      |
| Comm + 3.6% (w/w) Lintex1 70  |    |     |     |     |     |     |     |      |
| Omega-6 to omega-3 fatty acid ratio |    |     |     |     |     |     |     |      |
| Egg                           |    |     |     |     |     |     |     |      |
| Week 0                        | 22.44 | 23.29 | 23.17 | 23.66 | 0.738 | 0.701 | 0.309 | 0.737 |
| Week 1                        | 25.48\(^d\) | 12.24\(^c\) | 9.74\(^b\) | 5.57\(^a\) | 0.818 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |
| Week 2                        | 24.07\(^d\) | 8.78\(^b\) | 6.05\(^b\) | 4.45\(^a\) | 0.350 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |
| Week 3                        | 24.35\(^d\) | 10.24\(^c\) | 6.94\(^b\) | 3.82\(^a\) | 0.704 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |
| Week 4                        | 25.85\(^d\) | 9.81\(^c\) | 6.87\(^b\) | 4.16\(^a\) | 0.291 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |
| Serum                         |    |     |     |     |     |     |     |      |
| Week 0                        | 17.24 | 21.66 | 22.15 | 23.11 | 2.958 | 0.539 | 0.239 | 0.474 |
| Week 2                        | 25.10\(^d\) | 10.72\(^c\) | 8.48\(^b\) | 5.31\(^a\) | 1.039 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |
| Week 4                        | 19.23\(^d\) | 8.33\(^c\) | 5.66\(^b\) | 4.08\(^a\) | 0.667 | \(<0.001**\) | \(<0.001**\) | \(<0.001***\) |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.
\(^1\) Each value represents the least squares means of 5 replicates per group.
\(^2\) Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01, ***p-value<0.001).
\(^a-d\) Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Table 4. Serum proinflammatory cytokine (TNF-alpha, IL-1beta and IL-6) levels in experimental hens at week 0 and week 4\(^1\)

| Items              | Group                                      | SEM   | p-value\(^2\) |
|--------------------|--------------------------------------------|-------|---------------|
|                    | Commerical basal diet (C)                  |       |               |
| Feed composition   | C + 0.9% (w/w) Lintex170                   |       |               |
|                    | C + 1.8% (w/w) Lintex170                   |       |               |
|                    | C + 3.6% (w/w) Lintex170                   |       |               |
|                    | SEM                                        | Trt   | Lin           | Quad          |
|                    |                                             | 0.485 | 0.558         | 0.654         |
|                    |                                             | 0.380 | 0.119         | 0.545         |
| Serum pro-inflammatory cytokines (pg/mL) |                                             |       |               |
| TNF-alpha          | Week 0                                     | 27.13 | 23.54         | 39.35         |
|                    | Week 4                                     | 31.58\(^b\) | 23.34\(^{ab}\) | 10.55\(^a\)  |
|                    | Paired two-sided Student’s T-test (Week 0 vs. Week 4)\(^3\) | 0.787 | 0.992         | 0.436         | 0.043*         |
|                    |                                             | 0.380 | 0.119         | 0.545         |
| IL-1beta           | Week 0                                     | 137.67 | 84.07        | 123.47        |
|                    | Week 4                                     | 137.36 | 80.53        | 92.84         |
|                    | Paired two-sided Student’s T-test (Week 0 vs. Week 4)\(^3\) | 0.110 | 0.916         | 0.194         | 0.105         |
| IL-6               | Week 0                                     | 12.68  | 13.07        | 12.55         |
|                    | Week 4                                     | 15.05  | 12.79        | 12.68         |
|                    | Paired two-sided Student’s T-test (Week 0 vs. Week 4)\(^3\) | 0.337 | 0.473         | 0.123         | 0.257         |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect; TNF, Tumor necrosis factor; IL, Interleukin.

\(^1\) Each value represents the least squares means of 4 replicates per group.

\(^2\) Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01, ***p-value<0.001).

\(^3\) Unpaired two-sided Student’s T-tests were conducted to compare the values of Week 0 and Week 4 of each group (*p-value < 0.05).

\(^{ab}\) Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Table 5. Serum corticosterone levels in experimental hens at week 0 and week 4\(^1\)

| Items                  | Group          | p-value\(^2\) |
|------------------------|----------------|---------------|
|                        | C              | T1            | T2            | T3            | SEM | Trt | Lin | Quad |
| Feed composition       | Commercia1l basal diet (C) | C + 0.9% (w/w) Lintex170 | C + 1.8% (w/w) Lintex170 | C + 3.6% (w/w) Lintex170 |     |
|                        |                |               |               |               | SEM | Trt | Lin | Quad |
| Serum corticosterone (ng/mL) |               |               |               |               |     |
|                        | Week 0         | 16.81         | 13.05         | 11.45         | 22.28 | 4.340 | 0.347 | 0.331 | 0.160 |
|                        | Week 4         | 12.18         | 11.15         | 13.39         | 9.15  | 2.071 | 0.549 | 0.394 | 0.459 |
|                        | Paired two-sided Student's T-test p-value (Week 0 vs. Week 4)\(^3\) | 0.572 | 0.642 | 0.619 | 0.046* |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.

\(^1\) Each value represents the least squares means of 4 replicates per group.

\(^2\) Linear, and quadratic contrasts were tested.

\(^3\) Unpaired two-sided Student's T-tests were conducted to compare the values of Week 0 and Week 4 of each group (*p-value < 0.05).
Table 6. The ratios of heterophils to lymphocytes (H/L) in blood samples of experimental hens at week 0, 2 and 4

| Feed composition | Group | SEM | p-value<sup>2</sup> |
|------------------|-------|-----|---------------------|
|                  |       |     | Trt  | Lin  | Quad |
| Commercial basal diet (C) |       |     |      |      |      |
| Week 0           | 1.85  | 1.89 | 1.95 | 1.95 | 0.142 | 0.945 | 0.602 | 0.789 |
| Week 2           | 1.63  | 1.56 | 1.45 | 1.17 | 0.118 | 0.067 | 0.010*| 0.684 |
| Week 4           | 1.68<sup>b</sup> | 1.56<sup>b</sup> | 1.42<sup>ab</sup> | 1.21<sup>*</sup> | 0.097 | 0.021*| 0.002**| 0.846 |
| Paired two-sided Student's T-test p-value (Week 0 vs. Week 4)<sup>3</sup> | 0.144 | 0.077 | 0.015* | <0.001*** |

Heterophil:Lymphocyte ratio

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.
<sup>1</sup> Each value represents the least squares means of 5 replicates per group.
<sup>2</sup> Linear, and quadratic contrasts were tested (*p-value < 0.05, **p-value < 0.01, ***p-value <0.001).
<sup>3</sup> Unpaired two-sided Student's T-tests were conducted to compare the values of Week 0 and Week 4 of each group (*p-value < 0.05, **p-value < 0.01, ***p-value <0.001).
<sup>a-b</sup> Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Table 7. Effects of flaxseed in the diet on the performance of laying hens during 33-37 weeks of age.

| Items | Group | SE | p-value |
|-------|-------|----|---------|
|       |       | Trt | Lin | Quad |
| Feed composition | Commercial basal diet (C) | C+0.9% (w/w) Lintex170 | C+1.8% (w/w) Lintex170 | C+3.6% (w/w) Lintex170 |
| Week 0 to 1 Hen-day | | | | |
| Hen-day egg production (%) | 61.42 | 75.43 | 72.29 | 69.71 | 6.98 | 4 | 0.540 | 0.604 | 0.253 |
| Egg mass production (g day⁻¹ hen⁻¹) | 34.45 | 43.02 | 42.15 | 40.00 | 4.06 | 2 | 0.456 | 0.512 | 0.189 |
| Average egg weight (g/egg) | 55.59<sup>a</sup> | 57.10<sup>ab</sup> | 58.21<sup>b</sup> | 57.37<sup>ab</sup> | 0.46 | 0 | 0.005** | 0.015* | 0.004** |
| Feed intake (g day⁻¹ hen⁻¹) | 115.59 | 118.61 | 117.11 | 117.07 | 1.74 | 5 | 0.684 | 0.781 | 0.441 |
| Feed conversion ratio (g feed/g egg mass) | 2.08<sup>b</sup> | 2.08<sup>b</sup> | 2.01<sup>+</sup> | 2.04<sup>ab</sup> | 0.01 | 8 | 0.034* | 0.053 | 0.112 |
| Week 1 to 2 Hen-day | | | | |
| Hen-day egg production (%) | 81.14<sup>a</sup> | 90.57<sup>b</sup> | 86.86<sup>a</sup> | 84.57<sup>a</sup> | 1.62 | 0 | 0.003** | 0.670 | 0.003** |
| Egg mass production (g day⁻¹ hen⁻¹) | 46.82<sup>a</sup> | 53.40<sup>b</sup> | 51.81<sup>b</sup> | 49.15<sup>ab</sup> | 1.09 | 1 | 0.001** | 0.632 | <0.001** * |
| Average egg weight (g/egg) | 57.67<sup>a</sup> | 58.95<sup>bc</sup> | 59.64<sup>b</sup> | 58.10<sup>c</sup> | 0.28 | 9 | <0.001 *** | 0.553 | <0.001** * |
| Feed intake (g day⁻¹ hen⁻¹) | 119.53 | 119.97 | 119.94 | 119.95 | 0.22 | 2 | 0.434 | 0.272 | 0.295 |
| Feed conversion ratio (g feed/g egg mass) | 2.07<sup>a</sup> | 2.04<sup>ac</sup> | 2.01<sup>bc</sup> | 2.06<sup>ab</sup> | 0.01 | 0 | <0.001 *** | 0.820 | <0.001** * |
| Week 2 to 3 Hen-day | | | | |
| Hen-day egg production (%) | 91.43<sup>a</sup> | 93.14<sup>a</sup> | 95.71<sup>a</sup> | 96.57<sup>b</sup> | 1.24 | 8 | 0.029* | 0.005** | 0.329 |
| Egg mass production (g day⁻¹ hen⁻¹) | 53.15<sup>a</sup> | 55.74<sup>ab</sup> | 58.57<sup>b</sup> | 57.63<sup>ab</sup> | 1.21 | 3 | 0.021* | 0.014* | 0.048* |
| Average egg weight | 58.01<sup>a</sup> | 59.85<sup>ab</sup> | 61.19<sup>b</sup> | 59.67<sup>ab</sup> | 0.75 | 4 | 0.051 | 0.169 | 0.015* |
| Week 3 to 4 | Hen-day |   |   |   |   |   |   |   |   |   |   |
|------------|---------|---|---|---|---|---|---|---|---|---|---|
| Feed intake (g day⁻¹ hen⁻¹) | 119.96 | 119.95 | 119.99 | 119.94 | 0.02 | 2 | 0.385 | 0.580 | 0.275 |
| Feed conversion ratio (g feed/g egg mass) | 2.08 | 2.00 | 1.96 | 2.01 | 0.03 | 1 | 0.090 | 0.180 | 0.029* |
| Egg production (%) | 95.43a | 92.57b | 96.00a | 99.43c | 0.62 | 3 | <0.001*** | <0.001** | 0.002** |
| Egg mass production (g day⁻¹ hen⁻¹) | 56.13a | 56.03b | 59.26c | 59.82c | 0.64 | 4 | <0.001*** | <0.001** | 0.452 |
| Average egg weight (g/egg) | 58.83a | 60.52ab | 61.73b | 60.17b | 0.57 | 0 | 0.014* | 0.157 | 0.003** |
| Feed intake (g day⁻¹ hen⁻¹) | 119.89 | 119.71 | 119.96 | 119.94 | 0.12 | 7 | 0.520 | 0.505 | 0.807 |
| Feed conversion ratio (g feed/g egg mass) | 2.04a | 1.98ab | 1.94b | 1.99ab | 0.02 | 1 | 0.026* | 0.184 | 0.006** |

| Week 0 to 4 | Hen-day |   |   |   |   |   |   |   |   |   |
|------------|---------|---|---|---|---|---|---|---|---|
| Feed intake (g day⁻¹ hen⁻¹) | 118.74 | 119.56 | 119.25 | 119.22 | 0.47 | 2 | 0.673 | 0.669 | 0.402 |
| Feed conversion ratio (g feed/g egg mass) | 2.07a | 2.02bc | 1.98b | 2.03bc | 0.01 | 2 | <0.001*** | 0.018* | <0.001** |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.

1 Each value represents the least squares means of 10 replicate cages (5 hens per cage).
2 Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01, ***p-value<0.001).
3 Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Table 8. Effects of flaxseed in the diet on the egg quality of laying hens at 33-37 weeks of age1

| Items                        | Group          | SEM  | p-value² |
|------------------------------|----------------|------|---------|
|                              |                | Trt  | Lin     | Quad    |
| Feed composition             |                |      |         |         |
| Commercial basal diet (C)    | C              | 0.343| 0.309   | 0.086   |
|                             | T1 C + 0.9% Lintex170 | 0.442| 0.356   | 0.594   |
|                             | T2 C + 1.8% Lintex170 | 0.302| 0.026*  | 0.917   |
|                             | T3 C + 3.6% Lintex170 | 0.352| 0.509   | 0.920   |
| Egg quality                  |                |      |         |         |
| Albumen height (mm)          |                |      |         |         |
| Week 0                       | 11.57          | 0.343| 0.309   | 0.086   |
| Week 1                       | 10.53          | 0.442| 0.356   | 0.594   |
| Week 2                       | 9.46          | 0.302| 0.026*  | 0.917   |
| Week 3                       | 10.24          | 0.352| 0.509   | 0.920   |
| Week 4                       | 10.71          | 0.352| 0.509   | 0.920   |
| Haugh unit                   |                |      |         |         |
| Week 0                       | 106.66         | 1.270| 0.513   | 0.168   |
| Week 1                       | 101.83         | 1.871| 0.375   | 0.620   |
| Week 2                       | 96.172        | 1.347| 0.027*  | 0.961   |
| Week 3                       | 100.06         | 1.561| 0.579   | 0.929   |
| Week 4                       | 101.85         | 1.678| 0.499   | 0.694   |
| Eggshell thickness (mm)      |                |      |         |         |
| Week 0                       | 0.400          | 0.008| 0.918   | 0.815   |
| Week 1                       | 0.398          | 0.007| 0.172   | 0.452   |
| Week 2                       | 0.406          | 0.006| 0.698   | 0.360   |
| Week 3                       | 0.404          | 0.008| 0.601   | 0.734   |
| Week 4                       | 0.395          | 0.007| 0.596   | 0.830   |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect.

1 Each value represents the least squares means of 10 replicates.

2 Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01).

a-c Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Fig. 1. (A) Partial least squares discriminant analysis (PLS-DA) plot of lipid mediators obtained from the serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. (B) Variable importance in projection (VIP) plot of 15 lipid mediators (VIP scores Top 15) that were differentially regulated among groups. C, 0% (w/w) flaxseed; T1, C + 0.9% (w/w) flaxseed; T2, C + 1.8% (w/w) flaxseed; T3, C + 3.6% (w/w) flaxseed.
Fig. 2. Bar plots of relative concentrations of polyunsaturated fatty acids from the serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. AA (A), EPA (B), and DHA (C) were determined in the sera of laying hens fed diets containing flaxseed for 4 weeks and measured by LC-MS/MS with 5 replicates per group. AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; C, 0% (w/w) flaxseed; T1, C + 0.9% (w/w) flaxseed; T2, C + 1.8% (w/w) flaxseed; T3, C + 3.6% (w/w) flaxseed.
Fig. 3. Bar plots of relative concentrations of lipid mediators from serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. 9-HOTrE, ALA-derived lipid mediator (A), PGF3α, EPA-derived lipid mediator (B), and 4-HDoHE, DHA-derived lipid mediator (C) were determined in the sera of laying hens fed diets containing flaxseed for 4 weeks and measured by LC-MS/MS with 5 replicates per group. HOTrE, hydroxyoctadecatrichenoic acid; PGF, prostaglandin F; HDoHE, hydroxydocosahexaenoic acid; C, 0% (w/w) flaxseed; T1, C + 0.9% (w/w) flaxseed; T2, C + 1.8% (w/w) flaxseed; T3, C + 3.6% (w/w) flaxseed.
### Supplementary data

Table S1. Lipid mediator profile and relative concentration of week 4 serum samples from each group

| Lipid mediator | C     | T1    | T2    | T3    | SEM  | p-value<sup>2</sup> |
|----------------|-------|-------|-------|-------|------|----------------------|
|                |       | Trt   | Lin   | Quad  |      |                      |
| LXA5           | 0.42  | 0.26  | 0.49  | 0.36  | 0.071| 0.154 0.999 0.816    |
| LTB4 or 6t LTB4 or 12epi LTB4 or 6t,12epi LTB4 | 0.04  | 0.05  | 0.09  | 0.04  | 0.017| 0.189 0.849 0.095    |
| 6R-LXA4 or 6S-LXA4  | 0.13  | 0.14  | 0.19  | 0.16  | 0.030| 0.558 0.781 0.626    |
| 14R-LXA4        | 0.15  | 0.12  | 0.17  | 0.13  | 0.037| 0.801 0.774 0.792    |
| TXB1            | 0.04  | 0.03  | 0.04  | 0.05  | 0.014| 0.838 0.377 0.961    |
| LTD4 or 11t LTD4 or EXD4 | 0.07  | 0.06  | 0.05  | 0.07  | 0.022| 0.914 0.994 0.598    |
| Resolvin D3     | 1.14  | 0.65  | 1.01  | 1.19  | 0.360| 0.878 0.557 0.685    |
| Resolvin D2     | 0.18  | 0.17  | 0.17  | 0.18  | 0.029| 0.908 0.711 0.989    |
| Resolvin D1     | 0.03  | 0.03  | 0.03  | 0.03  | 0.011| 0.086 0.324 0.025*   |
| Resolvin E1     | 9.82  | 7.63  | 7.66  | 4.93  | 2.930| 0.773 0.318 0.753    |
| 6k-PGF1a        | 1580.60 | 3803.50 | 4063.19 | 3775.24 | 2002.4 | 0.875 0.787 0.545    |
| TXB1            | 8.45  | 4.95  | 18.66 | 6.14  | 9.277| 0.605 0.238 0.843    |
| TXB2            | 1098.34 | 757.89 | 1895.01 | 583.61 | 1021.5 | 0.728 0.293 0.939    |
| PGF2a or 11-beta| 0.65  | 0.52  | 0.84  | 0.97  | 0.300| 0.751 0.303 0.994    |
| PGF2a or 8-iso  |       |       |       |       |      |                      |
| PGF2a III       | 1.70  | 1.75  | 2.59  | 3.23  | 0.973| 0.618 0.204 0.865    |
| Delta-17 6k-PGF1a| 4.10  | 2.85  | 4.39  | 6.26  | 1.306| 0.390 0.117 0.504    |
| PGE3a           | 2.80<sup>a</sup> | 5.34<sup>a</sup> | 6.34<sup>a</sup> | 11.85<sup>b</sup> | 1.999| 0.165 0.038* 0.334    |
| 6,15 dk-dh-PGF1a| 6.14  | 5.20  | 9.79  | 4.98  | 6.717| 0.681 0.235 0.875    |
| 15k PGF2a       | 0.70  | 1.18  | 1.79  | 1.66  | 0.494| 0.375 0.184 0.303    |
| dh PGF2a        | 1.21  | 1.02  | 0.86  | 0.76  | 0.208| 0.476 0.266 0.459    |
| 19oh PGF2a or 20oh PGF2a | 1417.75 | 1960.31 | 1807.08 | 1789.84 | 978.22 | 0.847 0.843 0.568    |
| 2,3 dinor 11beta PGF2a | 6.77  | 18.76 | 3.87  | 15.90 | 5.735| 0.125 0.592 0.982    |
| tetranor-PGFFM  | 6.21  | 1.35  | 2.76  | 7.07  | 2.605| 0.450 0.430 0.167    |
| PGE1 or PGD1    | 28.19 | 21.30 | 25.04 | 84.41 | 22.356| 0.192 0.054 0.302    |
| PGE2 or 11beta  | 1150.81 | 846.97 | 908.72 | 2418.29 | 539.98 | 0.186 0.060 0.245    |
| PGE3 or PGD3    | 1561.95 | 2210.66 | 1558.75 | 2404.61 | 408.27 | 0.221 0.278 0.974    |
| dihomo PGE2 or dihomo PGD2 | 36.16  | 32.19 | 52.66 | 167.39 | 37.154| 0.063 0.012* 0.286    |
| 15k PGE2        | 0.76  | 0.78  | 1.92  | 2.74  | 0.606| 0.092 0.017* 0.798    |
| tetranor-PGEM   | 8.33  | 8.74  | 11.98 | 23.93 | 5.021| 0.133 0.024* 0.562    |
| PGK2            | 21.36 | 13.12 | 15.29 | 22.22 | 6.838| 0.789 0.656 0.374    |
| dihomo PGJ2 or dihomo 15d PGJ2 | 2.38  | 1.76  | 3.44  | 1.87  | 1.913| 0.694 0.250 0.875    |
|                        | 2.30 | 1.66 | 3.74 | 7.36 | 2.829 | 0.510 | 0.145 | 0.711 |
|------------------------|------|------|------|------|-------|-------|-------|-------|
| PGA2 or PGB2 or PGJ2  |      |      |      |      |       |       |       |       |
| 15d-PGA2 or 15d-PGJ2  | 4.96 | 2.09 | 1.18 | 2.88 | 1.395 | 0.469 | 0.549 | 0.167 |
| dkh PGE2 or dkh PGD2  | 2.84 | 2.48 | 5.09 | 7.37 | 3.101 | 0.662 | 0.224 | 0.986 |
| 5-iso PGF2a VI        | 1.00 | 1.02 | 1.41 | 2.23 | 0.402 | 0.137 | 0.023*| 0.776 |
| PD1 or 15I PD1 or     | 1.06 | 0.75 | 1.34 | 1.70 | 0.581 | 0.723 | 0.278 | 0.900 |
| 10S,17S-diHDoHE       |      |      |      |      |       |       |       |       |
| 12-oxoETE             | 1.17 | 1.56 | 0.94 | 1.19 | 0.253 | 0.607 | 0.503 | 0.428 |
| 15-oxoETE             | 6.93 | 7.14 | 2.31 | 2.20 | 2.179 | 0.065 | 0.046*| 0.102 |
| 9-oxoODE              | 0.80 | 1.24 | 0.32 | 0.43 | 0.283 | 0.180 | 0.094 | 0.630 |
| 13-oxoODE             | 6.37 | 6.22 | 2.14 | 2.32 | 2.027 | 0.283 | 0.100 | 0.454 |
| 15-oxoEDE             | 0.88 | 1.17 | 0.49 | 0.39 | 0.354 | 0.360 | 0.170 | 0.822 |
| 5,6 EET               | 0.60 | 0.80 | 0.29 | 0.56 | 0.176 | 0.261 | 0.3720| 0.100 |
| HXB3                  | 1.92 | 2.88 | 0.78 | 1.00 | 1.183 | 0.465 | 0.378 | 0.832 |
| 14,15 EpETE           | 1.05 | 0.82 | 0.37 | 0.44 | 0.300 | 0.190 | 0.124 | 0.159 |
| 16,17 EpDPE           | 1.12 | 1.51 | 0.75 | 1.11 | 0.354 | 0.564 | 0.617 | 0.692 |
| 5,6 diHETE            | 0.06 | 0.10 | 0.14 | 0.07 | 0.047 | 0.595 | 0.946 | 0.188 |
| 5,15 diHETE           | 0.04 | 0.09 | 0.08 | 0.04 | 0.025 | 0.571 | 0.556 | 0.200 |
| 12-HHT                | 6.31 | 4.94 | 6.75 | 0.81 | 5.183 | 0.914 | 0.492 | 0.858 |
| 11-HETE or 11,12 EET  | 52.43| 35.72| 36.96| 9.71 | 27.995| 0.994 | 0.942 | 0.811 |
| 9-HETE                | 0.39 | 0.28 | 0.31 | 0.17 | 0.206 | 0.998 | 0.911 | 0.903 |
| 9-HEPE                | 0.24a| 1.01b | 0.26a| 0.35b | 0.173 | 0.023*| 0.315 | 0.297 |
| 8-HDoHE               | 0.04a| 0.09a| 0.15a| 0.13a | 0.023 | 0.013*| 0.017*| 0.022*|
| 5-HETE                | 0.49 | 0.52 | 0.48 | 0.41 | 0.078 | 0.712 | 0.392 | 0.525 |
| 5-HEPE                | 0.13a| 0.20b | 0.39b | 0.23b | 0.058 | 0.045*| 0.242 | 0.025*|
| 7-HDoHE               | 0.23a| 0.44a| 0.43a| 0.90b | 0.099 | 0.216 | 0.148 | 0.304 |
| 4-HDoHE               | 0.08a| 0.17a| 0.20ab| 0.26b | 0.034 | 0.022*| 0.006**| 0.283 |
| 9-HOTrE               | 0.05a| 0.15b | 0.24b | 0.28b | 0.058 | 0.004<0.001*| 0.0908 |
| 5-HOTrE               | 0.28 | 0.29 | 0.27 | 0.29 | 0.055 | 0.762 | 0.788 | 0.633 |
| 15-HEPE               | 0.06 | 0.03 | 0.03 | 0.08 | 0.034 | 0.742 | 0.498 | 0.377 |
| 8-HETE or 8,9 EET     | 0.17 | 0.19 | 0.46 | 0.28 | 0.085 | 0.093 | 0.244 | 0.070 |
| 8-HETE               | 0.55 | 0.27 | 0.80 | 0.28 | 0.143 | 0.067 | 0.539 | 0.174 |
| 10-HDoHE              | 0.05 | 0.05 | 0.11 | 0.06 | 0.028 | 0.322 | 0.776 | 0.249 |
| 8-HETE               | 0.29 | 0.40 | 0.48 | 0.39 | 0.135 | 0.436 | 0.133 | 0.698 |
| 11-HDoHE              | 0.07 | 0.16 | 0.10 | 0.08 | 0.029 | 0.379 | 0.416 | 0.356 |
| 5,6 DHET              | 0.10 | 0.10 | 0.18 | 0.14 | 0.032 | 0.301 | 0.264 | 0.246 |
| 8,9 DHET              | 0.28 | 0.37 | 0.70 | 0.54 | 0.138 | 0.194 | 0.156 | 0.228 |
| 11,12 DHET            | 0.14 | 0.12 | 0.29 | 0.25 | 0.046 | 0.056 | 0.035*| 0.254 |
| 14,15 DHET            | 0.63 | 0.81 | 1.29 | 0.92 | 0.208 | 0.177 | 0.303 | 0.081 |
| tetrano 12-HETE       | 0.03 | 0.03 | 0.09 | 0.05 | 0.021 | 0.202 | 0.296 | 0.141 |
| 11-HEPE               | 0.09 | 0.11 | 0.14 | 0.09 | 0.035 | 0.649 | 0.278 | 0.545 |
| 13-HDoHE              | 0.05 | 0.05 | 0.12 | 0.23 | 0.086 | 0.441 | 0.122 | 0.746 |
| 16-HDoHE              | 0.07a| 0.16b | 0.42b | 0.25b | 0.070 | 0.018*| 0.058 | 0.021*|
| 15-HETE or 14,15 EET  | 3.05 | 2.35 | 2.18 | 0.75 | 1.664 | 0.973 | 0.958 | 0.645 |
|                | 17-HDoHE | 15-HETrE | 12-HETE | 14-HDoHE | 20-HETE | 19-HETE | 18-HETE | 17-HETE | 16-HETE | 17,18 EpETE | 9-HODE or 9,10 EpOME | 13-HODE or 12,13 EpOME | 13-HOTrE | 19,20 diHDPA | 9,10 diHOME | 12,13 diHOME | ADA          |
|----------------|----------|----------|----------|----------|----------|----------|----------|----------|----------|--------------|----------------------|------------------------|-----------|--------------|------------|-------------|--------------|
|                | 0.14     | 1.22     | 0.21 a   | 0.02 a   | 0.06     | 0.10     | 0.07     | 0.02     | 0.24     | 0.10 a       | 3.35                  | 6.26                   | 0.07 a   | 0.04         | 2.85       | 2.90        | 94.74        |
|                | 0.20     | 1.20     | 0.16 a   | 0.05 a   | 0.07     | 0.05     | 0.07     | 0.02     | 0.41     | 0.20 b       | 3.78                  | 9.31                   | 0.20 a   | 0.11         | 3.35       | 3.38        | 241.3        |
|                | 0.32     | 1.04     | 0.23 a   | 0.13 b   | 0.09     | 0.08     | 0.07     | 0.04     | 0.43     | 0.23 b       | 4.99                  | 8.90                   | 0.36 b   | 0.13         | 3.66       | 3.44        | 17.60        |
|                | 0.22     | 1.17     | 0.12 a   | 0.09 b   | 0.06     | 0.13     | 0.06     | 0.04     | 0.41     | 0.25 b       | 2.65                  | 6.29                   | 0.36 b   | 0.14         | 3.09       | 2.58        | 90.86        |
|                | 0.086    | 0.647    | 0.031    | 0.024    | 0.024    | 0.13     | 0.06     | 0.04     | 0.085    | 0.040        | 1.720                 | 2.093                  | 0.060    | 0.034        | 1.511      | 1.085       | 104.11       |
|                | 0.529    | 0.997    | 0.506    | 0.026    | 0.499    | 0.839    | 0.686    | 0.10     | 0.286    | 0.135        | 0.713                 | 0.579                  | 0.059    | 0.197        | 0.367      | 0.502       | 506.0        |
|                | 0.516    | 0.936    | 0.906    | 0.051    | 0.902    | 0.610    | 0.292    | 0.081    | 0.286    | 0.056        | 0.266                 | 0.247                  | 0.014*  | 0.069        | 0.107      | 0.212       | 660.0        |
|                | 0.244    | 0.872    | 0.922    | 0.036*   | 0.286    | 0.475    | 0.318    | 0.318    | 0.342    | 0.159        | 0.928                 | 0.620                  | 0.223    | 0.260        | 0.537      | 0.405       | 993.0        |

SEM, standard error of means; Trt, a treatment effect; Lin, a linear effect; Quad, a quadratic effect; LX, lipoxin; LT, leukotriene; EX, eoxin; HX, hepoxilin; PG, prostaglandin; TX, thromboxane; ETE, eicosatetraenoic acid; HDoHE, hydroxydocosahexaenoic acid; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid; HETrE, hydroxyeicosatrienoic acid; EpETE, epoxyeicosatetraenoic acid; EpOME, hydroxyoctadecadienoic acid; HOTrE, hydroxyoctadecatrienoic acid; DHET, dihydroxyeicosatrienoic acid; HDPA, hydroxydocosapentaenoic acid; HOME, hydroxyoctadecenoic acid; ADA, adrenic acid.

1 Each value represents the least squares means of 5 replicates.
2 Linear, and quadratic contrasts were tested (*p-value<0.05, **p-value<0.01, ***p-value<0.001).

a-b Means in a row without a common superscript letter differ significantly as analyzed by one-way ANOVA with post hoc TUKEY HSD test.
Fig. S1. Bar plots of relative concentrations of PUFA components from eggs and serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed after 4 weeks. The values of LA (A), AA (B), ALA (C), and DHA (D) were measured from 5 replicates of egg and serum samples from week 4. C, 0% (w/w) flaxseed; T1, 0.9% (w/w) flaxseed; T2, 1.8% (w/w) flaxseed; T3, 3.6% (w/w) flaxseed
Fig. S2. Bar plots of the relative concentrations of ALA-derived lipid mediator (13-HOTrE) in the serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. 13-HOTrE was determined in the sera of laying hens fed diets containing flaxseed for 4 weeks and measured by LC-MS/MS with 5 replicates per group. HOTrE, hydroxyoctadecatrienoic acid; C, 0% (w/w) flaxseed; T1, 0.9% (w/w) flaxseed; T2, 1.8% (w/w) flaxseed; T3, 3.6% (w/w) flaxseed.
Fig. S3. Bar plots of the relative concentrations of EPA-derived lipid mediators (5-HEPE, 17, 18-EpETE, 9-HEPE) in the serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. 5-HETE (A) and 9-HEPE (B) were determined in the sera of laying hens fed diets containing flaxseed for 4 weeks and measured by LC-MS/MS with 5 replicates per group. HEPE, hydroxyeicosapentaenoic acid; C, 0% (w/w) flaxseed; T1, 0.9% (w/w) flaxseed; T2, 1.8% (w/w) flaxseed; T3, 3.6% (w/w) flaxseed.
Fig. S4. Bar plots of relative concentrations of DHA-derived lipid mediators (14-HDoHE, 7-HDoHE, 8-HDoHE, 16-HDoHE) in the serum of laying hens fed 0, 0.9, 1.8 and 3.6% (w/w) flaxseed for 4 weeks. 8-HDoHE (A), 14-HDoHE (B), and 16-HDoHE (C) were determined in the sera of laying hens fed diets containing flaxseed for 4 weeks and measured by LC-MS/MS with 5 replicates per group. HDoHE, hydroxydocosahexaenoic acid; C, 0% (w/w) flaxseed; T1, 0.9% (w/w) flaxseed; T2, 1.8% (w/w) flaxseed; T3, 3.6% (w/w) flaxseed.