EVALUATION OF THE ANTI-INFLAMMATORY EFFECTS OF DIFFERENT TYPES AND CONCENTRATIONS OF OMEGA-3 POLYUNSATURATED FATTY ACIDS (PUFA) IN PULLET AND LAYING HEN DIETS

BY

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ABSTRACT

The dietary provision of omega-3 polyunsaturated fatty acids (PUFA), including alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), has gained considerable attention, particularly in poultry nutrition. While omega-3 PUFA supplementation in laying hens has primarily focused on producing omega-3 enriched eggs for human consumption, there are no established nutritional guidelines for their inclusion in laying hen diets. Additionally, the potential benefits of omega-3 PUFA in pullet feeding programs remain underexplored, despite pullets' susceptibility to immune stressors. This project aimed to determine how different types and levels of dietary omega-3 PUFA influence the health and productivity of pullets and laying hens, particularly under immune challenge. Using an established lipopolysaccharide (LPS) model, we evaluated immunomodulatory effects by analyzing oxylipin and their precursor fatty acids, along with the expression of immune receptors and cytokines in relevant tissues.

In the initial study, we characterized the acute inflammatory response to LPS in laying hens by measuring protein and mRNA levels of key pro- and anti-inflammatory mediators at 0, 1, 2, 3, 4, and 6 hours post-injection. Peak immune activation occurred at 2 hours post-challenge, establishing the optimal sampling time for subsequent studies.

In the second and third studies, birds were fed diets enriched with EPA and DHA from fish oil (FO), or with increasing levels of ALA from flaxseed oil (FSO) or DHA from algal biomass. Samples were collected at the 2-hour post-LPS time point to assess the immunomodulatory potential of these PUFA. FO supplementation led to increased plasma and liver levels of EPA- and DHA-derived oxylipins, while reducing omega-6 PUFA and their oxylipins. LPS challenge decreased total oxylipin levels by approximately 31% in

FO-fed birds. FO also decreased oxylipins associated with higher soluble epoxide hydrolase (sEH) activity under saline conditions but increased them under LPS challenge. While LPS induced all measured splenic cytokines, FO supplementation did not attenuate their expression.

In contrast, diets enriched with ALA via FSO resulted in higher plasma ALA, and ALA-derived oxylipins under both saline and LPS treatment, whereas algal biomass diet high in DHA had no effect on ALA levels. Increasing both ALA and DHA levels in diets significantly elevated plasma EPA and DHA levels, but only the DHA diet significantly enhanced EPA- and DHA-derived oxylipins and reduced plasma ARA. Similar fatty acid profiles were observed in the liver. Importantly, DHA supplementation at ≥0.4% suppressed splenic *IL-8* and *IL-10* expression.

Overall, different types and levels of dietary omega-3 PUFA distinctly influence inflammatory responses in poultry. Supplementation with omega-3 PUFA FSO, FO or algal biomass can beneficially modulate oxylipin synthesis following immune challenge in pullets and laying hens. Notably, DHA-enriched diets using algal biomass demonstrated selective immunomodulatory effects by downregulating the expression of pro-inflammatory cytokines. These findings suggest that incorporating omega-3 PUFA into poultry diets holds potential for enhancing immune resilience and supporting overall health.

Keywords: omega-3 PUFA, LPS, pullets, laying hens, immune response

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DEDICATION

In memory of Dr. James House,

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May Dr. House rest in peace.

FOREWORD

This thesis was prepared in accordance with the manuscript format and was composed of three manuscripts. The published manuscripts and manuscripts under preparation were listed as follows:

Chapter 4: Jing, M., **Li, S.**, Zhao, S., & House, J. D. (2024). Splenic gene expression of cytokines at multiple time points following lipopolysaccharide challenge in layers. *American Journal of Veterinary Research*, *85*(5):1-7.
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Chapter 5: **Li S.**, Jing M., Plaksii M., Zhao S., Yang C. House, J. D. &. Aukema H. M. The dietary effect of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) enriched fish oil (FO) on performance, fatty acid deposition, plasma oxylipins and cytokines in response to lipopolysaccharide (LPS) challenge in laying hens (Submitted to *Journal of Nutrition*)
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CONTRIBUTIONS OF AUTHORS

This thesis was primarily prepared by Shengnan Li, and reviewed, revised, and approved by both internal and external examiners, including Dr. Chengbo Yang (advisor), Dr. Anna Rogiewicz (committee member), Dr. Heather Blewett (committee member), and Dr. Xin Zhao (external examiner, McGill University).

All authors listed in the manuscripts presented in Chapters Four through Six have reviewed and approved the final versions of their respective chapters. Individual contributions to each manuscript are outlined as follows:

Chapter Four – Manuscript I:

Dr. James D. House and Shengnan Li designed the study. Shengnan Li and Shusheng Zhao conducted animal trials and collected samples. Mingyan Jing and Shengnan Li performed laboratory analyses. Mingyan Jing conducted the statistical analysis and wrote the manuscript.

Chapter Five – Manuscript II:

Dr. James D. House, Dr. Harold M. Aukema, and Dr. Mingyan Jing designed the study. Shengnan Li, Maryna Plaksii, and Shusheng Zhao performed the animal trial and sample collection. Shengnan Li conducted sample and statistical analyses. The manuscript was written by Dr. Harold M. Aukema and Shengnan Li. Dr. Chengbo Yang edited and reviewed the manuscript.

Chapter Six – Manuscript III:

Dr. James D. House, Dr. Harold M. Aukema, and Dr. Mingyan Jing designed the study. Shengnan Li, Zhongyan Wan, Maryna Plaksii, and Shusheng Zhao conducted the animal trial and sample collection. Shengnan Li performed the sample analysis, statistical analysis, and wrote the manuscript. Dr. Chengbo Yang edited and reviewed the manuscript.

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LIST OF ABBREVIATIONS

ALA	Alpha-linolenic acid
ARA	Arachidonic acid
BW	Body weight
CD14	Cluster of differentiation 14
COX	Cyclooxygenase
CYP	Cytochrome P450
DHA	Docosahexaenoic acid
DiHDPE	Dihydroxydocosapentaenoic acid
DiHETE	Dihydroxy-eicosatetraenoic acid
DiHETrE	Dihydroxy-eicosatrienoic acid
DiHOME	Dihydroxy-octadecaenoic acid
DPA	Docosapentaenoic acid
EET	Epoxy-eicosatrienoic acid
EFA	Essential fatty acids
ELOVL2	ELOVL fatty acid elongase 2
ELOVL5	ELOVL fatty acid elongase 5
EOME	Epoxy-octadecenoic acid
EP	Egg production
EPA	Eicosapentaenoic acid
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid

EpODE	Epoxy-octadecadienoic acid
EW	Egg weight
FA	Fatty acids
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 1
FI	Feed intake
FO	Fish oil
FSO	Flaxseed oil
GC-FID	Gas chromatography-flame ionization detector
GLA	γ-linolenic acid
GPR 120	G-protein coupled surface receptors 120
HDoHE	Hydroxy-docosahexaenoic acid
HEPE	Hydroxy-eicosapentaenoic acid
HETE	Hydroxy-eicosatetraenoic acid
HHTrE	Hydroxy-octadecatrienoic acid
HODE	Hydroxy-octadecadienoic acid
HOTrE	Hydroxy-octadecatrienoic acid
HpETE	Hydroperoxy-eicosatetraenoic acid
IFN-γ	Interferon-γ
IL-1β	Interleukin 1β
IL-2	Interleukin 2
IL-6	Interleukin 6
IL-8	Interleukin 8

IL-10	Interleukin 10
IV	Intravenous injection
LA	Linoleic acid
LBP	Lipopolysaccharide binding protein
LC-MS	Liquid chromatography mass spectrometer
LC-PUFA	Long chain polyunsaturated fatty acids
LPS	Lipopolysaccharide
LOX	Lipoxygenase
LT	Leukotrienes
MUFA	Monounsaturated fatty acids
MyD88	Myeloid differentiation primary response 88
NFκB	Nuclear factor-kappa B
oxoODE	Oxo-octadecadienoic acid
oxoETE	Oxo-eicosatetraenoic acid
PG	Prostaglandins
PLA2	Phospholipase A2
PLI	Post-lipopolysaccharide injection
PPARs	Peroxisome proliferator-activated receptors
PPAR-γ	Peroxisome proliferator-activated receptor-γ
PT	Protectin
PUFA	Polyunsaturated fatty acids
q-PCR	Quantitative PCR
RvD	Resolvin D

RvE	Resolvin E
sEH	Soluble epoxide hydrolase
SFA	Saturated fatty acids
TLR4	Toll like receptor 4
TMUFA	Total monounsaturated fatty acids
TNF-α	Tumor necrosis factor-α
TSFA	Total saturated fatty acids
TX	Thromboxanes

CHAPTER 1 GENERAL INTRODUCTION

Nutrition plays a crucial role in optimizing overall health and enhancing animal performance (1). In poultry, diets with adequate nutrition have been shown to positively influence the development and effectiveness of the immune system (2). Bacterial infections remain a major challenge in the global poultry industry, impacting bird health, productivity, and food safety. Common pathogens such as *Escherichia coli*, *Salmonella spp.*, *Clostridium perfringens*, and *Pasteurella multocida* are associated with significant economic losses due to increased mortality, decreased growth rates, reduced egg production, and condemnation at processing (3, 4). To mitigate these infections, the poultry industry has historically relied on antibiotics for both therapeutic and prophylactic purposes. These treatments have proven effective in controlling outbreaks and improving feed efficiency. However, rising concerns about antimicrobial resistance (AMR) and regulatory restrictions—such as bans on antibiotic growth promoters in the EU and increasing scrutiny in North America—have spurred interest in alternative strategies (5, 6).

Recently, there has been growing interest in incorporating feed components or additives with immune-regulatory properties into poultry feeding systems to strengthen immune defenses against these infectious challenges, including viruses, bacteria, and other pathogens (7, 8). Among these dietary components, essential fatty acids—particularly omega-3 and omega-6 polyunsaturated fatty acids (PUFA)—have been extensively studied for their health benefits in poultry and other animals (9, 10). Linoleic acid (LA), the precursor of omega-6 PUFA, has a dietary requirement of approximately 1.0% for pullets and layers in nutritional guidelines. In contrast, while recognized as essential, their dietary requirements for poultry have not yet been officially established.

Poultry products are widely consumed, and their fat composition can be successfully enriched with omega-3 PUFA, including α-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by feeding chickens on special diets (11). Current strategies for this practice include the provision of diets that are: 1) using diets high in ALA from plant-based sources, such as flaxseed or canola and their oils; 2) utilizing algae and marine products that are rich in DHA; or 3) supplementing feeds with marine or fish oil (FO), which are rich in both EPA and DHA, but not ALA (12). Nonetheless, the conversion of ALA to EPA and DHA is limited due to the β-oxidation of ALA, and only a small portion of this fatty acid (FA) is available for synthesis of the long chain (LC)-PUFA (Brenna et al., 2009). Therefore, direct supply of EPA and DHA in the diet may be an effective strategy for poultry to receive the LC-omega-3 PUFA.

Numerous research has provided evidence that dietary supplementation of omega-3 PUFA may modify the FA deposition in yolks and different biological tissues (13). However, it has not been well established as to whether and how the provision of omega-3 PUFA leads to health protective responses in poultry, especially for laying hens and pullets. Significant efforts have been made to determine their protective effects against inflammatory responses (8, 14). Cytokines are regulators of host responses to many immunological processes, including inflammation, which exert pro- and anti-inflammatory effects, and their levels reflect the status of inflammation (15, 16). Evidence of omega-3 PUFA treatment decreasing the pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, has been found in rats (17, 18), cell culture (19) and clinical studies (20, 21). However, studies on the biology of cytokines and their functions in regulating immune responses in avian systems have lagged behind research conducted in other species.

Recently, research has indicated that the mechanisms underlying the anti-inflammatory effects of omega-3 PUFA are attributed to the actions of the bioactive lipid mediators, known as oxylipins, which exert the resolution of inflammation (22). Through the main synthetic pathway, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP450), oxylipins are generated from either omega-3 or omega-6 PUFA with distinctly different and opposing effects (23). Omega-3 oxylipins, especially derived from EPA and DHA, including the new families of resolvins (E-series and D-series), protectins and maresins, known as the specialized pro-resolving mediators (SPM), promote anti-inflammation and pro-resolving properties (24). Oxylipins produced from LA (the precursor of omega-6 PUFA) and arachidonic acid (ARA; a product converted from LA), are mostly pro-inflammatory, such as prostaglandins (PG), thromboxanes (TX) and leukotrienes (LT) (25, 26). Meanwhile, AA-derived lipoxins and epoxyeicosatrienoic acid (EpETrE) have been reported as pro-resolving families (27).

Lipopolysaccharide (LPS), a potent pro-inflammatory substance found in the cell wall of gram-negative bacteria, has been used extensively in our laboratory to model bacterial infections experimentally in poultry, especially in laying hens (28-30). During LPS-induced inflammation, immunomodulatory cytokines and oxylipins are produced and serve as biomarkers of inflammatory status (15, 16). However, the effects of omega-3 PUFA on the contribution of the relationships between the supplementation of omega-3 PUFA, oxylipin profiles and cytokines in the immune status of laying hens and pullets have not been fully investigated. Lee et al. (16) reported that an ALA-enriched flaxseed diet (~14.4%

total omega-3 PUFA) enhanced the production of several omega-3 PUFA-derived lipid mediators while reducing serum levels of TNF-α, corticosterone, and the heterophil-to-lymphocyte ratio in laying hens. Additionally, ALA predominantly contributed to the production of ALA-derived oxylipins, while EPA- and DHA-derived oxylipins were primarily influenced by dietary DHA intake. However, the LPS challenge had minimal impact on these oxylipin profiles (31).

In this project, we first conducted a time course study to evaluate the cytokine expression induced by LPS challenge, aiming to establish the optimal time point of sample collection. Subsequently, we utilized diets containing EPA- and DHA-enriched FO, and examined its effect on performance, FA deposition, oxylipin profile, and expression of immune mediator in response to LPS challenge in laying hens. Furthermore, the increasing levels of either ALA (from FSO) or DHA (LC-omega-3 PUFA found in algae) were compared on measuring of performance, FA deposition, oxylipin profile, and expression of immune mediator in pullets challenged with LPS.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction to fatty acids (FA)

2.1.1 Structure and category of FA

FA are hydrocarbon chains with a methyl group at one end of the molecule and at least one carboxyl group (–C(=O)OH, –COOH, or –CO_2H) at the opposite end. There are various classifications of FA based on many characteristics. They can be categorized into a) saturated fatty acids (SFA, no double bonds), monounsaturated fatty acids (MUFA, single double bond) and PUFA (two or more double bonds), based on the number of double bonds; b) short chain fatty acids (2 to 4 carbon molecules), medium chain fatty acids (4 to 12 carbon molecules) and long chain fatty acids (more than 12 carbon molecules), based on the chain length; c) non-essential fatty acids and essential fatty acids, based on essentiality for humans and animals; or d) omega-6 PUFA and omega-3 PUFA, based on the position of the first double bond from the terminal methyl group (32).

2.2 Omega-3 and omega-6 PUFA

The omega-3 PUFA family is defined by a double bond beginning at the third carbon from the methyl end. Because vertebrates, including poultry, cannot insert double bonds more proximal to the methyl end than the ninth carbon atom (Δ-9 desaturase), omega-3 PUFA cannot be synthesized *de novo*, consequently, omega-3 PUFA have to be present in the diet. ALA is referred to as the essential precursor of the longer chain omega-3 PUFA, including EPA and DHA, because it is the metabolic precursor from which LC-omega-3 PUFA are synthesized (33). These features are shared by another family of FA, the omega-6 PUFA, whose precursor is LA, which is metabolized to ARA (34, 35). As in the case of omega-3

PUFA, the omega-6 PUFA is defined by a double bond beginning at the sixth carbon from the methyl end. Because neither of these FA can be synthesized *de novo*, ALA and LA are referred to as essential fatty acids for vertebrates.

2.2.1 Conversion of ALA and LA to long chain (LC)-PUFA

Liver is the major site of lipid metabolism in avian species (36) due to its key roles in regulating several metabolic fates, such as desaturation and elongation reactions of LA and ALA to LC-PUFA, β-oxidation and transportation to other tissues, including muscle or adipose tissues (37). All the reactions are catalyzed by an enzymatic system consisting in fatty acyl-CoA synthetases, Δ-6 and Δ-5 desaturases and respective elongases (38, 39). Key enzymes Δ5 and Δ6 desaturases are encoded by the genes fatty acid desaturase 1 (FADS1) and 2 (FADS2) respectively (40, 41), and two elongase enzymes derived from the genes Elongation of very long-chain fatty acid (ELOVL) 2 and ELOVL5 (39, 42). Particularly, Δ6 desaturase has been regarded as a major regulatory point in the conversion of plant-derived ALA to EPA or DHA in mammals (43). Gregory et al. (44) recently characterized the chicken elongase enzymes and have provided evidence that chickens have enriched expression of ELOVL5, compared to other animals examined to date. The conversion of LA and ALA to the LC-PUFA is illustrated in **Figure 2.1**, which shows that the primary product was ARA, and EPA and DHA respectively during the process (45).

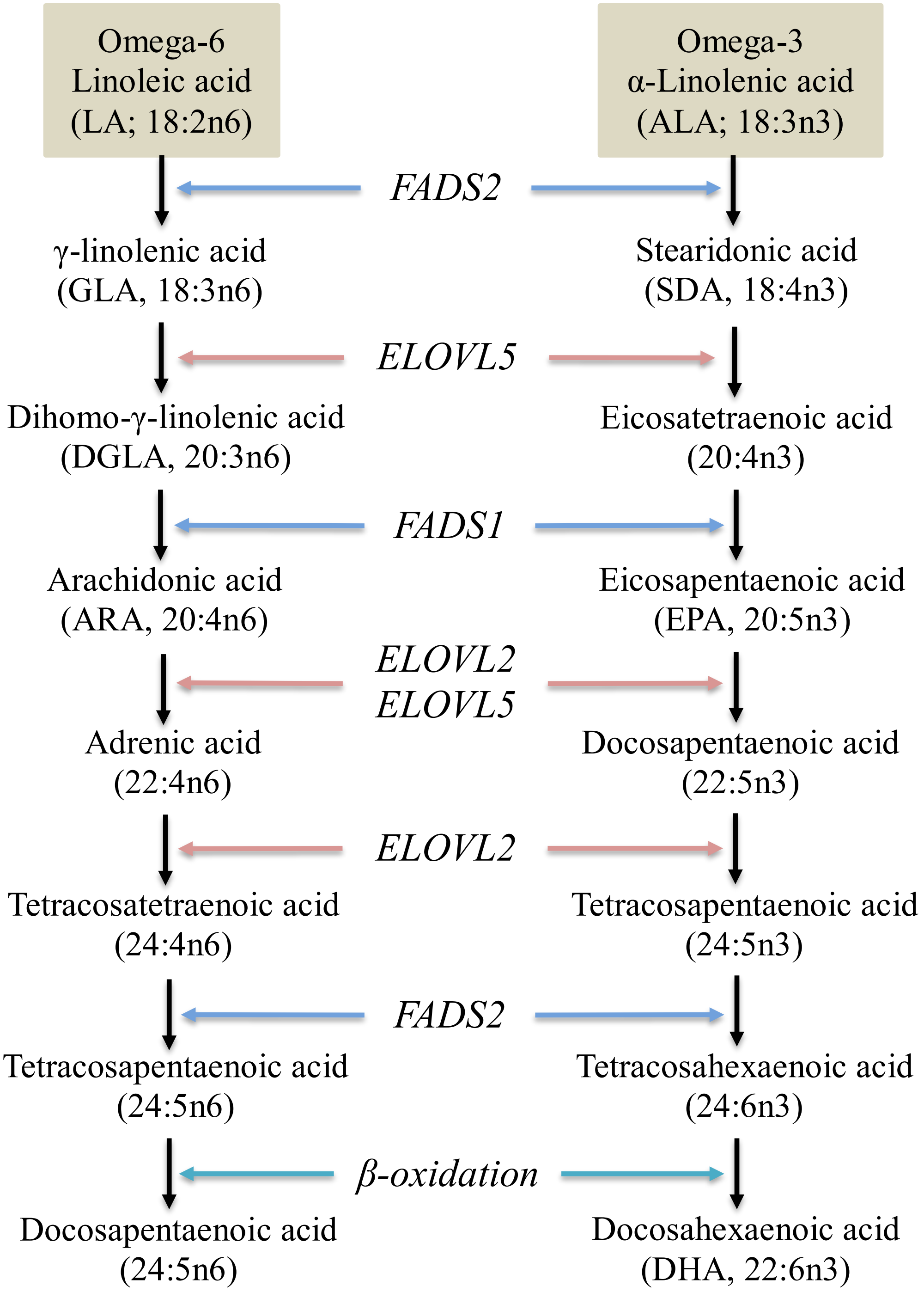


Figure 2.1. The general pathway for conversion of LA and ALA to LC-PUFA.

The conversion of ALA to long chain omega-3 PUFA (mainly EPA and DHA) is inefficient (46). One main reason is that there is a competition between LA and ALA for the desaturase and elongase enzymes to convert to final products as shown in **Figure 2.1** (47). Another factor limiting conversion is that high amounts of ALA (60%-85%) undergo β-oxidation to provide energy, but for other FA, including ARA, only around 30% is catabolized (48). Due to the low conversion efficiency of ALA to long-chain omega-3 PUFA, and the lack of sufficient dietary omega-3 LC-PUFA, the direct provision of preformed EPA and DHA is essential for both animals and humans. In particular, enriching hen eggs with omega-3 LC-PUFA has emerged as an effective and widely accepted strategy to enhance human intake of these essential fatty acids. As a result, dietary strategies to increase omega-3 PUFA content in diet have become a central focus in poultry nutrition research.

2.2.2 Dietary sources of omega-3 and omega-6 PUFA

A good dietary source useful for increasing omega-3 PUFA consumption is a greater consumption of vegetable derived omega-3 PUFA. ALA is found mainly in plant-based oils and/or oil food resources, and flaxseed (also known as linseed) is one of the richest sources. The ALA content of FSO accounts for more than 50% of total fatty acid (TFA). Other sources of ALA include canola oil and soybean oil, making up approximately 10% of the FA profile (49). Unfortunately, the bulk of the common crops are high LA producers, e.g. corn, soybean, while the most common vegetable oils such as soybean (7% ALA) and canola (9% ALA), do not contain ALA as most of the FA. Thus, the vegetable oils most widely consumed contain a high percentage of LA, such as corn oil (57%) or sunflower oil (71%), which competes directly with ALA for enzymes involved in elongation and desaturation. Fish oil (FO) is the common feed ingredients for animals with EPA- and DHA enriching, while marine products are the richest sources of the LC-omega-3 PUFA, predominantly enriched with DHA (50). The sources of omega-3 PUFA in selected plant and animal-based foods are briefly summarized in **Table 2.1** (50).

Table 2.1. Contents of omega-3 PUFA in selected plant and oils with Values are g/100g.

		Omega-3 PUFA		
		ALA	EPA	DHA
Plants and their oils	Flaxseed	22.8	-	-
	FSO	53.3	-	-
	Chia seed	17.6	-	-
	Canola	9.14	-	-
	Canola oil or rapeseed oil	9.1	-	-
	Walnuts	9.1	-	-
	Soybean or soybean oil	6.8	-	-
	Corn	1.16	-	-
Fish oil	Menhaden	-	13.17	8.56
	Salmon	-	13.20	18.23
	Sardine	-	10.14	10.66
	Cod liver	-	9.90	10.97
	Herring	-	6.27	4.21

However, specific requirements for livestock including poultry have yet to be clearly defined, despite numerous studies conducted on the enhancement of characteristics of animal products for human consumption.

2.3 Manipulating omega-3 PUFA into poultry diet

The inclusion of omega-3 PUFA in poultry diets has been practiced over the past years. Fish oil, safflower oil, linseed, fish meal, and algae (51, 52) are used in those feeding regimens. The dietary inclusion of omega-3 PUFA for poultry may directly influence their production, mainly the meat and eggs, which are demanded by human consumers (53). Fish and other types of seafood are generally considered to be major sources of omega-3 PUFA for humans, but they may not serve as primary foods in some countries, therefore, those enriched poultry products serve as good alternatives to fulfill the nutritional requirements of omega-3 PUFA. Studies have shown that the manipulation of omega-3 PUFA in poultry diets significantly improved growth and productive performance, immune response and anti-oxidative properties, meat quality as well as quality and nutritional values of eggs (54). Here, the beneficial effects are mainly focused on the characteristics of poultry products, including meat and eggs, and not the bird’s health.

2.3.1 Dietary enrichment for laying hens

Appropriate feeding strategies play a crucial role in enhancing the nutritional value of eggs in laying hens. Numerous studies have demonstrated that dietary modifications can significantly enrich the omega-3 PUFA content in eggs. Current strategies for this practice include the provision of diets that are: 1) high in ALA, derived from plant sources (flaxseed and oil); 2) high in EPA and/or DHA, derived from marine or algal sources; or 3) a combination of both approaches (12). It is generally recognized that the endogenous conversion efficiency of plant-based precursor fatty acids to DHA is low and not as efficient as preformed DHA (55). A number of studies have demonstrated that when flaxseed is added to hens' feed, yolk ALA levels increase proportional to the percentage of flaxseed addition. Amounts of DHA in yolk increase as well, albeit to a lesser extent and not in linear response to the level of flaxseed (56-58). Our previous study showed that feeding ALA-rich hemp products to laying hens increased the content of egg yolk DHA (approx. 55 mg/egg) which however eventually reached a plateau with further increases in dietary ALA levels (59). Several factors including activities of rate limiting desaturase and elongase enzymes, total amount and their relative proportions of omega−3 PUFA in the diet, and age, strain as well as gender of animals have been shown to influence ALA conversion rate (60-63). However, the precise underlying mechanisms for limited DHA deposition remain incompletely elucidated.

In contrast to the traditional use of plant-based sources of ALA to derive eggs enriched in omega-3, principally DHA, the use of marine products (fish oil and algae product) leads to higher levels of egg yolk DHA content (64, 65). Neijat et al. (13) demonstrated that hens fed DHA-rich diets incorporated three times more DHA into egg yolks compared to ALA-fed hens (179 ± 5.55 mg vs. 66.7 ± 2.25 mg per yolk, respectively) at the highest dietary inclusion levels (0.6% FSO and algal DHA). Marine oils, such as FO, typically contain both EPA and DHA and, depending on the source, EPA intake by hens can be similar to that of the DHA intake. However, when examining the fatty acid profile of the table eggs, DHA is preferentially deposited over EPA (66). Lawlor et al. (2010) reported that egg yolk total omega-3 LC-PUFA levels increased in linear fashion, but DHA predominated, reaching levels of 160 mg/egg compared to 40 mg/egg for EPA, when hens fed FO supplemented diets providing EPA and DHA with a maximum inclusion level of 38 and 25 g per kg EPA and DHA, respectively. This may be related to enriched expression of ELOVL5 in chickens as discussed above, however, the specific mechanisms regulating long chain PUFA enrichment of eggs are still not clearly understood. In summary, the supplementation of omega-3 PUFA in poultry diets can effectively modulate lipid profiles and enhance FA deposition in egg yolks. This approach offers a promising strategy for fortifying animal-derived foods with omega-3 PUFA, contributing to improved nutritional value for human consumption.

Although enriching laying hen diets with omega-3 precursors is considered one of the most effective strategies for enhancing the functional FA profile of eggs, the impact of such supplementation on production parameters requires careful evaluation. A meta-analysis from Irawan et al. (67) reported that the performance responses of laying hens to dietary fat and oil supplementation have generally been inconsistent and appear to be independent of factors such as age, strain, or trial duration. The effects of dietary FA sources on production performance remain conflicting. Some studies have reported a decline in egg production (EP) and egg weight (EW) following supplementation (68, 69). In contrast, other research has shown increases in EP and EW (70, 71), while many studies have found no significant effects on production parameters (72-74). These discrepancies highlight the need for further investigation to fully understand the interactions between dietary FA supplementation and laying hen performance.

2.3.2 Dietary enrichment for pullets

The effects of early lipid exposure through hatching eggs on tissue PUFA composition in post-hatch chicks have been extensively studied. Breeder hens were fed diets enriched with either FO or sunflower oil, serving as sources of omega-3 and omega-6 PUFA, respectively, to produce omega-3-enriched and omega-3-depleted eggs. The total omega-3 PUFA concentration was 0.9% in omega-3-depleted eggs, compared to 4.1% in omega-3-enriched eggs (75, 76). Subsequently, chicks hatched from these eggs were fed diets devoid of LC-PUFA to mimic commercial feeding conditions. Despite the post-hatch dietary deficiency in omega-3 PUFA, chicks from omega-3-enriched eggs retained higher levels of EPA, DHA, and total omega-3 PUFA across various tissues and cells. Similarly, chicks from omega-6 PUFA-enriched eggs exhibited greater retention of ARA in tissues such as the liver, heart, brain, spleen, and duodenum(75, 76).

Moreover, early supplementation of omega-3 PUFA through the maternal diet and hatching eggs provides a significant advantage, as the enriched chicks had greater DHA reserves available during the critical early post-hatch period. For instance, DHA levels remained higher in the liver, spleen, bursa, and cardiac ventricle of chicks hatched from omega-3 PUFA-enriched eggs up to 14–28 days post-hatch, compared to those hatched from omega-3 PUFA-depleted eggs (77). In the duodenum, DHA content peaked at day 14 in chicks from omega-3 PUFA-enriched eggs.

While previous feeding strategies aimed at improving growth, health, and productivity have largely focused on the pre-hatch period, it is equally important to address nutritional needs during the post-hatch phase to optimize overall performance and immune system development in birds.

2.4 Inflammatory process

Inflammation is a critical and natural component of the host's defense mechanism, essential for combating pathogenic organisms and responding to injury (78). It creates a hostile environment for pathogens, facilitates their destruction, and triggers metabolic changes within the host. This complex process involves numerous cell types and an extensive array of chemical mediators (79).

The inflammatory response begins with an increased blood supply to the affected site, accompanied by heightened vascular wall permeability (78). This allows plasma and large molecules to cross the endothelium, delivering soluble mediators to the site of inflammation. Leukocytes (white blood cells) migrate from the bloodstream into the surrounding tissue, guided by chemical signals released at the site of inflammation. These signals act as chemoattractants and upregulate adhesion molecules on the endothelium, enabling leukocytes to tether transiently before moving into the tissue. Once at the site of inflammation, these activated leukocytes release various chemical mediators (80).

The mediators produced during inflammation depend on factors such as the cell type, the nature of the inflammatory stimulus, the anatomical site, and the stage of the response (81). These mediators include lipids (e.g., PG, LT), endocannabinoids, platelet-activating factor), peptides (e.g., cytokines, chemokines), reactive oxygen species (e.g., superoxide anion, hydrogen peroxide), amino acid derivatives (e.g., histamine, nitric oxide), and enzymes (e.g., matrix proteases) (82). Notably, lipid mediators and cytokines will be introduced in detail in the next two sections (2.5 and 2.6).

The influx of cells and the presence of diverse inflammatory mediators lead to the characteristic signs of inflammation: redness, swelling, heat, pain, and loss of function.

While inflammation is designed to neutralize pathogens, the cellular activities and chemical mediators involved can inadvertently damage host tissues. However, inflammation is typically self-limiting, resolving rapidly due to various negative feedback mechanisms. These include the secretion of anti-inflammatory cytokines and pro-resolving lipid mediators, the downregulation of receptors for inflammatory signals, and the activation of regulatory cells that suppress pro-inflammatory responses (80).

When these regulatory mechanisms fail, inflammation may become excessive, inappropriate, or chronic, leading to irreparable tissue damage. This pathological inflammation can result in the onset of disease. Thus, understanding the balance between pro-inflammatory and anti-inflammatory processes is crucial for managing inflammation and its associated risks.

2.5 Oxylipins: lipid mediators of PUFA

2.5.1 Formation of oxylipins

In addition to desaturation and chain elongation, polyunsaturated fatty acids (PUFA) can undergo oxidation, leading to the formation of highly bioactive metabolites known as oxylipins. This process involves one or more mono- or dioxygen-dependent enzymatic reactions that regulate various physiological functions, including inflammation, immunity, reproduction, and development(83). Oxylipin biosynthesis is initiated by cellular activation, as PUFA are not readily available in free form but are predominantly stored at the sn-2 position of phospholipid membranes(84). The enzyme phospholipase A2 (PLA2) plays a key role in this process by hydrolyzing membrane phospholipids to release PUFA into their free forms (85). Once liberated, these PUFA are enzymatically oxidized into distinct oxylipin classes, including eicosanoids and docosanoids, through three major pathways:

cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP) enzymes (86).

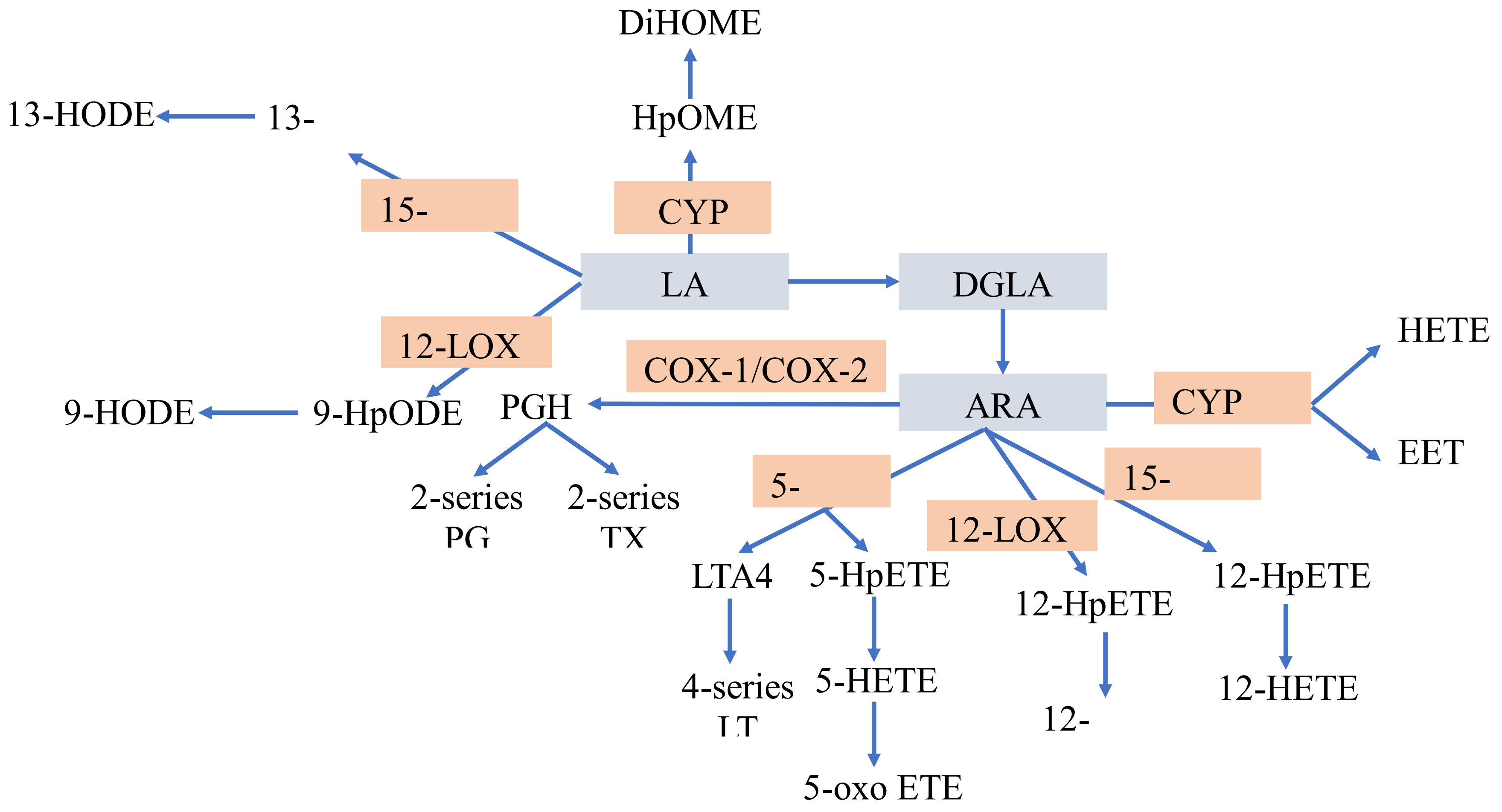
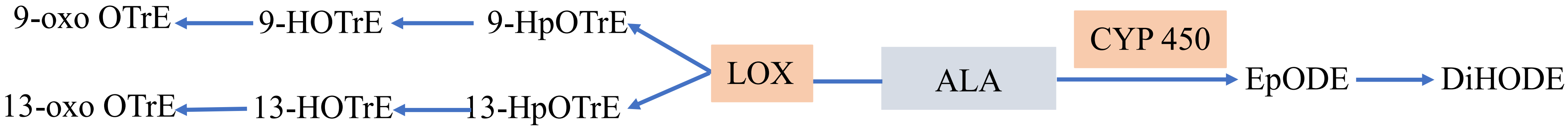
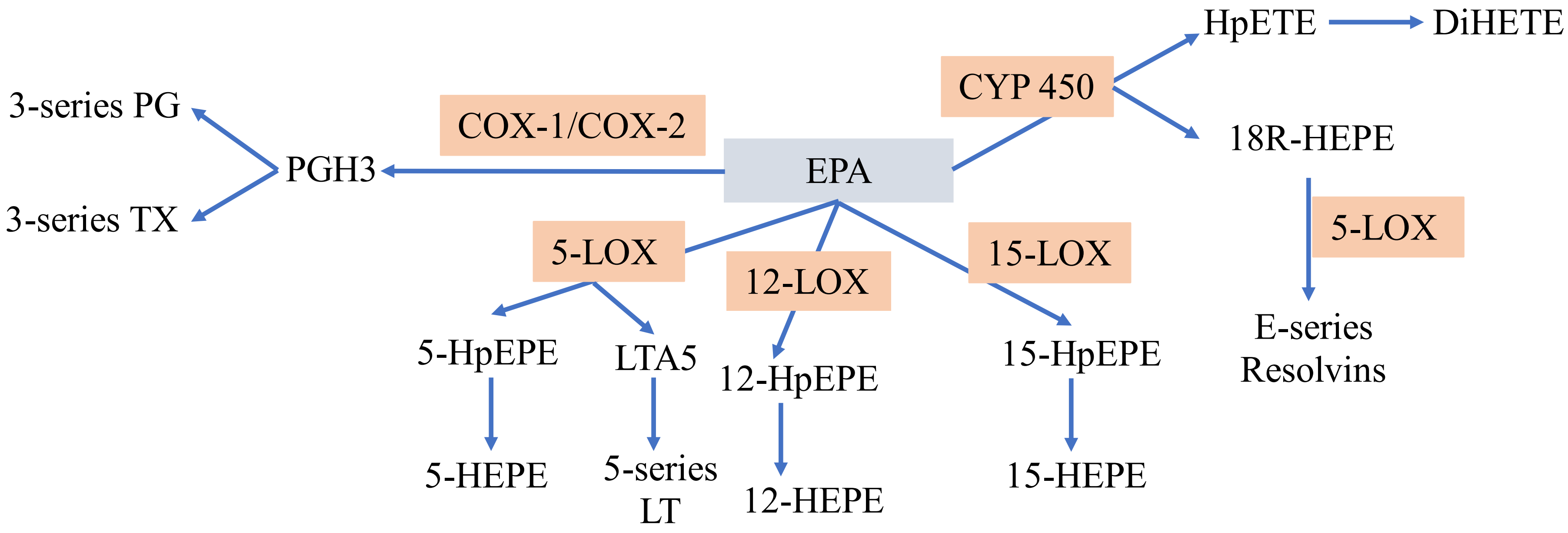


Figure 2.2. LA- and ARA–derived oxylipins and their metabolisms.

(A)


9-oxo OTrE
9-HOTrE
9-HpOTrE
13-oxo OTrE
13-HOTrE
13-HpOTrE
LOX
ALA
CYP 450
EpODE
DiHODE

(B)


HpETE
DiHETE
CYP 450
3-series PG
COX-1/COX-2
18R-HEPE
PGH3
EPA
3-series TX
5-LOX
15-LOX
5-LOX
12-LOX
5-HpEPE
LTA5
12-HpEPE
15-HpEPE
E-series
Resolvins
5-HEPE
5-series
LT
12-HEPE
15-HEPE

(C)

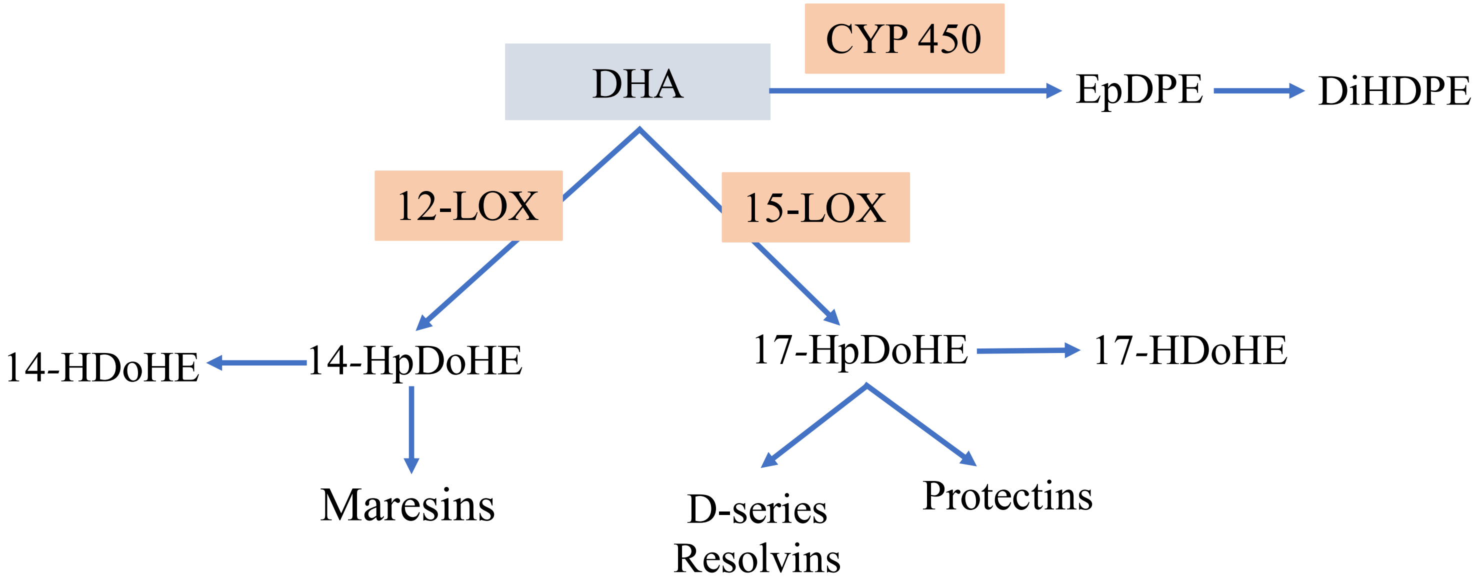


Figure 2.3. ALA- (A), EPA- (B) and DHA- (C) derived oxylipins and their metabolisms.

2.5.2 Oxylipins derived from omega-6 PUFA

Metabolism of the ARA initially generates PGH2, followed by the rapid conversion to other 2-series PG (PGE2, PGF2 and PGD2) and TX (TXA2 and TXB2) via the COX pathway. While COX-1 is said to be constitutively expressed and COX-2 is induced in inflammatory cells by classical inflammatory stimuli, resulting in a large increase of PG. ARA yields hydroperoxy derivatives, hydroperoxyl-eicosatetrae (HpETE) via the LOX pathway, which are further rapidly converted to hydroxy FA, hydroxy- eicosatetraenoic acid (HETE), via glutathione peroxidase (87). Different kinds of HpETE can be metabolized via several other routes. For example, 5-hydroperoxy-eicosatetraenoic acid (5-HpETE) converts to 5-HETE and 4-series LT, including LTC4, LTB4, LTD4, and LTE4 by the 5-LOX pathway. These PG, particularly PGE2 and LT are recognized as mediators and regulators of inflammation (88, 89). Metabolism of AA via CYP v-hydroxylase activity leads to the conversion of HETE that the hydroxy group is at the omega or methyl end of the FA (e.g., 20-HETE), while CYP epoxygenase activity generates epoxy FA, epoxy-eicosatrienoic acid (EpETrE) and further converted to dihydroxy FA, dihydroxy-eicosatetraenoic acid (DiHETE) via soluble epoxide hydrolase (sHE) (90-92). LA produces hydroperoxy FA oxylipins through the LOX pathway and they are rapidly metabolized to hydroxy-octadecadienoic acid (HODE), then further converted to oxo-octadecadienoic acid (oxo-ODE). Meanwhile, HODE can continue to trihydroxy FA, such as 9,10,13-trihydroxy-octadecenoic acid (triHOME). LA also can produce via the epoxygenase activity of CYP to epoxygenated FA [e.g., 12,13-epoxy-octadecenic acid (12,13-EpOME)], which are metabolized via sEH activity to form dihydroxy FA [e.g., 12,13-dihydroxy-octadecenoic acid (DiHOME)] (93). LA- and ARA–derived oxylipins are presented in **Figure 2.2**.

2.5.3 Oxylipin derived from omega-3 PUFA

ALA (**Figure 2.3A**) can be metabolized to hydroxy-octadecatrienoic acid (HOTrE; e.g., 9-HOTrE) via the LOX pathway, followed by further metabolizing to keto FA [e.g., 9-oxo octadecatrienoic acid (9 oxo-OTrE)]. ALA also can generate epoxygenated FA, epoxy-octadecadienoic acid (EpODE) via CYP epoxygenase activity, which can be continued converted to dihydroxy FA, dihydroxyoctadecadienoic acid (DiHODE) via sEH (94). However, there is little functional information available on ALA-derived oxylipins and, therefore, this needs further investigation. EPA (**Figure 2.3B**) can be metabolized via the COX pathway, resulting in 3-series PG (e.g., PGE3) and TX (e.g., TXA3), which is less potent or produced less efficiently than ARA oxylipins (95). EPA can produce hydroperoxy eicosapentaenoic acid (HpEPE) and can be further converted to hydroxy eicosapentaenoic acid (HEPE) by the LOX pathway, and 5-series LT (e.g., LTB5). Metabolism of EPA via CYP epoxygenase activity results in epoxy eicosatetraenoic acid (EpETE; e.g., 5,6-EpETE) (96), which can be further metabolized to dihydroxy eicosatetraenoic acid (e.g., 5,6-DiHETE) by sEH (97). Especially, EPA can produce hydroxy FA which has hydroxy groups in the positions of 18–20 carbon through the v-hydroxylase activity of the CYP, such as 18-HEPE. Importantly, E -series resolvins can be formed by 18-HEPE (**Figure 2.4**) (98-100). DHA (**Figure 2.3C**) can form hydroxy docosahexaenoic acid (HDoHE) by the LOX activity with a hydroperoxy intermediate hydroperoxy-docosahexaenoic acid (HpDoHE). Notably, 14-HpDoHE can be further developed to generate maresins (101) or to 14-HDoHE with further formation of dihydroxy-docosahexaenoic acid (DiHDoHE, and 17-HpDoHE can produce D-series resolvins and protectins (**Figure 2.5**).

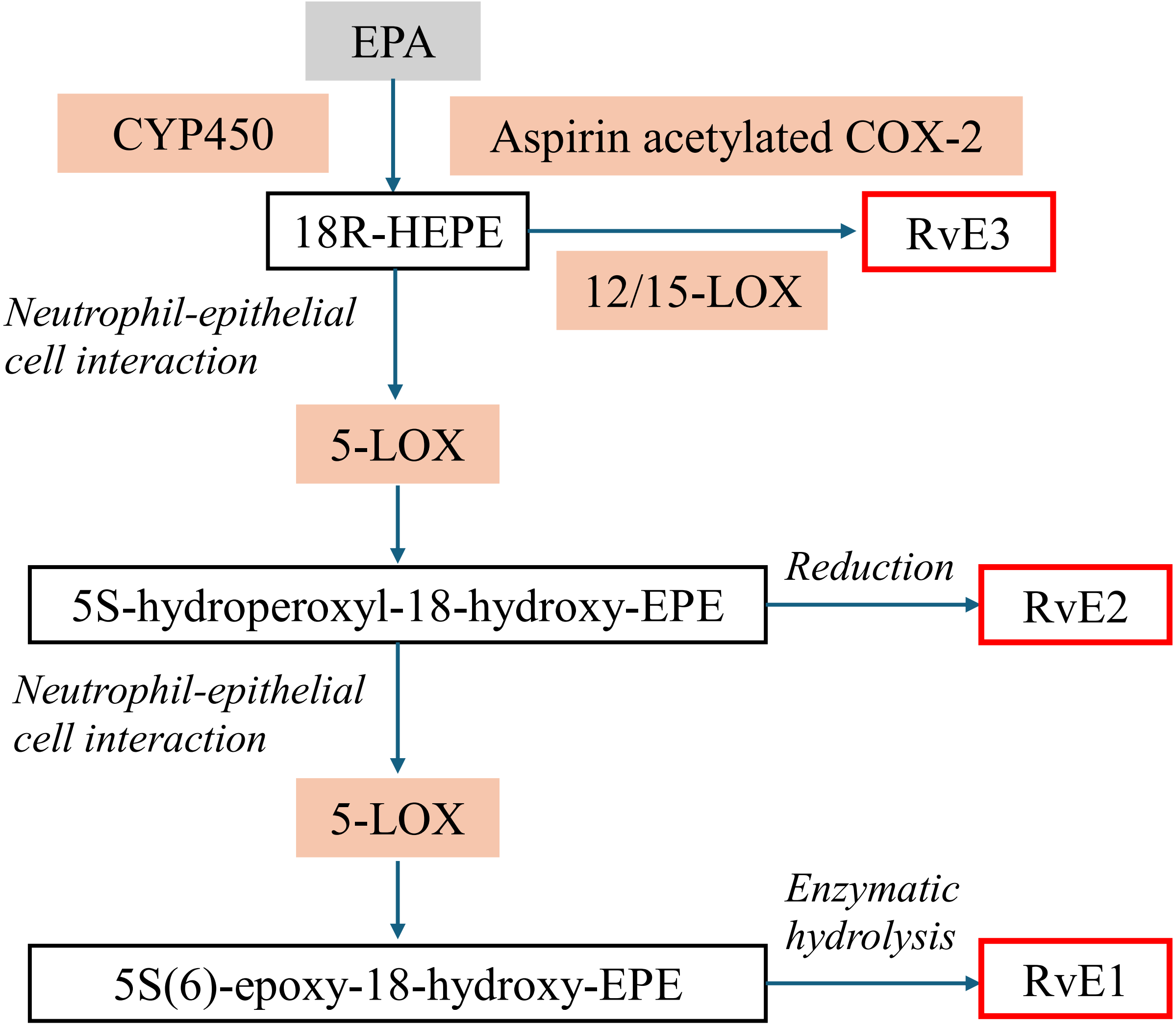


Figure 2.4. Biosynthesis of E-series resolvins via EPA.

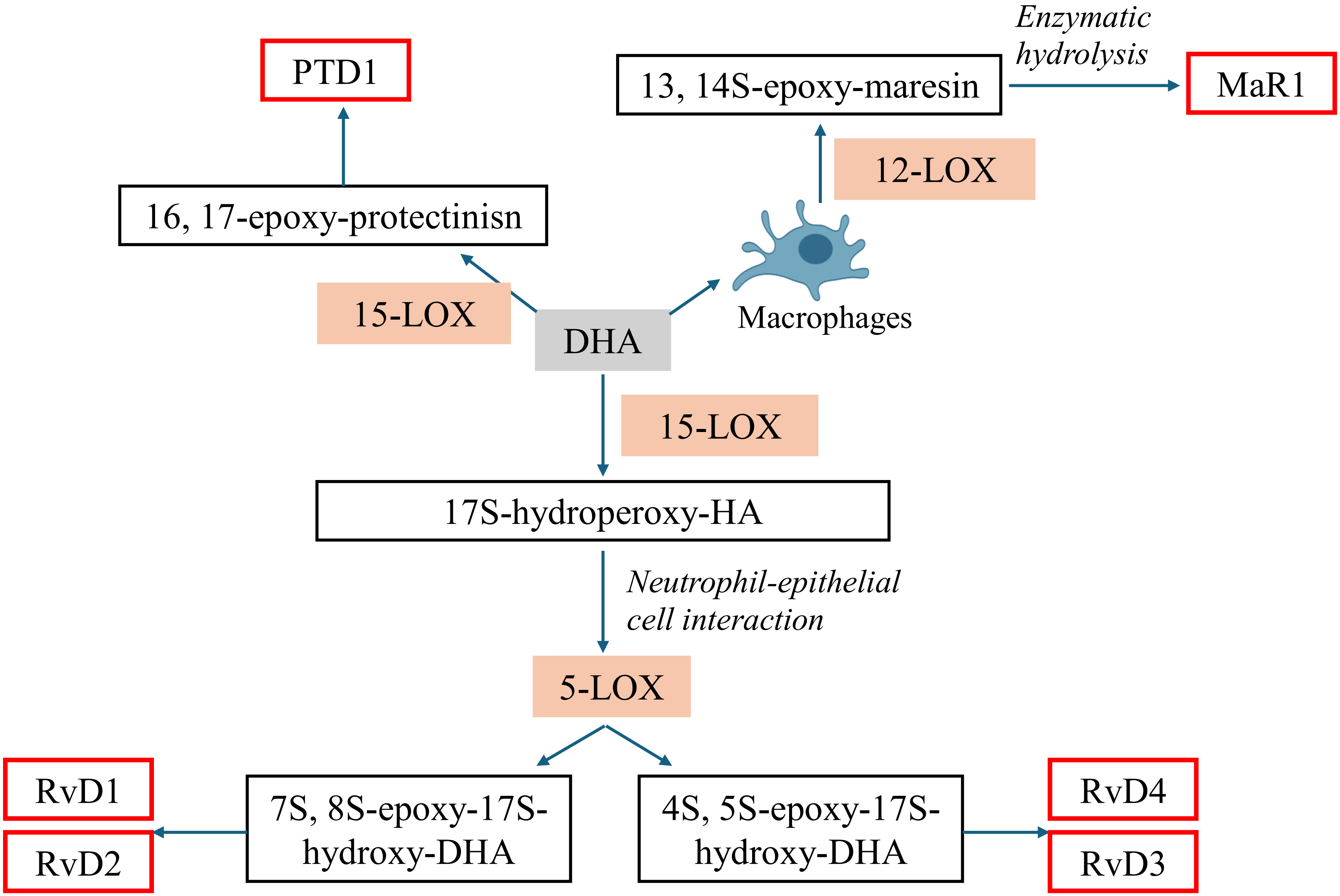

PTD1
Enzymatic hydrolysis
13, 14S-epoxy-maresin
MaR1
12-LOX
16, 17-epoxy-protectinisn
15-LOX
DHA
Macrophages
15-LOX
17S-hydroperoxy-HA
Neutrophil-epithelial cell interaction
5-LOX
RvD1
RvD2
7S, 8S-epoxy-17S-hydroxy-DHA
4S, 5S-epoxy-17S-hydroxy-DHA
RvD4
RvD3

Figure 2.5. Biosynthesis of D-series resolvins, maresins, and protectins via DHA.

2.5.4 Omega-3 PUFA and oxylipins in poultry

There is strong scientific evidence that the metabolism of oxylipins can be modified by the dietary intake of omega-3 PUFA in avian species. Studies in laying hens showed that consumption of FO or flaxseed reduced the concentrations of PGE2 and expression of COX-2 in ovaries since the laying hen is considered a good model of spontaneous ovarian cancer that recapitulates the human disease. This result was confirmed to reduce the incidence and severity of ovarian cancer (102, 103). In addition, the administration of fish oil significantly decreased the contents of plasma PGE2 and TXB2 in broiler chickens (104). It has also been indicated that modulating maternal dietary omega-3 PUFA in progeny birds can reduce pro-inflammatory eicosanoid production in their offspring. Broiler chicks hatched from a lower ratio of omega-6 to omega-3 PUFA (<0.8) enriched eggs had the lowest level of cardiac PGE2 (105). Similarly, the production of LTB4 and the ratio of LTB4 to LTB5 by thrombocytes from chicks that hatched from a high ratio of omega-6 to omega-3 PUFA (15.0) eggs was higher than those chicks hatched from lower ratio eggs (76). The concentration of PGE2 was greater, whereas TXA3 was lower in the heart tissue of chicks hatched from high omega-3 PUFA enriched eggs when the maternal diet contains 3.5% of sunflower or FO (106). The results indicate dietary intake of omega-3 and omega-6 PUFA can modify the lipid metabolites in the tissues of chickens. However, to date, few documents reported the relationship between the production of oxylipins and dietary supplementation of omega-3 PUFA in laying hens and pullets, which needs to be further investigated.

2.6 Cytokines and transcription factors involved in inflammatory process

2.6.1 Overview of cytokines

Cytokines are a diverse group of small proteins primarily produced and released by cells involved in the inflammatory and immune systems, although they are not exclusively limited to these functions (107, 108). Acting through specific receptors, cytokines modulate the activity of their originating cells (autocrine signaling) or other target cells (paracrine signaling) (109). This group includes TNF, various IL, interferons, chemokines, and lymphokines.

Cytokines are synthesized by a wide variety of cells, such as monocytes, macrophages, T and B lymphocytes, and mast cells, as well as non-immune cells, including endothelial cells, fibroblasts, and adipocytes (110). Notably, a single cytokine may be produced by multiple cell types. Elevated levels of specific cytokines, including TNF, IL-1β, IL-6, and IL-8, are a hallmark of many inflammatory diseases, underscoring their critical role in the pathophysiology of inflammation and immune-related conditions (78, 111).

2.6.2 Inflammatory signaling and transcription factor activation

Several mechanisms have been proposed to explain the protective effects of omega-3 PUFA against inflammation. The effects of omega-3 PUFA on cytokines and other inflammation-related proteins are largely mediated through changes in the expression of genes encoding these proteins. This indicates that omega-3 PUFA influences the signaling pathways regulating gene expression in inflammatory cells (112). Nuclear factor-kappa B (NFκB) is a widely expressed inducible transcription factor that involves the synthesis and/or activities of several pro-inflammatory cytokines, adhesion molecules and COX-2.

Initially, NFκB exists as a trimer in the cytosol, with one of the subunits being the inhibitory subunit of NFκB (IκB). Then NFκB can be activated by a signaling cascade triggered by the inflammatory stimuli, including in response to LPS. This activity involves the process of phosphorylation of IκB by the IκB kinase (IKK) complex, subsequently resulting in the IκB degradation. Meanwhile, the remaining NFκB dimer translocates to the nucleus where the position up-regulates inflammatory gene expression, including TNF-α, IL-1β, and IL-6, thereby leading to their induction (113). In addition, peroxisome proliferator-activated receptor-γ (PPAR-γ), another transcription factor, is involved in the anti-inflammatory effects of omega-3 PUFA (114). PPAR-γ potentially inhibits the translocation of NFκB to the nucleus and subsequently inhibits its activation. Therefore, the production of pro-inflammatory cytokines can be reduced under endotoxin stimulation. Furthermore, ARA- and EPA-derived eicosanoids and other lipid mediators derived from DHA can bind to and regulate PPAR-γ, which is a key mechanism between the omega-3 PUFA and inflammatory processes (115).

Besides, the mechanism of the influence of omega-3 PUFA on NFκB activation is identified by G-protein coupled receptor (GPR120). GPR120 is known as a G-protein coupled cell membrane receptor, bonded with LC-PUFA, and expressed on macrophages. GPR120 acts as a synthetic agonist that modulates macrophage responses to endotoxin stimulation by preserving cytosolic IκB levels and suppressing the expression of pro-inflammatory cytokines such as TNF-α and IL-6 (116). Oh, et al. (116) reported that EPA and DHA, but not ARA, improved GPR120-mediated gene activation. These findings suggest that EPA and DHA may have an inhibitory effect on NFκB via GPR120 by blocking NFκB activations. Taken together, omega-3 PUFA appear to inhibit NFκB activation through at least two distinct but potentially interconnected mechanisms: activation of GPR120 and activation of PPAR-γ.

2.6.4 Omega-3 PUFA and cytokines

Few studies have investigated the effects of omega-3 PUFA on inflammatory cytokine expression in laying hens and pullets compared to other species. Sijben et al. (117) demonstrated that dietary PUFA derived from FO enhanced the mRNA level of IL-2 but not Interferon (IFN)-γ in chicks at 37 or 38 days of age following a *Salmonella typhimurium* LPS challenge at 2 hours post-injection. In contrast, a subsequent study by Sijben et al. (45) found that mRNA expression of IFN-γ was significantly higher in chickens fed an FO-enriched diet compared to those fed diets enriched with corn oil (CO), linseed oil (LO), or beef tallow (BT) after a similar *Salmonella typhimurium* LPS challenge. Additionally, Lee et al. (16) reported that dietary supplementation with 3.6% flaxseed significantly reduced the pro-inflammatory cytokine, TNF-α, in laying hens.

Some studies have also explored the effects of dietary omega-3 PUFA supplementation on cytokine expression in broilers. For example, Ibrahim et al. (118) demonstrated that enriching broiler diets with omega-3 PUFA significantly increased the splenic mRNA expression of IFN-γ and IL-1β, particularly in FO-supplemented groups with dietary omega-6 to omega-3 ratios of 1.5:1 and 4:1, and in LO-supplemented groups with ratios of 1:1 and 2.5:1. Furthermore, the expression of IL-6 was elevated in the LO-supplemented groups, and IL-2 expression increased with reduced omega-6 to omega-3 ratios, achieved by increasing dietary levels of FO and LO. Maroufyan et al. (119) further reported that supplementation with omega-3 PUFA using combinations of tuna oil, sunflower oil, and palm oil at 8%, 11.5%, and 16.5% enrichment levels increased IL-2 and IFN-γ expression in plasma at pre-challenge and two days post-challenge. Notably, both cytokines were significantly higher at a 0.5% omega-3 PUFA level compared to an 8.0% level before the challenge. These findings collectively highlight the complex and dose-dependent effects of omega-3 PUFA on cytokine regulation in poultry.

2.7 Lipopolysaccharide (LPS)

LPS has been widely used to model bacterial infections experimentally in poultry and other animals as it is a major component of the cell wall of Gram-negative bacteria and a highly efficient pro-inflammatory substance (120). Intravenous injection (IV) administration of LPS can lead to the development of symptoms mimicking those of Gram-negative sepsis, including fever, neutrophil release, and an increase in acute-phase proteins, such as inflammatory cytokines IL-6, IL-1β and TNF-α (121). Structurally, LPS consists of three regions: an outer polysaccharide domain (commonly referred to as the O-antigen), a unique core oligosaccharide, and a lipid-rich inner region known as lipid A, which is primarily responsible for its endotoxic effects (122). LPS recognition and signaling involve several key molecules. The process begins when LPS binds to the acute-phase protein LPS-binding protein (LBP), which facilitates its transfer to cluster of differentiation 14 (CD14) (123, 124), This LPS–CD14 complex then interacts with toll-like receptor 4 (TLR4) and its co-receptor myeloid differentiation protein 2 (MD-2), forming a multi-component receptor complex. The lipid A region of LPS plays a critical role in this interaction, initiating activation of the TLR4/MD-2 signaling cascade and triggering downstream immune responses. (125, 126). This activation initiates the MyD88-dependent pathway, resulting in the phosphorylation and degradation of IκB, the inhibitor of NFκB. Consequently, NFκB is released, translocates into the nucleus, and promotes the transcription of genes involved in inflammation. (127-129). These include pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), adhesion molecules, acute-phase proteins, nitric oxide, and prostaglandins, all of which contribute to the amplification of the immune response (130-132).

2.7.1 The use of LPS on avian species

Currently, the optimum dose of LPS for inducing an antibody response in laying hens is considered to be 8 mg/kg BW via intravenous injection, based on studies by Gehad, et al. (133). In that study, administration of LPS at 8 mg/kg BW elicited significant clinical signs of sickness and produced a markedly greater antibody response seven days post-injection compared to lower doses (0, 0.5, 1, 2, and 4 mg/kg BW) in 8-week-old Cornell K-strain chickens. This dosage has also been applied in our previous layer experiments (28-30). In mammals, TNF-α is the first cytokine to appear following LPS administration (within 90 minutes to 2 hours), followed by interleukin-6 (IL-6) at approximately 3 to 4 hours post-injection. Importantly, the timing of plasma cytokine appearance in mammals is not significantly influenced by LPS dosage (134-136). However, in poultry, particularly laying hens, the time course of cytokine expression following LPS exposure remains poorly characterized.(137). Gehad, et al. (133) reported that plasma TNF-α activity increased at 6 hours post-LPS injection, which is notably later than the peak observed in mammals. This delay may reflect species-specific differences in immune response kinetics between birds and mammals. Additionally, IL-1 activity in plasma was significantly elevated at 1, 3, and 6 hours post-injection compared to saline-treated controls, with a peak observed at 3 hours. Despite these findings, the temporal expression patterns of cytokines following LPS administration, both at the protein and gene expression levels, remain incompletely characterized in laying hens and warrant further investigation.

2.8 Summary

Overall, dietary omega-3 PUFA has been shown to elicit protective immune responses by modulating the production of oxylipins and their precursor FA and cytokines. However, relevant situations inpullets and laying hens remain inadequately investigated. In particular, the potential impact of different types and levels of omega-3 PUFA in poultry diets has not been thoroughly explored. Thus, further research is needed to establish omega-3 dietary requirements for both pullets and laying hens, addressing gaps in current feeding and management guidelines that overlook the importance of these nutrients. A deeper understanding of the relationship between omega-3 PUFA status and immune challenges will facilitate the development of management strategies aimed at enhancing hen health and welfare, improving immunocompetence, and ultimately reducing disease risks in the layer industry.

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

3.1.1 General hypothesis

Different types and levels of dietary omega-3 PUFA (ALA, EPA, DHA) exert distinct immunomodulatory effects in poultry by altering fatty acid composition, oxylipin profiles, and cytokine responses following an LPS-induced immune challenge.

3.1.2 Specific Hypotheses

1. The gene expression of pro- (e.g., IL-1β, IL-6, TNF-α) and anti-inflammatory (e.g., IL-10) cytokines, as well as plasma IL-6 protein levels, will reach a peak within 2–4 hours after intravenous LPS injection in laying hens.2. Dietary supplementation with FO (rich in EPA and DHA) will modulate immune responses in LPS-challenged laying hens by shifting plasma oxylipin profiles toward anti-inflammatory mediators, enhancing the deposition of omega-3 PUFA in tissues and attenuating the splenic expression of pro-inflammatory cytokines.

3. Diets enriched with DHA-rich algal biomass will more effectively increase plasma LC-PUFA-derived oxylipins and their precursor fatty acids in tissues, and suppress pro-inflammatory cytokine expression in response to LPS compared to diet enriched with FSO (ALA source).

3.2 Objectives

The overall objective of this thesis is to investigate the immunomodulatory properties of different types and levels of omega-3 PUFA by 1) comparing the FSO (a source of ALA) to algal biomass (performed DHA) in pullet diets; 2) evaluating FO high in EPA and DHA in layer diets when chickens experienced the maximum inflammatory response.

Thus, the above hypotheses will be examined with the following specific objectives:

1). To identify the specific time point at which the gene expression of pro- and anti-inflammatory cytokines, and the plasma protein level of IL-6, reach their peak in laying hens following LPS administration, by sampling at 0, 1, 2, 4, and 6 hours post-injection;

2). To determine the effect of FO rich in EPA and DHA in layers' diet on performance; FA deposition in yolk, liver and plasma in laying hens after LPS challenge; and the production of oxylipins in plasma and cytokines in spleen of layers subjected to an LPS challenge; and

3). To identify the inclusion of either FSO (as a source of ALA), or algal DHA biomass (as a source of preformed DHA) in the diets of pullets on performance; FA profiles in liver and plasma in pullets subjected to an LPS challenge; and plasma oxylipin profiles and splenic expression of inflammatory cytokines in pullets exposed to an LPS challenge.

CHAPTER 4 MANUSCRIPT I

THE TIME COURSE STUDY: SPLENIC GENE EXPRESSION OF CYTOKINES AFTER THE LPS CHALLENGE IN LAYING HENS[1]

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[1] Jing M, Li S, Zhao S, House JD. Splenic gene expression of cytokines at multiple time points following lipopolysaccharide challenge in layers. Am J Vet Res. 2024;85(5):1-7.

4.1 Abstract

Background: Disturbances in homeostasis due to infection, trauma, or immunological disorders activate immune cells that release cytokines such as interleukin (IL), interferon (IFN), and tumor necrosis factor-alpha (TNF-α), initiating inflammatory responses. While cytokine research in mammals is well-established, there is limited understanding of cytokine regulation in avian species, especially in laying hens, despite emerging evidence of similar functions in poultry.

Objective: To investigate inflammatory responses to lipopolysaccharide (LPS) injection in layers.

Materials and Methods: Thirty 39-week-old laying hens were randomly assigned to two treatment groups after 1 week adaptation. The first group received an injection of 8 mg/kg LPS, while the second group was injected with sterile saline. At the beginning of the study, three hens served as baseline controls (time 0) for both the saline and LPS groups. Blood and spleen samples were collected at six time points: 0 hours (pre-injection) and 1, 2-, 3-, 4-, and 6-hour post-injection.

Results: LPS administration significantly elevated splenic mRNA expression levels of *IL-1β*, *IL-2, IL-6, IL-8*, *IL-10, IFN-γ*, and *TNF-α* ($P < 0.001$), as well as serum IL-6 levels ($P < 0.01$) compared to saline injection. Cytokine gene expression peaked rapidly, reaching maximum levels within 2 hours of post-LPS injection. Over time, the differences between saline- and LPS-treated groups diminished. Similarly, serum IL-6 concentration peaked 2 hours after LPS administration. Among the cytokines, *IL-6* exhibited the most pronounced LPS-induced upregulation, followed by *IL-1β* and *IL-8*, whereas *TNF-α* showed the least response.

Conclusions: Laying hens reached maximum immune response at 2-hour post LPS challenge.

Keywords: spleen, cytokines, LPS challenge, laying hens, serum

4.2 Introduction

Disturbances in homeostasis are caused by infection, trauma, or immunological disorders activate various cell types, including leukocytes, fibroblasts, and endothelial cells. Upon activation, these cells release cytokines such as interleukin (IL), interferon (IFN), and tumor necrosis factor (TNF)-α. These cytokines bind to specific receptors on the surface or within target cells, initiating inflammatory responses (138, 139). Cytokines are essential molecules in the inflammatory cascade, acting as mediators that coordinate the immune system's communication network. Despite the significant progress in understanding cytokine biology in mammals, research on the roles of cytokines in regulating inflammatory responses in avian species remains limited. However, emerging studies suggest that chicken cytokines exhibit similar biological activity and functionality to those described in mammals (140).

Lipopolysaccharide (LPS), a key component of the cell wall of gram-negative bacteria, is a potent inducer of inflammation and is widely used as a model to mimic bacterial infection and provoke acute immune challenges in poultry (141). LPS triggers inflammatory responses through toll-like receptor (TLR) 4, which is expressed on the surface of leukocytes such as monocytes, macrophages, and heterophils (142). This receptor-ligand interaction activates macrophages and other immune cells, prompting the production and secretion of cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, and IFN-γ (143, 144). These cytokines exert both pro- and anti-inflammatory effects and serve as critical biomarkers for monitoring immune responses and assessing physiological or pathological processes. Numerous studies have demonstrated that LPS administration enhances cytokine production in chickens, primarily in broilers (144-146). However, most investigations have focused on single time points, overlooking the temporal dynamics of cytokine expression during the inflammatory response (147-149). Since cytokine expression varies over time, optimizing the duration of LPS challenges is essential for accurate quantification of these mediators. Furthermore, limited research has examined multiple immunoregulatory molecules in response to bacterial infection over various time intervals, particularly in laying hens.

The early stages of inflammation are critical for the immune system to mount an effective response against microbial infections (150). Therefore, this study focused on the acute phase of the inflammatory response in laying hens following LPS administration. Splenic mRNA expressions of cytokine levels, including *IL-1β, IL-2, IL-6, IL-8, IL-10, TNF-α, IFN-γ*, and *TLR4*, were evaluated at multiple time points (0, 1, 2-, 3-, 4-, and 6-hour post-injection). This research aims to provide valuable insights into LPS-induced inflammation models in layers, offering a foundation for studying the effects of dietary nutrients, management practices, and exogenous treatments on poultry immune function. Ultimately, these findings could support advancements in health monitoring and the overall welfare of poultry flocks.

4.3 Materials and methods

4.3.1 Experimental animals

A total of 33 Dekalb White layers, 39 weeks of age, were obtained from the main flock of the Poultry Barn, University of Manitoba. The birds were housed individually in cages measuring 38 cm in width and 52 cm in depth, providing a total floor space of 1,976 cm^2 per bird. Each cage was equipped with an independent feeder, a nipple drinker, and a perch. The hens were maintained under a 16-hour light and 8-hour dark photoperiod, with feed and water provided *ad libitum*. All experimental procedures involving the use and care of animals were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee (Protocol F18-025 [AC11383]). The management of the birds adhered to the guidelines established by the Canadian Council on Animal Care.

4.3.2 Experimental design

After a 1-week adaptation period, 30 birds (40 weeks old) were randomly assigned to two groups. The first group received an intravenous (IV) injection of *Escherichia coli* LPS (serotype 0111:B4, Sigma Aldrich) at a dose of 8 mg/kg body weight via the brachial wing vein, while the second group was injected with sterile saline. In addition, three untreated birds served as control animals, providing baseline data (time 0) for both saline- and LPS-treated groups. Following injection, the general behavior of each bird was monitored. At time points 0 (before injection) and 1, 2-, 3-, 4-, and 6-hour post-injection, rectal body temperature was recorded, and approximately 5 mL of blood was drawn from the brachial wing vein. Birds were then euthanized by CO_2 asphyxiation, and spleens were collected. Blood samples were placed in serum separator tubes and allowed to clot at room temperature for 2 hours. Serum was subsequently separated by centrifugation at 1,000 × g for 20 minutes and stored at −80 °C until analysis. Spleen samples were flash-frozen in liquid nitrogen immediately after excision and stored at −80 °C for subsequent gene expression analysis.

The LPS dosage used in this study was determined based on previous experiments conducted by our group (28, 29) and other researchers (151). Considering the challenge posed by venipuncture at the brachial vein, which often leads to hematomas that complicate repeated blood sampling, the study employed serial bird sacrifice at specified time points.

This approach, widely used in poultry research (152-154), is a viable alternative to repeated blood sampling. To minimize animal usage in alignment with ethical considerations and statistical requirements, three birds were allotted per time point. This number was deemed sufficient for statistical analysis (155) and is consistent with sample sizes reported in similar studies on chickens (152, 156).

4.3.3 RNA isolation and cDNA synthesis

Total RNA was extracted from approximately 10 mg of spleen with the commercial kit (RNeasy Mini kit; Qiagen) in accordance with the manufacturer's protocol, which included on-column genomic DNA digestion (RNase-Free DNase Set for use with RNeasy/QIAamp Columns; Qiagen). Total RNA concentration was measured at an optical density of 260 nm using a NanoDrop^{TM} 2000 spectrophotometer (Thermo Fisher Scientific, Ottawa, Canada), and RNA purity was verified by the ratio of absorbance at 260 and 280 nm. In addition, RNA integrity was verified by visualization in an agarose gel. Reverse transcription of 1 µg of total RNA in a 20-µL reaction was conducted using cDNA synthesis kit (SuperScript VILO; Invitrogen). The generated cDNAs were stored at −20 °C and used as the templates in the following quantitative real-time PCR (qRT-PCR) analysis.

4.3.4 qRT-PCR analysis

qRT-PCR was conducted to measure the relative mRNA expression levels of target genes, including *IL-1β*, *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IFN-γ*, *TNF-α*, and *TLR4*. The sequences of the primers used are provided in **Table 4.1**, and their specificity was confirmed using NCBI BLASTN. qRT-PCR was performed on a real-time PCR system (StepOne Real-Time PCR System; Applied Biosystems), using real-time PCR SYBR master mix (Fast SYBR Green

Master Mix; Applied Biosystems). The amplification protocol consisted of an initial denaturation at 95 °C for 20 seconds, followed by 40 cycles of denaturation at 95 °C for 3 seconds, and combined annealing and extension at 61 °C for 30 seconds. A melting curve analysis was performed with the following steps: 95 °C for 15 seconds, 60 °C for 1 minute, and 95 °C for 15 seconds.

Each sample was run in duplicate, and the mean values were used for subsequent analysis. The relative expression of target genes was calculated using the $2^{-\Delta\Delta Ct}$ method (157), with β-actin as the reference gene. The stability of β-actin expression was confirmed by analyzing its delta cycle threshold (ΔCt) values, which remained consistent across all samples (data not shown).

Table 4.1. Primers used for qRT-PCR.

Genes[1]	**Primer sequences (5’ to 3’)[2]**	**Amplicon length (bp)**	**GenBank access No.**
IL-1β	F: TACACCCGCTCACAGTCCTT R: CAATGTTGAGCCTCACTTTCTG	130	NM_204524
IL-2	F: ACACCAACTGAGACCCAGGAGT R: GACCCGTAAGACTCTTGAGGTTC	160	NM_204153
IL-6	F: GCAGGACGAGATGTGCAAGA R: GGTAGGTCTGAAAGGCGAACA	176	NM_204628
IL-8	F: GATTGAACTCCGATGCCAGTG R: TGTCCAGAATTGCCTTGATGAT	197	NM_205018
IL-10	F: CAAGCAGATCAAGGAGACGTTC R: TCAGCAGGTACTCCTCGATGTAGT	104	NM_001004414
TNF-α	F: GTCTGCTCCTAGTGGCTTTCCT R: CTACGGGTTGCTGCACATACA	212	AY765397
IFN-γ	F: GCTGACGGTGGACCTATTATTGT R: ACGCCATCAGGAAGGTTGTT	188	NM_205149
TLR-4	F: CCAAACACCACCCTGGACTTG R: TGTATGGATGTGGCACCTTGAA	120	NM_001030693
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	NM_205518

[1] IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin 10; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4

4.3.5 Measurement of serum IL-6 concentrations

Serum concentrations of IL-6 were measured by the ELISA (chicken IL-6 ELISA kit; LSBio) following the user manual. Standard curves and samples were run duplicate.

4.3.6 Statistical analysis

All data were analyzed using a completely randomized design, with individual birds serving as the experimental units. The study included 11 treatment groups: baseline (time 0; pre-injection) and time points 1, 2-, 3-, 4-, and 6- hour post-injection for both LPS- and saline-treated groups. The mRNA expression of genes in response to time and injection type was evaluated using one-way ANOVA with the PROC MIXED procedure of SAS (version 9.4; SAS Institute Inc.).

For serum IL-6, data from the saline-treated groups, which served as a validation for the LPS challenge, were excluded from the statistical analysis as they were below the limit of detection. Tukey's multiple range test was used for post-hoc comparisons, with statistical significance set at $P < 0.05$.

4.4 Results

4.4.1 Measurement of body temperature

As described earlier, three birds were left untreated and served as baseline controls, representing the time 0 control for both saline- and LPS-treated groups. No adverse effects of LPS injections on bird behavior were observed. Furthermore, rectal temperature did not differ significantly ($P > 0.05$) among the groups, averaging 40.7 °C for the baseline controls, 40.3 °C for the saline-injected group, and 40.1 °C for the LPS-injected group (**Figure 4.1**).

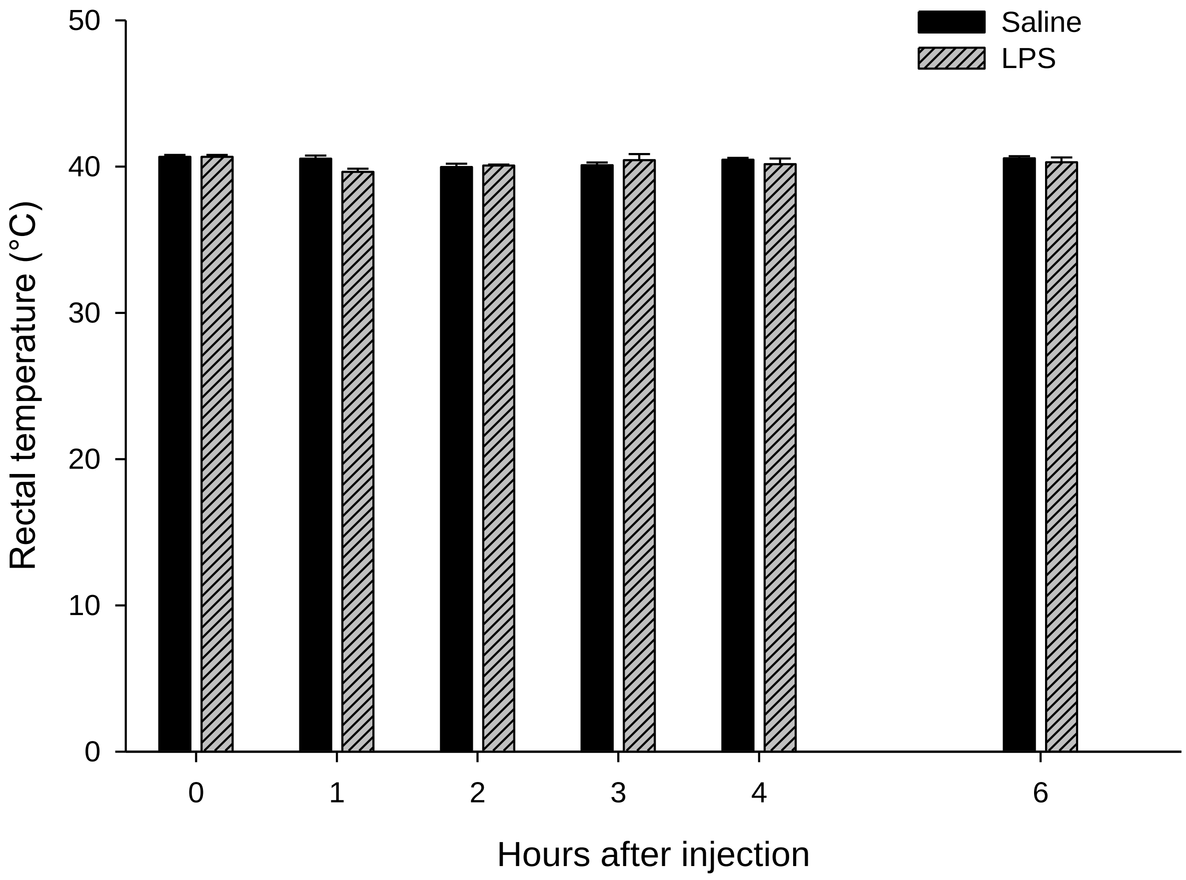


Figure 4.1. Changes over time in the rectal temperature of LPS and saline-injected laying hens (Means ± SEM, n = 3).

4.4.2 Relative mRNA expression of inflammation regulatory genes in spleen

The mRNA expression of cytokines, including *IL-1β*, *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IFN-γ*, and *TNF-α*, was significantly elevated ($P < 0.001$) in the spleens of LPS-injected birds compared to saline-injected birds. As expected, no differences were observed between the baseline and saline-injected groups ($P > 0.05$; **Table 4.2**). The largest differences in cytokine expression between the LPS- and saline-treated groups occurred within the first 2 hours post-injection, with these differences diminishing over time. At the 1-hour post-LPS injection, *IL-1β* mRNA expression reached its peak and subsequently declined but remained higher than the saline control levels until 6 hours. *IL-2*, *IL-6*, and *IL-8* expression showed significant changes to only up to 3 hours post-LPS injection, with the most pronounced alterations occurring between 1 and 2 hours. Meanwhile, *IL-10*, *IFN-γ*, and *TNF-α* expression remained elevated up to 4 hours after LPS administration, with the largest changes detected at 2 hours.

Among the cytokines measured, *IL-6* exhibited the most substantial LPS-induced increase in mRNA expression (1,725-fold), followed by *IL-1β* (281-fold), *IL-8* (258-fold), *IFN-γ* (39-fold), *IL-10* (37-fold), and *IL-2* (16-fold). The lowest increase was observed for *TNF-α* (4-fold). Notably, *TLR4* mRNA levels were not affected by LPS injection ($P > 0.05$).

Table 4.2 mRNA expression profile of inflammation regulatory genes at different time points following LPS challenge in layers[1].

	Time						SEM	Treatment		SEM	*P* values		
	Baseline (0 h)	1 h	2 h	3 h	4 h	6 h		LPS	Saline		Time	Treatment	Time*Treatment^{2}
IL-1β	12.09^{c}	7.88^{a}	8.78^{a}	10.00^{b}	9.96^{b}	11.10^{bc}	0.26	7.95^{a}	11.98^{b}	0.15	< 0.001	< 0.001	< 0.001
	1.00	***18.57***	***9.95***	***4.26***	***4.37***	***1.99***		***16.28***	***1.00***				
IL-2	15.53^{c}	13.78^{a}	14.05^{ab}	15.15^{bc}	15.70^{c}	15.50^{c}	0.27	13.85^{a}	16.05^{b}	0.16	< 0.001	< 0.001	< 0.001
	1.00	***3.38***	***2.79***	***1.31***	***0.89***	***1.02***		***4.59***	***1.00***				
IL-6	13.93^{c}	8.67^{a}	7.95^{a}	11.42^{ab}	11.63^{abc}	13.15^{bc}	0.64	8.30^{a}	13.95^{b}	0.42	< 0.001	< 0.001	< 0.001
	1.00	***38.24***	***62.97***	***5.68***	***4.93***	***1.72***		***50.41***	***1.00***				
IL-8	10.76^{c}	6.40^{a}	8.14^{ab}	9.18^{bc}	9.48^{c}	9.18^{bc}	0.31	7.41^{a}	10.31^{b}	0.20	< 0.01	< 0.001	< 0.01
	1.00	***20.53***	***6.17***	***3.00***	***2.43***	***3.00***		***7.47***	***1.00***				
IL-10	11.40^{bc}	9.75^{ab}	9.27^{a}	10.97^{abc}	11.15^{abc}	12.30^{c}	0.44	9.39^{a}	12.22^{b}	0.25	< 0.001	< 0.001	< 0.01
	1.00	***3.14***	***4.38***	***1.35***	***1.19***	***0.54***		***7.10***	***1.00***				
INF-γ	12.48^{b}	9.96^{ab}	9.82^{a}	10.87^{b}	11.67^{b}	11.98^{b}	0.28	9.86^{a}	12.41^{b}	0.20	< 0.001	< 0.001	< 0.001
	1.00	**5.73**	**6.32**	**3.07**	**1.75**	**1.42**		***5.85***	***1.00***				
TNF-α	6.29^{b}	5.82^{ab}	5.55^{a}	5.42^{a}	5.76^{ab}	6.05^{ab}	0.15	5.09^{a}	6.54^{b}	0.09	< 0.01	< 0.001	< 0.001
	1.00	***1.38***	***1.67***	***1.82***	***1.44***	***1.18***		***2.74***	***1.00***				
TLR4	8.34^{ab}	7.68^{a}	8.39^{ab}	8.29^{ab}	8.46^{b}	8.35^{ab}	0.16	8.09^{a}	8.41^{b}	0.09	< 0.05	< 0.05	0.345
	1.00	***1.58***	***0.97***	***1.04***	***0.92***	***1.00***		***1.25***	***1.00***				

[1] Data are presented as least squares means of ΔCt and their SEM (*n* = 3). ΔCt = Ct (target gene) – Ct (β-actin), where target gene refers to IL-1β, IL-2, IL-6, IL-8, IL-10, INF-γ, TNF-α and TLR4, and β-actin is used as an internal control for gene expression normalization. Bold italic values are the fold changes in splenic mRNA expression relative to baseline (pre injection) for time effect, and saline for

treatment effect, based on $2^{-\Delta\Delta Ct}$ method. Means are compared using Tukey's multiple range test. Values with different letters in the same row indicate significant differences ($P < 0.05$), same for Table 3a and Table 3b.

[2] When the $P_{\text{time} \times \text{treatment}}$ is significant, probability for F tests and comparisons of means for time effect were carried out separately for LPS or saline injection, as shown in Table 4.3 and Table 4.4.

Table 4.3 Splenic mRNA abundance of inflammation regulatory genes at different time points after LPS administration.

	Time						SEM	*P* values
	Baseline (0 h)	1 h	2 h	3 h	4 h	6 h		
IL-1β	12.09^{e}	3.96^{a}	5.93^{b}	7.90^{c}	8.03^{cd}	9.82^{d}	0.39	< 0.001
	1.00	***281.36***	***71.82***	***18.24***	***16.64***	***4.83***		
IL-2	15.53^{c}	11.58^{a}	12.20^{ab}	14.11^{bc}	14.85^{c}	14.84^{c}	0.46	< 0.001
	1.00	***15.50***	***10.09***	***2.68***	***1.60***	***1.62***		
IL-6	13.93^{d}	3.63^{a}	3.18^{a}	7.99^{b}	9.78^{bc}	11.26^{c}	0.45	< 0.001
	1.00	***1256.52***	***1725.02***	***61.23***	***17.67***	***6.33***		
IL-8	10.76^{c}	2.75^{a}	6.30^{b}	7.92^{b}	8.31^{b}	8.38^{b}	0.50	< 0.001
	1.00	***257.76***	***21.96***	***7.15***	***5.46***	***5.22***		
IL-10	11.40^{bc}	8.00^{ab}	6.20^{a}	9.85^{bc}	9.25^{abc}	11.66^{c}	0.75	< 0.01
	1.00	***10.59***	***36.66***	***2.93***	***4.43***	***0.84***		
INF-γ	12.48^{d}	8.12^{ab}	7.21^{a}	9.40^{abc}	10.14^{bcd}	11.78^{cd}	0.59	< 0.001
	1.00	***20.52***	***38.58***	***8.47***	***5.06***	***1.62***		
TNF-α	6.29^{c}	5.44^{abc}	4.35^{ab}	4.30^{a}	4.65^{ab}	5.50^{bc}	0.25	< 0.001
	1.00	***1.79***	***3.82***	***3.96***	***3.12***	***1.72***		

Table 4.4 Splenic mRNA abundance of inflammation regulatory genes at different time points after saline administration.

	Time						SEM	*P* values
	Baseline (0 h)	1 h	2 h	3 h	4 h	6 h		
IL-1β	12.09	11.80	11.63	12.10	11.89	12.37	0.35	0.734
	1.00	***1.23***	***1.38***	***1.00***	***1.15***	***0.82***		
IL-2	15.53	15.97	15.91	16.18	16.54	16.17	0.27	0.259
	1.00	***0.74***	***0.77***	***0.64***	***0.50***	***0.64***		
IL-6	13.93	13.71	12.73	14.85	13.47	15.03	1.38	0.844
	1.00	***1.16***	***2.30***	***0.53***	***1.38***	***0.47***		
IL-8	10.76	10.05	9.97	10.43	10.65	9.98	0.46	0.708
	1.00	***1.64***	***1.74***	***1.26***	***1.08***	***1.72***		
IL-10	11.40	11.51	12.33	12.10	13.04	12.94	0.46	0.110
	1.00	**0.93**	**0.52**	**0.62**	**0.32**	**0.34**		
INF-γ	12.48	11.80	12.44	12.33	13.21	12.18	0.36	0.224
	1.00	***1.60***	***1.03***	***1.11***	***0.61***	***1.24***		
TNF-α	6.29	6.20	6.75	6.54	6.87	6.60	0.17	0.119
	1.00	***1.06***	***0.73***	***0.84***	***0.67***	***0.81***		

4.4.3 Concentration of IL-6 in serum

Additionally, serum IL-6 levels were elevated following LPS administration, mirroring the mRNA expression patterns observed in the spleen. Serum IL-6 reached its maximum concentration of 2 hours of post-LPS injection and subsequently declined. However, no statistically significant differences were observed among the 3-, 4-, 6-, and 0-hour time points ($P < 0.01$; **Figure 4.2**).

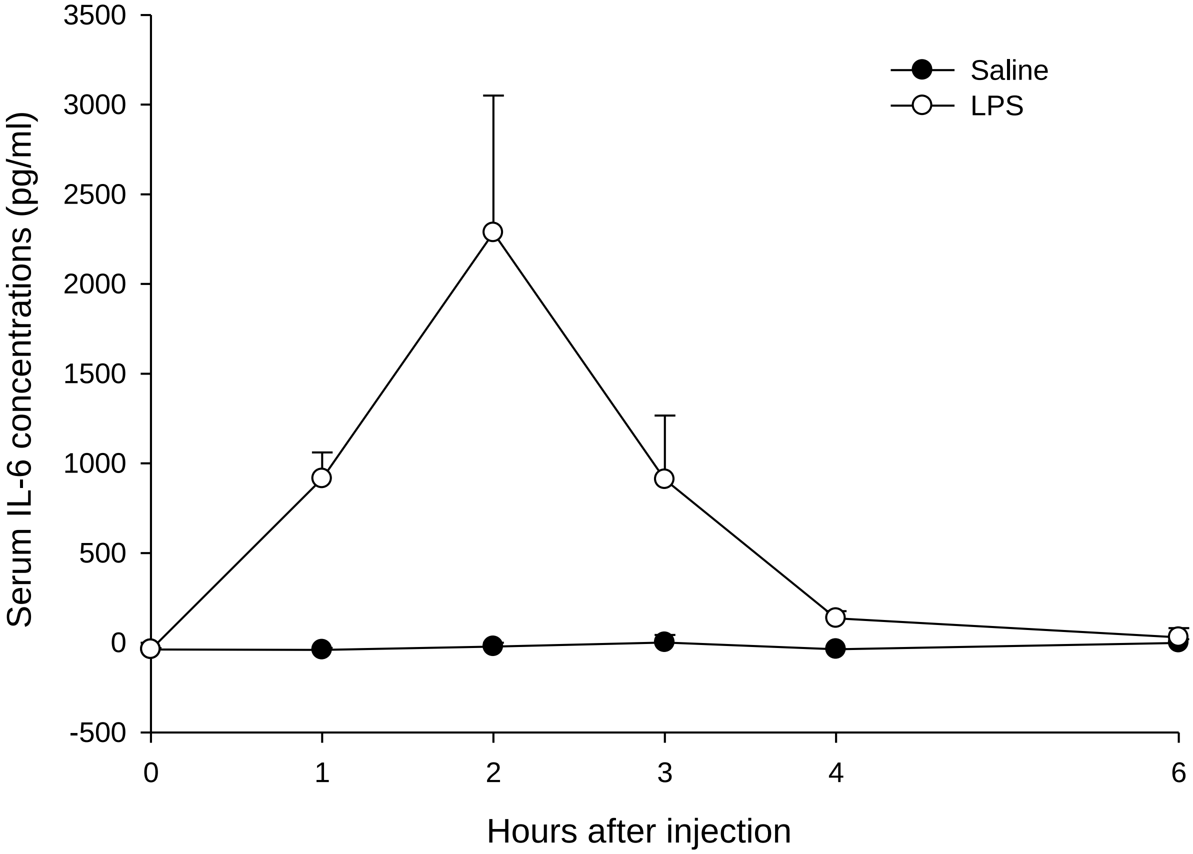


Figure. 4.2. Changes over time in the concentration of serum IL-6 in LPS and saline-injected laying hens (Means ± SEM, *n* = 3).

4.5 Discussion

LPS is a well-established model of inflammation commonly used in both in vivo and in vitro studies (158, 159). Its effects include the altered production of various mediator molecules, with cytokines being particularly critical. Cytokines can be broadly categorized as either proinflammatory or anti-inflammatory, depending on their roles. Proinflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8, TNF-α, and IFN-γ promote the initiation and propagation of inflammation, whereas anti-inflammatory cytokines like IL-6 and IL-10 suppress immune responses and inhibit inflammation. Notably, IL-6 exhibits both pro- and anti-inflammatory properties (160). The current study investigated the temporal inflammatory profile in laying hens following an acute immune challenge induced by LPS. Specifically, changes in serum cytokine concentrations and splenic mRNA expression of cytokines and *TLR4* were assessed over a 6-hour post-LPS injection. The results demonstrated that LPS administration induced an inflammatory response in the layers, as evidenced by increased cytokine expression. However, LPS injection did not alter the rectal temperature of the birds. Previous studies have reported mixed effects of LPS on body temperature in chickens. Leshchinsky and Klasing (153) observed a significant increase in body temperature in layers, but not in broilers, after LPS injection. Another study (161) found that broilers initially exhibited a decrease in body temperature below normal, followed by a subsequent increase above normal after LPS administration. These discrepancies may stem from variations in ambient temperature, LPS dosage, age, genetic background, or route of administration used in different studies. Chickens are generally considered resistant to the harmful effects of LPS, which may also contribute to these differences (162).

TLR4 is a key receptor for recognizing gram-negative bacteria and mediating the inflammatory response. It is best known as the primary receptor for LPS (142). Binding of LPS to TLR4 triggers a signaling cascade that activates nuclear factor kappa B (NFκB), ultimately leading to the production of various cytokines and other inflammatory mediators (143). The expression of TLR4 mRNA has been documented in chicken tissues (29). However, in the current study, LPS injections did not significantly alter TLR4 mRNA levels compared to the saline controls. Similarly, Munyaka et al. (29) reported no changes in TLR4 expression in the spleen or cecal tonsils of LPS-challenged laying hens. In contrast, other studies have found that LPS significantly upregulated TLR4 gene expression in the spleen (141) and jejunal mucosa of broilers (144). These inconsistencies may reflect differences in experimental conditions, including bird species, tissue types, and environmental factors.

TNF-α, IL-1β, IL-6, and IL-8 are critical inflammatory mediators commonly produced by monocytes and macrophages during the early phase of inflammation. Among these, TNF-α is recognized as the first cytokine to appear during the inflammatory response in mammals, typically peaking in plasma following LPS injection (163). TNF-α also activates monocytes and macrophages to release additional proinflammatory molecules, including IL-1β, IL-6, and IL-8, through feedback regulation (164). In cases of sepsis, TNF-α appears within 1.5 to 2 hours post-LPS injection, followed by IL-1β and IL-6 (163). In the current study, *TNF-α* mRNA levels in the spleen reached their maximum 2 to 3 hours after LPS injection, which occurred later than the peak levels for IL-1β and IL-6. This delayed response is consistent with findings by Gehad et al. (151), who reported that plasma TNF-α activity in chickens peaked at 6 hours post-LPS injection. Differences in response timing could be attributed to variations in the traits studied (protein levels in plasma vs. mRNA levels in spleen) or animal variability. Furthermore, the magnitude of LPS-induced *TNF-α* mRNA increase was the lowest among all the cytokines examined, suggesting that TNF-α may not be a sensitive biomarker of inflammation in chickens, warranting further investigation.

IL-1β and IL-8 play key roles in the innate immune response by recruiting inflammatory cells (165). For example, previous studies have linked the deteriorated performance of LPS-challenged chickens to the increased release of IL-1β (165), while exposure of intestinal epithelia to LPS was shown to stimulate IL-8 secretion, exacerbating intestinal inflammation (158). IL-6, a cytokine with both pro- and anti-inflammatory roles, promotes T-helper (Th) 17 cell differentiation and enhances the ability of heterophils to eliminate pathogens (165, 166). In this study, splenic mRNA expressions of *IL-1β*, *IL-6*, and *IL-8* peaked at 1-hour post-LPS injection. Although *IL-6* mRNA expression was slightly higher at 2 hours than at 1 hour, the difference was not statistically significant. These results confirm the early expression of these cytokines during the initial stages of inflammation. The difference between saline and LPS treatments diminished over time, reflecting the birds' adaptation or recovery following the immune challenge. Previous studies have reported elevated circulating levels or mRNA expression of *IL-1β*, *IL-6*, and *IL-8* at 2 to 4 hours post-LPS administration in chickens (141, 144, 148). For example, Nakamura et al. (156) observed that serum IL-6 concentrations increased from 1 hour to 2 days, peaking at 3 hours post-LPS administration. Similarly, De Boever et al. (161) reported that plasma IL-6 concentrations peaked 3 hours after LPS injection and returned to baseline within 9 hours in broilers. The highest mRNA expression of *IL-1β* in the spleen was detected at 2 hours post-LPS injection (117). Collectively, these findings suggest that LPS induces rapid and transient increases in these inflammatory mediators, with the level of stimulation decreasing over time, indicating recovery or adaptation to the immune challenge. Importantly, the current study highlights that the magnitude of LPS-induced mRNA expression increases was highest for *IL-6*, followed by *IL-1β* and *IL-8*. This suggests that these three cytokines, particularly IL-6, are reliable biomarkers of acute inflammation in layers. Given its significant role, IL-6 has been consistently chosen as a key cytokine in studies investigating LPS-induced immune responses in poultry (156, 161).

IL-2 and IFN-γ are proinflammatory cytokines produced by differentiated Th1 cells, which play a pivotal role in pathogen elimination and the propagation of autoimmune responses (167). IL-2 serves as a key autocrine growth factor, central to adaptive immunity, while IFN-γ activates natural killer cells, promotes macrophage activation, and induces nitric oxide secretion, thereby amplifying inflammation (168). In this study, significant changes in *IL-2* and *IFN-γ* mRNA levels were observed only within the first 2 hours post-LPS administration. These findings align with previous reports, such as the study by Leshchinsky and Klasing (154), which demonstrated a significant increase in *IFN-γ* mRNA expression from 0 to 1-hour post-LPS injection, with elevated levels persisting before declining at 3 hours. Similarly, other studies have reported increased mRNA expression or circulating concentrations of *IL-2* and *IFN-γ* within 2 hours of LPS challenge in broiler chickens (148). Sijben et al. (117) further explored the temporal dynamics of *IL-2* and *IFN-γ* mRNA expression in the spleen at multiple time points (0, 2, 3, 4, 6, 8, 16, and 24 hours post-LPS injection) and found peak transcription levels at 2 hours. Taken together, these findings suggest that the optimal window for detecting IL-2 and IFN-γ is within the first few hours following LPS administration, emphasizing their rapid and transient roles in the early immune response.

IL-10 is a well-known anti-inflammatory cytokine, with its immunoregulatory role demonstrated in chickens. Produced primarily by regulatory T cells and Th2 cells (169), IL-10 plays a critical role in maintaining immune homeostasis by suppressing excessive inflammation. It achieves this by inhibiting the production of proinflammatory cytokines such as TNF-α, IL-1β, IL-2, and IL-6 and by modulating Th1 cell activity through the inhibition of IFN-γ production (170). A recent study reported elevated serum levels and higher ileal *IL-10* mRNA expression in pigeons injected intraperitoneally (IP) with LPS (171). Consistent with this, the present study demonstrated that LPS administration upregulated *IL-10* mRNA expression in the spleen, highlighting the balance between pro- and anti-inflammatory responses crucial for maintaining immune homeostasis and performance in birds.

Interestingly, the mRNA expression of *IL-10* peaked at 2 hours post-LPS injection and then rapidly returned to baseline by 3 hours, suggesting that LPS-induced *IL-10* expression occurs early and is transient. Previous studies in broilers also reported elevated circulating IL-10 concentrations at 2 hours post-LPS administration. However, findings from Echeverry et al. (159) indicated an upregulated *IL-10* mRNA expression in chicken B cells at 4 hours post-LPS challenge. Notably, in the current study, *IL-10* mRNA expression appeared to trend higher at 4 hours compared to 3 or 6 hours post-LPS administration. These observations underline the need for further investigations into the temporal dynamics of IL-10 expression following LPS challenge to better understand its regulatory role in avian immune responses.

4.6 Conclusion

In conclusion, IV administration of LPS induced a marked inflammatory response in laying hens, as evidenced by increased expression of cytokines such as *TNF-α*, *IFN-γ*, *IL-1β*, *IL-2*, *IL-6*, *IL-8*, and *IL-10*. The study demonstrated that cytokine expression levels peaked rapidly, reaching their maximum between 1 and 2 hours post-LPS administration, and subsequently returned to baseline levels by 6 hours. This pattern suggests that layers can adapt to or mitigate the immune challenges associated with bacterial infections over time. The findings emphasize the importance of timing in detecting inflammation in a clinical setting, with the first few hours post-LPS administration identified as the optimal window for measuring inflammatory responses. Notably, the magnitude of the LPS-induced cytokine expression was greatest for IL-6, indicating its potential as a distinct biomarker for inflammation in layers. However, further research involving larger sample sizes is warranted to validate this finding.

This 6-hour challenge model provides valuable insights into the temporal dynamics of immune activation in chickens. The knowledge generated from this study may have direct clinical applications in identifying multiple inflammatory biomarkers and evaluating the effects of exogenous treatments (e.g., nutrients, feed additives, immunomodulators) on the avian immune system.

BRIDGE TO CHAPTER 5

The findings from Chapter 4 demonstrated that laying hens exhibited peak immune responsiveness at 2 hours following lipopolysaccharide (LPS) administration, as evidenced by the rapid induction of selected pro- and anti-inflammatory cytokines. This time point was identified as the optimal window to capture the acute phase of the immune response, thus providing a rationale for subsequent investigations. Building upon this, Chapter 5 focuses on evaluating the anti-inflammatory potential of dietary fish oil (FO), high in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), by assessing its modulatory effects on immune markers at 2 hours post-LPS challenge in laying hens. This approach aims to determine whether FO supplementation can attenuate the early immune response during acute inflammation in laying hens.

CHAPTER 5 MANUSCRIPT II

THE DIETARY EFFECT OF EICOSAPENTAENOIC ACID (EPA) AND DOCOSAHEXAENOIC ACID (DHA) FROM FISH OIL (FO) ON PERFORMANCE, FATTY ACID DEPOSITION, PLASMA OXYLIPINS AND CYTOKINES IN RESPONSE TO LIPOPOLYSACCHARIDE (LPS) CHALLENGE IN LAYING HENS

5.1 Abstract

Background Including fish oil (FO) in laying hen diets is an attractive strategy to increase egg and tissue omega-3 PUFA, while simultaneously protecting the hen from potential immune challenges.

Objectives: This study was designed to compare the effects of a standard hen diet with one containing FO, rich in both eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), on the levels of PUFA in egg and tissue, plasma oxylipins, and splenic cytokine expression in LPS-challenged laying hens.

Methods: Twenty-four 40-week-old Dekalb White layers were randomly assigned to either control (no EPA or DHA) or a FO (0.4 g/100g EPA and DHA) diets. After 6 weeks, hens were challenged with LPS (8 mg/kg body weight; iv) or saline 2 hours before termination. FA were analyzed by GC, free oxylipins by HPLC/MS/MS, and cytokines by qRT-PCR.

Results: Egg yolk, liver and plasma were enriched in omega-3 PUFA and reduced in omega-6 PUFA in the FO group. Interestingly, LPS increased levels of liver ALA as well as plasma ALA and DHA in hens fed FO diet. These FO-fed hens exhibited higher concentrations of plasma oxylipins derived from EPA and DHA, while showing lower concentrations of omega-6 oxylipins. In control hens, LPS increased the level of oxylipins by approximately 14%, particularly octadecanoids derived from ALA and linoleic acid (LA). In contrast, in hens fed FO diet, LPS caused a decrease of about 31% in oxylipins derived from both omega-6 and omega-3 PUFA. Consistent with these putatively anti-inflammatory effects, FO decreased oxylipins associated with higher soluble epoxide hydrolase (sEH) activity in saline treated hens. However, FO increased these oxylipins in

LPS treated hens. LPS induced the expression of splenic cytokines, and this was not altered by diet.

Conclusions: Dietary EPA and DHA enriches omega-3 PUFA in yolk and in liver and plasma of layers and modulates the effects of LPS on plasma oxylipins in a manner that is largely, but not completely, indicative of an anti-inflammatory effect.

Keywords: FO, EPA, DHA, oxylipins, LPS, inflammation, laying hens

5.2 Introduction

Omega-3 PUFA, including α-linolenic acid (ALA), long-chain PUFA (LC-PUFA) EPA and DHA, have well-documented health benefits for humans, as reviewed (9, 172, 173). A key strategy to enhance the dietary intake of omega-3 PUFA in humans is the enrichment of animal-derived products, such as eggs, through feed supplementation (174, 175). Strategies for this practice include: 1) using diets high in ALA from plant-based sources such as flaxseed or canola; 2) utilizing algal products, which are particularly high in DHA; or 3) supplementing feeds with marine or fish oil (FO), which are rich in both EPA and DHA, but not ALA (12). All three approaches effectively increase the total omega-3 PUFA content and nutritional value of eggs, but they result in varying proportions of individual omega-3 PUFA (176-179).

The health benefits of omega-3 PUFA are largely attributed to their anti-inflammatory properties, mediated in part through bioactive lipid mediators known as oxylipins (180). The main synthetic pathways, cyclooxygenase (COX), cytochrome P450 (CYP450), generate oxylipins from either omega-3 or omega-6 PUFA with generally opposing effects (181). Omega-3 oxylipins, especially those derived from EPA and DHA have anti-inflammatory and pro-resolving properties, while oxylipins derived from linoleic acid (LA) and arachidonic acid (ARA), are generally pro-inflammatory, although it is not always this straightforward as reviewed (22, 182). For example, epoxy-PUFA derived via the CYP450 pathway and their subsequent diol products formed via soluble epoxide hydrolase (sEH) exhibit anti- and pro-inflammatory properties, respectively, so their balance influences the overall inflammatory effect (183-186).

Given that omega-3 PUFA are recognized for their immunomodulatory properties, maintaining a balanced intake of omega-6 and omega-3 PUFA is crucial for regulating immune function, particularly during disease challenges or other stressors that trigger a robust acute-phase response (14). Modern poultry feeds are typically high in omega-6 and deficient in omega-3 PUFA, largely due to the reliance on omega-6-rich ingredients and the absence of official omega-3 PUFA requirements in poultry nutrition guidelines. In commercial poultry production, the environment comprises multiple interacting stressors, including external factors such as temperature, lighting, and social or behavioral conditions, as well as internal challenges such as exposure to pathogens and toxins, as summarized by Ncho et al (187). These stressors encompass various physical, physiological, and infectious factors that may act independently or synergistically, potentially compromising the immune system (188-190). LPS is a potent pro-inflammatory substance found in the cell wall of gram-negative bacteria that has been used extensively to model bacterial infections experimentally in poultry (28-30, 191). During LPS-induced inflammation, immunomodulatory cytokines and oxylipins are produced and serve as biomarkers of inflammatory status (15, 192). Several studies have examined the potential immunomodulatory properties of dietary omega-3 PUFA in birds to overcome an immune challenge elicited by LPS (191, 193).

However, not all dietary omega-3 PUFA have equal effects on expression of oxylipins and cytokines. Generally, ALA, EPA, and DHA primarily increased their respective oxylipins, while also reducing omega-6 PUFA-derived oxylipins in the following order of effectiveness: DHA > EPA > ALA (194, 195). DHA can reduce the expression of multiple pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-α), IL-6, monocyte chemoattractant protein (MCP)-1, and IL-10, whereas EPA exhibits a more pronounced effect on cytokine ratios, such as TNF-α/IL-10 and MCP-1/IL-10 (196). Additionally, both EPA and DHA can modify the specialized pro-resolving mediators (SPM) lipidome in plasma by replacing omega-6 ARA derivatives with their respective metabolites, including a five-fold increase in 18-hydroxy-EPA (induced by EPA) and a three-fold increase in 17- and 14-hydroxy-DHA (induced by DHA) shown in this study. Furthermore, da Cunha de Sá et al. (197) demonstrated that different EPA:DHA ratios differentially affect adipose tissue metabolism. FO rich in EPA, but not DHA, effectively reversed obesity-induced metabolic alterations by preventing changes in TNF-α, IL-10, and resistin secretion in adipose tissue. Further, in our prior studies with dietary ALA or with dietary algal-derived DHA- enriched diets, after a 4-hour LPS challenge, ALA supplementation increased all quantified ALA-derived oxylipins, while DHA supplementation elevated nearly all quantified EPA- and DHA-derived oxylipins (31). However, these diets were not compared to the standard laying hen diet low in omega-3 PUFA, so it is unclear how oxylipins change compared to hens provided a commercial diet. Due to the unique effects of individual fatty acids and the prohibitively high cost and limited commercial availability of purified EPA in poultry feed, the objective of the current study was to evaluate the effects of a fish oil containing both EPA and DHA compared to a commercial diet with low omega-3 PUFA content, in laying hens challenged with LPS, on plasma oxylipins and their precursor PUFA and on splenic expression of representative pro- and anti-inflammatory cytokines.

5.3 Materials and methods

The experimental protocol, including details of animal utilization and welfare, was reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee. Additionally, all the layers were fed and managed according to the recommendations established by the Canadian Council on Animal Care (198).

5.3.1 Animals and experimental design

A total of 24 forty-week-old Dekalb White laying hens were received from a local hatchery (Steinbach Hatchery & Feed Ltd., Steinbach, MB, Canada) and raised, fed, and managed in the Poultry Metabolism Unit at the University of Manitoba. Birds were randomly housed in cages individually (38 × 52 cm to provide 1,976 cm^2 area space per bird) with independent feeders, nipple waterers, and perches. Water and feed were supplied *ad libitum*, and light was provided for 15 hours throughout the entire experimental period (week 0-8 starter, week 9-12 grower and week 13-16 finisher). All birds experienced a 2-wk adaptation period by feeding commercial layer diets, followed by random assignment to one of two diets (12 hens per diet): a commercial laying hen diet (control, 0% EPA and DHA) or a diet containing FO (0.4% EPA and DHA). Menhaden oil was sourced from Dyets, Inc. (Bethlehem, PA, USA). Both diets were formulated to meet or exceed the nutrient requirement based on the Dekalb White commercial product guide (North American Version). The ingredients and nutrient composition of the experimental diets are presented in **Table 5.1,** and the analyzed FA profile in experimental diets is provided in **Table 5.2**.

Table 5.1 Ingredients and nutrient composition of experimental laying hen diets[1].

	Control diet	FO diet[2]
Ingredient %		
Corn (whole grain)	60.36	42.22
Soybean Meal	24.18	21.99
Wheat	0.000	19.92
Corn Oil	2.148	0.000
Vitamin Premix	1.000	1.000
Mineral Premix	0.500	0.500
Biophos[3]	1.533	1.520
Salt	0.280	0.265
Limestone	9.899	9.938
DL-Methionine	0.096	0.100
Lysine	0.011	0.039
Menhaden Oil (Dyets)	0.000	2.500
Total	100.00	100.00
Calculated nutrients		
AMEn (Poultry; Kcal/kg)	2815.7	2816.7
Crude Fat (%)	4.60	4.63
Crude Protein (%)	17.00	17.00
Calcium (%)	4.00	4.00
Total Phosphorus (%)	0.64	0.63
Available Phosphorus (%)	0.45	0.45
Calculated fatty acids[4]		
LA (%)	1.77	1.00
ALA (%)	0.04	0.05
EPA (%)	0.00	0.17
DHA (%)	0.00	0.13
Total Omega-3 (%)	0.04	0.36
Analyzed nutrients		
AMEn (Poultry; Kcal/kg)	2931.2	2978.6
Crude Fat (%)	4.39	5.16
Crude Protein (%)	16.72	18.24
Calcium (%)	4.46	4.49
Phosphorus (%)	0.58	0.59

[1] Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3,000 IU of vitamin D3, 150 IU of vitamin E, 3 mg of vitamin K3 (as menadione), 0.02 mg of vitamin B12, 0.2 mg of biotin, 6.5 mg of riboflavin, 4 mg of folic acid, 10 mg of calcium pantothenate, 39.9 mg of niacin, 2.2 mg of thiamine, 4.5 mg of pyridoxine, 1,000 mg of choline chloride, 125 mg antioxidant (ethoxyquin), 66 mg of manganese, 70 mg of zinc, 80 mg of iron, 10 mg of copper, 0.3 mg of selenium, 0.4 mg of iodine.

[2] FO, fish oil.

[3] Biophos, a feed-grade monocalcium phosphate, contained 21% P and 18% Ca (Landmark Feeds, Winnipeg, Manitoba, Canada).

[4] LA, Linoleic acid; ALA, α-linolenic acid; EPA, Eicosapentaenoic acid; DHA, Docosahexaenoic acid.

Table 5.2. Analyzed fatty acid composition of fish oil and experimental diets in laying hens[1].

	Menhaden oil[2]	Control diet	FO[3] diet
Analyzed fatty acids (% by wt)			
TSFA	15.1	0.56	0.90
TMUFA	8.68	1.04	0.77
LA (18:2n-6)	0.86	2.26	1.22
GLA (18:3n-6)	0.31	0.00	0.01
ARA (20:4n-6)	0.49	0.00	0.02
ALA (18:3n-3)	0.96	0.08	0.11
EPA (20:5n-3)	6.98	0.00	0.22
DPA (22:5n-3)	1.08	0.00	0.03
DHA (22:6n-3)	5.67	0.00	0.18
Omega-3 PUFA	14.7	0.08	0.54

[1] ARA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; TMUFA, total monounsaturated fatty acids (sum of palmitoleic acid and oleic acid); TSFA, total saturated fatty acids (sum of myristic acid, palmitic acid and stearic acid).

[2] Menhaden oil, dietary source of fish oil.

[3] FO, fish oil.

5.3.2 Sample collection and measurements

Feed intake (FI) and body weight (BW) were recorded weekly throughout the entire experiment (6 weeks). Egg weight (EW) and EP were measured and recorded daily. On the final day of the feeding period, eggs were collected from all birds, and yolks were separated and stored at -20℃ for subsequent FA analysis. At termination, all birds in each treatment (*n* = 12) were randomly assigned to receive either 8 mg/kg body weight of *Escherichia coli* (*E. coli*) derived LPS (serotype 0111: B4, Sigma Aldrich Inc., St. Louis, MO; *n* = 6) or sterile saline (*n* = 6) by intravenous injection, as described (137, 151). The feed was withdrawn after injection. All birds were euthanized by CO_2 asphyxiation 2 hours post LPS injection, since cytokines peak 2 hours post-challenge in laying hens following an LPS challenge (199). Plasma, liver, and spleen samples were collected and stored at -80℃ until further analysis.

5.3.3 Fatty acids extraction and analysis

Total lipids from plasma, liver, egg yolk, and experimental diets were extracted as described (200). Briefly, 1g of egg yolk, liver samples, experimental diets (finely ground), and 100 µL plasma were pre-conditioned with chloroform/methanol (2:1, by volume) and 5mg/mL internal standard C13:0 (Nu-Chek Prep, Inc., Elysian, MN, USA) for FA extraction. Aliquots of a known volume (to contain approximately 50 mg lipid) were dried under nitrogen and methylated using 3 mL of boron trifluoride. The final methylated FA of the samples were extracted into iso-octane and were determined using a Varian 450 GC with flame ionization detector (FID) (GC-FID; Agilent Technologies Canada Inc., ON), equipped with a DB225MS column (30 m × 0.25 mm diameter and 0.25 mm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario). The settings of equipment and calculations of FA contents in all samples were described previously (31).

5.3.4 Oxylipin extraction in plasma

One mL of water (pH 3), 10 µL of antioxidant solution, and 100 µL of deuterated internal standard (Cayman Chemical, Ann Arbor, MI) were added to 200 µL of plasma sample and then adjusted to pH < 3 for oxylipin extraction. Samples were applied to Strata-X solid phase extraction columns (33u, 60 mg/3mL; Phenomenex, CA) before elution of free oxylipins with 10% methanol. All samples were dried under nitrogen and reconstituted in 100 µL of a mobile phase of water-acetonitrile-formic acid (70:30:0.02, v/v/v, LC-MS grade). Oxylipins were determined using high-performance liquid chromatography mass spectrometry (HPLC-MS/MS; QTRAP 6500; Sciex, ON, Canada). Further details of HPLC-MS/MS parameters, deuterated standards used, standard curve slopes and oxylipin retention times are provided in (201, 202).

5.3.5 Total RNA extraction and reverse transcription

Total RNA was isolated from spleen samples (10 ± 0.5 g) using the RNeasy Mini kit (Qiagen Canada Inc., Mississauga, ON, Canada), with genomic DNA contamination eliminated using a DNase set kit (Qiagen Canada Inc., Mississauga, ON, Canada) as per the manufacturer's instructions. Subsequently, 1 µg of RNA was utilized for cDNA synthesis employing the Superscript® VILO™ cDNA Synthesis Kit (Invitrogen Canada Inc., Burlington, ON, Canada), following the manufacturer's protocol. The cDNA samples were stored at –20°C for further qRT-PCR analysis.

5.3.6 qRT-PCR analysis

Quantitative real-time PCR was performed using the Step One Thermo Cycler (Applied Biosystems). PCR primers for studied genes were designed using Primer Premier 5 software and are summarized in **Table 5.3** (199). A melting curve program was used to check the specificity of each PCR product and to optimize the primer concentrations. The target mRNA abundance was normalized based on the expression of the housekeeping gene, β-actin and relative mRNA expression was calculated according to the $2^{-\Delta\Delta CT}$ method. Each reaction was run in duplicate using Step One Software (Applied Biosystems, Canada). Real-time PCR efficiencies were acquired according to the formula: $E = 10-1/\text{slope of standard curve}$ (203). The express PCR efficiency of all primers used in this project was between 95% and 100%. Each sample for each gene was run in duplicate using Step One Software (Applied Biosystems), along with the controls without cDNA that were conducted.

Table 5.3. Primer sequences for quantitative real-time PCR.

Genes[1]	Primer sequences (5’ to 3’)[2]	Amplicon length (bp)	GenBank access No.
IL-1β	F: TACACCCGCTCACAGTCCTT R: CAATGTTGAGCCTCACTTTCTG	130	NM_204524
IL-2	F: ACACCAACTGAGACCCAGGAGT R: GACCCGTAAGACTCTTGAGGTTC	160	NM_204153
IL-6	F: GCAGGACGAGATGTGCAAGA R: GGTAGGTCTGAAAGGCGAACA	176	NM_204628
IL-10	F: CAAGCAGATCAAGGAGACGTTC R: TCAGCAGGTACTCCTCGATGTAGT	104	NM_001004414
TNF-α	F: GTCTGCTCCTAGTGGCTTTCCT R: CTACGGGTTGCTGCACATACA	212	AY765397
IFN-γ	F: GCTGACGGTGGACCTATTATTGT R: ACGCCATCAGGAAGGTTGTT	188	NM_205149
TLR4	F: CCAAACACCACCCTGGACTTG R: TGTATGGATGTGGCACCTTGAA	120	NM_001030693
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	NM_205518

[1] IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin10; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4.

[2] F = forward; R = reverse.

5.3.7 Statistical analysis

Data was analyzed as a completely randomized design, using R version 4. 3. 3. For the repeated-measures analysis of performance data, the model is: $y_{ijk} = \mu + d_i + a_{ij} + w_k + d_{wik} + e_{ijk}$, where y_{ijk} is an observation on a performance measure, including BW, FI, and EW; μ represents the population mean, d_i is the effect of the i'th diet (either control or 2.5% FO diet; i = 1, 2), a_{ij} represents the effect of the j'th hen in the i'th dietary treatment, w_k is the effect of the k'th week (k = 1, 2, … 6), d_{wik} is the interaction of diet and week, and e_{ijk} is the residual error. Treatment, week, and their interaction are considered fixed effects, and hen and the residual error are considered random effects.

For oxylipin patterns in response to both dietary treatment and LPS challenge, principal component analysis (PCA) was first performed, using R version 4. 3. 3 FactoMineR and factoextra packages. Factor loadings were generated to examine the weight of the correlations of oxylipins to the principal components derived. Then for oxylipins and other single-measure outcomes, including FA and relative mRNA expressions, a two-factor factorial model was used: $y_{ijk} = \mu + d_i + c_j + dc_{ij} + e_{ijk}$, where y_{ijk} is a single-measure observation on a hen, μ is the population mean, d_i is the effect of the i'th diet (i = 1, 2), c_j is the effect of the j'th challenge level (either LPS or saline injection; j = 1, 2), dc_{ij} is the interaction of the two factors, and e_{ijk} is the residual error due to the k'th hen in the ij'th combination of diet and challenge. All effects are considered fixed effects except the residual error. The Shapiro-Wilk and Bartlett's test were used for assessing normal distribution and homogeneity of variance, and if necessary, data were log-transformed to achieve these two assumptions within each group. The missing value was observed in oxylipin data, due to the unsuccessful blood collection from one bird in the control/saline group. All data were presented as least squares means (LSM) ± SE, adjusted using Tukey's *post hoc* test to compare for significant differences (*P* < 0.05).

5.4 Results

5.4.1 Hen performance

Laying hen performance, including FI, BW, EW, rate of EP, and feed conversion ratio (FCR), were not affected by diet (**Table 5.4**). At the end of the feeding period, the hens provided the control diet consumed 106.0 g feed/day, had an average BW of 1.77 kg, produced eggs with a mean weight of 61.7 g, had a 97.2% EP rate, and exhibited a FCR of 1.75. Hens provided the FO diet consumed 102.4 g/day of feed, had an average BW of 1.73 kg, produced eggs with a mean weight of 61. 9 g had a 96.0% EP rate and exhibited a FCR of 1.76.

Table 5.4. Performance of hens (from 42 to 48 weeks of age) provided either the control or fish oil diet[1].

	FI (g/hen/day)	BW (kg)	Egg production (%)	EW (g/egg)	FCR (g/g)[2]
Diet effect					
Control	106.0	1.77	97.2	61.7	1.75
2.5% FO[3]	102.4	1.73	96.0	61.9	1.76
SEM	1.765	0.028	1.748	0.854	0.047
Week effect					
Week 1	96.9	1.75	92.9^{b}	60.6	1.75^{ab}
Week 2	103.4	1.74	95.2^{ab}	61.1	1.79^{ab}
Week 3	111.6	1.76	96.4^{ab}	62.3	1.86^{a}
Week 4	108.0	1.75	98.2^{ab}	62.4	1.76^{ab}
Week 5	103.6	1.75	98.2^{ab}	62.1	1.71^{ab}
Week 6	101.6	1.76	98.8^{a}	62.4	1.65^{b}
SEM	1.866	0.020	1.731	0.660	0.046
P values					
Diet	0.1665	0.2657	0.6349	0.8833	0.0528
Week	<.0001	0.4809	0.0154	<.0001	0.0070
Diet × week	0.0002	0.1708	0.9762	0.0172	0.3837

[1] Data are presented as least squares means and their SEM ($n = 12$). Values with different superscripts within a column are significantly different at $P < 0.05$.

[2] FI, feed intake; BW, body weight; EW, egg weight; FCR, feed conversion ratio, g of feed consumed per g of egg produced.

[3] FO, fish oil.

5.4.2 Fatty acid composition of yolks, liver, and plasma

Egg yolks from hens that were fed a diet containing FO had higher concentrations of total and individual omega-3 PUFA, including ALA, EPA, DPA, and DHA, compared to those from hens fed the control diet ($P < 0.0001$; **Table 5.5**). Additionally, the FO diet resulted in lower concentrations of total and individual omega-6 PUFA, including LA and ARA, leading to a lower omega-6 to omega-3 PUFA ratio ($P < 0.0001$; **Table 5.5**).

Table 5.5. Fatty acid composition of egg yolk (mg/yolk) of laying hens provided either the control or fish oil diet[1].

	Control diet	FO diet[3]	*P* values
SFA, mg/yolk			
14:0	15.66±0.805	31.10±1.343	<.0001
16:0	1241.2±38.36	1265.8±35.91	0.6443
18:0	426.5±17.57	399.2±11.11	0.2042
MUFA, mg/yolk			
16:1	106.9±5.486	171.2±8.145	<.0001
18:1	1661.6±49.07	1475.1±42.47	0.0090
Omega-3 PUFA, mg/yolk			
18:3n-3	24.48±1.038	40.61±1.887	<.0001
20:5n-3	1.510±0.538	19.44±1.953	<.0001
22:5n-3	3.124±0.354	15.16±1.205	<.0001
22:6n-3	58.71±5.502	159.44±6.823	<.0001
Omega-6 PUFA, mg/yolk			
18:2n-6	962.8±28.21	725.2±28.14	<.0001
18:3n-6	7.484±0.316	7.220±0.279	0.5370
20:4n-6	83.36±7.408	39.03±1.380	<.0001
Totals[2], mg/yolk			
SFA	1683.4±54.22	1696.1±46.78	0.8610
MUFA	1768.4±51.58	1646.4±47.37	0.0953
Omega-3 PUFA	87.83±6.970	234.7±9.658	<.0001
Omega-6 PUFA	1053.7±28.96	771.5±29.48	<.0001
Omega-6/Omega-3	12.55±0.738	3.344±0.181	<.0001

[1] Data are presented as least squares means and their SE (n = 12). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

[3] FO, fish oil.

In liver, hens that were fed an FO diet exhibited higher concentrations of individual omega-3 LC-PUFA as well as total omega-3 PUFA compared to those fed the control diet. Conversely, the concentrations of ARA and the omega-6 to omega-3 ratio were lower in hens fed the FO diet when compared to the control treatment ($P < 0.0001$; **Table 5.6**). Additionally, there was an interaction between diet and injection regarding ALA, as the FO/LPS group showed higher ALA levels than both the FO/saline ($P < 0.05$) and the control/LPS groups ($P < 0.01$; **Table 5.6**).

Table 5.6. Fatty acid composition in liver (mg/g) of laying hens provided either the control or fish oil diet followed by saline or LPS challenge[1].

	Control diet		FO diet[3]		*P* values		
	Saline	LPS	Saline	LPS	Diet	Injection	Diet*Injection
SFA, mg/g							
14:0	0.188±0.047^{b}	0.089±0.014	0.183±0.036	0.234±0.028^{a}	0.0497	0.4823	0.0369
16:0	13.18±2.362	8.575±0.768	10.10±1.575	11.07±0.762	0.3803	0.3219	0.1059
18:0	6.935±1.376	4.635±0.236	5.126±0.794	4.964±0.286	0.4101	0.2051	0.1793
MUFA, mg/g							
16:1	0.852±0.173	0.483±0.078	0.734±0.119	1.008±0.146^{a}	0.1426	0.7277	0.0257
18:1	15.98±5.315	7.546±2.554	9.207±3.536	12.42±1.457	0.1824	0.3696	0.1460
Omega-3 PUFA, mg/g							
ALA (18:3n-3)	0.163±0.031	0.135±0.015	0.186±0.025	0.255±0.018^{a,b}	0.0058	0.3916	0.0478
EPA (20:5n-3)	0.015±0.003	0.010±0.002	0.178±0.011	0.197±0.015	<.0001	0.2227	0.0620
DPA (22:5n-3)	0.030±0.003	0.029±0.005	0.114±0.005	0.135±0.009	<.0001	0.1093	0.0693
DHA (22:6n-3)	0.887±0.088	0.780±0.073	1.854±0.129	2.218±0.100	<.0001	0.7089	0.0576
Omega-6 PUFA, mg/g							
LA (18:2n-6)	9.285±1.509	6.874±0.485	7.118±0.828	7.234±0.394	0.3383	0.2280	0.1866
GLA (18:3n-6)	0.061±0.009	0.045±0.005	0.053±0.005	0.056±0.004	0.7986	0.2978	0.1702
ARA (20:4n-6)	1.887±0.056	1.959±0.130	1.229±0.107	1.078±0.089	<.0001	0.6938	0.2767
Totals[2], mg/g							
SFA	20.30±3.764	13.30±0.999	15.40±2.389	16.27±1.056	0.3375	0.3162	0.1140
MUFA	16.83±5.413	8.029±2.609	9.940±3.640	13.43±1.592	0.2280	0.3533	0.1340
Omega-3 PUFA	1.095±0.093	0.954±0.079	2.332±0.147	2.805±0.124	<.0001	0.2550	0.4538
Omega-6 PUFA	11.23±1.564	8.878±0.462	8.400±0.872	8.367±0.406	0.0962	0.2251	0.2374
Omega-6/Omega-3	10.51±1.436	9.570±0.736	3.588±0.247	3.016±0.209	<.0001	0.2731	0.7271

[1] Data are presented as least squares means and their SE (n = 6). Superscript of a indicates a significant difference ($P < 0.05$) between treatments under the same injection; b indicates a significant difference ($P < 0.05$) between LPS and saline injections under the same diet. Superscripts of a and b are on the higher number.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

[3] FO, fish oil.

Similar effects were observed in plasma. The levels of LA ($P = 0.0371$), ARA ($P < 0.0001$), total omega-6 PUFA ($P = 0.0216$), and the omega-6 to omega-3 ratio ($P < 0.0001$) were all lower in layers given the FO diet compared to those fed the control diet (**Figure 5.1A-D**; **Table 5.7**). Interactions between diet and injection were observed with individual and total omega-3 PUFA. Specifically, plasma levels of ALA, DPA, DHA, and total omega-3 PUFA were higher in the FO/LPS group compared to both the FO/saline and control/LPS groups ($P < 0.05$; **Figure 5.2A, C-E**; **Table 5.7**). Additionally, hens fed the FO diet when compared to control diet exhibited higher plasma EPA concentrations when challenged with either LPS or saline ($P < 0.05$; **Figure 5.2B**; **Table 5.7**).

Table 5.7. Fatty acid composition in plasma (mg/mL) of laying hens provided either the control or fish oil diet followed by saline or LPS challenge[1].

	Control diet		FO diet[3]		*P* values		
	Saline	LPS	Saline	LPS	Diet	Injection	Diet*Injection
SFA, mg/mL							
14:0	0.071±0.010	0.055±0.0128	0.059±0.015	0.128±0.027^{a,b}	0.1075	0.1561	0.0270
16:0	5.548±0.705	4.868±1.239	2.953±0.707	5.667±1.118	0.3827	0.3244	0.1079
18:0	2.002±0.234	1.845±0.476	1.015±0.265^{a}	1.888±0.331^{b}	0.2002	0.3257	0.1638
MUFA, mg/mL							
16:1	0.442±0.067	0.358±0.090	0.297±0.072	0.661±0.158^{b}	0.4673	0.2069	0.0498
18:1	8.093±1.006	7.228±1.815	3.889±1.055	7.419±1.589	0.1850	0.3723	0.1484
Omega-3 PUFA, mg/mL							
ALA (18:3n-3)	0.108±0.019	0.099±0.022	0.096±0.024	0.179±0.026^{a,b}	0.3480	0.1290	0.0499
EPA (20:5n-3)	0.009±0.003	0.004±0.001	0.047±0.010^{a}	0.108±0.031^{a}	<.0001	0.9710	0.0166
DPA (22:5n-3)	0.017±0.004	0.013±0.005	0.029±0.007	0.071±0.020^{a,b}	0.0006	0.3921	0.0250
DHA (22:6n-3)	0.318±0.054	0.235±0.056	0.392±0.089	0.815±0.176^{a,b}	0.0076	0.3829	0.0195
Omega-6 PUFA, mg/mL							
LA (18:2n-6)	4.559±0.636	4.141±0.948	2.293±0.521	3.686±0.523	0.0371	0.2712	0.0758
GLA (18:3n-6)	0.035±0.005	0.032±0.009	0.019±0.004	0.034±0.005	0.1981	0.2745	0.0605
ARA (20:4n-6)	0.496±0.045	0.454±0.105	0.143±0.029	0.214±0.025	<.0001	0.3501	0.0647
Totals[2], mg/mL							
SFA	7.621±0.937	6.767±1.727	4.027±0.981	7.683±1.475	0.3408	0.3196	0.1168
MUFA	8.535±1.062	7.586±1.904	4.186±1.113	8.080±1.746	0.2298	0.3551	0.1356
Omega-3 PUFA	0.452±0.079	0.350±0.082	0.563±0.128	1.172±0.250^{a,b}	0.0079	0.2623	0.0214
Omega-6 PUFA	5.089±0.680	4.627±1.061	2.455±0.553	3.934±0.552	0.0216	0.2747	0.0727
Omega-6/Omega-3	11.56±0.916	13.66±1.082	4.496±0.342	3.660±0.328	<.0001	0.9928	0.3030

[1] Data are presented as least squares means and their SE ($n = 6$). Superscript of a indicates a significant difference ($P < 0.05$) between treatments under the same injection; b indicates a significant difference ($P < 0.05$) between LPS and saline injections under the same diet. Superscripts of a and b are on the higher number.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids.

[3] FO, fish oil.

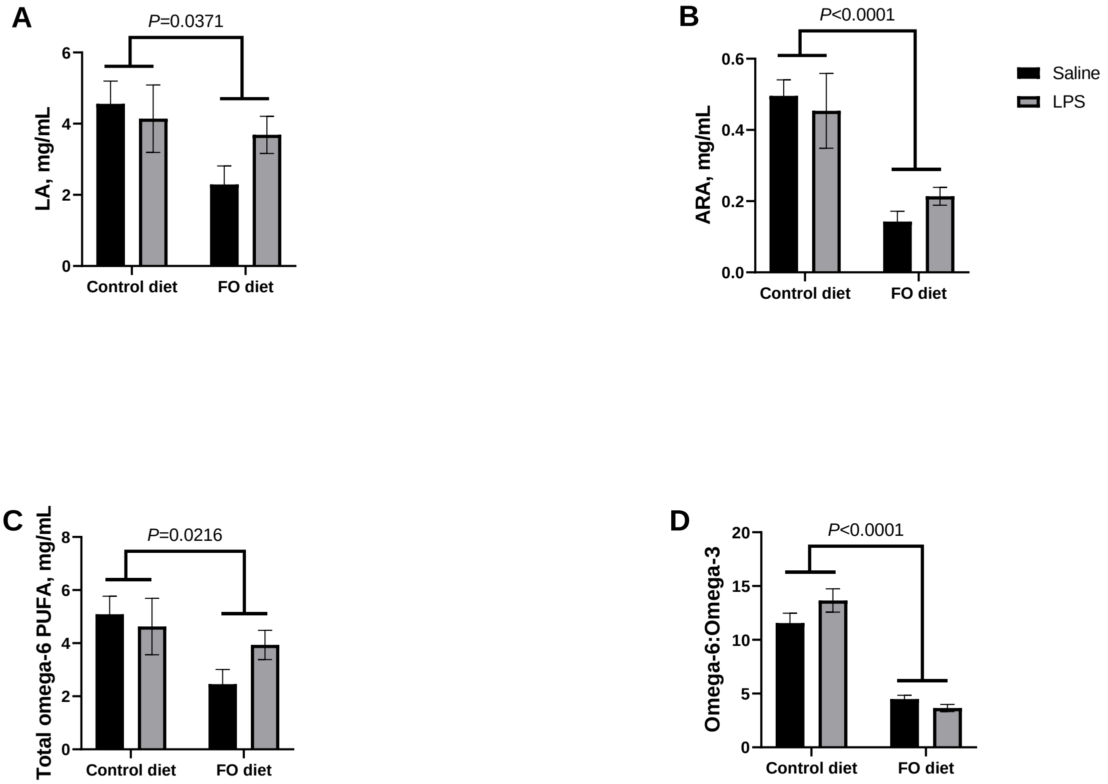


Figure 5.1. The concentration of total LA (A), ARA (B), total omega-6 PUFA (C) and omega-6:omega-3 PUFA (D) in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge.

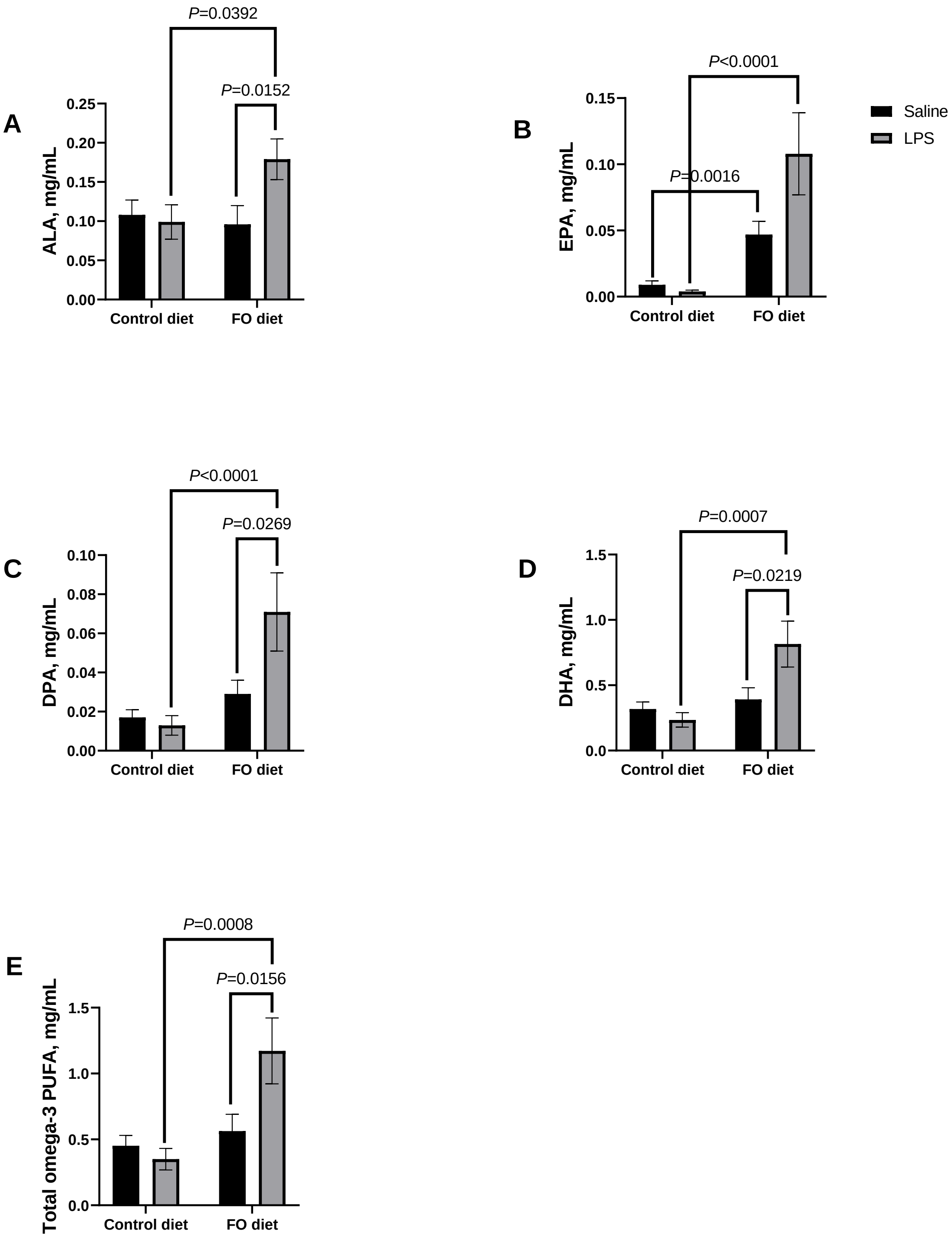
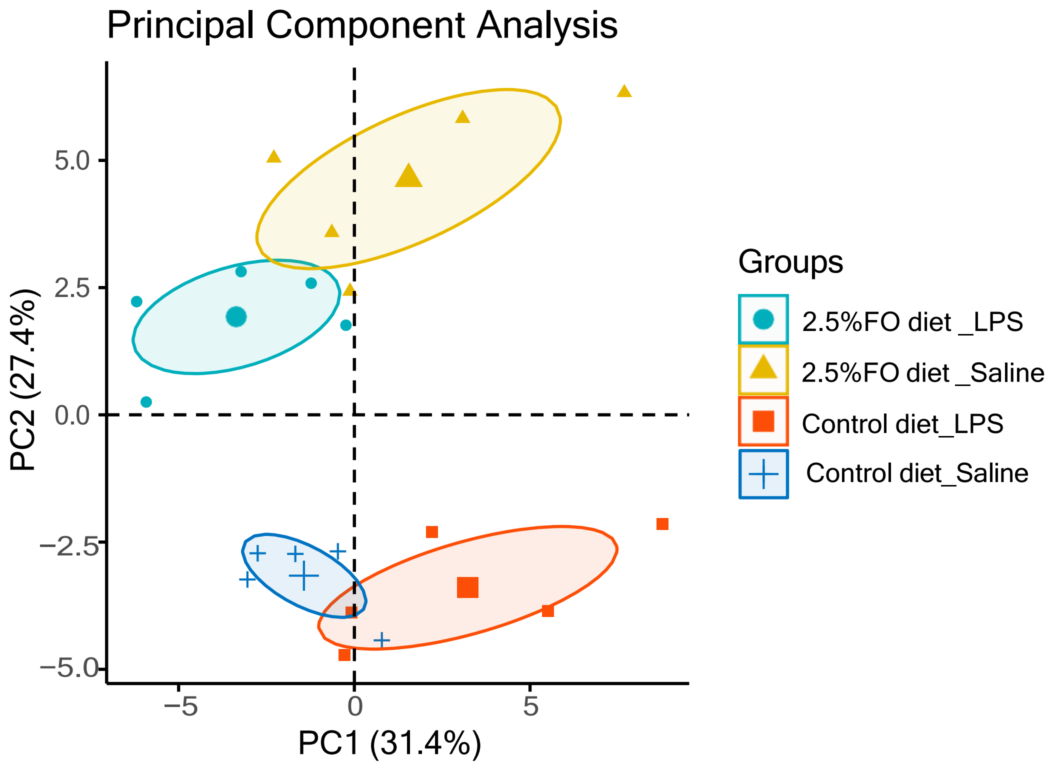

A
P=0.0392
P=0.0152
ALA, mg/mL
0.25
0.20
0.15
0.10
0.05
0.00
Control diet
FO diet
B
P<0.0001
P=0.0016
EPA, mg/mL
0.15
0.10
0.05
0.00
Control diet
FO diet
■ Saline
▨ LPS
C
P<0.0001
P=0.0269
DPA, mg/mL
0.10
0.08
0.06
0.04
0.02
0.00
Control diet
FO diet
D
P=0.0007
P=0.0219
DHA, mg/mL
1.5
1.0
0.5
0.0
Control diet
FO diet
E
P=0.0008
P=0.0156
Total omega-3 PUFA, mg/mL
1.5
1.0
0.5
0.0
Control diet
FO diet

Figure 5.2. The concentration of total ALA (A), EPA (B), DPA (C) and DHA (D) and total omega-3 PUFA (E) in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge.

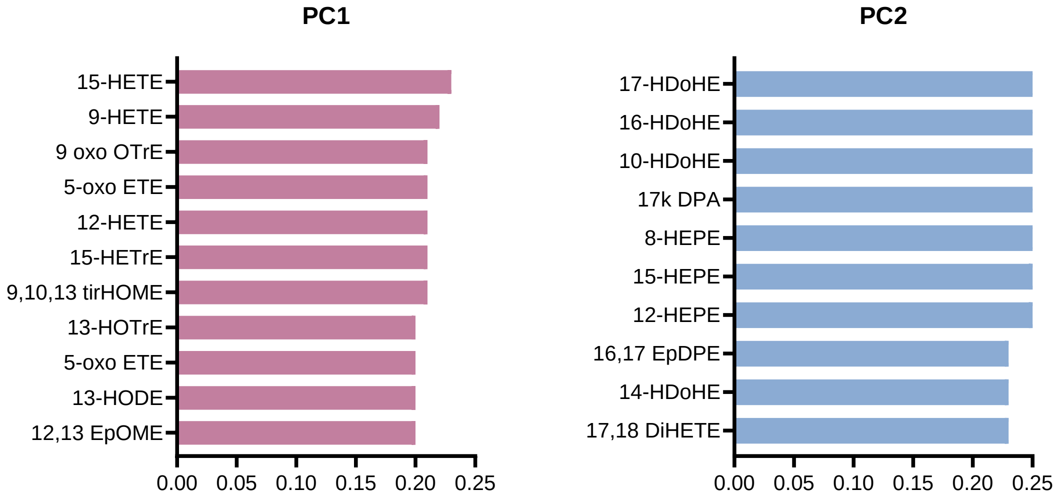
5.4.3 Principal Component Analysis of plasma oxylipins

The PCA of plasma oxylipins in all four groups revealed a clear pattern associated with diet (**Figure 5.3A**). This was particularly evident in PC2 separation, which was associated with EPA and DHA oxylipins **(Figure 5.3B; Table 5.8A)**. In contrast, the separation of groups in PC1 was less distinct, as the patterns observed in the LPS and saline groups were in opposite directions for the two diet groups. Therefore, the PCA of the control and FO diets were performed separately. For the PCA of the control diet, the top PC1 loadings related to the saline group included oxylipins derived from omega-6 PUFA (LA, DGLA, ARA), as well as from ALA. In terms of PC2, the top negative loadings associated with LPS were sEH products, specifically DiHETrE and DiHOME, while the top positive PC2 loading was an sEH substrate, 19,20-EpDoPE (**Figure 5.3C-D, Table 5.8B**). In the PCA for the FO diet, the top PC1 loadings associated with saline were primarily related to ARA oxylipins. The separation along PC2 was less pronounced, however, three sEH substrates, namely EpODE, EpETrE, and EpDoPE, appeared among the top negative loadings (**Figure 5.3E-F; Table 5.8C)**, while the top positive loadings were oxylipins originated from LA and ALA.

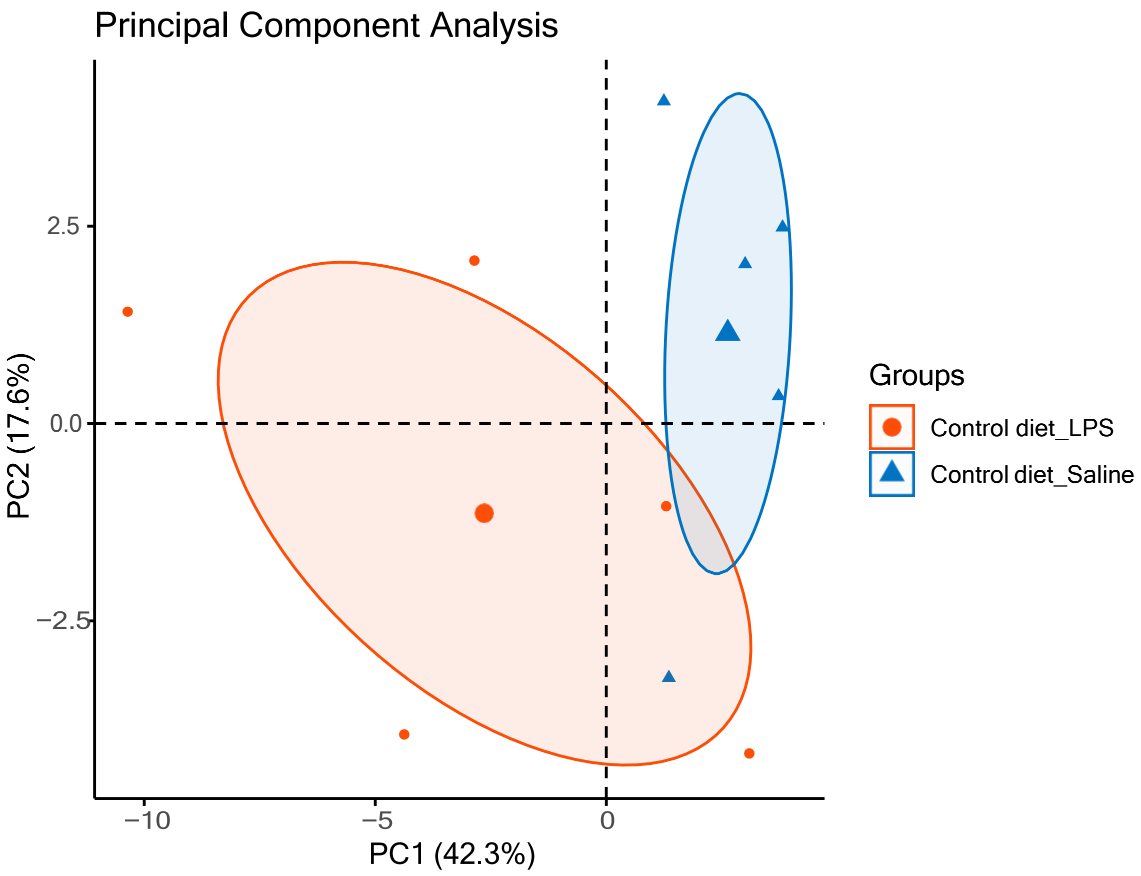
（A）


Principal Component Analysis
PC2 (27.4%)
PC1 (31.4%)
5.0
2.5
0.0
−2.5
−5.0
−5
0
5
Groups
2.5%FO diet _LPS
2.5%FO diet _Saline
Control diet_LPS
Control diet_Saline

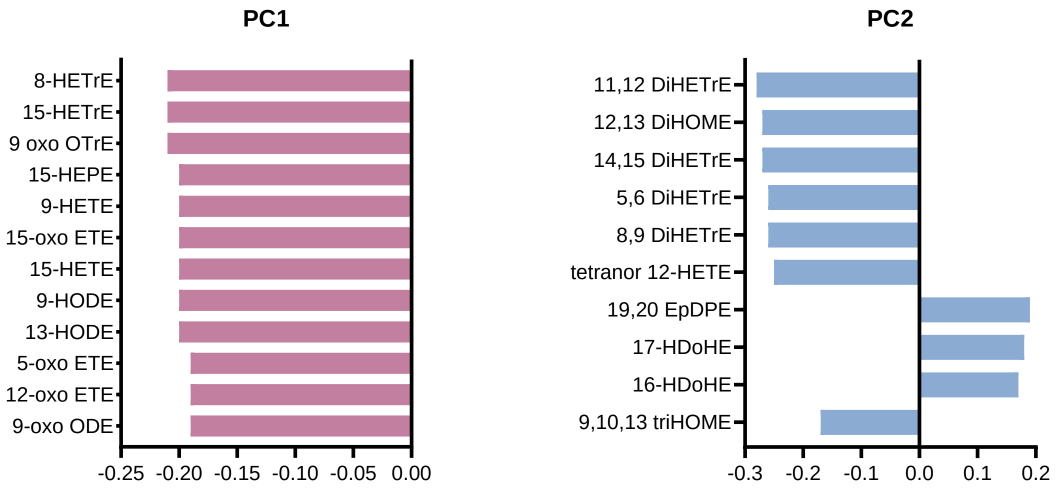
（B）


PC1
15-HETE
9-HETE
9 oxo OTrE
5-oxo ETE
12-HETE
15-HETrE
9,10,13 tirHOME
13-HOTrE
5-oxo ETE
13-HODE
12,13 EpOME
0.00
0.05
0.10
0.15
0.20
0.25
PC2
17-HDoHE
16-HDoHE
10-HDoHE
17k DPA
8-HEPE
15-HEPE
12-HEPE
16,17 EpDPE
14-HDoHE
17,18 DiHETE
0.00
0.05
0.10
0.15
0.20
0.25

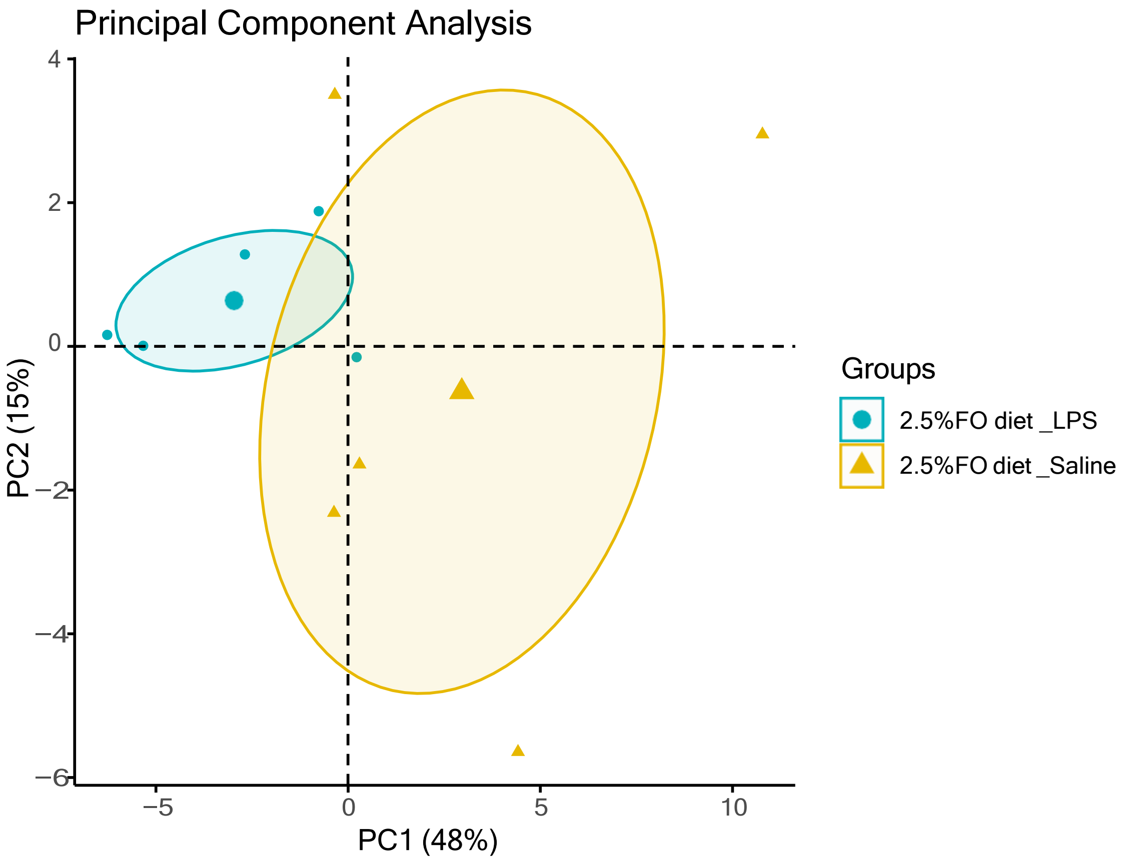
(C)


Principal Component Analysis
PC2 (17.6%)
2.5
0.0
−2.5
−10
−5
0
PC1 (42.3%)
Groups
Control diet_LPS
Control diet_Saline

(D)


PC1
8-HETrE
15-HETrE
9 oxo OTrE
15-HEPE
9-HETE
15-oxo ETE
15-HETE
9-HODE
13-HODE
5-oxo ETE
12-oxo ETE
9-oxo ODE
-0.25
-0.20
-0.15
-0.10
-0.05
0.00
PC2
11,12 DiHETrE
12,13 DiHOME
14,15 DiHETrE
5,6 DiHETrE
8,9 DiHETrE
tetranor 12-HETE
19,20 EpDPE
17-HDoHE
16-HDoHE
9,10,13 triHOME
-0.3
-0.2
-0.1
0.0
0.1
0.2

(E)


Principal Component Analysis
4
2
0
−2
−4
−6
PC2 (15%)
−5
0
5
10
PC1 (48%)
Groups
2.5%FO diet _LPS
2.5%FO diet _Saline

(F)

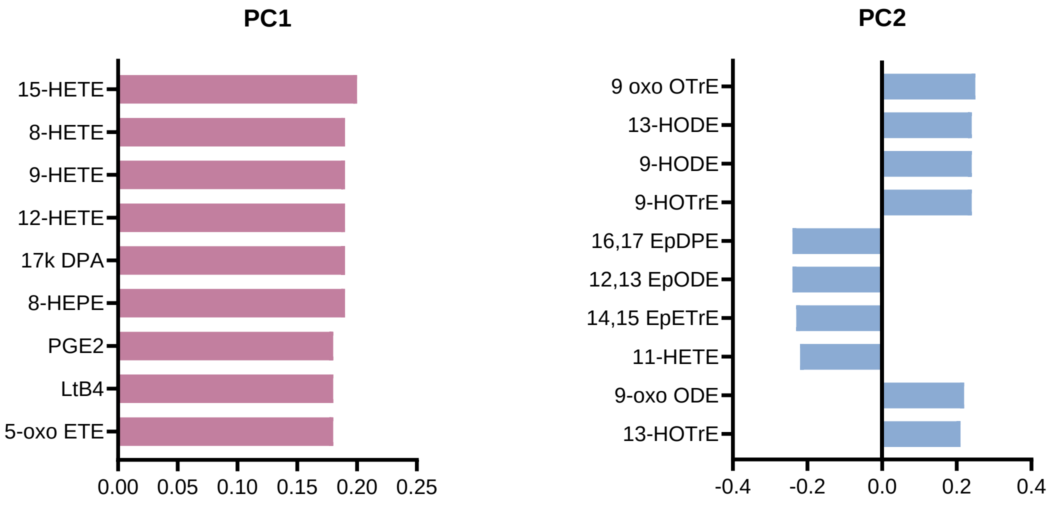


Figure 5.3. Multivariate analysis of oxylipins. (A) PCA of oxylipins in plasma of laying hens provided either the control or FO diet followed by saline or LPS challenge. (B) Loading coefficients of correlations for top 10 oxylipins to PC1 and PC2 shown in (A). (C) PCA of oxylipins in plasma of laying hens provided a control diet followed by saline or LPS challenge. (D) Loading coefficients of correlations for top 10 oxylipins to PC1 and PC2 shown in (C). (E) PCA of oxylipins in plasma of laying hens provided FO diet followed by saline or LPS challenge. (F) Loading coefficients of correlations for top 10 oxylipins to PC1 and PC2 shown in (E).

Table 5.8 A full list of correlations between selected variables and the principal component score from PCA for oxylipin profile in plasma of laying hens provided either the control or fish oil diet (A); control diet only (B); and fish oil diet only (C) followed by saline or LPS challenge.

(A)

Oxylipins[1]	**PC1**	**PC2**
12,13 diHOME	0.18	-0.08
12,13 EpOME	0.20	0.05
13-HODE	0.20	-0.00
9,10 DiHOME	0.19	-0.07
9,10 EpOME	0.13	0.12
9,10,13 triHOME	0.21	-0.06
9-HODE	0.08	0.01
9-oxo ODE	0.16	0.05
15-HETrE	0.21	0.02
8-HETrE	0.19	0.03
11,12 DiHETrE	0.05	-0.14
11b PGE2	0.07	-0.16
11-HETE	0.16	0.07
12-HETE	0.21	-0.03
12-oxoETE	0.18	-0.12
14,15 DiHETrE	0.10	-0.05
14,15 EpETrE	0.12	0.08
15-HETE	0.23	0.02
15k PGE2	0.08	-0.13
15-oxo ETE	0.21	-0.06
5,6 DiHETrE	0.11	-0.08
5,6 EpETrE	0.11	-0.05
5-oxo ETE	0.20	-0.07
8,9 DiHETrE	0.06	-0.04
8,9 EpETrE	0.19	0.09
8-HETE	0.18	0.09
9-HETE	0.22	0.07
LTB4	-0.06	-0.07
PGE2	0.14	-0.07
tetranor 12-HETE	0.15	-0.10
TXB2	0.08	0.14
12,13 EpODE	0.11	0.08
13-HOTrE	0.20	-0.07
9 oxoOTrE	0.21	-0.02
9-HOTrE	0.18	-0.07
12-HEPE	0.05	0.25
14,15 DiHETE	0.01	0.22
15-HEPE	0.07	0.25
17,18 DiHETE	-0.02	0.23
8-HEPE	0.05	0.26

17k DPA	0.03	0.26
10-HDoHE	0.03	0.26
14-HDoHE	0.07	0.23
16,17 DiHDoPE	-0.11	0.17
16,17 EpDPE	-0.01	0.23
16-HDoHE	0.03	0.26
17-HDoHE	0.04	0.26
19,20 DiHDoPE	-0.11	0.16
19,20 EpDPE	0.01	0.22

(B)

Oxylipins[1]	**PC1**	**PC2**
12,13 diHOME	-0.11	-0.27
12,13 EpOME	-0.18	-0.11
13-HODE	-0.20	-0.07
9,10 DiHOME	-0.14	-0.14
9,10 EpOME	-0.16	-0.14
9,10,13 triHOME	-0.16	-0.17
9-HODE	-0.20	-0.02
9-oxo ODE	-0.19	0.06
15-HETrE	-0.21	0.04
8-HETrE	-0.21	0.08
11,12 DiHETrE	0.07	-0.28
11b PGE2	0.04	-0.13
11-HETE	-0.16	0.16
12-HETE	-0.16	-0.11
12-oxoETE	-0.19	-0.09
14,15 DiHETrE	0.02	-0.27
14,15 EpETrE	-0.10	0.06
15-HETE	-0.20	0.08
15k PGE2	0.13	-0.10
15-oxo ETE	-0.20	0.09
5,6 DiHETrE	0.03	-0.26
5,6 EpETrE	0.02	0.07
5-oxo ETE	-0.19	0.14
8,9 DiHETrE	0.06	-0.26
8,9 EpETrE	-0.17	-0.02
8-HETE	-0.14	0.16
9-HETE	-0.20	0.06
LTB4	0.08	-0.01
PGE2	0.04	-0.10
tetranor 12-HETE	-0.10	-0.25
TXB2	0.07	0.16
12,13 EpODE	-0.08	-0.07
13-HOTrE	-0.19	-0.16
9 oxoOTrE	-0.21	-0.07
9-HOTrE	-0.17	-0.17
12-HEPE	-0.17	0.09
14,15 DiHETE	-0.01	-0.16
15-HEPE	-0.20	-0.04
17,18 DiHETE	0.00	-0.16
8-HEPE	-0.17	0.12
17k DPA	-0.09	0.06
10-HDoHE	-0.09	0.06

14-HDoHE	-0.15	-0.12
16,17 DiHDoPE	0.11	-0.02
16,17 EpDPE	0.07	0.15
16-HDoHE	-0.16	0.17
17-HDoHE	-0.14	0.18
19,20 DiHDoPE	0.06	-0.14
19,20 EpDPE	0.08	0.19

(C)

Oxylipins[1]	**PC1**	**PC2**
12,13 diHOME	0.15	-0.02
12,13 EpOME	0.17	0.14
13-HODE	0.12	0.24
9,10 DiHOME	0.16	-0.05
9,10 EpOME	0.14	0.04
9,10,13 triHOME	0.17	-0.17
9-HODE	0.10	0.24
9-oxo ODE	0.17	0.22
15-HETrE	0.16	0.01
8-HETrE	0.12	-0.11
11,12 DiHETrE	0.14	0.14
11b PGE2	0.10	-0.17
11-HETE	0.14	-0.22
12-HETE	0.19	-0.09
12-oxoETE	0.10	0.10
14,15 DiHETrE	0.11	0.11
14,15 EpETrE	0.13	-0.23
15-HETE	0.20	-0.03
15k PGE2	0.14	0.08
15-oxo ETE	0.17	0.10
5,6 DiHETrE	0.17	0.03
5,6 EpETrE	0.13	-0.03
5-oxo ETE	0.18	0.10
8,9 DiHETrE	0.12	0.21
8,9 EpETrE	0.17	-0.15
8-HETE	0.19	-0.09
9-HETE	0.19	-0.08
LTB4	0.18	-0.03
PGE2	0.18	-0.03
tetranor 12-HETE	0.10	0.17
TXB2	0.16	-0.15
12,13 EpODE	0.11	-0.24
13-HOTrE	0.11	0.21
9 oxoOTrE	0.12	0.25
9-HOTrE	0.08	0.24
12-HEPE	0.16	-0.08
14,15 DiHETE	0.12	0.14
15-HEPE	0.17	0.07
17,18 DiHETE	0.07	0.15
8-HEPE	0.19	-0.07
17k DPA	0.19	0.03
10-HDoHE	0.16	-0.10

14-HDoHE	0.15	-0.02
16,17 DiHDoPE	-0.09	0.13
16,17 EpDPE	-0.08	-0.24
16-HDoHE	0.16	-0.04
17-HDoHE	0.15	-0.14
19,20 DiHDoPE	-0.11	0.15
19,20 EpDPE	0.11	-0.09

[1] DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxy-eicosadocosapentaenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxy-octadecenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LTB, leukotriene B; oxo ETE, oxo-eicosatetraenoic acid; oxo ODE, oxo-octadecadienoic acid; oxo OTrE, oxo-octadecatrienoic acid; PG, prostaglandin; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane.

5.4.4 Plasma omega-3 oxylipins

Consistent with the results of the PCA conducted on all four groups, further analyses revealed that the FO diet, when compared to the control diet, led to increased levels of all quantified EPA and DHA oxylipins in hens. This was evident in both the main effects and interaction effects, where the FO diet resulted in elevated oxylipin levels in both the saline and LPS groups. Regarding ALA oxylipins, the FO diet increased one type in the saline-treated group and decreased one in the LPS group (**Figure 5.4**; **Table 5.9)**.

Specific analyses of the effects of LPS further revealed the oxylipins that were dependently altered by the diet. For omega-3 oxylipins, interactions showed that three ALA, one EPA, one DHA, and total ALA oxylipins were elevated by LPS in hens fed the control diet, but these levels remained unaltered in those fed the FO diet. Further, one ALA, two EPA, three DHA, and total DHA oxylipins were lower in response to LPS only in hens fed the FO diet, except for two DHA sEH products, which were higher in response to LPS challenge in hens fed the FO diet (**Figure 5.4**; **Table 5.9**).

5.4.5 Plasma omega-6 oxylipins

The FO diet resulted in lower levels of four ARA oxylipins (main effects only). In addition, interaction effects showed that two LA-, total LA- and five ARA- oxylipins were also lower in hens fed the FO diet, but only when challenged with LPS. In contrast, in unchallenged hens, the FO diet increased one LA and one ARA oxylipin (both sEH substrates), while decreasing another ARA oxylipin, LtB_4. With respect to the LPS effects, three LA and six

ARA oxylipins (five of which were sEH substrates) were lower in LPS-challenged hens only when they were fed the FO diet. In hens consuming the control diet, LPS caused varying effects, as it increased two LA oxylipins (including 1 sEH substrate) and decreased LtB_4 (**Figure 5.5**; **Table 5.9**).

			FO Effects			**LPS Effects**	
		Saline	Main	LPS	Control	Main	FO
Precursor PUFA	**Oxylipin**						
ALA	9-HOTrE			↓	↑		
ALA	13-HOTrE			↓	↑		
ALA	9 oxoOTrE			↓	↑		
ALA	12,13 EpODE	↑		↓			↓
	Total ALA oxylipins				↑		
EPA	8-HEPE	↑		↑			↓
EPA	12-HEPE	↑		↑			↓
EPA	15-HEPE	↑		↑	↑		
EPA	14, 15 DiHETE		↑				
EPA	17, 18 DiHETE		↑				
	Total EPA oxylipins		↑				
DPA	17k DPA		↑				
DHA	10-HDoHE	↑		↑			↓
DHA	14-HDoHE	↑		↑	↑		
DHA	16-HDoHE	↑		↑			↓
DHA	17-HDoHE	↑		↑			↓
DHA	16, 17 EpDPE		↑			↓	
DHA	16, 17 DiHDoPE	↑		↑			↑
DHA	19,20 EpDPE		↑				
DHA	19,20 DiHDoPE	↑		↑			↑
	Total DHA oxylipins	↑		↑			↓

↑↓: higher/lower in either the FO group or the LPS challenge group as the main effect.

↑↓: higher/lower in the FO/saline group or the FO/LPS group, compared to the Control/saline group or Control/LPS challenge group, respectively.

↑↓: higher/lower in the Control/LPS group or the FO/LPS group, compared to the Control/saline group or FO/saline challenge group, respectively.

Figure 5.4. Differences of omega-3 oxylipins in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge.

		FO Effects			**LPS Effects**		
		Saline	Main	LPS	Control	Main	FO
LA	9-HODE						
LA	13-HODE				↑		
LA	9 oxoODE						↓
LA	9, 10, 13 triHOME			↓			
LA	9, 10 EpOME	↑			↑		↓
LA	9, 10 diHOME						
LA	12, 13 EpOME			↓			↓
LA	*12,13 diHOME*						
	Total LA oxylipins			↓			
DGLA	8-HETrE						
DGLA	*15-HETrE*						
ARA	PGE2						
ARA	11b PGE2		↓				
ARA	15k PGE2		↓				
ARA	TXB2						
ARA	LTB4	↓			↓		
ARA	8-HETE						
ARA	9-HETE			↓			↓
ARA	11-HETE			↓			↓
ARA	12-HETE			↓			↓
ARA	15-HETE						
ARA	tetranor 12-HETE			↓			
ARA	5 oxo ETE						
ARA	12 oxo ETE		↓				
ARA	15 oxo ETE						
ARA	5,6 EpETrE			↓			↓
ARA	5,6 DiHETrE						
ARA	8,9 EpETrE	↑					↓
ARA	8,9 DiHETrE						
ARA	11,12 DiHETrE		↓				
ARA	14, 15 EpETrE						↓
ARA	14, 15 DiHETrE						

↑↓: higher/lower in either the FO group or the LPS challenge group as the main effect.

↑↓: higher/lower in the FO/saline group or the FO/LPS group, compared to the Control/saline group or Control/LPS challenge group, respectively.

↑↓: higher/lower in the Control/LPS group or the FO/LPS group, compared to the Control/saline group or FO/saline challenge group, respectively.

Figure 5.5. Differences of omega-6 oxylipins in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge.

Table 5.9. Oxylipin compositions (ng/mL) in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge[1].

		Control diet		FO diet[3]		*P* values		
	Pathway	Saline	LPS	Saline	LPS	Diet	Injection	Diet*Injection
LA[2], ng/mL								
9-HODE	LOX	177.9±33.70	334.9±83.25	299.2±76.05	185.9±43.67	0.8287	0.7321	0.0469
13-HODE	LOX	192.2±41.78	357.6±75.41^{b}	293.2±52.86	204.4±39.43	0.6369	0.4907	0.0325
9 oxoODE	LOX	116.4±27.39	280.9±113.5	348.5±127.9^{b}	121.9±44.30	0.0951	0.0239	0.0267
9, 10, 13 TriHOME	LOX	121.6±20.66	225.8±38.84^{a}	150.9±30.01	97.53±29.94	0.1247	0.4172	0.0202
9, 10 EpOME	CYP-e	15.07±1.984	33.23±4.427^{b}	52.19±14.77^{a,b}	20.32±6.807	0.0701	0.0030	0.0028
9, 10 DiHOME	CYP-e	4.294±0.607	5.679±0.728	4.011±0.944	2.959±0.619	0.0586	0.8239	0.1177
12, 13 EpOME	CYP-e	30.57±3.561	58.11±7.935^{a}	59.76±17.24^{b}	26.48±7.824	0.9083	0.7867	0.0101
12,13 DiHOME	CYP-e	5.057±0.704	8.127±0.643	5.180±1.337	4.531±0.639	0.0664	0.1884	0.0510
DGLA[2], ng/mL								
8-HETrE	LOX	0.691±0.047	1.105±0.211	0.953±0.118	0.788±0.170	0.8558	0.4165	0.0710
15-HETrE	LOX	1.528±0.128	2.735±0.682	2.128±0.295	1.779±0.331	0.1460	0.1327	0.0742
ARA[2], ng/mL								
PGE2	COX	0.513±0.040	0.472±0.053	0.425±0.090	0.298±0.055	0.0517	0.1975	0.5004
11b PGE2	COX	9.919±0.651	10.40±1.242	6.344±1.139	5.677±0.868	0.0008	0.9250	0.5762
15k PGE2	COX	1.953±0.238	1.658±0.160	1.088±0.312	1.053±0.294	0.0115	0.5303	0.6206
TXB2	COX	16.35±6.763	3.038±1.902	35.58±14.41	6.828±2.751	0.3379	0.8402	0.4758
LTB4	LOX	1.425±0.522^{a,b}	0.253±0.039	0.447±0.024	0.576±0.070	0.4232	0.0546	0.0135
8-HETE	LOX	4.027±0.468	4.340±0.713	5.613±1.410	2.747±0.459	0.9967	0.1549	0.0815
9-HETE	LOX	2.480±0.196	3.371±0.821^{a}	4.080±1.177^{b}	1.556±0.399	0.0412	0.0183	0.0381
11-HETE	LOX	2.231±0.174	2.230±0.463^{a}	3.075±0.724^{b}	1.047±0.155	0.0138	0.0038	0.0206

12-HETE	LOX	1.598±0.177	3.233±0.984^{a}	2.201±0.510^{b}	1.100±0.209	0.0058	0.0154	0.0131
15-HETE	LOX	4.941±0.462	6.049±1.415	5.881±1.083	3.531±0.675	0.4327	0.5358	0.0968
tetranor 12-HETE	LOX	0.201±0.056	0.292±0.022^{a}	0.211±0.043	0.131±0.016	0.0662	0.8864	0.0383
5 oxo ETE	LOX	5.709±1.234	7.837±2.320	4.375±1.150	2.976±0.939	0.0568	0.8123	0.2597
12 oxo ETE	LOX	15.86±1.614	37.49±7.986	10.90±2.182	14.03±4.149	0.0075	0.4666	0.2020
15 oxo ETE	LOX	3.050±0.391	5.212±1.382	2.483±0.942	2.019±0.638	0.0569	0.3678	0.1711
5,6 EpETrE	CYP-e	1.113±0.146	1.108±0.092^{a}	1.169±0.075^{b}	0.477±0.134	0.0001	0.0002	0.0016
5,6 DiHETrE	CYP-e	0.215±0.040	0.506±0.037	0.178±0.039	0.131±0.011	0.1172	0.4228	0.5786
8,9 EpETrE	CYP-e	0.206±0.024	0.299±0.023	0.361±0.064^{a,b}	0.183±0.052	0.6668	0.3544	0.0077
8,9 DiHETrE	CYP-e	0.329±0.063	0.331±0.075	0.334±0.077	0.233±0.025	0.4747	0.4409	0.4304
11,12 DiHETrE	CYP-e	0.459±0.120	0.612±0.148	0.328±0.083	0.239±0.025	0.0169	0.2700	0.2242
14, 15 EpETrE	CYP-e	0.240±0.010	0.232±0.023	0.395±0.116^{b}	0.125±0.045	0.8890	0.0257	0.0361
14, 15 DiHETrE	CYP-e	0.603±0.067	0.616±0.046	0.532±0.107	0.562±0.024	0.3729	0.7609	0.9029
ALA^{2}, ng/mL								
9-HOTrE	LOX	7.956±1.401	21.79±3.209^{a,b}	11.46±2.312	10.06±2.451	0.1099	0.0210	0.0064
13-HOTrE	LOX	9.421±2.034	26.73±5.161^{a,b}	14.55±2.231	11.11±1.593	0.1084	0.0391	0.0040
9 oxoOTrE	LOX	14.73±3.854	54.57±11.89^{a,b}	34.81±11.03	19.32±6.222	0.4063	0.1902	0.0067
12,13 EpODE	CYP-e	1.176±0.158	1.959±0.359^{a}	2.636±0.766^{a,b}	0.903±0.156	0.0099	0.0007	0.0011
EPA^{2}, ng/mL								
8-HEPE	LOX	0.467±0.050	0.564±0.174	2.532±0.235^{a,b}	1.661±0.192^{a}	<.0001	0.0433	0.0145
12-HEPE	LOX	0.365±0.014	0.586±0.135	2.391±0.234^{a,b}	1.248±0.237^{a}	<.0001	0.0828	0.0025
15-HEPE	LOX	1.027±0.052	2.572±0.584^{b}	7.376±1.016^{a}	5.539±0.917^{a}	<.0001	0.4000	0.0127
14, 15 DiHETE	CYP-e	4.835±1.311	4.488±0.334	24.18±6.803	22.87±2.265	<.0001	0.9769	0.9474
17, 18 DiHETE	CYP-e	7.624±2.724	6.630±1.084	35.86±6.185	34.25±4.340	<.0001	0.8428	0.8004
DPA^{2}, ng/mL								
17k DPA	LOX	49.61±10.66	54.82±11.07	311.7±40.94	204.6±24.49	<.0001	0.1048	0.1486

DHA, mg/mL								
10-HDoHE	LOX	2.990±0.227	3.009±0.221	9.227±1.134^{a,b}	5.976±0.430^{a}	<.0001	0.0114	0.0368
14-HDoHE	LOX	6.909±0.401	11.08±2.021^{b}	21.19±2.665^{a}	16.00±1.957^{a}	0.0485	0.0302	0.0166
16-HDoHE	LOX	5.540±0.274	5.751±0.979	15.44±1.297^{a,b}	11.37±1.043^{a}	<.0001	0.0654	0.0432
17-HDoHE	LOX	25.97±1.363	25.95±4.843	82.18±5.542^{a,b}	50.44±4.696^{a}	<.0001	0.0024	0.0024
16, 17 EpDPE	CYP-e	6.213±0.764	5.311±0.471	25.11±4.011	13.05±2.119	<.0001	0.0102	0.0776
16, 17 DiHDoPE	CYP-e	0.287±0.047	0.216±0.036	0.608±0.065^{a}	1.174±0.150^{a,b}	<.0001	0.0044	0.0071
19,20 EpDPE	CYP-e	1.553±0.173	1.245±0.099	4.144±0.520	3.283±0.646	<.0001	0.1021	0.7783
19,20 DiHDoPE	CYP-e	0.578±0.134	0.496±0.103	1.362±0.135^{a}	2.484±0.344^{a,b}	<.0001	0.0023	0.0091
Totals, ng/mL								
LA		744.0±130.6	1477.8±340.0^{a}	1292.2±293.2	709.2±174.0	0.6645	0.7662	0.0178
ARA		73.43±9.186	89.28±13.98	86.01±22.88	45.52±10.44	0.3174	0.4267	0.0806
ALA		33.29±6.637	105.1±19.54^{a,b}	63.45±15.08	41.39±9.844	0.2387	0.0882	0.0035
EPA		14.32±4.052	14.84±1.851	72.34±14.07	65.56±5.997	<.0001	0.7629	0.5506
DHA		50.04±2.788	53.06±7.151	159.3±12.10^{a,b}	103.8±9.138^{a}	<.0001	0.0071	0.0033

[1] Data are presented as least squares means and their SE ($n = 5$). Superscript of a indicates a significant difference ($P < 0.05$) between treatments under the same injection; b indicates a significant difference ($P < 0.05$) between LPS and saline injections under the same diet. Superscripts of a and b are on the higher number.

[2] COX, cyclooxygenase; CYP-e, cytochrome P450 epoxygenase; CYP-h, cytochrome P450 hydrolase; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETE, dihydroxy-eicosatetraenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxy-eicosadocosapentaenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxy-octadecenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HETrE, hydroxy-eicosatrienoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LTB, leukotriene B; LOX, lipoxygenase; oxo ETE, oxo-eicosatetraenoic acid; oxo ODE, oxo-octadecadienoic acid; oxo OTrE, oxo-octadecatrienoic acid; PG, prostaglandin; triHOME, trihydroxy-octadecenoic acid; TX, thromboxane.

[3] FO, fish oil.

5.4.6 Plasma PUFA epoxides and corresponding diols

Both PCA and ANOVA indicated changes in oxylipins that were substrates and products of sEH in response to LPS challenge and FO presence in the diet. The enzyme CYP450 epoxygenase converts FA to their epoxide derivatives, producing epoxy-PUFA oxylipins, which are subsequently metabolized to dihydroxy-PUFA (diols) by sEH activity. The ratio of sEH products (diols) to substrates (epoxides) has been used to estimate *in vivo* sEH activity, with increased activity being associated with increased inflammation. These ratios were calculated for all seven pairs of diols/epoxides that were quantifiable in plasma. In saline-treated hens, the FO diet resulted in three lower ratios, while in LPS-treated hens, six of seven ratios were higher. Regarding LPS treatment, one ratio was lower in hens fed the control diet, whereas all seven ratios were higher in hens fed the FO diet (**Table 5.10**).

Table 5.10. Precursor PUFA epoxides to their corresponding diols (ng/mL) in plasma of laying hens provided either the control or fish oil diet followed by saline or LPS challenge[1].

		Control diet		2.5% FO diet		*P* values		
Precursor FFA[2]	Diol/Epoxide[3]	Saline	LPS	Saline	LPS	Diet	Injection	Diet*Injection
LA	12.13.DiHOME/ 12.13.EpOME	0.166±0.012^{a}	0.135±0.012	0.086±0.017	0.206±0.013^{a,b}	0.7520	0.0049	<.0001
LA	9.10.DiHOME/ 9.10.EpOME	0.287±0.016^{a,b}	0.196±0.013	0.116±0.017	0.282±0.030^{a,b}	0.0486	0.0805	<.0001
ARA	14.15.DiHETrE/ 14.15.EpETrE	2.561±0.377^{a}	2.630±0.485	1.271±0.149	5.586±0.867^{a,b}	0.8289	0.0003	0.0003
ARA	5.6.DiHETrE/ 5.6.EpETrE	0.212±0.056	0.161±0.018	0.133±0.025	0.290±0.039^{a,b}	0.5082	0.1786	0.0133
ARA	8.9.DiHETrE/ 8.9.EpETrE	1.790±0.506	1.403±0.557	0.737±0.083	2.473±0.546^{b}	0.9264	0.1139	0.0095
DHA	16.17.DiHDoPE/ 16.17.EpDPE	0.049±0.008	0.049±0.010	0.036±0.004	0.110±0.007^{a,b}	0.0054	0.0001	0.0001
DHA	19.20.DiHDoPE/ 19.20.EpDPE	0.377±0.069	0.338±0.065	0.381±0.014	1.047±0.189^{a,b}	0.0020	0.0182	0.0063

[1] Data are presented as least squares means and their SE ($n = 5$). Superscript of a indicates a significant difference ($P < 0.05$) between treatments under the same injection; b indicates a significant difference ($P < 0.05$) between LPS and saline injections under the same diet. Superscripts of a and b are on the higher number.

[2] FFA, free fatty acids.

[3] DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EpDPE, epoxy-eicosadocosapentaenoic acid; EpETrE, epoxyeicosatrienoic acid; EpOME, epoxy-octadecenoic acid.

5.4.7 Relative mRNA expression

The administration of LPS greatly increased the mRNA expression of all measured splenic cytokines, either as overall main effects or simple effects in both diet groups (**Figure 5.6A-C**). However, it did not affect the levels of *TLR4*. The effects of dietary FO inclusion were less pronounced, only resulting in an increase in *TNF-α* expression, and this was observed only in unchallenged hens ($P < 0.0001$; **Figure 5.6D**; **Table 5.11**).

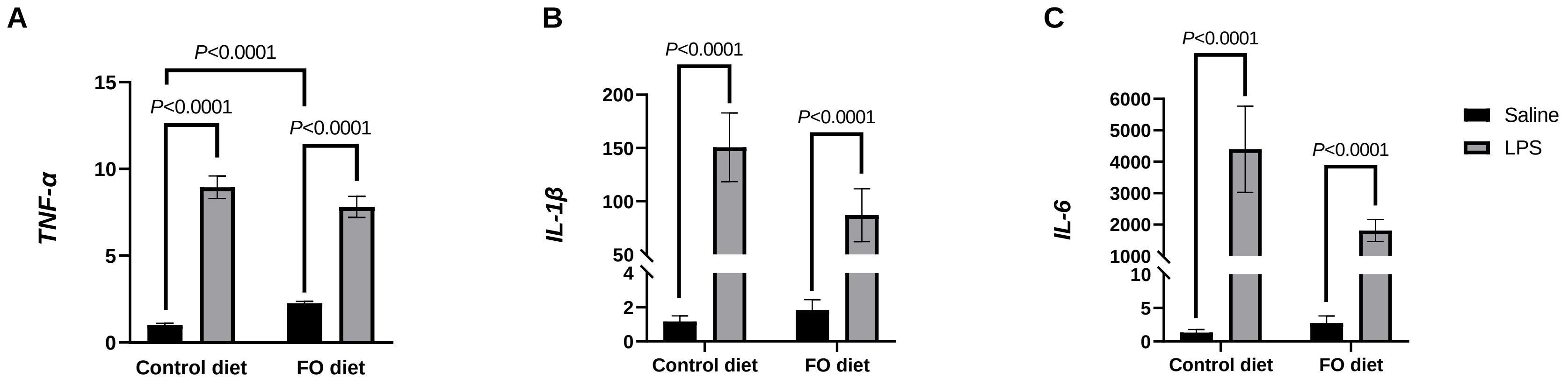


Figure 5.6. Relative mRNA expression of *IL-2* (A), *IL-10* (B), *IFN-γ* (C), *TNF-α* (D), *IL-1β* (E), and *IL-6* (F) in the spleen of laying hens provided either the control or fish oil diet followed by saline or LPS challenge.

Table 5.11. Relative mRNA expression of cytokines and receptor in the spleen of laying hens provided either the control or fish oil diet followed by saline or LPS challenge[1].

	Control diet		FO diet[3]		*P* values		
Genes[2]	Saline	LPS	Saline	LPS	Diet	Injection	Diet*Injection
IL-1β	1.172±0.326	150.69±32.17[b]	1.848±0.598	86.88±24.79[b]	0.6523	<.0001	0.0386
IL-2	1.192±0.312	20.90±7.525	1.070±0.171	13.24±3.238	0.7854	<.0001	0.7532
IL-6	1.349±0.437	4394.5±1369.3[b]	2.761±1.047	1805.5±346.8[b]	<.0001	<.0001	0.0401
IL-10	1.031±0.095	43.59±12.45	0.811±0.238	23.03±3.970	0.7495	<.0001	0.8394
TNF-α	1.018±0.089	8.944±0.650[b]	2.262±0.107[a]	7.811±0.611[b]	0.0002	<.0001	<.0001
IFN-γ	1.030±0.117	29.02±4.720	1.689±0.437	27.49±6.261	0.4934	<.0001	0.2440
TLR4	1.008±0.058	1.528±0.125	1.843±0.414	1.604±0.184	0.0787	0.5634	0.1358

[1] Data are presented as the least squares means and their SE based on $2^{-\Delta\Delta Ct}$ method ($n = 6$). Beta-actin is used as an internal control for gene expression normalization. Superscript of a indicates a significant difference ($P<0.05$) between treatments under the same injection; b indicates a significant difference ($P<0.05$) between LPS and saline injections under the same diet. Superscripts of a and b are on the higher number.

[2] IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-10, interleukin10; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4.

[3] FO, fish oil.

5.5. Discussion

The data presented herein provide essential insights into the plasma oxylipin profiles and their precursor PUFA compositions in laying hens, highlighting their modulation by both dietary FO and by an LPS-mediated inflammatory challenge. Dietary FO led to higher plasma levels of EPA and all quantified EPA and DHA oxylipins, and lower levels of omega-6 PUFA and ~42% of omega-6 oxylipins, in both saline and LPS-challenged hens, suggesting a reduced inflammatory state with FO feeding. LPS effects on oxylipins were markedly influenced by diet, as LPS challenge resulted in ~14% of plasma oxylipins (particularly the octadecanoids) being elevated in control-fed hens, while ~31% of oxylipins were reduced when provided with the FO diet. Consistent with putatively anti-inflammatory effects of dietary FO, oxylipins indicative of higher sEH activity were lower in FO compared to control diet hens that were unchallenged. However, when challenged with LPS, plasma oxylipins indicative of higher sEH activity were higher with FO feeding, indicating a possible response to inflammation and increased vasoconstriction. Additionally, cytokine expressions were not altered by the FO diet under the immune challenge, indicating that oxylipin effects appear to be separate from altered cytokine levels. Our previous study found that feeding ALA increased ALA and EPA concentrations, leading to higher levels of their respective oxylipins, while having little effect on DHA-derived oxylipins. In contrast, increasing dietary DHA, but not ALA, resulted in greater accumulation of EPA and DHA in the liver and plasma, along with elevated levels of their oxylipins (31). These findings demonstrated that the type of omega-3 PUFA in the diet strongly influences tissue fatty acid composition and subsequent oxylipin production. However, oxylipins do not always mimic their fatty acid precursors completely. For example, plasma EPA and oxylipins from EPA and DHA were increased by dietary FO in both saline and LPS-challenged hens, but plasma DHA only increased in LPS-challenged hens provided the FO diet. Discrepancies between oxylipins and their precursor PUFA have been observed in plasma in rats (201) and humans (204), and therefore must be measured directly to determine their effects. Oxylipins derived from EPA and DHA predominantly exhibit anti-inflammatory properties, as previously reviewed (205, 206).

In contrast, oxylipins derived from LA and ARA are generally pro-inflammatory, although their full physiological significance remains unclear (182). While several studies have observed that omega-3 PUFA supplementation reduces total omega-6 PUFA levels in plasma and various tissues, the impact on omega-6-derived oxylipins is variable. Some studies reported minimal effects of ALA, DHA, or a combination of EPA and DHA on ARA-derived oxylipins (26, 204, 207). In contrast, other studies found that DHA supplementation had a more pronounced effect in reducing circulating omega-6 PUFA oxylipins, including pro-inflammatory eicosanoids derived from ARA (194). In the present study, we observed that the FO diet lowered plasma levels of individual omega-6 oxylipins, with most ARA-derived hydroxy-eicosatetraenoic acids (HETE) significantly reduced in

LPS-challenged hens only when provided the FO diet. HETE derived from ARA via LOX pathways are primarily characterized as pro-inflammatory, although some, such as 12-HETE, have been noted to exhibit anti-inflammatory properties (208).

Some retro-conversion from EPA and DHA to ALA may occur in chicken plasma and liver, as suggested by the slight elevation of ALA in both liver and plasma with FO feeding. This observation aligns with findings showing increased ALA levels in the liver and kidney of rats fed diets enriched with either DHA or EPA (194). Notably, EPA and DHA are more efficiently converted into bioactive lipid mediators with well-established roles in resolving inflammation (80, 115). Therefore, under inflammatory conditions such as LPS challenge, the chicken body may preferentially utilize EPA and DHA for the synthesis of immunomodulatory oxylipins, rather than ALA. This metabolic prioritization could explain the observed shift in oxylipin profiles, where ALA-derived oxylipins were reduced in the plasma of LPS-challenged hens fed the FO diet, despite an elevation in their ALA precursor. Additionally, most ALA-derived oxylipins were elevated in LPS-challenged hens fed the control diet. This increase, along with the concurrent rise in some LA-derived oxylipins in the same group, and their reduction in FO-fed hens, suggests that dietary fish oil may mitigate the LPS-induced production of these octadecanoid oxylipins. Further studies are required to determine whether these oxylipins are inflammatory mediators that are reduced when inflammation is mitigated by FO feeding or whether they impart anti-inflammatory responses, as evidence for ALA oxylipins suggests they may have anti-inflammatory properties (180, 209, 210), and LA oxylipins have been shown to exhibit both pro- and anti-inflammatory responses (182).

CYP450 epoxygenases convert PUFA into bioactive epoxides (epoxy-PUFA oxylipins), which generally possess anti-inflammatory properties. These epoxides are subsequently metabolized by sEH into diols, a process that often reduces their bioactivity or may even result in pro-inflammatory effects (211-213). The balance between epoxides and diols is thus a critical determinant of inflammatory outcomes, with the diol-to-epoxide ratio frequently used as an indirect indicator of *in vivo* sEH activity. While FO, rich in EPA and DHA, is widely considered anti-inflammatory, its modulatory effects on lipid mediator pathways, such as CYP450-sEH, can vary depending on the immune status (214, 215). In this study, FO-fed hens under non-inflammatory (saline-treated) conditions exhibited lower diol/epoxide ratios, which are typically associated with less inflammation (214, 215). However, during acute immune activation induced by LPS, the FO diet was associated with increased sEH activity, as indicated by greater conversion of epoxides to diols. This appears to be contradictory to the expected anti-inflammatory effect of FO, but sEH metabolites also are implicated in vasoconstrictive responses, which can reduce blood flow and limit immune cell infiltration into tissues under LPS-induced systemic inflammation, thereby reducing the risk of collateral tissue damage. Indeed, LPS induces vasodilation (216, 217), so the vasoconstrictive effects of sEH metabolites in FO-feed chickens herein may be a protective response. And as with the apparent discrepancies in the role of octadecanoids in the inflammatory response, it remains unclear whether elevated diol/epoxide ratios are a cause or consequence of inflammation in LPS-challenged hens. Additionally, since plasma concentrations only serve as proxies for changes occurring in tissues, further studies examining oxylipin dynamics in relevant organs are necessary to fully elucidate the modulatory effects of FO on inflammatory pathways (218).

Overall, the findings herein largely indicate that FO supplementation may attenuate the acute inflammatory response by modulating the production of pro-resolving oxylipins. This contrasts with the lack of effect of dietary FO in attenuating the rise in cytokines in response to LPS challenge, suggesting that oxylipin effects are distinct from cytokine responses. Cytokines are classified based on their function as either pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines, including IL-1β, IL-2, IL-6, TNF-α, and IFN (interferon)-γ, promote the onset and progression of autoimmune inflammation. In contrast, anti-inflammatory cytokines, such as IL-10, play a crucial role in suppressing inflammatory responses (219, 220). Several studies have reported elevated circulating levels and increased mRNA expression of both pro-inflammatory and anti-inflammatory cytokines in chickens following LPS administration (144, 149). The current findings consistently revealed that LPS increased all cytokine expressions 2 hours post exposure in both diet groups, but that there were minimal effects of dietary FO. Although several studies have explored the impact of dietary FO on cytokine expression following inflammatory challenge, the findings remain inconsistent, with some reporting modulatory effects while others show limited or no significant changes (14). FO may alleviate the elevation of splenic expression of *IL-6* and *TNF-α* in broilers induced by LPS through down-regulating nuclear factor kappa B (NFκB) expression (120, 221). In contrast, Wils-Plotz and Klasing (14) reported that FO supplementation failed to modulate the splenic expression of cytokines after LPS challenge. These discrepancies across studies may be attributed to differences in tissue type analyzed, LPS dosage, and duration of dietary intervention. Additionally, our study assessed only splenic gene expression, which may not fully capture the systemic effects of dietary FO. Therefore, the relationships of oxylipins to other factors, such as leukocyte function and circulating cytokine levels, should be considered in future studies (26).

The observed changes in fatty acid profiles in yolk, liver and plasma demonstrate the metabolic incorporation and preferential deposition of EPA and DHA from dietary FO. These findings are consistent with previous poultry studies that reported the incorporation of dietary omega-3 PUFA into egg yolks and tissues, contributing to a favorable reduction in the omega-6 to omega-3 PUFA ratio (65, 74, 222). These modifications have implications for improving the nutritional quality of poultry products, particularly eggs, as a source of omega-3 PUFA for human consumption (65, 223). The similar production parameters between the control and FO groups demonstrate that these benefits are feasible without compromising performance.

5.6 Conclusion

In conclusion, dietary FO in laying hens challenged with LPS effectively increased omega-3 PUFA and decreased the omega-6 PUFA in egg yolk, hen liver and plasma, and increased plasma EPA and DHA oxylipins while reducing omega-6 oxylipins. Increased omega-3 and decreased omega-6 oxylipins are associated with anti-inflammatory effects, highlighting opportunities to enhance the dietary omega-3 PUFA profile in eggs while maintaining laying hen performance and potentially protecting bird health and welfare in the face of infection. At the same time, several changes in oxylipins such as an increase in ALA oxylipins in LPS challenged hens provided control diets, or an increase in putative sEH activity in LPS challenged hens provided dietary FO, indicates that the relationship of oxylipins to inflammation requires further investigation.

BRIDGE TO CHAPTER 6

During the rearing phase, pullets are routinely exposed to multiple stressors such as vaccination, handling, environmental heat, and dietary transitions. These stressors, whether occurring independently or concurrently, can activate physiological stress responses, compromise immune function, and adversely affect growth performance and overall welfare. Given the known immunomodulatory and anti-inflammatory properties of omega-3 polyunsaturated fatty acids (PUFA) and their bioactive metabolites (e.g., oxylipins), the following chapter investigates the effects of dietary omega-3 PUFA supplementation on immune resilience in pullets. This approach aims to improve health outcomes and support optimal development during this critical period.

CHAPTER 6 MANUSCRIPT III

THE EFFECT OF INCREASING LEVELS OF OMEGA-3 PUFA FROM EITHER FLAXSEED OIL OR PREFORMED DOCOSAHEXAENOIC ACID (DHA) ON PERFORMANCE, FATTY ACID COMPOSITION, PLASMA OXYLIPINS AND IMMUNE MEDIATORS IN RESPONSE TO LIPOPOLYSACCHARIDE (LPS) CHALLENGE IN PULLET

6.1 Abstract

Background: Current guidelines for omega-3 PUFA supplementation in pullets are limited. Given pullets' susceptibility to infections, dietary omega-3 PUFA may provide an opportunity to enhance immune defenses. LPS are potent stimulators of inflammation and have been commonly used to model bacterial infection in poultry. However, the effects of different types and levels of omega-3 PUFA on immune responses during a lipopolysaccharide (LPS) challenge remain unclear.

Objectives: The study aimed to determine the effect of increasing levels of omega-3 PUFA from either alpha-linolenic acid (ALA)-rich flaxseed oil (FSO; ALA diet) or docosahexaenoic acid (DHA)-rich DHA algal biomass (DHA diet) on the alteration of FA composition in biological tissues, plasma oxylipins and expression of cytokines of pullets challenged with LPS compared to the control diet.

Materials and Methods: A total of 315, one-day-old Dekalb White pullets were raised in a cage-rearing system (98 cm deep × 68 cm wide × 37 cm high) and were randomly assigned to one of seven treatment diets, including a control, and 0.2%, 0.4%, or 0.6% of total dietary omega-3 PUFA, as either ALA-rich FSO or DHA-rich algal biomass. Each cage was considered an experimental unit with five pullets and nine cages per treatment. At termination (16th week), 2 birds from the same cage (7 cages were randomly selected per treatment) were challenged with either *Escherichia coli*-derived LPS (8 mg/kg; i.v. injection; n = 7) or saline (n = 7). Liver, spleen, and plasma were collected two hours post-challenge for subsequent analyses.

Results: Increasing dietary omega-3 supplementation led to predictable responses in fatty acid (FA) concentrations of plasma and liver. The levels of plasma ALA- and DHA-derived oxylipins and their total amounts were significantly elevated in pullets fed diets enriched with their respective precursor PUFA, regardless of whether they received saline or LPS treatment. EPA-derived oxylipins and their totals also increased in response to both precursor PUFA-enriched diets, with the diet containing DHA-rich algal biomass diet showing a greater effect than the ALA-rich FSO diet. The LPS challenge significantly increased individual and total LA oxylipin levels, while the DHA diet reduced some individual LA oxylipins and their totals. Furthermore, pullets fed the diet containing 0.6% DHA showed significantly lower *interleukin* (*IL)-8* expression, while those on the 0.4% DHA diet exhibited significantly lower *IL-10* expression compared to the other treatments.

Conclusions: The results indicated that dietary intake of ALA and DHA uniquely affected FA deposition and oxylipin synthesis under the administration of LPS in pullets, without impacting their performance.

Key words: FSO, ALA, DHA, oxylipins, LPS, pullets

6.2 Introduction

Dietary interventions using omega-3 PUFA inclusion have been documented to result in the enrichment of animal-derived products, particularly eggs, with health benefits for human consumption (65, 178, 224, 225). This approach involves formulating diets for animals that are rich in omega-3 PUFA. Current strategies include providing diets rich in alpha-linolenic acid (ALA), which derived from plant sources and their oils, such as flaxseed and canola (12, 59, 224, 226). Studies have demonstrated that adding flaxseed to hens' feed increases the yolk ALA levels proportionally to the percentage of flaxseed inclusion. Our previous study showed that feeding ALA-rich hemp products to laying hens increased the content of DHA in egg yolk (approx. 55 mg/egg), which however eventually plateaued with further increases in dietary ALA levels (59). In contrast, the inclusion of marine or algal sources in the hens' diet has been shown to substantially increase long chain (LC)-PUFA in a linear fashion, including both DHA and EPA, with DHA levels predominating (178). However, scientific evidence suggests that the conversion of ALA to EPA and particularly DHA varies significantly depending on the source of FA. Furthermore, the conversion to DHA is often limited due to the distinct desaturation and elongation pathways involved (12). Therefore, directly supplying EPA and DHA through the diet may be a more effective strategy for achieving sufficient levels of LC-PUFA.

In addition to FA deposition in yolks and biological tissues, PUFA can also be oxidized into a large family of bioactive lipid mediators known as oxylipins (22). These lipids mediators are generated through enzymatic processes, including those involving cyclooxygenases (COX), lipoxygenases (LOX), and cytochrome P450 (CYP), as well as non-enzymatic processes (227-229). Typically, these three enzymes act on intracellular PUFA after phospholipase A_2 (PLA_2) releases them from the membrane phospholipid. Therefore, enriching certain PUFA in cellular membranes (for example, through increased dietary intake) is expected to enhance the production of oxylipins derived from those PUFA (26).

Oxylipins mediate a variety of physiological processes, including inflammation (112, 230). During an inflammatory response, cytokines are released to regulate the immune reaction, and oxylipins can function as either pro- or anti-inflammatory mediators, depending on their PUFA origin and the enzymatic pathway involved in their synthesis (231) (24, 232, 233). Pro-inflammatory oxylipins, primarily derived from omega-6 PUFA, stimulate the expression of cytokines such as IL-1β, IL-6, and TNF-α, while anti-inflammatory oxylipins derived from omega-3 PUFA reduce the expression of these cytokines (234, 235). This distinction is particularly important in young, developing pullets, which are susceptible to stress factors such as vaccination, environmental handling, temperature shifts, and dietary transitions (236). These stressors can negatively affect growth performance, development, and health in young laying pullets, potentially negatively impacting their overall lifetime egg production (EP) (237). The rapid development of the digestive, immune, and integumentary systems during the early life stages makes this a critical opportunity for intervention.

Given the anti-inflammatory effects of omega-3 PUFA and their metabolites, dietary supplementation with omega-3 PUFA is expected to enhance the immune defense mechanisms in pullets subjected to an immune challenge. In this study, we used LPS, a component of the cell wall of gram-negative bacteria (120), as an experimental bacterial infection model to induce the immune response in pullets. Therefore, the objectives of this study were to determine the effects of including either FSO as a source of ALA, or algal biomass rich in DHA as a source of preformed DHA in the diets of pullets on 1) the growth performance during the starter, grower, and finisher phases; 2) the FA compositions and oxylipin profiles of tissues subjected to an LPS challenge; and 3) the expression of pro- and inflammatory cytokines and their receptors in pullets exposed to an LPS challenge.

6.3 Material and methods

All research methods and procedures, including details of animal utilization and welfare were reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee. Additionally, all the pullets were fed and managed strictly followed the recommendations established by Canadian Council on Animal Care (198).

6.3.1 Chickens and experimental design

A total of 315, day-old Dekalb White pullets were obtained from local hatchery (Steinbach Hatchery & Feed Ltd., Steinbach, MB, Canada). All pullets were raised and divided into groups of 5 birds/cage (98 cm deep × 68 cm wide × 37 cm high) in a cage-rearing system at the Small Animal Research Facility of the Department of Animal Science, University of Manitoba. Each cage was considered as an experimental replicate with 9 replicates (45 pullets) per treatment. Pullets were randomly assigned to 1 of 7 treatment diets (10 hens/treatment), including a control, and 0.2%, 0.4%, or 0.6% of total dietary omega-3 PUFA, as either ALA-rich FSO obtained from Dyets, Inc. (Bethlehem, PA, USA) or DHA-rich algal biomass, a source of a dried whole-cell algae product (DHAgold^{TM} S17-B, DSM Nutritional Products, North America, Parsippany, NJ, USA) for starter (week 0-8), grower (week 9-12) and finisher (week 13-16) stages. All diets were formulated to meet or exceed the nutrient requirement based on the Dekalb White commercial product guide (North American Version). The ingredients and nutrient composition of the experimental diets are presented in **Table 6.1** and the analyzed FA profile in experimental diets is provided in **Table 6.2**.

Table 6.1. Ingredients and nutrient composition of experimental diets for pullets including Starter (0-4 weeks), Grower (9-12 weeks) and Finisher (13-16 weeks) containing increasing levels of total omega-3 PUFA[1].

Total omega-3 fatty acids (% of diet)[2]	Starter (week 0-8)							Grower (week 9-12)							Finisher (week 13-16)						
	Control diet	ALA diet			Algal DHA diets			Control diet	ALA diet			Algal DHA diets			Control diet	ALA diet			Algal DHA diets		
		0.20	0.40	0.60	0.20	0.40	0.60		0.20	0.40	0.60	0.20	0.40	0.60		0.20	0.40	0.60	0.20	0.40	0.60
Ingredients (%)																					
Corn	53.37	53.36	53.36	53.11	52.61	53.02	53.10	52.11	52.10	52.10	51.25	51.40	51.82	49.85	52.12	52.12	52.11	50.11	51.36	51.77	46.61
Soybean Meal	26.68	26.68	26.68	26.64	26.62	26.77	26.78	18.33	18.32	18.32	18.13	18.20	18.35	18.40	14.18	14.17	14.17	13.87	14.11	14.28	14.10
Wheat	0.000	0.000	0.000	0.000	0.000	0.000	0.000	22.98	22.99	23.00	23.87	22.99	21.61	20.27	26.89	26.90	26.91	28.00	26.90	25.52	24.24
Wheat Middlings	13.487	13.494	13.504	13.745	13.474	12.099	11.124	0.000	0.000	0.000	0.000	0.000	0.000	2.424	0.000	0.000	0.000	0.983	0.000	0.000	5.741
Corn Oil	0.995	0.700	0.327	0.000	0.885	0.484	0.151	0.869	0.577	0.205	0.000	0.762	0.361	0.000	0.811	0.521	0.149	0.000	0.707	0.306	0.000
VM Premix	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500	1.500
Salt	0.390	0.390	0.390	0.390	0.391	0.391	0.391	0.390	0.390	0.390	0.390	0.390	0.391	0.389	0.391	0.391	0.391	0.390	0.391	0.392	0.388
Limestone	1.786	1.786	1.786	1.787	1.785	1.779	1.776	1.893	1.893	1.893	1.896	1.892	1.887	1.885	2.225	2.225	2.225	2.230	2.223	2.218	2.220
Biophos[2]	1.379	1.379	1.379	1.378	1.383	1.391	1.401	1.444	1.444	1.443	1.441	1.448	1.457	1.462	1.481	1.481	1.481	1.477	1.485	1.494	1.494
lysine	0.171	0.171	0.171	0.171	0.171	0.171	0.194	0.234	0.235	0.235	0.236	0.236	0.237	0.236	0.219	0.219	0.219	0.220	0.219	0.219	0.219
DL-Methionine	0.218	0.218	0.218	0.218	0.218	0.217	0.222	0.172	0.172	0.172	0.173	0.172	0.170	0.168	0.141	0.141	0.141	0.142	0.141	0.139	0.137
Threonine	0.022	0.022	0.022	0.023	0.022	0.019	0.028	0.087	0.087	0.087	0.089	0.087	0.084	0.081	0.045	0.045	0.045	0.047	0.044	0.041	0.039
Flax oil[3]	0.000	0.295	0.668	1.039	0.000	0.000	0.000	0.000	0.292	0.664	1.034	0.000	0.000	0.000	0.000	0.290	0.662	1.031	0.000	0.000	0.000
DHAGold[4]	0.000	0.000	0.000	0.000	0.941	2.142	3.339	0.000	0.000	0.000	0.000	0.929	2.130	3.329	0.000	0.000	0.000	0.000	0.923	2.124	3.322
Calculated nutrients																					
AMEn (Poultry; Kcal/kg)	2850	2850	2850	2850	2850	2850	2850	2750	2750	2750	2750	2750	2750	2750	2700	2700	2700	2700	2700	2700	2700

Crude Fat (%)	3.700	3.700	3.700	3.744	3.900	3.900	3.966	3.700	3.700	3.700	3.862	3.900	3.900	3.900	3.700	3.700	3.700	3.900	3.900	3.900	3.900
Crude Protein (%)	20.50	20.50	20.50	20.50	20.50	20.50	20.50	18.30	18.30	18.30	18.29	18.28	18.28	18.37	17.00	17.00	17.00	17.02	17.00	17.00	17.20
Calcium (%)	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.100	1.200	1.200	1.200	1.200	1.200	1.200	1.200
Total Phosphorus (%)	0.737	0.737	0.737	0.738	0.745	0.747	0.752	0.791	0.791	0.791	0.795	0.798	0.801	0.803	0.812	0.812	0.812	0.818	0.820	0.823	0.824
Available Phosphorus (%)	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450	0.450
Calculated fatty acids[5]																					
Linoleic Acid (%)	1.836	1.713	1.558	1.430	1.760	1.510	1.300	1.870	1.749	1.594	1.537	1.797	1.547	1.300	1.885	1.765	1.610	1.578	1.813	1.563	1.321
Linolenic Acid (%)	0.041	0.199	0.399	0.599	0.040	0.035	0.032	0.043	0.199	0.399	0.599	0.042	0.037	0.034	0.044	0.199	0.399	0.599	0.043	0.038	0.035
DHA (%)	0.000	0.000	0.000	0.000	0.160	0.364	0.568	0.000	0.000	0.000	0.000	0.158	0.362	0.566	0.000	0.000	0.000	0.000	0.157	0.361	0.565
Omega-3 (%)	0.041	0.200	0.400	0.600	0.200	0.400	0.600	0.043	0.200	0.400	0.600	0.200	0.400	0.600	0.044	0.200	0.400	0.600	0.200	0.400	0.600
Analyzed nutrients																					
AMEn (Poultry; Kcal/kg)	3156	3142	3120	3099	3133	3160	3143	2984	2992	2984	3004	3001	2999	2999	3044	3041	3050	3067	3054	3072	3011
Crude Fat (%)	4.280	4.310	3.520	3.720	4.070	4.260	4.430	3.330	3.540	3.550	3.790	4.040	3.930	3.70	4.000	4.080	4.170	4.180	4.150	4.740	4.000
Crude Protein (%)	20.65	21.19	20.31	20.43	20.39	20.87	20.15	18.21	18.55	17.70	18.06	17.43	17.80	17.57	16.97	16.45	16.83	16.72	16.90	16.89	15.71
Calcium (%)	0.840	1.220	0.870	1.150	0.950	1.050	1.110	1.130	1.130	1.270	1.240	1.230	1.200	1.24	1.360	1.240	1.350	1.380	1.350	1.450	1.610
Phosphorus (%)	0.660	0.730	0.700	0.750	0.720	0.730	0.760	0.760	0.780	0.770	0.780	0.760	0.790	0.78	0.770	0.790	0.780	0.820	0.800	0.800	0.740

[1] Provided per kilogram of diet, vitamin-mineral premix contained: 12,000 IU of vitamin A; 2,500 IU of vitamin D3, 30 IU of vitamin E, 3 mg of vitamin K3 (as menadione), 0.03 mg of vitamin B12, 0.2 mg of biotin, 7 mg of riboflavin, 1 mg of folic acid, 12 mg of calcium pantothenate, 40 mg of niacin, 2.5 mg of thiamine, 5 mg of pyridoxine, 1,000 mg of choline chloride, 125 mg antioxidant (ethoxyquin), 85 mg of manganese, 80 mg of zinc, 80 mg of iron, 10 mg of copper, 0.3 mg of selenium, 1 mg of iodine.

[2] Biophos, a feed grade monocalcium phosphate, contained 21% P and 18% Ca (Landmark Feeds, Winnipeg, Manitoba, Canada).

[3] Flaxseed oil, obtained from Dyets, Inc. (Bethlehem, PA, USA). Specification provided (%): Fat, LA and ALA are 100, 16.0 and 57.0, respectively.

[4] DHAgold™ S17-B dried whole-cell algae product supplied by DSM Nutritional Products (North America, Parsippany, NJ, USA). Specification provided (%): Fat and DHA are 40 and 17, respectively.

[5] LA, Linoleic acid; ALA, α-linolenic acid; DHA, Docosahexaenoic acid.

Table 6.2. Analyzed FA composition of experimental diets for pullets including Starter (0-4 weeks), Grower (9-12 weeks) and Finisher (13-16 weeks) containing increasing levels of total omega-3 PUFA[1].

Total omega-3 fatty acids (% of diet)[1]	Starter (week 0-8)							Grower (week 9-12)							Finisher (week 13-16)						
	Control diet	FSO diet			Algal DHA diets			Control diet	FSO diet			Algal DHA diets			Control diet	FSO diet			Algal DHA diets		
		0.20	0.40	0.60	0.20	0.40	0.60		0.20	0.40	0.60	0.20	0.40	0.60		0.20	0.40	0.60	0.20	0.40	0.60
SFA	0.580	0.559	0.549	0.550	0.724	0.881	1.001	0.595	0.597	0.557	0.543	0.726	0.837	1.033	0.597	0.589	0.589	0.605	0.722	0.830	0.983
MUFA	0.933	0.896	0.870	0.865	0.904	0.801	0.689	0.925	0.929	0.847	0.824	0.895	0.754	0.692	0.948	0.933	0.912	0.938	0.901	0.769	0.656
LA (18:2n-6)	2.124	1.980	1.838	1.774	2.061	1.837	1.572	2.126	2.161	1.803	1.709	2.053	1.738	1.589	2.053	1.953	1.825	1.827	2.038	1.774	1.544
GLA (18:3n-6)	0.000	0.000	0.000	0.000	0.001	0.002	0.003	0.000	0.000	0.000	0.000	0.001	0.002	0.003	0.000	0.000	0.000	0.000	0.001	0.002	0.003
ARA (20:4n-6)	0.000	0.000	0.001	0.001	0.002	0.005	0.007	0.000	0.000	0.001	0.001	0.002	0.004	0.007	0.000	0.000	0.000	0.000	0.002	0.004	0.006
ALA (18:3n-3)	0.096	0.230	0.446	0.648	0.100	0.097	0.088	0.099	0.102	0.414	0.573	0.099	0.091	0.088	0.100	0.237	0.411	0.611	0.100	0.091	0.086
EPA (20:5n-3)	0.000	0.000	0.000	0.000	0.005	0.011	0.016	0.000	0.000	0.000	0.000	0.005	0.010	0.018	0.001	0.001	0.001	0.001	0.005	0.010	0.017
DPA (22:5n-3)	0.001	0.000	0.000	0.000	0.001	0.003	0.004	0.000	0.000	0.000	0.000	0.001	0.002	0.005	0.000	0.000	0.000	0.000	0.001	0.003	0.004
DHA (22:6n-3)	0.002	0.002	0.001	0.001	0.198	0.463	0.687	0.001	0.000	0.000	0.000	0.186	0.409	0.715	0.001	0.000	0.000	0.001	0.174	0.390	0.676
Omega-3 PUFA	0.099	0.232	0.448	0.650	0.304	0.574	0.795	0.101	0.103	0.415	0.575	0.292	0.512	0.825	0.102	0.238	0.412	0.613	0.279	0.493	0.783

[1] ARA, arachidonic acid; ALA, α-linolenic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; TMUFA, total monounsaturated fatty acids (sum of palmitoleic acid and oleic acid); TSFA, total saturated fatty acids (sum of myristic acid, palmitic acid and stearic acid).

6.3.2 Sample collection and measurement

The growth performance measurements, i.e., FI and BW were recorded weekly throughout the whole experiment. At termination (week 16), two birds from the same cage of seven cages in each treatment were randomly challenged with either *Escherichia coli* (*E. coli*)-derived LPS (8 mg/kg; i.v. injection; $n = 7$) or saline ($n = 7$). All birds were euthanized by CO_2 asphyxiation 2-hour post-injection, based on the protocol established in a pilot study from our lab (Jing et al., 2024). Tissue samples including liver, plasma and spleen were collected and stored at -80℃ for subsequent analyses.

6.3.3 Fatty acid extraction and analysis

Total lipids from all samples were extracted as described (200). The final methylated FA of the samples were extracted into iso-octane and determined using a Varian 450 GC with a flame ionization detector (FID) (GC-FID; Agilent Technologies Canada Inc., ON), equipped with a DB225MS column (30 m × 0.25 mm diameter and 0.25 mm film thickness; Agilent Technologies Canada Inc., Mississauga, Ontario). The equipment settings and the calculations for determining the FA contents have been previously described (31).

6.3.4 Oxylipin extraction and analysis

One mL of water (pH 3), 10 µL of antioxidant solution and 100 µL of deuterated internal standard (Cayman Chemical, Ann Arbor, MI) were added to 200 µL of plasma sample and then adjusted to pH < 3 for oxylipin extraction. Samples were applied to Strata-X solid phase extraction columns (33u, 60 mg/3mL; Phenomenex, CA) before elution of free oxylipins with 10% methanol. All samples were dried under nitrogen and reconstituted in 100 µL of a mobile phase of water-acetonitrile-formic acid (70:30:0.02, v/v/v, LC-MS grade). Oxylipins were determined using high-performance liquid chromatography-mass spectrometry (HPLC-MS/MS; QTRAP 6500; Sciex, ON, Canada) as described (PMID 28826940 and 28667077) and quantified using the stable isotope dilution method (238).

6.3.5 Total RNA extraction and reverse transcription

Total RNA was isolated from spleen samples (10 ± 0.5 g) using the RNeasy Mini kit (Qiagen Canada Inc., Mississauga, ON, Canada), with genomic DNA contamination eliminated with DNase set kit (Qiagen Canada Inc., Mississauga, ON, Canada) according to the manufacturer's protocol. Subsequently, 1 µg of RNA in a 20 µL reaction was performed for cDNA synthesis using the Superscript® VILO™ cDNA Synthesis Kit (Invitrogen Canada Inc., Burlington, ON, Canada). The cDNA samples were stored at –20°C for further quantitative real-time PCR analysis.

6.3.6 qRT-PCR analysis

The relative mRNA expression levels of studied genes including *IL-1β*, *IL-2*, *IL-6*, *IL-8*, *IL-10*, *IFN-γ*, *TNF-α*, and *TLR4* were conducted using the OneStep Real-Time Detection System (Applied Biosystems, Canada). The levels of target mRNA were normalized to the expression of the housekeeping gene, β-actin, and relative mRNA expression was determined using the $2^{-\Delta\Delta CT}$

method (157). The amplification conditions were followed according to previously described (31). PCR primers for studied genes are summarized in **Table 6.3** and their specificity was confirmed using NCBI BLASTN (199).

Table 6.3. Primer sequences for quantitative real-time PCR.

Genes[1]	**Primer sequences (5' to 3')**[2]	**Amplicon length (bp)**	**GenBank access No.**
IL-1β	F: TACACCCGCTCACAGTCCTT R: CAATGTTGAGCCTCACTTTCTG	130	NM_204524
IL-2	F: ACACCAACTGAGACCCAGGAGT R: GACCCGTAAGACTCTTGAGGTTC	160	NM_204153
IL-6	F: GCAGGACGAGATGTGCAAGA R: GGTAGGTCTGAAAGGCGAACA	176	NM_204628
IL-8	F: GATTGAACTCCGATGCCAGTG R: TGTCCAGAATTGCCTTGATGAT	197	NM_205018
IL-10	F: CAAGCAGATCAAGGAGACGTTC R: TCAGCAGGTACTCCTCGATGTAGT	104	NM_001004414
TNF-α	F: GTCTGCTCCTAGTGGCTTTCCT R: CTACGGGTTGCTGCACATACA	212	AY765397
IFN-γ	F: GCTGACGGTGGACCTATTATTGT R: ACGCCATCAGGAAGGTTGTT	188	NM_205149
TLR4	F: CCAAACACCACCCTGGACTTG R: TGTATGGATGTGGCACCTTGAA	120	NM_001030693
β-Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	NM_205518

[1] IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin10; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4.

[2] F = forward; R = reverse.

6.3.7 Statistical analysis

The experiment was a completely randomized design with dietary treatment and experimental unit (cages with 5 birds per cage; 9 cages per treatment) regarded as the fixed and the random effects, respectively. All data were analyzed by the MIXED procedure of SAS (version 9.4; SAS Inst. Inc., Cary, NC, USA). For the repeated-measures analysis of performance data, the model is: $y_{ijk} = \mu + d_i + a_{ij} + w_k + d_{wik} + e_{ijk}$, where y_{ijk} is an observation on a performance measure, including BW, FI, and EW; μ represents the population mean, d_i is the effect of the i'th diet (i = 1, 2, … 7), a_{ij} represents the effect of the j'th hen in the i'th dietary treatment, w_k is the effect of the k'th week (k = 1, 2, … 16), d_{wik} is the interaction of diet and week, and e_{ijk} is the residual error. Treatment, week, and their interaction are considered fixed effects, and hen and the residual error are considered random effects. For single-measure outcomes on hens (plasma biochemistry, FA, oxylipins and mRNA expressions) we used a two-factor factorial model (dietary treatment and LPS administration): $y_{ijk} = \mu + d_i + c_j + dc_{ij} + e_{ijk}$, where y_{ijk} is a single-measure observation on a hen, μ is the population mean, d_i is the effect of the i'th diet (i = 1, 2, … 7), c_j is the effect of the j'th challenge level (either LPS or saline injection; j = 1, 2), dc_{ij} is the interaction of the two factors, and e_{ijk} is the residual error due to the k'th hen in the ij'th combination of diet and challenge. The Shapiro-Wilk test was used for assessing normal distribution. All data were presented as least squares means (LSM) ± SE, adjusted using Tukey's *post hoc* test to compare for significant differences ($P < 0.05$).

6.4 Results

6.4.1 Growth performance

Overall, the growth performance of pullets was satisfactory, with no negative effects of the experimental diets on BW or FI. This was reflected in a significant main effect of age (week) on FI ($P < 0.0001$). Consequently, a significant diet × week interaction was observed on BW ($P < 0.05$; **Table 6.4**).

Table 6.4. Performance of pullets (from 20 to 28 weeks of age) consuming diets containing increasing levels of total omega-3 derived from either ALA diets (from FSO) or algal DHA diets[1].

	Control	ALA diet			Algal DHA diet			P values		
		0.2	0.4	0.6	0.2	0.4	0.6	Diet	Week	Diet*Week
BW								0.0820	<0.0001	0.0039
Week 1	69.54	70.92	71.25	69.59	72.03	70.27	71.29			
Week 2	118.9	121.3	123.8	120.8	124.2	126.8	124.8			
Week 3	192.0	190.4	193.5	186.9	197.2	198.4	198.3			
Week 4	287.1	281.6	285.1	278.1	291.6	295.4	295.7			
Week 5	385.6	378.4	381.5	373.4	391.6	392.6	395.8			
Week 6	490.0	480.3	480.0	473.0	493.9	495.1	497.7			
Week 7	600.4	587.9	587.6	579.9	603.4	601.2	605.1			
Week 8	689.7	691.8	677.7	670.6	692.4	687.4	699.3			
Week 9	786.4	778.3	774.6	757.3	783.6	779.8	788.5			
Week 10	871.3	864.6	869.2	841.4	868.7	857.3	872.0			
Week 11	951.5	947.7	928.6	920.6	943.7	939.0	948.2			
Week 12	1019.9	1016.1	1006.8	988.8	1016.7	1010.6	1028.6			
Week 13	1093.6	1090.9	1080.1	1056.7	1086.4	1074.0	1100.0			
Week 14	1158.7	1156.5	1147.8	1118.9	1143.9	1130.3	1162.2			
Week 15	1214.8	1215.4	1201.6	1169.0	1208.1	1186.7	1222.9			
Week 16	1275.3	1276.8	1262.9	1243.8	1267.6	1228.6	1279.7			
SEM	8.090	8.168	8.090	8.491	8.092	8.657	8.136			
FI								0.1249	<0.0001	0.0514
Week 1	11.80	12.70	12.17	13.12	12.78	12.25	12.11			
Week 2	19.18	19.08	19.87	19.18	20.31	20.04	19.79			
Week 3	20.32	21.20	20.49	19.82	20.40	19.58	20.21			
Week 4	31.59	32.21	31.96	30.26	32.17	32.42	31.36			
Week 5	40.64	40.08	39.06	38.66	39.95	40.09	39.56			

Week 6	46.01	45.24	42.73	45.11	45.69	44.67	42.64
Week 7	48.55	49.34	49.14	18.59	49.53	48.87	48.94
Week 8	54.84	53.29	52.02	52.45	53.30	52.07	51.97
Week 9	60.68	59.99	58.95	57.40	59.60	58.02	58.67
Week 10	62.93	61.92	62.01	60.58	60.01	57.94	60.61
Week 11	65.13	62.54	63.13	59.90	60.61	60.21	60.08
Week 12	68.05	67.05	68.11	67.91	66.19	62.72	64.93
Week 13	75.33	74.33	73.65	71.68	72.40	69.82	73.64
Week 14	77.53	73.09	74.79	72.26	74.23	70.53	70.65
Week 15	77.62	77.36	75.94	73.41	75.47	72.70	71.94
Week 16	77.55	81.13	74.27	76.48	75.82	71.32	71.28
SEM	1.236	1.278	1.248	1.281	1.253	1.248	1.252

[1] Data are presented as least squares means and their SEM (*n* = 9). FI: feed intake; BW: body weight.

6.4.2 Omega-3 PUFA composition in liver and plasma

Increasing dietary omega-3 supplementation yielded similar responses in total and individual omega-3 PUFA in both liver and plasma. Compared with the control group, the level of ALA was increased in the liver and plasma from pullets fed diets containing increasing levels of ALA-rich FSO (*P* < 0.0001; **Figure 6.1 A-B**), but not those fed DHA--rich algal biomass. The levels of liver and plasma EPA and DPA were increased in both diets fed pullets (*P* < 0.0001; **Figure 6.1 C-D**; **Table 6.5, 6.6**). DHA levels were also increased in both diets fed pullets, with the DHA diet contributing more than the ALA diet (*P* < 0.0001) in both the liver and plasma (**Figure 6.1 E-F**). Furthermore, total omega-3 PUFA levels in both liver and plasma were significantly increased (*P* < 0.0001; **Figure 6.1 G-H**) in response to diets enriched with either ALA-rich FSO or DHA-rich algal biomass, compared to the control group. However, the DHA diet resulted in greater omega-3 PUFA accumulation in the liver than the ALA diet, whereas no such difference was observed in plasma.

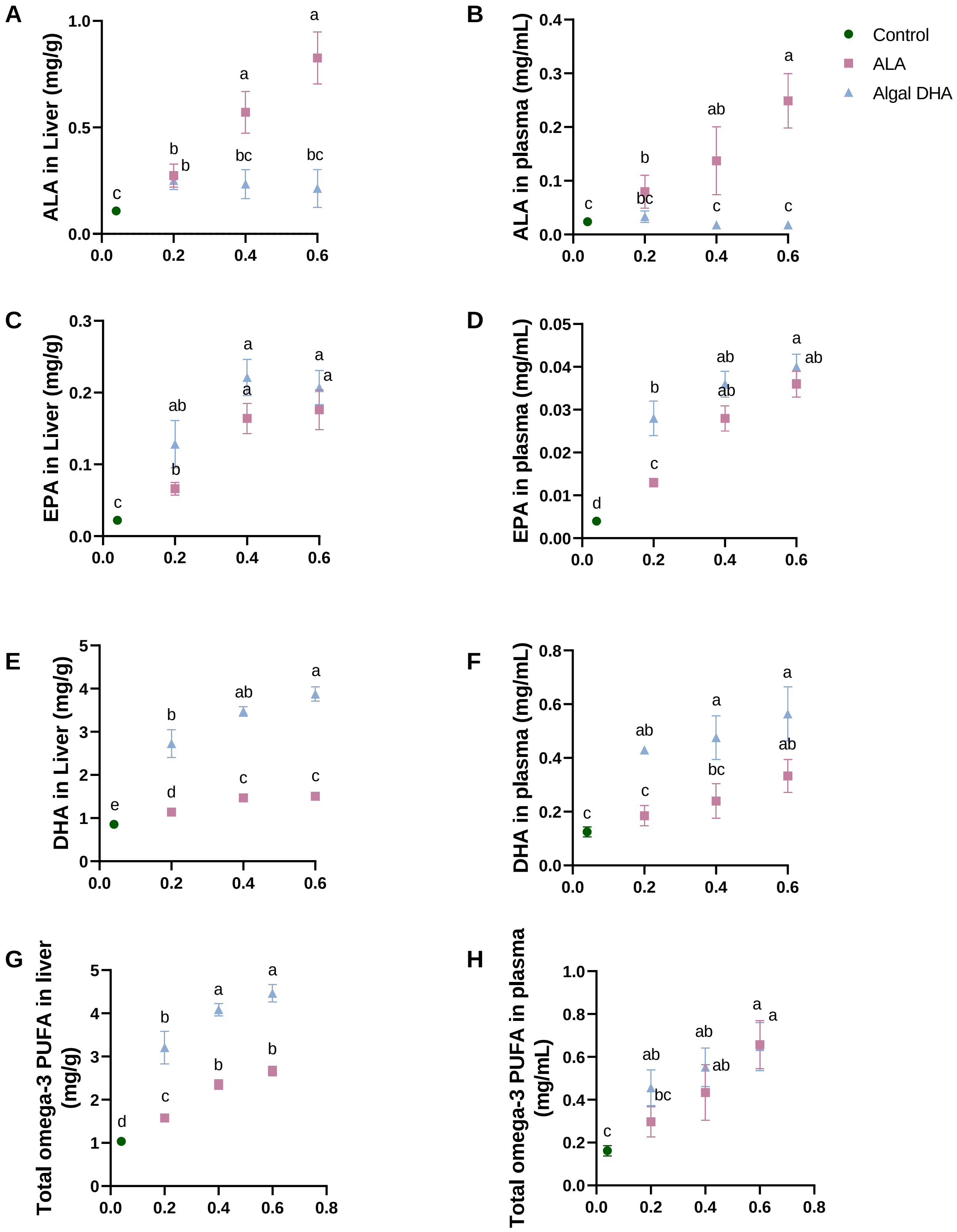

A
ALA in Liver (mg/g)
1.0
0.5
0.0
0.0
0.2
0.4
0.6
c
b
b
a
bc
a
bc
B
ALA in plasma (mg/mL)
0.4
0.3
0.2
0.1
0.0
0.0
0.2
0.4
0.6
c
b
bc
ab
c
a
c
Control
ALA
Algal DHA
C
EPA in Liver (mg/g)
0.3
0.2
0.1
0.0
0.0
0.2
0.4
0.6
c
ab
b
a
a
a
a
D
EPA in plasma (mg/mL)
0.05
0.04
0.03
0.02
0.01
0.00
0.0
0.2
0.4
0.6
d
b
c
ab
ab
a
ab
E
DHA in Liver (mg/g)
5
4
3
2
1
0
0.0
0.2
0.4
0.6
e
b
d
ab
c
a
c
F
DHA in plasma (mg/mL)
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
c
ab
c
a
bc
a
ab
G
Total omega-3 PUFA in liver (mg/g)
5
4
3
2
1
0
0.0
0.2
0.4
0.6
0.8
d
b
c
a
b
a
b
H
Total omega-3 PUFA in plasma (mg/mL)
1.0
0.8
0.6
0.4
0.2
0.0
0.0
0.2
0.4
0.6
0.8
c
ab
bc
ab
ab
a
a

Figure 6.1. Individual and total omega-3 PUFA in liver (mg/g) and plasma (mg/mL) of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from either ALA or DHA following the LPS challenge.

6.4.3 Omega-6 PUFA composition and ratios in liver and plasma

In the liver, supplementation with either FSO rich in ALA or algal biomass rich in DHA- had minimal effect on omega-6 PUFA, except ARA (**Table 6.2A)**. The levels of ARA and the ratio of omega-6 to omega-3 PUFA were decreased in the liver of pullets that received both diets compared to the control group, with the DHA diet having a more pronounced effect ($P<0.0001$). In plasma, ARA level decreased in pullets fed the DHA diet compared to those fed the control diet, but not in the group fed the ALA diet ($P<0.0001$; **Table 6.2B**). The omega-6 to omega-3 PUFA ratios were also decreased in the plasma from pullets that were fed both diets compared to the control group, again with the DHA diet showing the greater effect ($P<0.0001$). Additionally, an injection effect was observed in plasma ARA levels, with LPS resulting in higher levels ($P<0.05$; **Table 6.6**). No interaction effects of diet and injection were observed on omega-6 PUFA in either the liver or plasma.

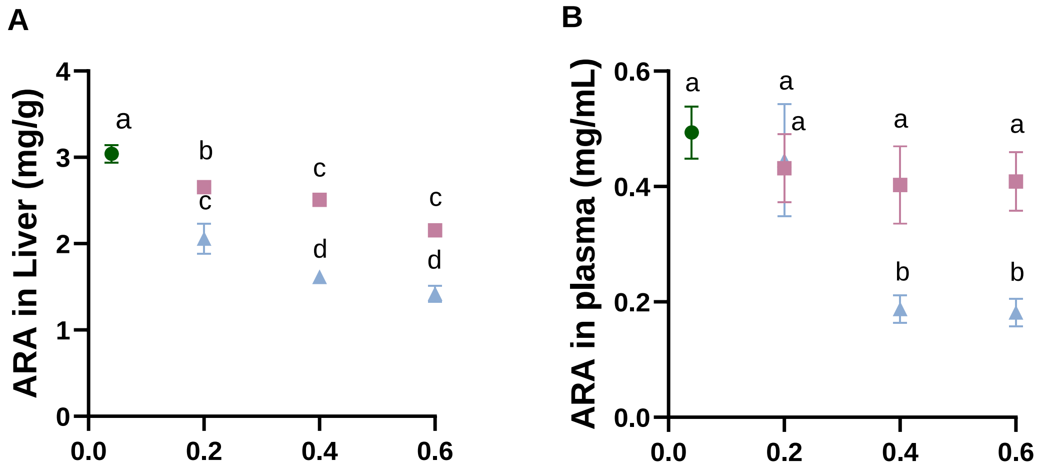


Figure 6.2. ARA in liver (mg/g) and plasma (mg/mL) of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from either ALA or DHA following the LPS challenge.

Table 6.5. Liver fatty acid composition (mg/g) of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from either ALA or DHA following the LPS challenge[1].

	Diet effect							Injection effect		P values		
	Control	0.2%ALA	0.4%ALA	0.6%ALA	0.2%DHA	0.4%DHA	0.6%DHA	LPS	Saline	Diet	Injection	Diet* Injection
SFA, mg/g												
14:0	0.138±0.03^{b}	0.160±0.04^{b}	0.177±0.04^{ab}	0.341±0.06^{ab}	0.341±0.06^{ab}	0.378±0.08^{a}	0.417±0.10^{a}	0.245±0.03	0.265±0.03	0.0026	0.3500	0.6067
16:0	12.12±2.177	13.90±3.433	15.03±3.327	14.93±2.619	23.67±4.008	22.38±4.248	23.00±4.884	16.84±1.941	18.65±1.941	0.0488	0.2935	0.4084
18:0	7.554±0758	7.719±0.932	8.647±1.021	8.025±0.807	11.02±1.065	11.36±1.363	11.39±1.509	9.13±0.602	9.64±0.602	0.0219	0.4269	0.3668
MUFA, mg/g												
16:1	1.036±0.257	1.552±0.581	1.485±0.421	1.606±0.435	2.100±0.462	1.805±0.426	1.940±0.529	1.489±0.243	1.804±0.243	0.7151	0.2030	0.6484
18:1	16.22±3.705	19.50±5.468	22.41±5.792	22.51±4.832	35.22±7.256	28.76±6.000	30.22±7.616	23.64±3.176	26.31±3.176	0.4178	0.3332	0.6640
Omega-6 PUFA, mg/g												
LA (18:2n-6)	5.917±0.673	6.051±0.715	6.643±0.967	6.676±0.798	11.168±1.651	8.855±1.938	7.138±1.546	7.561±0.682	7.425±0.682	0.3755	0.8571	0.3758
GLA (18:3n-6)	0.036±0.005	0.034±0.004	0.035±0.005	0.035±0.004	0.057±0.010	0.042±0.009	0.038±0.007	0.039±0.004	0.040±0.004	0.9191	0.5827	0.7317
ARA (20:4n-6)	3.043±0.100^{a}	2.654±0.075^{b}	2.508±0.072^{b}	2.154±0.044^{c}	2.058±0.175^{c}	1.615±0.075^{d}	1.422±0.092^{d}	2.232±0.052	2.182±0.052	<.0001	0.4860	0.3341
Omega-3 PUFA, mg/g												
ALA (18:3n-3)	0.108±0.019^{c}	0.274±0.054^{b}	0.571±0.098^{a}	0.826±0.122^{a}	0.250±0.042^{b}	0.234±0.068^{bc}	0.214±0.089^{bc}	0.353±0.039	0.355±0.039	<.0001	0.7731	0.4485
EPA (20:5n-3)	0.022±0.003^{c}	0.066±0.009^{b}	0.164±0.021^{a}	0.176±0.028^{a}	0.128±0.033^{ab}	0.221±0.025^{a}	0.207±0.024^{a}	0.133±0.012	0.148±0.012	<.0001	0.6981	0.5207
DPA (22:5n-3)	0.049±0.004^{b}	0.098±0.011^{a}	0.148±0.015^{a}	0.157±0.019^{a}	0.104±0.024^{ab}	0.156±0.015^{a}	0.172±0.018^{a}	0.130±0.009	0.122±0.009	<.0001	0.4136	0.1819
DHA (22:6n-3)	0.858±0.046^{e}	1.138±0.043^{d}	1.468±0.072^{c}	1.505±0.061^{c}	2.725±0.326^{b}	3.472±0.111^{ab}	3.872±0.169^{a}	2.203±0.081	2.094±0.081	<.0001	0.3190	0.2784
Totals, mg/g												
SFA	19.003±2.933	21.78±4.380	23.852±4.382	23.130±3.445	35.028±5.255	34.116±5.662	34.801±6.469	26.21±2.559	28.56±2.559	0.0400	0.2021	0.4041
MUFA	17.251±3.954	21.054±6.044	23.892±6.206	24.106±5.221	37.321±7.700	30.561±6.413	32.156±8.129	25.13±3.407	28.11±3.407	0.4345	0.3197	0.6648
Omega-6 PUFA	8.994±0.654	8.740±0.685	9.186±0.930	8.862±0.777	13.283±1.619	10.512±1.880	8.598±1.499	9.831±0.663	9.647±0.663	0.3738	0.5885	0.8081
Omega-3 PUFA	1.037±0.047^{d}	1.575±0.059^{c}	2.350±0.115^{b}	2.665±0.114^{b}	3.207±0.380^{b}	4.084±0.145^{a}	4.465±0.200^{a}	2.820±0.098	2.719±0.098	<.0001	0.515	0.1960
Omega-6/3 PUFA	8.737±0.539^{a}	5.482±0.253^{b}	3.842±0.249^{c}	3.270±0.146^{cd}	4.543±0.652^{bcd}	2.527±0.386^{de}	1.935±0.293^{e}	4.273±0.212	4.395±0.212	<.0001	0.8865	0.9738

[1] Data are presented as least squares means and their SEM (*n* = 7). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; TMUFA, total monounsaturated fatty acids (sum of palmitoleic acid and oleic acid); TSFA, total saturated fatty acids (sum of myristic acid, palmitic acid and stearic acid); Total n-6, total omega-6 polyunsaturated fatty acid (sum of LA, GLA and ARA); Total n-3, total omega-3 polyunsaturated fatty acid (sum of ALA, EPA, DPA and DHA).

Table 6.6. Plasma fatty acid composition (mg/mL) of pullets (16-week-old) fed control diet and diets with increasing levels of total omega-3 PUFA derived from either ALA or DHA following the LPS challenge[1].

	Diet effect							Injection effect		P values		
	Control	0.2%ALA	0.4%ALA	0.6%ALA	0.2%DHA	0.4%DHA	0.6%DHA	LPS	Saline	Diet	Injection	Diet* Injection
SFA, mg/g												
14:0	0.019±0.003	0.023±0.006	0.024±0.008	0.031±0.006	0.039±0.011	0.023±0.006	0.028±0.005	0.026±0.004	0.027±0.004	0.6485	0.4778	0.3643
16:0	1.798±0.355	2.286±0.713	2.403±0.907	3.193±0.700	3.382±0.976	1.695±0.485	1.920±0.605	2.311±0.378	2.454±0.378	0.6318	0.2739	0.3558
18:0	1.132±0.150	1.203±0.230	1.273±0.298	1.537±0.252	1.540±0.353	0.840±0.148	0.927±0.223	1.118±0.132	1.297±0.132	0.2679	0.3381	0.4785
MUFA, mg/g												
16:1	0.125±0.026	0.198±0.068	0.132±0.039	0.270±0.058	0.245±0.077	0.112±0.046	0.117±0.042	0.161±0.029	0.182±0.029	0.2789	0.3246	0.2333
18:1	2.609±0.617	3.390±1.173	3.553±1.447	5.178±1.300	4.920±1.478	1.949±0.640	2.342±0.922	3.351±0.606	3.490±0.606	0.4230	0.4624	0.3763
Omega-6 PUFA, mg/g												
LA (18:2n-6)	1.273±0.226^{ab}	1.613±0.526^{ab}	1.503±0.553^{ab}	1.930±0.369^{a}	1.940±0.563^{a}	0.878±0.234^{ab}	0.761±0.215^{b}	1.409±0.220	1.419±0.220	0.0359	0.2353	0.2493
GLA (18:3n-6)	0.010±0.002^{ab}	0.011±0.003^{ab}	0.009±0.003^{ab}	0.012±0.002^{a}	0.012±0.004^{ab}	0.005±0.001^{b}	0.005±0.001^{b}	0.009±0.001	0.010±0.001	0.0131	0.2376	0.3379
ARA (20:4n-6)	0.494±0.045^{a}	0.432±0.059^{a}	0.403±0.067^{a}	0.409±0.051^{a}	0.374±0.071^{ab}	0.188±0.024^{b}	0.182±0.024^{b}	0.321±0.031^{b}	0.388±0.031^{a}	<.0001	0.0406	0.5825
Omega-3 PUFA, mg/g												
ALA (18:3n-3)	0.024±0.005^{cd}	0.080±0.031^{bc}	0.137±0.063^{ab}	0.249±0.051^{a}	0.023±0.008^{cd}	0.018±0.006^{d}	0.018±0.005^{d}	0.083±0.017	0.074±0.017	<.0001	0.5464	0.2293
EPA (20:5n-3)	0.004±0.0004^{d}	0.013±0.001^{c}	0.028±0.003^{ab}	0.036±0.003^{ab}	0.025±0.003^{b}	0.036±0.003^{ab}	0.040±0.003^{a}	0.022±0.030	0.030±0.001	<.0001	0.0510	0.4201
DPA (22:5n-3)	0.009±0.0008^{c}	0.0186±0.002^{b}	0.028±0.003^{ab}	0.039±0.004^{a}	0.022±0.004^{b}	0.022±0.003^{b}	0.025±0.004^{ab}	0.021±0.002	0.026±0.002	<.0001	0.1369	0.8785
DHA (22:6n-3)	0.125±0.019^{c}	0.185±0.038^{bc}	0.240±0.064^{bc}	0.333±0.061^{ab}	0.430±0.112^{ab}	0.476±0.081^{a}	0.564±0.101^{a}	0.321±0.037	0.351±0.037	<.0001	0.3111	0.4029
Totals, mg/g												
SFA	2.949±0.500	3.512±0.943	3.700±1.209	4.761±0.956	4.961±1.335	2.557±0.632	2.875±0.834	3.455±0.511	3.778±0.511	0.5407	0.2564	0.3938
MUFA	2.734±0.643	3.589±1.240	3.685±1.476	5.448±1.356	5.165±1.544	2.061±0.685	2.459±0.963	3.512±0.632	3.671±0.632	0.4148	0.4559	0.3681
Omega-6 PUFA	1.777±0.264^{ab}	2.056±0.580^{ab}	1.915±0.620^{ab}	2.350±0.419^{a}	2.325±0.633^{ab}	1.071±0.252^{ab}	0.948±0.239^{b}	1.739±0.289	1.816±0.289	0.0113	0.2061	0.2969
Omega-3 PUFA	0.162±0.024^{c}	0.297±0.070^{bc}	0.434±0.129^{ab}	0.657±0.112^{a}	0.455±0.084^{ab}	0.552±0.090^{ab}	0.648±0.112^{a}	0.436±0.050	0.479±0.050	<.0001	0.2326	0.4299
Omega-6/3 PUFA	10.772±0.280^{a}	6.593±0.185^{b}	4.316±0.177^{c}	3.525±0.060^{c}	3.198±0.228^{c}	1.807±0.108^{d}	1.378±0.090^{e}	4.396±0.095	4.630±0.095	<.0001	0.5785	0.1509

[1] Data are presented as least squares means and their SEM (*n* = 7). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; GLA, gamma-linolenic acid; LA, linoleic acid; TMUFA, total monounsaturated fatty acids (sum of palmitoleic acid and oleic acid); TSFA, total saturated fatty acids (sum of myristic acid, palmitic acid and stearic acid); Total n-6, total omega-6 polyunsaturated fatty acid (sum of LA, GLA and ARA); Total n-3, total omega-3 polyunsaturated fatty acid (sum of ALA, EPA, DPA and DHA).

6.4.4 Supplementation of ALA and DHA effects on omega-3 oxylipins

Compared to the control group, ALA- and DHA-derived oxylipins, as well as their totals, were significantly increased in the plasma from pullets fed diets enriched with their respective PUFA precursor. These increases were observed as either main effects (**Table 6.7**) or interaction effects (**Table 6.8**), where diets enriched with ALA or DHA elevated their respective oxylipin derivatives in both saline- and LPS-treated groups (**Figure 6.3, 6.5**). In addition, individual EPA-derived oxylipins and their totals were significantly increased with both precursor PUFA-enriched diets compared to the control group. However, the DHA diet contributed more substantially to these increases than the ALA diet (**Figure 6.4**).

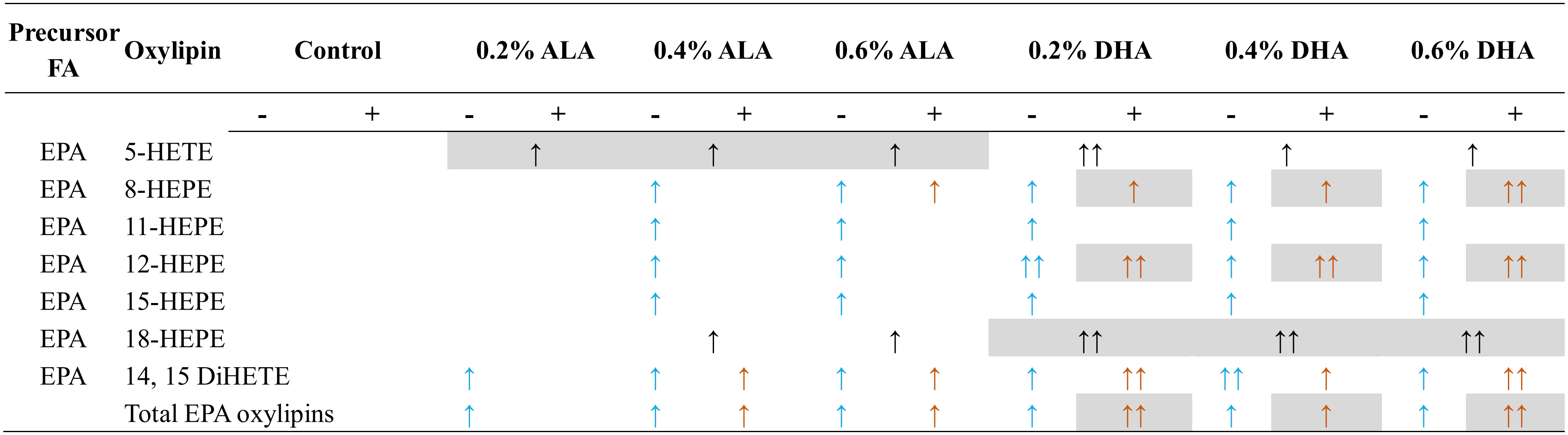
Precursor FA	Oxylipin	Control		0.2% ALA		0.4% ALA		0.6% ALA		0.2% DHA		0.4% DHA		0.6% DHA	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+
ALA	9-HOTrE							↑							
ALA	13-HOTrE					↑	↑	↑	↑						
ALA	9 oxo OTrE			↑		↑		↑							
ALA	12,13 EpODE			↑		↑		↑	↑						
	Total ALA oxylipins					↑	↑	↑	↑						

↑: higher compared to control as main effect

↑: higher in saline-treated dietary treatment group compared to control as interaction effect

↑: higher in LPS-treated dietary treatment group compared to control as interaction effect

Figure 6.3. Changes of individual and total ALA oxylipins in plasma of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following either LPS or Saline injection.



Precursor FA	Oxylipin	Control		0.2% ALA		0.4% ALA		0.6% ALA		0.2% DHA		0.4% DHA		0.6% DHA	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+
EPA	5-HETE			↑		↑		↑		↑↑		↑		↑	
EPA	8-HEPE					↑		↑	↑	↑	↑	↑	↑	↑	↑↑
EPA	11-HEPE					↑		↑		↑		↑		↑	
EPA	12-HEPE					↑		↑		↑↑	↑↑	↑	↑↑	↑	↑↑
EPA	15-HEPE					↑		↑		↑		↑		↑	
EPA	18-HEPE					↑		↑		↑↑		↑↑		↑↑	
EPA	14, 15 DiHETE			↑		↑	↑	↑	↑	↑	↑↑	↑↑	↑	↑	↑↑
	Total EPA oxylipins			↑		↑	↑	↑	↑	↑	↑↑	↑	↑	↑	↑↑

↑: increased compared to control as main effect

↑↑: significantly higher between different sources under same concentration

↑: higher in saline-treated dietary treatment group compared to control as interaction effect

↑: higher in LPS-treated dietary treatment group compared to control as interaction effect

▨ : increased with the graded level of omega-3 PUFA

Figure 6.4. Changes of individual and total EPA oxylipins in plasma of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following either LPS or Saline injection.

Precursor FA	Oxylipin	Control		0.2% ALA		0.4% ALA		0.6% ALA		0.2% DHA		0.4% DHA		0.6% DHA	
		-	+	-	+	-	+	-	+	-	+	-	+	-	+
DHA	10-HDoHE									↑	↑	↑	↑	↑	↑
DHA	14-HDoHE							↑		↑↑		↑↑		↑↑	
DHA	16-HDoHE									↑	↑	↑	↑	↑	↑
DHA	17-HDoHE									↑	↑	↑	↑	↑	↑
DHA	16, 17 EpDPE									↑		↑		↑	
DHA	16, 17 DiHDoPE									↑		↑		↑	
DHA	19,20 EpDPE							↑		↑↑		↑↑		↑↑	
DHA	19,20 DiHDoPE					↑		↑		↑↑		↑↑		↑↑	
	Total DHA oxylipins									↑	↑	↑	↑	↑	↑

↑: increased compared to control as main effect

↑↑**:** significantly higher between different sources under same concentration

↑: higher in saline-treated dietary treatment group compared to control as interaction effect

↑: higher in LPS-treated dietary treatment group compared to control as interaction effect

▨ : increased with the graded level of omega-3 PUFA

Figure 6.5. Changes of individual and total DHA oxylipins in plasma of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following either LPS or Saline injection.

6.4.5 Supplementation of ALA and DHA effects on omega-6 oxylipins

Nine LA-derived oxylipins were detected in plasma, of which eight individual oxylipins and total LA oxylipins were significantly increased following the LPS challenge. A diet effect was observed for five individual oxylipins and total LA oxylipins, with their levels significantly reduced in pullets fed the DHA diet (**Figure 6.6**; **Table 6.7**).

Twenty-four ARA-derived oxylipins were identified, of which 10 individual oxylipins and total ARA oxylipins increased, while two individual oxylipins decreased in response to the LPS challenge. A significant dietary effect was observed for 14 individual ARA oxylipins and their totals. Plasma ARA oxylipin levels were significantly lower in pullets fed the DHA diet compared to the control group. In contrast, the ALA diet had minimal impact on omega-6 oxylipins (**Figure 6.6; Table 6.7**).

Precursor FA	**Oxylipin**	**Control**	**0.2% ALA**	**0.4% ALA**	**0.6% ALA**	**0.2% DHA**	**0.4% DHA**	**0.6% DHA**
LA	9-HODE						↓	↓
LA	9 oxo ODE						↓	
LA	13 oxo ODE						↓	↓
LA	9, 12, 13 triHOME						↓	↓
	Total LA oxylipins						↓	↓
ARA	PGE2						↓	↓
ARA	15k PGE2						↓	↓
ARA	12-HHTrE						↓	
ARA	5-HETE				↓		↓	↓
ARA	8-HETE					↓		
ARA	12-HETE				↓		↓	
ARA	15-HETE				↓	↓	↓	↓
ARA	5 oxo ETE						↓	↓
ARA	15 oxo ETE					↓	↓	↓
ARA	14, 15 DiHETrE				↓		↓	↓
ARA	20cooh AA						↓	↓
	Total ARA oxylipins						↓	↓

Figure 6.6. Changes of individual and total omega-6 oxylipins in plasma of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following either LPS or Saline injection.

Table 6.7. The composition of plasma oxylipins derived from omega-3 and omega-6 PUFA (ng/mL) of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following the LPS challenge1.

	Diet effect							Challenge effect		P values		
	Control	0.2%ALA	0.4%ALA	0.6%ALA	0.2%DHA	0.4%DHA	0.6%DHA	LPS	Saline	Diet	Injection	Diet* Injection
LA, ng/mL												
9-HODE	117.9±15.95^{a}	94.94±12.81^{ab}	76.83±9.128^{ab}	68.12±7.127^{abc}	95.22±11.22^{ab}	57.38±5.499^{bc}	50.51±5.589^{c}	103.9±5.496^{a}	56.32±5.496^{b}	0.0019	<.0001	0.0512
9 oxo ODE	66.03±14.67^{a}	41.86±7.025^{ab}	48.60±14.28^{ab}	43.99±15.11^{ab}	36.56±4.046^{ab}	22.19±3.283^{b}	24.81±4.847^{ab}	53.68±5.222^{a}	27.47±5.222^{b}	0.0161	<.0001	0.2967
13-HODE	132.4±17.47	121.3±15.37	99.05±11.23	80.67±8.317	126.0±13.24	78.72±6.977	72.64±9.892	131.6±6.572^{a}	71.49±6.572^{b}	0.0080	<.0001	0.2091
9,10 EpOME	12.95±1.959	12.75±1.948	8.937±1.564	6.567±1.047	11.57±1.630	10.32±1.227	10.59±1.755	12.22±0.867^{a}	8.830±0.867^{b}	0.0536	0.0075	0.0549
9,10 diHOME	4.823±0.692	4.136±0.450	3.838±0.366	3.331±0.366	5.484±0.701	4.486±0.424	4.662±1.016	6.261±0.332^{a}	2.528±0.332^{b}	0.1781	<.0001	0.2277
12, 13 EpOME	23.70±3.365	23.12±3.343	16.93±2.860	13.59±2.260	21.06±2.544	17.87±2.021	18.85±2.892	20.77±1.494	17.85±1.494	0.1982	0.1722	0.0188
12, 13 diHOME	10.88±1.253	10.54±1.199	10.99±1.474	9.152±1.057	13.49±1.807	12.37±1.094	14.19±2.719	17.10±0.861^{a}	6.220±0.861^{b}	0.2271	<.0001	0.1297
13 oxo ODE	35.09±6.035^{a}	30.86±7.969^{ab}	22.97±6.151^{ab}	24.74±8.090^{ab}	22.68±2.423^{ab}	13.53±2.793^{b}	13.19±3.148^{b}	32.78±3.029^{a}	13.81±3.029^{b}	0.0109	<.0001	0.3142
9,12,13 triHOME	43.21±6.051^{a}	37.82±3.421^{a}	33.82±3.353^{ab}	25.97±2.584^{abc}	39.28±4.777^{a}	21.36±1.418^{c}	21.55±2.593^{bc}	38.09±1.998^{a}	25.63±1.998^{b}	0.0006	<.0001	0.1680
Total LA	512.9±67.04^{a}	431.0±48.28^{ab}	381.8±43.92^{ab}	313.41±40.49^{ab}	432.3±43.29^{ab}	270.5±22.76^{b}	267.09±32.61^{b}	477.7±23.78	267.7±23.77	0.0033	<.0001	0.2462
DGLA, ng/mL												
8-HETrE	0.541±0.081	0.512±0.064	0.507±0.100	0.397±0.036	0.470±0.057	0.371±0.040	0.440±0.040	0.503±0.034	0.422±0.034	0.6068	0.1096	0.0616
15-HETrE	1.210±0.216	1.113±0.136	1.167±0.214	0.692±0.080	0.914±0.130	0.858±0.086	0.980±0.140	1.058±0.081	0.923±0.081	0.1053	0.2463	0.2776
ARA, ng/mL												
PGE2	0.580±0.077^{ab}	0.581±0.072^{a}	0.482±0.046^{ab}	0.300±0.023^{bc}	0.359±0.043^{abc}	0.263±0.023^{c}	0.261±0.034^{c}	0.432±0.027^{a}	0.375±0.027^{b}	0.0001	0.0150	0.5625
11b PGE2	10.83±1.416^{ab}	11.34±1.392^{a}	8.638±0.824^{ab}	5.821±0.584^{b}	7.298±0.839^{ab}	4.996±0.492^{b}	5.294±0.652^{b}	8.254±0.509^{a}	7.238±0.509^{b}	0.0006	0.0497	0.6261
15k PGE2	2.242±0.339^{a}	2.176±0.364^{a}	1.591±0.180^{ab}	1.410±0.180^{abc}	1.533±0.184^{abc}	0.917±0.109^{bc}	0.870±0.148^{c}	1.601±0.125	1.467±0.125	0.0018	0.1855	0.6223
dhk PGF2a	1.730±0.188	1.606±0.261	1.330±0.238	1.311±0.195	1.311±0.195	0.539±0.047	0.406±0.029	1.944±0.092^{a}	0.242±0.092^{b}	0.0098	<.0001	0.1948
TXB2	1.710±0.285	1.858±0.419	1.667±0.269	1.639±0.398	1.029±0.215	0.737±0.128	0.630±0.149	1.935±0.153^{a}	0.714±0.153^{b}	0.0135	<.0001	0.3311
5,6 EpETrE	0.787±0.124	0.846±0.143	0.759±0.085	0.525±0.094	0.673±0.115	0.689±0.064	0.686±0.100	0.583±0.057^{b}	0.835±0.056^{a}	0.5607	0.0026	0.1042
5,6 DiHETrE	0.157±0.021	0.133±0.014	0.130±0.017	0.109±0.010	0.159±0.020	0.128±0.011	0.143±0.011	0.134±0.008	0.140±0.008	0.2331	0.6440	0.0671
11, 12 DiHETrE	0.301±0.041	0.263±0.036	0.235±0.025	0.190±0.022	0.320±0.050	0.222±0.029	0.204±0.036	0.300±0.019^{a}	0.195±0.018^{b}	0.1796	0.0003	0.3495
12 HHTrE	0.725±0.208^{a}	0.836±0.322^{a}	0.593±0.114^{a}	0.544±0.177^{ab}	0.882±0.565^{ab}	0.215±0.030^{b}	0.294±0.074^{ab}	0.771±0.145^{a}	0.391±0.145^{b}	0.0008	0.0007	0.2561
12 oxo ETE	8.385±1.340	7.225±1.316	7.598±1.444	5.940±0.861	6.701±0.849	7.139±1.058	8.054±1.126	7.854±0.621	6.730±0.621	0.7545	0.2055	0.1713
8,9 EpETrE	0.308±0.045	0.345±0.053	0.308±0.052	0.208±0.029	0.279±0.067	0.237±0.027	0.247±0.037	0.257±0.025	0.295±0.025	0.3021	0.2917	0.2998
8,9 DiHETrE	0.230±0.029	0.209±0.028	0.190±0.017	0.184±0.030	0.251±0.033	0.178±0.026	0.192±0.031	0.252±0.015^{a}	0.158±0.015^{b}	0.6158	<.0001	0.1136
14, 15 EpETrE	0.270±0.044	0.252±0.042	0.244±0.032	0.154±0.027	0.230±0.0508	0.178±0.021	0.209±0.035	0.171±0.020^{b}	0.268±0.020^{a}	0.1915	0.0009	0.1747
14, 15 DiHETrE	0.598±0.049^{a}	0.569±0.049^{ab}	0.496±0.032^{ab}	0.417±0.025^{b}	0.525±0.046^{ab}	0.409±0.032^{b}	0.390±0.028^{b}	0.547±0.021^{a}	0.426±0.021^{b}	0.0048	0.0001	0.3154
5-HETE	4.523±0.639^{a}	2.953±0.325^{abc}	3.000±0.289^{ab}	2.239±0.193^{bc}	2.950±0.318^{abc}	2.023±0.184^{c}	2.232±0.231^{bc}	2.881±0.183	2.810±0.183	0.0050	0.6812	0.0513
5 oxo ETE	3.449±0.576^{a}	2.542±0.290^{a}	2.328±0.248^{ab}	1.654±0.164^{ab}	2.127±0.311^{ab}	1.362±0.162^{b}	1.390±0.213^{b}	1.846±0.166^{b}	2.397±0.166^{a}	0.0010	0.0635	0.0800
8-HETE	3.545±0.418^{a}	2.359±0.232^{ab}	2.525±0.246^{ab}	1.839±0.199^{b}	1.994±0.310a^{b}	1.821±0.261^{b}	2.426±0.379^{ab}	2.480±0.162	2.237±0.162	0.0303	0.2920	0.0867
9-HETE	2.649±0.386	1.555±0.162	1.874±0.206	1.464±0.190	1.455±0.224	1.346±0.211	1.618±0.237	1.830±0.129	1.587±0.129	0.1520	0.1869	0.2755
11-HETE	1.443±0.139^{a}	1.218±0.116^{a}	1.181±0.108^{a}	0.906±0.086^{ab}	1.024±0.229^{ab}	0.692±0.087^{b}	0.781±0.096^{b}	1.066±0.070	1.004±0.070	0.0003	0.2129	0.0047
12-HETE	1.454±0.177^{a}	0.929±0.102^{ab}	1.482±0.206^{a}	0.759±0.083^{b}	0.919±0.149^{ab}	0.740±0.104^{b}	0.933±0.111^{ab}	1.039±0.075	1.023±0.075	0.0082	0.7790	0.1686

tetranor 12-HETE	0.264±0.144	0.139±0.047	0.238±0.062	0.140±0.044	0.239±0.048	0.134±0.025	0.206±0.078	0.304±0.039^{a}	0.085±0.039^{b}	0.4372	<.0001	0.9708
15-HETE	4.343±0.513^{a}	3.060±0.334^{ab}	3.397±0.432^{ab}	2.250±0.115^{bc}	2.406±0.241^{bc}	1.629±0.166^{c}	1.693±0.231^{c}	2.686±0.170	2.679±0.170	<.0001	0.4889	0.0795
15 oxo ETE	2.199±0.293^{a}	1.568±0.242^{ab}	1.612±0.260^{ab}	1.307±0.155^{ab}	1.104±0.118^{bc}	0.684±0.084^{cd}	0.627±0.109^{d}	1.372±0.105	1.228±0.105	<.0001	0.1374	0.3917
20cooh AA	69.68±16.89a	30.70±10.33^{abc}	14.77±3.749^{abc}	18.65±5.632^{abc}	28.84±6.608^{ab}	11.30±1.465^{bc}	11.17±2.251^{c}	37.62±4.466^{a}	9.556±4.466^{b}	0.0033	<.0001	0.2447
Total ARA	102.4±18.81^{a}	75.26±10.50^{ab}	56.66±5.900^{abc}	49.96±6.011^{bc}	64.03±7.263^{ab}	38.57±2.629^{c}	40.95±4.006^{c}	78.15±4.994^{a}	44.09±4.994^{b}	0.0002	<.0001	0.0597
ALA, ng/mL												
9 oxo OTrE	4.187±1.112^{b}	8.429±0.942^{a}	23.38±7.693^{a}	24.34±10.69^{a}	3.384±0.415^{b}	1.943±0.281^{b}	2.756±0.418^{b}	14.55±2.681^{a}	4.995±2.681^{b}	<.0001	<.0001	0.0979
12, 13EpODE	0.693±0.064	1.777±0.300	2.257±0.387	2.927±0.523	0.666±0.106	0.674±0.073	0.855±0.105	1.526±0.149	1.288±0.149	<.0001	0.0025	0.0062
9-HOTrE	3.629±0.787^{bc}	7.351±1.210^{ab}	10.53±1.966^{ab}	16.13±6.065^{a}	3.471±0.558^{c}	2.227±0.215^{c}	2.389±0.262^{c}	8.824±0.889^{a}	4.756±0.889^{b}	<.0001	0.0061	0.1374
13-HOTrE	3.925±0.805^{bc}	7.571±1507^{b}	15.95±2.615^{a}	19.68±6.713^{a}	3.821±0.664^{bc}	2.800±0.278^{c}	3.170±0.255^{c}	11.20±1.504^{a}	5.066±1.504^{b}	<.0001	<.0001	0.0112
Total ALA	12.43±2.690	25.13±3.071	52.12±11.40	63.08±20.91	11.34±1.591	7.644±0.732	9.169±0.881	36.10±4.897	15.59±4.897	<.0001	<.0001	0.0479
EPA, ng/mL												
5-HEPE	0.078±0.023^{c}	0.202±0.022^{b}	0.493±0.103^{ab}	0.507±0.123^{a}	0.464±0.065^{a}	0.810±0.100^{a}	1.028±0.099^{a}	0.581±0.045	0.442±0.045	<.0001	0.7491	0.0580
8-HEPE	0.122±0.017	0.223±0.026	0.461±0.076	0.553±0.187	0.488±0.069	0.789±0.113	1.065±0.072	0.576±0.051	0.481±0.051	<.0001	0.6892	0.0069
15-HEPE	0.343±0.081	0.546±0.095	1.337±0.212	1.217±0.264	1.311±0.180^{a}	2.137±0.313	3.005±0.247	1.500±0.115	1.328±0.115	<.0001	0.7945	0.0205
14, 15 DiHETE	0.814±0.118	1.942±0.146	3.458±0.270	3.978±0.200	5.143±0.735	6.761±0.588	10.23±1.330	5.617±0.338	3.618±0.338	<.0001	<.0001	0.0155
11-HEPE	0.258±0.098	0.333±0.068	0.561±0.089	0.567±0.117	0.608±0.104	0.823±0.141	1.017±0.099	0.632±0.056	0.559±0.056	0.0002	0.2985	0.0136
12-HEPE	0.092±0.013	0.206±0.034	0.412±0.051	0.523±0.068	0.523±0.068	0.749±0.094	1.074±0.046	0.472±0.033	0.527±0.033	<.0001	0.0605	0.0003
18-HEPE	0.323±0.058^{d}	0.576±0.100^{d}	1.710±0.298^{bc}	1.456±0.296^{c}	1.566±0.217^{c}	2.682±0.301^{ab}	3.827±0.274^{a}	1.875±0.128	1.584±0.128	<.0001	0.3301	0.1177
Total EPA	2.030±0.219	4.028±0.335	8.432±0.922	8.719±1.021	10.10±1.080	14.75±1.259	21.24±1.724	11.25±0.563	8.548±0.563	<.0001	0.0033	0.0125
DPA, ng/mL												
17k DPA	4.558±0.775^{c}	8.893±1.164^{bc}	21.50±4.779^{bc}	17.10±3.398^{b}	27.57±7.120^{b}	58.36±6.789^{a}	63.42±8.958^{a}	29.95±2.951	27.60±2.951	<.0001	0.3760	0.2926
DHA, ng/mL												
10-HDoHE	1.373±0.169^{d}	1.672±0.143^{cd}	2.288±0.174^{c}	2.361±0.221^{c}	5.252±0.566^{b}	8.242±1.071^{ab}	13.20±1.557^{a}	5.314±0.405	4.510±0.405	<.0001	0.2630	0.0012
14-HDoHE	3.077±0.387^{d}	3.536±0.334^{cd}	4.796±0.449^{cd}	5.110±0.552^{c}	10.80±1.228^{b}	21.00±2.443^{a}	34.61±4.521^{a}	12.92±2.108	13.808±2.108	<.0001	0.5789	0.5053
16-HDoHE	2.396±0.254^{d}	2.787±0.239^{cd}	3.961±0.310^{cd}	3.930±0.412d	8.193±0.886^{bc}	12.66±1.640^{b}	18.43±1.943^{a}	7.981±0.558	6.977±0.558	<.0001	0.4173	0.0009
17-HDoHE	10.42±1.146^{b}	11.49±1.004^{b}	16.45±1.228^{b}	15.69±1.886^{b}	33.53±3.730^{b}	48.90±5.640^{b}	64.61±6.878^{a}	30.09±2.025	27.36±2.025	0.0001	0.2048	0.0009
16, 17EpDPE	4.272±0.507^{c}	6.691±0.854^{c}	7.319±0.879^{c}	6.596±1.193^{c}	18.90±2.703^{b}	38.59±3.783^{a}	55.36±8.779^{a}	18.65±2.039^{b}	20.70±2.039^{a}	<.0001	0.0025	0.0665
16, 17DiHDoPE	0.112±0.020^{c}	0.205±0.021^{c}	0.212±0.028^{c}	0.234±0.022^{c}	0.848±0.097^{b}	1.184±0.140^{ab}	1.771±0.300^{a}	0.810±0.070^{a}	0.495±0.810^{b}	<.0001	<.0001	0.3170
19, 20EpDPE	0.989±0.104^{d}	1.670±0.178^{cd}	1.878±0.214^{cd}	1.628±0.173^{c}	4.307±0.532^{b}	8.341±0.851^{a}	12.32±1.643^{a}	4.237±0.395b	4.658±0.395a	<.0001	0.0076	0.2653
19, 20DiHoPE	0.248±0.026^{d}	0.382±0.038^{cd}	0.487±0.029^{c}	0.502±0.056^{c}	1.264±0.177^{b}	1.868±0.197^{ab}	2.470±0.336^{a}	1.242±0.088^{a}	0.821±0.088^{b}	<.0001	0.0003	0.2873
Total DHA	25.93±2.421	31.98±2.680	42.33±2.945	41.44±3.410	94.08±9.475	162.0±15.95	234.0±25.16	94.26±6.423	88.24±6.423	<.0001	0.1231	0.0026

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[1] Data are presented as least squares means and their SEM (*n* = 6). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] ARA, arachidonic acid; ALA, α-linolenic acid; COX, cyclooxygenase; CYP-e, cytochrome P450 epoxygenase; CYP-h, cytochrome P450 hydrolase; DHA, docosahexaenoic acid; DiHDoPE, dihydroxy-docosapentaenoic acid; DiHETrE, dihydroxy-eicosatrienoic acid; DiHOME, dihydroxy-octadecenoic acid; EPA, eicosapentaenoic acid; EpDoPE, epoxy-eicosadocosapentaenoic acid; EpOME, epoxy-octadecenoic acid; HDoHE, hydroxy-docosahexaenoic acid; HEPE, hydroxy-eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; HOTrE, hydroxy-octadecatrienoic acid; LA, linoleic acid; LOX, lipoxygenase; oxoETE, oxo-eicosatetraenoic acid; oxoODE, oxo-octadecadienoic acid; oxoOTrE , oxo-octadecatrienoic acid; PG, prostaglandin; TriHOME, trihydroxy-octadecenoic acid; TX, thromboxane.

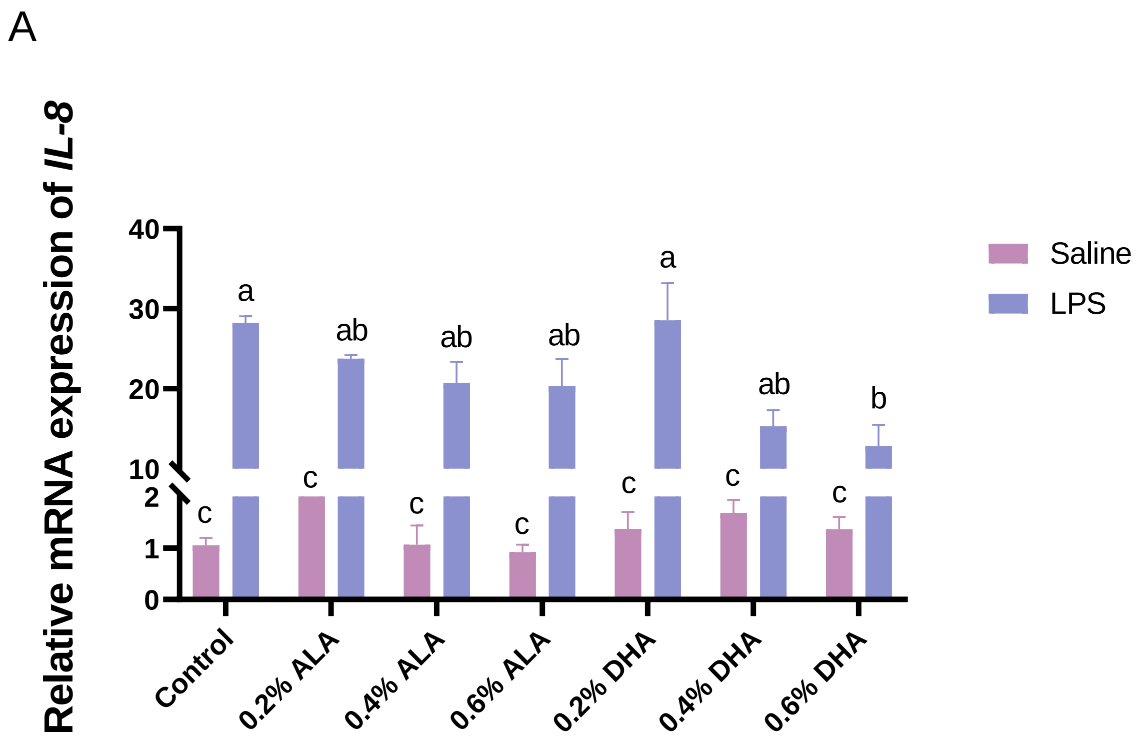
Table 6.8. Interaction effects oxylipins derived from omega-3 and omega-6 PUFA (ng/mL) of pullets (16-week-old) fed the control diet and diets with increasing levels of total omega-3 PUFA derived from ALA or DHA following the LPS challenge.

		Control	0.2% ALA	0.4% ALA	0.6% ALA	0.2% DHA	0.4% DHA	0.6% DHA	P Values		
									Diet	Injection	Diet*Injection
ALA oxylipins											
13-EpODE	Saline	0.526 ± 0.099^{b}	1.632 ± 0.300^{a}	3.023 ± 0.648^{a}	2.439 ± 0.879^{a}	0.589 ± 0.118^{b}	0.470 ± 0.089^{b}	0.341 ± 0.071^{b}	<.0001	0.0025	0.0062
	LPS	0.860 ± 0.082^{b}	1.923 ± 0.519^{ab}	1.493 ± 0.425^{b}	3.416 ± 0.568^{a}	0.742 ± 0.177^{b}	0.879 ± 0.117^{b}	1.370 ± 0.198^{ab}			
13-HOTrE	Saline	2.831 ± 0.536^{b}	5.343 ± 1.004^{ab}	11.89 ± 1.684^{a}	10.11 ± 1.441^{a}	2.220 ± 0.313^{b}	2.164 ± 0.466^{bc}	0.908 ± 0.213^{c}	<.0001	<.0001	0.0112
	LPS	5.018 ± 1.518^{b}	9.798 ± 2.843^{ab}	20.02 ± 4.952^{a}	29.25 ± 13.35^{a}	5.421 ± 1.029^{b}	3.436 ± 0.303^{b}	5.432 ± 0.463^{b}			
Total ALA	Saline	8.921 ± 1.772^{bc}	18.75 ± 3.580^{ab}	36.65 ± 6.294^{a}	29.34 ± 3.766^{a}	6.782 ± 2.962^{c}	5.859 ± 1.270^{cd}	2.828 ± 0.707^{d}	<.0001	<.0001	0.0479
	LPS	15.95 ± 5.081^{bc}	31.50 ± 4.990^{ab}	67.59 ± 21.91^{a}	96.82 ± 41.64^{a}	15.90 ± 2.962^{bc}	9.430 ± 0.732^{c}	15.51 ± 1.614^{bc}			
EPA oxylipins											
11-HEPE	Saline	0.133 ± 0.027^{c}	0.301 ± 0.055^{bc}	0.730 ± 0.127^{ab}	0.499 ± 0.132^{ab}	0.576 ± 0.147^{ab}	1.046 ± 0.271^{a}	0.627 ± 0.150^{ab}	0.0002	0.2985	0.0136
	LPS	0.329 ± 0.193^{b}	0.364 ± 0.124^{b}	0.393 ± 0.124^{b}	0.634 ± 0.194^{ab}	0.641 ± 0.146^{ab}	0.600 ± 0.078^{ab}	1.407 ± 0.131^{a}			
12-HEPE	Saline	0.089 ± 0.016^{c}	0.235 ± 0.053^{bc}	0.610 ± 0.096^{a}	0.470 ± 0.118^{ab}	0.518 ± 0.092^{a}	0.887 ± 0.159^{a}	0.882 ± 0.068^{a}	<.0001	0.0605	0.0003
	LPS	0.094 ± 0.019^{e}	0.178 ± 0.044^{de}	0.215 ± 0.035^{cde}	0.413 ± 0.127^{bcd}	0.528 ± 0.100^{bc}	0.610 ± 0.101^{ab}	1.267 ± 0.615^{a}			
14,15 DiHETE	Saline	0.565 ± 0.121^{d}	1.799 ± 0.211^{c}	3.145 ± 0.436^{bc}	2.793 ± 0.258^{bc}	3.807 ± 0.879^{abc}	7.206 ± 0.787^{a}	6.008 ± 1.063^{ab}	<.0001	<.0001	0.0155
	LPS	1.063 ± 0.203^{d}	2.085 ± 0.201^{cd}	3.771 ± 0.318^{bc}	5.163 ± 0.305^{b}	6.479 ± 1.178^{b}	6.315 ± 0.874^{b}	14.44 ± 2.437^{a}			
15-HEPE	Saline	0.287 ± 0.064^{c}	0.639 ± 0.176^{bc}	1.463 ± 0.214^{ab}	1.225 ± 0.392^{ab}	1.239 ± 0.209^{ab}	2.604 ± 0.600^{a}	1.837 ± 0.391^{a}	<.0001	0.7945	0.0205
	LPS	0.399 ± 0.149^{d}	0.453 ± 0.069^{cd}	1.211 ± 0.366^{bc}	1.211 ± 0.354^{bc}	1.383 ± 0.294^{bc}	1.670 ± 0.180^{ab}	4.173 ± 0.302^{a}			
8-HEPE	Saline	0.128 ± 0.022^{c}	0.256 ± 0.042^{bc}	0.545 ± 0.071^{ab}	0.391 ± 0.066^{ab}	0.456 ± 0.081^{ab}	0.926 ± 0.194^{a}	0.669 ± 0.126^{ab}	<.0001	0.6892	0.0069
	LPS	0.116 ± 0.026^{d}	0.192 ± 0.030^{cd}	0.377 ± 0.135^{bcd}	0.716 ± 0.369^{bc}	0.520 ± 0.113^{bc}	0.652 ± 0.115^{ab}	1.462 ± 0.071^{a}			
Total EPA	Saline	1.564 ± 0.214^{e}	4.142 ± 0.645^{d}	8.581 ± 1.209^{bc}	6.946 ± 1.036^{cd}	8.484 ± 1.279^{bc}	16.70 ± 2.126^{a}	13.52 ± 2.189^{ab}	<.0001	0.0033	0.0125
	LPS	2.496 ± 0.383^{c}	3.915 ± 0.13^{c}	8.283 ± 1.391^{b}	10.59 ± 1.760^{b}	11.72 ± 1.741^{b}	12.80 ± 1.350^{b}	28.96 ± 2.663^{a}			
DHA oxylipins											
10-HDoHE	Saline	1.467 ± 0.238^{d}	1.725 ± 0.264^{cd}	3.144 ± 0.279^{bc}	2.855 ± 0.416^{bcd}	5.281 ± 0.748^{ab}	9.458 ± 1.930^{a}	7.642 ± 1.617^{a}	<.0001	0.2630	0.0012
	LPS	1.280 ± 0.240^{c}	1.618 ± 0.110^{c}	1.433 ± 0.209^{c}	1.868 ± 0.147^{c}	5.223 ± 0.851^{b}	7.026 ± 0.930^{b}	18.75 ± 2.662^{a}			
16-HDoHE	Saline	2.629 ± 0.361^{e}	2.860 ± 0.454^{de}	5.151 ± 0.544^{bcd}	4.832 ± 0.793^{cde}	8.424 ± 1.330^{abc}	10.26 ± 2.211^{ab}	14.69 ± 3.003^{a}	<.0001	0.4173	0.0009
	LPS	2.162 ± 0356^{c}	2.713 ± 0.151^{c}	2.771 ± 0.296^{c}	3.027 ± 0.226^{c}	7.959 ± 1.171^{b}	10.63 ± 1.320^{b}	26.60 ± 3.196^{a}			

17-HDoHE	Saline	11.78±1.743^{c}	12.02±1.788^{c}	21.59±2.085^{bc}	20.42±3.674^{bc}	35.11±5.585^{ab}	54.72±9.427^{a}	35.90±8.334^{ab}	0.0001	0.2048	0.0009
	LPS	3.055±1.489^{c}	10.96±0.913^{c}	11.31±1.300^{c}	10.96±0.856^{c}	31.96±4.944^{b}	43.08±6.193^{b}	93.32±10.94^{a}			
Total DHA	Saline	27.92±3.804^{d}	33.83±4.993^{cd}	56.11±4.850^{bc}	50.67±6.593^{cd}	101.9±14.52^{ab}	181.2±26.00^{a}	152.1±34.46^{a}	<.0001	0.1231	0.0026
	LPS	23.95±2.995^{c}	30.13±1.946^{c}	28.55±3.342^{c}	32.22±1.745^{c}	86.30±12.17^{b}	142.7±18.50^{b}	316.0±36.68^{a}			

6.4.6 Relative splenic mRNA expressions of cytokines

The LPS challenge significantly increased the mRNA expression of all measured splenic cytokines, either as an overall main effect or through interaction effects (**Table 6.9**). Interaction effects were specifically observed for splenic mRNA expressions of *IL-8* (**Figure 6.1A**) and *IL-10* **(Figure 6.1B).** Pullets fed the 0.6% DHA diet showed significantly lower *IL-8* expression, while those on the 0.4% DHA diet exhibited significantly lower *IL-10* expression after the LPS challenge, compared to other treatments.


A
Relative mRNA expression of *IL-8*
40
30
20
10
2
1
0
a
ab
ab
ab
a
ab
b
c
c
c
c
c
c
c
Control
0.2% ALA
0.4% ALA
0.6% ALA
0.2% DHA
0.4% DHA
0.6% DHA
Saline
LPS

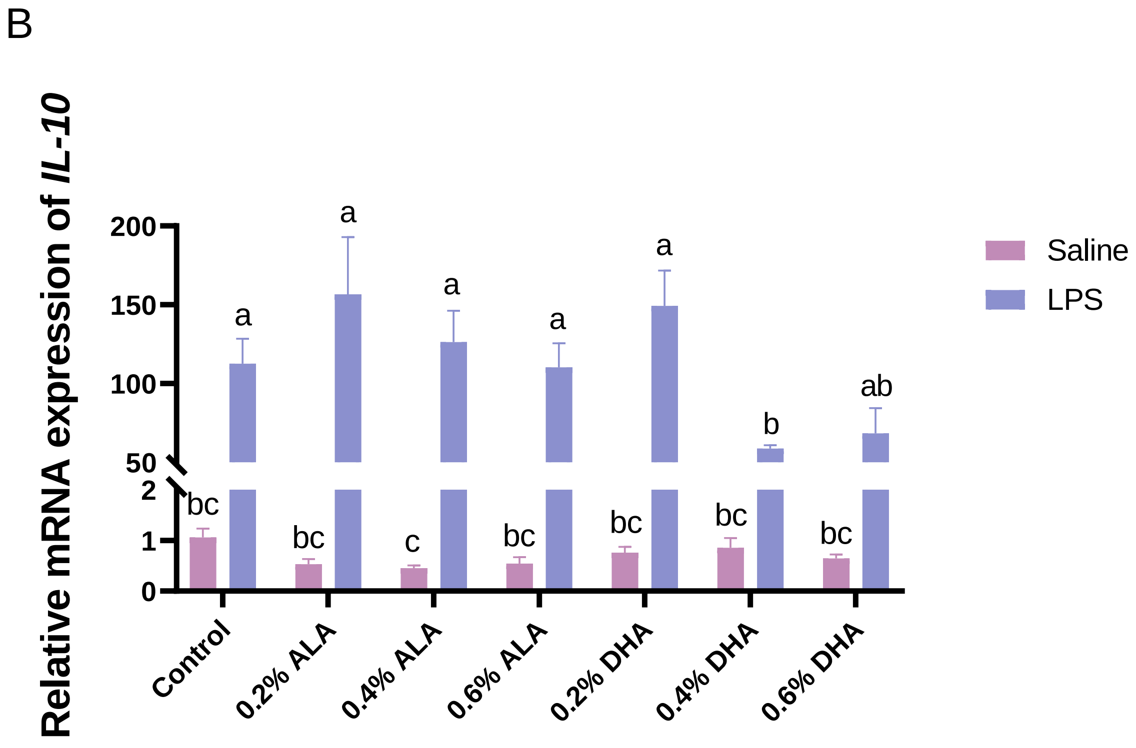


Figure 6.7. Relative mRNA expression of *IL-8* (A) and *IL-10* (B) in the spleen of laying hens fed diets containing increasing levels of total omega-3 derived from either ALA or DHA diets following LPS challenge.

Table 6.9. Relative mRNA expression of pro- and anti-inflammatory cytokines in the spleen of laying hens fed the control diet and diets with increasing levels of total omega-3 derived from ALA or DHA following LPS challenge[1].

	Diet effect							Injection effect		P values		
Genes[2]	Control	0.2%ALA	0.4%ALA	0.6%ALA	0.2%DHA	0.4%DHA	0.6%DHA	LPS	Saline	Diet	Injection	Diet* Injection
IL-1β	32.15±6.660	29.74±2.381	41.43±7.918	33.55±7.455	49.46±6.568	44.65±12.35	47.84±5.108	78.43±5.721^{a}	1.233±0.125^{b}	0.0886	<.0001	0.0522
IL-2	15.47±2.172	15.96±5.787	14.74±2.580	9.843±0.929	22.10±3.677	18.40±5.012	16.46±4.704	31.39±2.889^{a}	0.886±0.054^{b}	0.1464	<.0001	0.1486
IL-6	353.7±45.18^{ab}	412.9±54.50^{a}	420.0±56.84^{a}	358.6±49.97^{a}	420.2±46.68^{a}	200.6±33.44^{b}	216.4±48.24^{b}	679.6±39.00^{a}	1.074±0.200^{b}	0.7694	<.0001	0.2059
IL-8	14.64±0.412	12.97±3.599	10.90±1.327	10.64±1.518	14.96±2.315	8.491±1.013	7.092±1.351	21.39±1.388^{a}	1.379±0.170^{b}	0.2638	<.0001	0.0208
IL-10	56.85±7.865^{a}	78.57±18.13^{a}	63.43±9.927^{a}	55.45±6.882^{ab}	75.08±11.18^{a}	29.78±1.122^{c}	34.58±8.000^{bc}	111.8±7.464^{a}	0.693±0.050^{b}	0.0115	<.0001	<.0001
TNF-α	2.935±0.154	3.202±0.357	2.977±0.246	2.875±0.191	3.088±0.243	3.260±0.241	2.789±0.411	5.025±0.148^{a}	1.011±0.148^{b}	0.8905	<.0001	0.8536
IFN-γ	59.49±6.73	56.28±8.94	56.74±8.10	41.50±5.33	89.30±12.57	83.91±9.112	68.82±13.81	129.02±5.09^{a}	1.274±5.09^{b}	0.0316	<.0001	0.7076
TLR4	1.225±0.063	1.131±0.072	1.145±0.051	1.193±0.063	1.351±0.087	1.121±0.051	1.204±0.080	1.497±0.036^{a}	0.895±0.036^{b}	0.2483	<.0001	0.0039

[1] Data are presented as the least squares means and their SE based on $2^{-\Delta\Delta Ct}$ method (*n* = 6). Beta-actin is used as an internal control for gene expression normalization. Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-8, interleukin 8; IL-10, interleukin10; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha; TLR4, toll-like receptor 4.

6.5 Discussion

This study investigated the effects of dietary supplementation with ALA-rich FSO and DHA-rich algal biomass on performance, omega-3 and omega-6 PUFA profiles, oxylipin levels, and immune responses in pullets, with a particular focus on their response to an LPS challenge. The results provide critical insights into how dietary omega-3 PUFA sources influence FA metabolism, inflammatory mediator production, and immune gene expression.

Dietary omega-3 PUFA supplementation did not affect pullet growth performance under standard management conditions. However, the significant interaction between diet and week regarding BW may indicate gradual adaptation to dietary changes, particularly in diets enriched with omega-3 PUFA. Notably, FI increased overtime, stabilizing after week 13, which aligns with the natural growth trajectory of pullets as they reached the growth target according to the guidebook management.

In this study, supplementation with both ALA- and DHA-enriched diets significantly increased omega-3 PUFA concentrations in the liver and plasma. This finding supports previous research from our lab (13, 178) and confirms that dietary omega-3 PUFA are efficiently incorporated into tissues. This is consistent with the competitive enzymatic pathways shared by omega-3 and omega-6 PUFA (80). Additionally, the significant reduction in ARA in the liver and plasma, with a more pronounced effect in the DHA-rich algal biomass group, reflects the inhibitory effect of dietary DHA on ARA metabolism, likely through the suppression of Δ-6 desaturase activity (239). The LPS-induced increase in plasma ARA suggests an acute inflammatory response, as ARA serves as a precursor for pro-inflammatory eicosanoids (231). However, the FA deposition in the liver does not always reflect the effects plasma levels, especially regarding the feeding of ALA (194). Thus, this study confirms previous findings on the relationship between dietary omega-3 supplementation and omega-3 accumulation patterns in the tissues of laying hens, including egg yolk. While most of the past research has focused on egg yolk enrichment, this study now turns attention to the potential benefits of omega-3 supplementation on bird health and inflammatory responses when faced with an inflammatory challenge.

Dietary omega-3 PUFA intake has been shown to significantly influence the lipidome in various species (76, 178, 224, 240). In the current study, we provided a comprehensive and novel characterization of the plasma oxylipin profile in laying hens, focusing on oxylipins derived from LA, ARA, ALA, EPA, and DHA. As expected, dietary supplementation with ALA resulted in higher plasma concentrations of ALA and a corresponding increase in total ALA-derived oxylipins, including individual oxylipins, compared to supplementation with DHA. Although information on the effects of FSO supplementation on ALA oxylipin accumulation in layers is limited, findings from human studies align with our results. For instance, studies have demonstrated that higher doses of FSO and/or longer supplementation periods lead to increased levels of ALA-derived oxylipins in humans (207, 241). Furthermore, although the functional roles of ALA-derived oxylipins in poultry are not well-documented, recent evidence suggests that increased ALA oxylipins, such as 9- and 13-HOTrE, in THP-1 macrophages treated with ALA and LPS, are associated with suppressed production of pro-inflammatory cytokines, including TNF-α, IL-1β, and IL-6 (180). Our study confirmed that dietary ALA supplementation had minimal effects on DHA-derived oxylipins, which is consistent with our previous study in laying hens (31), underscoring the limited metabolic conversion of ALA to DHA in avian species.

On the other hand, supplementation with increasing levels of DHA, but not ALA, resulted in higher concentrations of DHA in both the liver and plasma, along with increased levels of DHA-derived oxylipins. These findings are consistent with previous studies (194). Interestingly, the DHA diet also elevated EPA-derived oxylipins. While a study by Metherel et al. (242) where the compound-specific isotope analysis was used, reported that retro-conversion of DHA to EPA is a minor contributor to EPA levels in rats, limited evidence of such retro-conversion has been observed in chickens. Neijat et al. (178) further confirmed the absence of DHA-to-EPA retro-conversion, even with elevated dietary omega-3 PUFA levels in both ALA- and DHA-fed groups. The increased concentration of EPA in the DHA diet was likely due to its direct presence in the algal biomass rather than metabolic retro-conversion. This dietary EPA contributed to the observed increases in plasma and liver EPA levels, which subsequently led to elevated EPA-derived oxylipins. The increases in omega-3-derived oxylipins, particularly those derived from EPA and DHA, are noteworthy because these mediators play critical roles in resolving inflammation and promoting tissue repair (243, 244) (100). Their elevation in both saline- and LPS-treated groups suggests that dietary omega-3 supplementation provides a baseline anti-inflammatory reservoir that can be mobilized during immune challenges.

Our study demonstrated that dietary supplementation with DHA-rich algal biomass significantly reduced the levels of omega-6 oxylipins, particularly those derived from ARA. This observation aligns with findings from previous studies, which show that the inclusion of omega-3 PUFA in the diet decreases both ARA and AA-derived oxylipins in plasma and liver in rats, with DHA exerting a greater effect compared to ALA (194). The stronger potency of DHA compared to ALA in reducing ARA-derived oxylipins suggests that DHA plays a more prominent role in this process. This effect has been previously observed in various tissues, including rat myocardium, where DHA-rich fish oil feeding significantly reduced ARA-derived oxylipins (245, 246). These findings further showcased the distinct influence of DHA on modulating the omega-6 oxylipin profile and its potential to mitigate inflammatory processes. In our study, the LPS challenge significantly induced the production of some LA-derived and ARA-derived oxylipins, despite no observable effect on their precursor PUFA levels in either the liver or plasma. These findings align with previous studies, which have shown that LPS stimulates the innate immune response by activating monocytes and macrophages. This activation includes the upregulation of COX-2, a key enzyme involved in the production of pro-inflammatory eicosanoids (234, 247, 248). These results highlight the role of LPS in triggering inflammatory pathways through enhanced oxylipin biosynthesis, regardless of changes in precursor PUFA concentrations. However, the mechanisms underlying these processes, particularly in chickens, including pullets, remain underexplored and warrant further investigation to clarify species-specific inflammatory responses.

The LPS challenge significantly increased splenic cytokine expression, confirming the expected immune response (142, 249). Lower expressions of *IL-8* and *IL-10* in specific DHA treatments suggest a dose-dependent effect of DHA on modulating cytokine expression, as DHA has been shown to downregulate pro-inflammatory cytokines while maintaining or enhancing anti-inflammatory cytokine responses (250, 251). This finding highlights the immunomodulatory potential of DHA-enriched diets.

6.6 Conclusion

This study demonstrates that the differential effects of ALA- and DHA-enriched diets on oxylipin profiles have important implications for the health and productivity of pullets. DHA-rich algal biomass appears to be more effective than ALA-rich FSO in enhancing the production of anti-inflammatory omega-3 oxylipins while suppressing pro-inflammatory omega-6 oxylipins. This suggests that DHA diets could be a valuable strategy for mitigating inflammation-related challenges in poultry, such as those induced by environmental stressors or pathogenic infections. In addition, dietary supplementation with DHA by using algal biomass was more effective than ALA from FSO in modulating cytokine expression in response to LPS challenge, particularly at dietary DHA concentrations of 0.4% or higher. The findings highlight the need for further exploration of the functional consequences of changes in oxylipin profiles, particularly in relation to immune cell function, tissue repair, and systemic inflammatory responses. Additionally, investigating the temporal dynamics of oxylipin production and their interactions with cytokines during immune challenges could provide a deeper understanding of the mechanisms behind the anti-inflammatory effects of dietary omega-3 PUFA.

CHAPTER 7 GENERAL DISCUSSION AND CONCLUSION

7.1 General discussion

Essential fatty acids, such as LA (precursor of omega-6 PUFA) and ALA (precursor of omega-3 PUFA), are integral components of poultry feeding systems (54). These FA are considered essential because hens lack the necessary desaturase enzymes to insert double bonds beyond the Δ-9 carbon, a process that can only occur in plants. Once a double bond is introduced at the 3rd or 6th carbon (from the CH_3 end), hens can elongate the carbon chain and add further double bonds to produce LC-PUFA of 20 or 22 carbons. This synthesis primarily occurs in the liver and involves three key steps: Δ-6 desaturation, chain elongation, and Δ-5 desaturation. Through this pathway, ALA is converted into EPA, which is further elongated to DPA and finally converted to DHA via Δ-6 desaturation, chain elongation, and peroxisomal β-oxidation of DPA (252). Similarly, LA undergoes the same pathway, with ARA being the primary metabolite produced.

In typical breeder hen diets, LA constitutes over 50% of TFA, while ALA makes up only ~3–3.5%, reflecting the predominance of corn and other omega-6 PUFA-rich dietary fat sources. This imbalance in dietary omega-6 and omega-3 PUFA results in a lack of LC-PUFA in commercial hatching eggs (253). Oils from corn, sunflower, and safflower are major sources of LA, while limited economical and practical sources of omega-3-rich oils are available for poultry feeding.

Common plant-based sources of omega-3 PUFA include flaxseed, canola, and chia. Flaxseed and FSO, due to their high fat (>38%) and ALA (>50%) content, along with other nutritional benefits (e.g., metabolizable energy, protein), are the most extensively studied ingredients for their ability to enhance the omega-3 PUFA content in eggs (254, 255). Hens that are fed flaxseed primarily incorporate ALA into eggs, while marine sources, such as FO, are more effective at enriching eggs with LC- omega-3 PUFA, such as EPA, DPA, and DHA. Previous studies conducted in our lab using hempseed (7% ALA) and hempseed oil (18% ALA) showed low conversion efficiencies of ALA to DHA in egg yolk (3.5% and 12.5%, respectively). However, the use of FSO improved this conversion efficiency, resulting in 11% DHA and 29% total omega-3 PUFA content in eggs (32). Despite this improvement, the low conversion rate of ALA to DHA indicates the need for long-chain PUFA-enriched sources, such as FO and marine products, in poultry diets. Consequently, our research utilized FSO (high in ALA), EPA- and DHA-rich FO, and DHA-rich algal biomass in pullet and laying hen diets to investigate their effects on omega-3 PUFA deposition and immune responses.

Based on previous studies conducted in our lab with hempseed products (13, 59, 178), an optimal daily intake of 500–600 mg of total omega-3 PUFA per hen, primarily ALA, was identified as the ideal range to achieve the highest DHA enrichment in eggs. This intake level was determined for hens consuming approximately 100 g of feed per day. Although flaxseed and FSO result in greater DHA incorporation into egg yolk (up to 90 mg/yolk;

(58, 256), compared to hempseed products (~50 mg/yolk) (13, 59), the efficiency of DHA deposition from flaxseed-based diets begins to decline within a similar ALA intake range as observed with hempseed ingredients (256, 257). For the current study, a daily omega-3 PUFA intake of 600 mg/hen was used as a reference to determine the highest supplementation level in laying hen diets. In study 2, diets were formulated to provide 0.4% total omega-3 PUFA, primarily from EPA- and DHA-rich FO. In study 3, pullet diets included four levels of total omega-3 PUFA supplementation (ranging from 0.03% to 0.6%) derived from either ALA-rich FSO or DHA-rich algal biomass.

The digestive, immune, and integumentary (skin and feathers) systems undergo rapid growth and development during the first six weeks of a modern pullet's life (258). Pullets experiencing higher levels of stress during this critical growth phase are more likely to exhibit poor body uniformity, which can negatively impact their lifetime EP (237). Common stressors during the rearing period include vaccination, handling, heat exposure, and feed changes, which may occur individually or simultaneously. These stressors can activate stress responses, impair immunity, and adversely affect growth and overall well-being (259). In addition, laying hens reared in intensive commercial conditions are frequently exposed to pathogen-associated molecular patterns, such as LPS from gram-negative bacteria and lipoteichoic acid from gram-positive bacteria. The inhalation of endotoxins from environmental gram-negative bacteria has been identified as a significant health issue in poultry. The administration of LPS in chickens is known to stimulate the immune system by activating NFκB, triggering the release of acute-phase proteins and proinflammatory cytokines, upregulating TLR2 and TLR4 expression, and modulating antibody responses (260). Clinical symptoms commonly observed include fever, anorexia, and reduced growth (260, 261).

Given these challenges, this project employed LPS as a model for bacterial infection to experimentally challenge pullets and laying hens. In this study, laying hens received an intravenous injection of *E. coli* LPS at 8 mg/kg BW. Chickens are known to be more resistant to endotoxins from gram-negative bacteria (151), and this LPS dosage is sufficient to induce an acute-phase response, as demonstrated in our previous studies (28-30). Previous research has shown that LPS induces the release of proinflammatory cytokines such as IFN-γ, IL-1β, and IL-6 (but not TNF-α), with peak levels occurring 3–4 hours post-LPS injection at both the mRNA expression level in the spleen and the protein expression level in plasma (151, 153, 154, 156). However, Li et al. (31) reported no significant effects of diet or diet-LPS interaction on IFN-γ expression. In this project, a time-course study was conducted, revealing that most cytokine expressions peaked at 2 hours post-LPS challenge. Therefore, the 2-hour time point was selected as the sample collection time for subsequent studies (Study 2 and Study 3).

While omega-3 and omega-6 PUFA share the same metabolic pathway, each family exhibits distinctly different and sometimes opposing biological effects. Plasma concentrations, though commonly measured, serve only as a proxy for biological changes occurring in tissues (31, 218). Importantly, blood oxylipin profiles do not necessarily reflect those in tissues, as highlighted by Leng et al. (201). Future research should consider analyzing other tissues, such as the liver, to gain a more comprehensive understanding of oxylipin dynamics. Additionally, it is important to note that free oxylipins represent only a fraction of the total oxylipins present in tissues. Although free oxylipins are often considered the most active form and are frequently studied, esterified oxylipins also exhibit biological functions, as reported by O'Donnell and Murphy (262). The presence of esterified oxylipins, especially in tissues like the heart, may influence the interpretation of data. However, evidence from Schebb et al. (263) suggests that dietary modulation affects both free and esterified oxylipins in a similar manner.

Moreover, EPA- and DHA-derived eicosanoids and docosanoids, including resolvins (E-series and D-series), protectins, and maresins, play protective, anti-inflammatory, and pro-resolving roles in various inflammatory disease models (232, 233). These compounds may not be detectable in sufficient quantities at a single time point, such as 2 hours post-LPS challenge, underscoring the need for multi-timepoint studies to capture their temporal dynamics during the inflammatory response (264).

In addition to modulating the profile of lipid mediators involved in inflammatory processes, omega-3 PUFA influences the production of proinflammatory cytokines (265). Numerous studies have demonstrated that omega-3 PUFA, particularly EPA and DHA, can attenuate classic proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 in both *in vivo* and *in* *vitro* models in response to LPS (228, 266, 267). Our study only reported splenic gene expression of cytokines, which might not provide a comprehensive view of the effects of dietary FO interventions. Other factors, such as leukocyte function and circulating cytokine levels, should also be considered (26). Another limitation is the use of a single time-point approach. Michaeli, et al. (268) reported that the response times for plasma cytokines, including TNF-α and IL-6, differed between the control and FO treatments in healthy men following an LPS challenge. Therefore, assessing multiple time points during the acute phase of inflammation might reveal temporal changes between lipid mediators and cytokine biomarkers.

Overall, this study confirmed that dietary DHA and the combination of EPA+DHA suppressed inflammation through the production of LC-PUFA-derived oxylipins and cytokines in response to an LPS challenge. Additionally, omega-3 PUFA have been shown to exert immunomodulatory effects, influencing the activation of cellular and humoral immunity (269). Therefore, future research should investigate the effects of different types of omega-3 PUFA on cellular and humoral immune responses using an alternative immune challenge, such as vaccination.

7.2 General conclusion

Overall, omega-3 PUFA plays protective and anti-inflammatory roles by regulating oxylipin biosynthesis and influencing the production of receptors and cytokines, particularly under LPS-induced immune challenges, with LC-PUFA showing notable efficacy. Including omega-3 PUFA in the diet represents a promising strategy to enhance the overall health and immune function of laying hens. A deeper understanding of the relationship between dietary omega-3 PUFA intake and its effects on immunological responses could help reduce disease risks in the poultry industry.

CHAPTER 8 FUTURE DIRECTIONS

Future directions include:

To compare the impact of a control diet, modeled on commercial nutrition guidelines for both pullets, laying hens and breeding hens, against diets that contain three different types of omega-3 PUFA diets on:

1). Overall health and productivity in pullets and laying hens. In conjunction with performance parameters (EW, EP, BW, FI and feed efficiency) in both pullets and layers, serum antibodies will be measured, with blood samples obtained on before the vaccination as a baseline.

2). Cell-mediated and humoral immune response after vaccination. The proliferative responses, lymphocyte phenotypes will be measured in plasma and cytokine production will be detected in spleen in pullets and laying hens. Antibody titer responses and levels of certain virus induced by vaccine will be detected in serum and tissues respectively in pullets and laying hens.

3). Total antibodies in progeny/offspring. Total IgA levels will be measured in egg yolk.

CHAPTER 9 LITERATURE CITED

1. Jones DR, Anderson KE, Davis GS. The Effects of Genetic Selection on Production Parameters of Single Comb White Leghorn Hens. Poult Sci. 2001;80(8):1139-43.
2. Faluyi OB, Adebayo IA, Agbede JO. Influence of Hypo and Hyper Methionine Supplementation on Growth Performance and Immunological Responses to Newcastle Disease Vaccinations of Broiler Chickens. African journal of food, agriculture, nutrition, and development : AJFAND. 2017;15(5).
3. Wigley P. Salmonella Enterica in the Chicken: How It Has Helped Our Understanding of Immunology in a Non-Biomedical Model Species. Front Immunol. 2014;5:482.
4. Nhung NT, Chansiripornchai N, Carrique-Mas JJ. Antimicrobial Resistance in Bacterial Poultry Pathogens: A Review. Front Vet Sci. 2017;4:126.
5. Casewell M, Friis C, Marco E, McMullin P, Phillips I. The European Ban on Growth-Promoting Antibiotics and Emerging Consequences for Human and Animal Health. J Antimicrob Chemother. 2003;52(2):159-61.
6. Van Boeckel TP, Pires J, Silvester R, Zhao C, Song J, Criscuolo NG, et al. Global Trends in Antimicrobial Resistance in Animals in Low- and Middle-Income Countries. Science. 2019;365(6459):1266-.
7. Korver DR. Implications of Changing Immune Function through Nutrition in Poultry. Anim Feed Sci Technol. 2012;173(1-2):54-64.
8. Swiatkiewicz S, Arczewska-Wlosek A, Jozefiak D. The Relationship between Dietary Fat Sources and Immune Response in Poultry and Pigs: An Updated Review. Livest Sci. 2015;180:237-46.
9. Shahidi F, Ambigaipalan P. Omega-3 Polyunsaturated Fatty Acids and Their Health Benefits. Annu Rev Food Sci Technol. 2018;9(1):345-81.
10. Baker EJ, Miles EA, Burdge GC, Yaqoob P, Calder PC. Metabolism and Functional Effects of Plant-Derived Omega-3 Fatty Acids in Humans. Prog Lipid Res. 2016;64:30-56.
11. Konieczka P, Czauderna M, Smulikowska S. The Enrichment of Chicken Meat with Omega-3 Fatty Acids by Dietary Fish Oil or Its Mixture with Rapeseed or Flaxseed—Effect of Feeding Duration. Anim Feed Sci Technol. 2017;223:42-52.
12. Fraeye I, Bruneel C, Lemahieu C, Buyse J, Muylaert K, Foubert I. Dietary Enrichment of Eggs with Omega-3 Fatty Acids: A Review. Food Research International. 2012;48(2):961-9.
13. Neijat M, Suh M, Neufeld J, House JD. Increasing Levels of Dietary Hempseed Products Leads to Differential Responses in the Fatty Acid Profiles of Egg Yolk, Liver and Plasma of Laying Hens. Lipids. 2016;51(5):615-33.
14. Wils-Plotz EL, Klasing KC. Effects of Immunomodulatory Nutrients on Growth Performance and Immune-Related Gene Expression in Layer Chicks Challenged

with Lipopolysaccharide. Poult Sci. 2017;96(3):548-55.

15. Kolobaric N, Drenjancevic I, Matic A, Susnjara P, Mihaljevic Z, Mihalj M. Dietary Intake of N-3 Pufa-Enriched Hen Eggs Changes Inflammatory Markers' Concentration and Treg/Th17 Cells Distribution in Blood of Young Healthy Adults-a Randomised Study. Nutrients. 2021;13(6):1851.
16. Lee S-M, Kim HK, Lee H-B, Kwon O-D, Lee E-B, Bok J-D, et al. Effects of Flaxseed Supplementation on Omega-6 to Omega-3 Fatty Acid Ratio, Lipid Mediator Profile, Proinflammatory Cytokines and Stress Indices in Laying Hens. J Appl Anim Res. 2021;49(1):460-71.
17. Li Y, Zhao F, Wu Q, Li M, Zhu Y, Song S, et al. Fish Oil Diet May Reduce Inflammatory Levels in the Liver of Middle-Aged Rats. Sci Rep. 2017;7(1):6241.
18. Dang R, Zhou X, Tang M, Xu P, Gong X, Liu Y, et al. Fish Oil Supplementation Attenuates Neuroinflammation and Alleviates Depressive-Like Behavior in Rats Submitted to Repeated Lipopolysaccharide. Eur J Nutr. 2018;57(3):893-906.
19. Baker EJ, Valenzuela CA, De Souza CO, Yaqoob P, Miles EA, Calder PC. Comparative Anti-Inflammatory Effects of Plant- and Marine-Derived Omega-3 Fatty Acids Explored in an Endothelial Cell Line. Biochimica et biophysica acta Molecular and cell biology of lipids. 2020;1865(6):158662-.
20. Ellulu MS, Khaza'ai H, Patimah I, Rahmat A, Abed Y. Effect of Long Chain Omega-3 Polyunsaturated Fatty Acids on Inflammation and Metabolic Markers in Hypertensive and/or Diabetic Obese Adults: A Randomized Controlled Trial. Food Nutr Res. 2016;60:29268.
21. Jamilian M, Samimi M, Mirhosseini N, Afshar Ebrahimi F, Aghadavod E, Taghizadeh M, et al. A Randomized Double-Blinded, Placebo-Controlled Trial Investigating the Effect of Fish Oil Supplementation on Gene Expression Related to Insulin Action, Blood Lipids, and Inflammation in Gestational Diabetes Mellitus-Fish Oil Supplementation and Gestational Diabetes. Nutrients. 2018;10(2):163.
22. Gabbs M, Leng S, Devassy JG, Monirujjaman M, Aukema HM. Advances in Our Understanding of Oxylipins Derived from Dietary Pufas. Adv Nutr. 2015;6(5):513-40.
23. Fabian CJ, Kimler BF, Hursting SD. Omega-3 Fatty Acids for Breast Cancer Prevention and Survivorship. Breast Cancer Res. 2015;17(1):62.
24. Serhan CN, Petasis NA. Resolvins and Protectins in Inflammation Resolution. Chem Rev. 2011;111(10):5922-43.
25. Araujo P, Belghit I, Aarsæther N, Espe M, Lucena E, Holen E. The Effect of Omega-3 and Omega-6 Polyunsaturated Fatty Acids on the Production of Cyclooxygenase and Lipoxygenase Metabolites by Human Umbilical Vein Endothelial Cells. Nutrients. 2019;11(5):966.
26. Pauls SD, Rodway LR, Sidhu KK, Winter T, Sidhu N, Aukema HM, et al. Oils Rich in Alpha-Linolenic Acid or Docosahexaenoic Acid Have Distinct Effects on Plasma Oxylipin and Adiponectin Concentrations and on Monocyte Bioenergetics in

Women with Obesity. J Nutr. 2021;151(10):3053-66.

27. Brouwers H, Jonasdottir HS, Kuipers ME, Kwekkeboom JC, Auger JL, Gonzalez-Torres M, et al. Anti-Inflammatory and Proresolving Effects of the Omega-6 Polyunsaturated Fatty Acid Adrenic Acid. J Immunol. 2020;205(10):2840-9.
28. Jing M, Munyaka PM, Tactacan GB, Rodriguez-Lecompte JC, O K, House JD. Performance, Serum Biochemical Responses, and Gene Expression of Intestinal Folate Transporters of Young and Older Laying Hens in Response to Dietary Folic Acid Supplementation and Challenge with Escherichia Coli Lipopolysaccharide. Poult Sci. 2014;93(1):122-31.
29. Munyaka PM, Tactacan G, Jing M, O K, House JD, Rodriguez-Lecompte JC. Immunomodulation in Young Laying Hens by Dietary Folic Acid and Acute Immune Responses after Challenge with Escherichia Coli Lipopolysaccharide. Poult Sci. 2012;91(10):2454-63.
30. Munyaka PM, Tactacan G, Jing M, O K, House JD, St Paul M, et al. Response of Older Laying Hens to an Escherichia Coli Lipopolysaccharide Challenge When Fed Diets with or without Supplemental Folic Acid. Poult Sci. 2013;92(1):105-13.
31. Li S, Jing M, Mohamed N, Rey-Dubois C, Zhao S, Aukema HM, et al. The Effect of Increasing Concentrations of Omega-3 Fatty Acids from Either Flaxseed Oil or Preformed Docosahexaenoic Acid on Fatty Acid Composition, Plasma Oxylipin, and Immune Response of Laying Hens. J Nutr. 2023;153(7):2105-16.
32. Neijat M. Omega-3 Fatty Acid Enrichment of Chicken Eggs: Regulation of Long Chain Polyunsaturated Fatty Acid Metabolism in Laying Hens. 2014.
33. Simopoulos A. Evolutionary Aspects of Diet, Essential Fatty Acids and Cardiovascular Disease. European Heart Journal Supplements. 2001;3(suppl-D):D8-D21.
34. Calder PC. Mechanisms of Action of (N-3) Fatty Acids. J Nutr. 2012;142(3):592S-9S.
35. Simopoulos AP. An Increase in the Omega-6/Omega-3 Fatty Acid Ratio Increases the Risk for Obesity. Nutrients. 2016;8(3):128.
36. Leveille GA, Romsos DR, Yeh Y, O'Hea EK. Lipid Biosynthesis in the Chick. A Consideration of Site of Synthesis, Influence of Diet and Possible Regulatory Mechanisms. Poult Sci. 1975;54(4):1075-93.
37. Scorletti E, Byrne CD. Omega-3 Fatty Acids, Hepatic Lipid Metabolism, and Nonalcoholic Fatty Liver Disease. Annu Rev Nutr. 2013;33(1):231-48.
38. Horton JD, Shah NA, Warrington JA, Anderson NN, Park SW, Brown MS, et al. Combined Analysis of Oligonucleotide Microarray Data from Transgenic and Knockout Mice Identifies Direct Srebp Target Genes. Proc Natl Acad Sci U S A. 2003;100(21):12027-32.
39. Wang Y, Botolin D, Christian B, Busik J, Xu J, Jump DB. Tissue-Specific, Nutritional, and Developmental Regulation of Rat Fatty Acid Elongases. J Lipid Res. 2005;46(4):706-15.

40. Cho HP, Nakamura M, Clarke SD. Cloning, Expression, and Fatty Acid Regulation of the Human Delta-5 Desaturase. J Biol Chem. 1999;274(52):37335-9.
41. Cho HP, Nakamura MT, Clarke SD. Cloning, Expression, and Nutritional Regulation of the Mammalian Delta-6 Desaturase. J Biol Chem. 1999;274(1):471-7.
42. Leonard AE, Pereira SL, Sprecher H, Huang YS. Elongation of Long-Chain Fatty Acids. Prog Lipid Res. 2004;43(1):36-54.
43. James MJ, Ursin VM, Cleland LG. Metabolism of Stearidonic Acid in Human Subjects: Comparison with the Metabolism of Other N-3 Fatty Acids. Am J Clin Nutr. 2003;77(5):1140-5.
44. Gregory MK, Geier MS, Gibson RA, James MJ. Functional Characterization of the Chicken Fatty Acid Elongases. J Nutr. 2013;143(1):12-6.
45. Sijben JW, Klasing KC, Schrama JW, Parmentier HK, van der Poel JJ, Savelkoul HF, et al. Early in Vivo Cytokine Genes Expression in Chickens after Challenge with Salmonella Typhimurium Lipopolysaccharide and Modulation by Dietary N--3 Polyunsaturated Fatty Acids. Dev Comp Immunol. 2003;27(6-7):611-9.
46. Domenichiello AF, Kitson AP, Bazinet RP. Is Docosahexaenoic Acid Synthesis from Alpha-Linolenic Acid Sufficient to Supply the Adult Brain? Prog Lipid Res. 2015;59:54-66.
47. Wood KE, Mantzioris E, Gibson RA, Ramsden CE, Muhlhausler BS. The Effect of Modifying Dietary La and Ala Intakes on Omega-3 Long Chain Polyunsaturated Fatty Acid (N-3 Lcpufa) Status in Human Adults: A Systematic Review and Commentary. Prostaglandins Leukot Essent Fatty Acids. 2015;95:47-55.
48. Barcelo-Coblijn G, Murphy EJ. Alpha-Linolenic Acid and Its Conversion to Longer Chain N-3 Fatty Acids: Benefits for Human Health and a Role in Maintaining Tissue N-3 Fatty Acid Levels. Prog Lipid Res. 2009;48(6):355-74.
49. Burdge GC, Calder PC. Dietary Alpha-Linolenic Acid and Health-Related Outcomes: A Metabolic Perspective. Nutr Res Rev. 2006;19(1):26-52.
50. Saini RK, Keum YS. Omega-3 and Omega-6 Polyunsaturated Fatty Acids: Dietary Sources, Metabolism, and Significance - a Review. Life Sci. 2018;203:255-67.
51. Poorghasemi M, Seidavi A, Qotbi AA, Laudadio V, Tufarelli V. Influence of Dietary Fat Source on Growth Performance Responses and Carcass Traits of Broiler Chicks. Asian-Australas J Anim Sci. 2013;26(5):705-10.
52. Samman S, Kung FP, Carter LM, Foster MJ, Ahmad ZI, Phuyal JL, et al. Fatty Acid Composition of Certified Organic, Conventional and Omega-3 Eggs. Food Chem. 2009;116(4):911-4.
53. Mousa SA, Abdel-Rahe SM, Abdel-Rahe HA, Sadeek ALS. Effect of Dietary Fat Sources and Antioxidant Types on Growth Performance and Carcass Quality of Japanese Quails. International Journal of Poultry Science. 2017;16(11):443-50.
54. Alagawany M, Elnesr SS, Farag MR, Abd El-Hack ME, Khafaga AF, Taha AE, et al. Omega-3 and Omega-6 Fatty Acids in Poultry Nutrition: Effect on Production

Performance and Health. Animals (Basel). 2019;9(8):573.

55. Plourde M, Cunnane SC. Extremely Limited Synthesis of Long Chain Polyunsaturates in Adults: Implications for Their Dietary Essentiality and Use as Supplements. Appl Physiol Nutr Metab. 2007;32(4):619-34.

56. Aymond WM, Van Elswyk ME. Yolk Thiobarbituric Acid Reactive Substances and N-3 Fatty Acids in Response to Whole and Ground Flaxseed. Poult Sci. 1995;74(8):1388-94.

57. Ferrier LK, Caston L, Leeson S, James Squires E, Celi B, Thomas L, et al. Changes in Serum Lipids and Platelet Fatty Acid Composition Following Consumption of Eggs Enriched in Alpha-Linolenic Acid (Lna). Food Res Int. 1992;25(4):263-8.

58. Scheideler SE, Froning GW. The Combined Influence of Dietary Flaxseed Variety, Level, Form, and Storage Conditions on Egg Production and Composition among Vitamin E-Supplemented Hens. Poult Sci. 1996;75(10):1221-6.

59. Gakhar N, Goldberg E, Jing M, Gibson R, House JD. Effect of Feeding Hemp Seed and Hemp Seed Oil on Laying Hen Performance and Egg Yolk Fatty Acid Content: Evidence of Their Safety and Efficacy for Laying Hen Diets. Poult Sci. 2012;91(3):701-11.

60. Cachaldora P, García-Rebollar P, Alvarez C, Méndez J, De Blas JC. Double Enrichment of Chicken Eggs with Conjugated Linoleic Acid and N-3 Fatty Acids through Dietary Fat Supplementation. Anim Feed Sci Technol. 2008;144(3-4):315-26.

61. Fredriksson S, Elwinger K, Pickova J. Fatty Acid and Carotenoid Composition of Egg Yolk as an Effect of Microalgae Addition to Feed Formula for Laying Hens. Food Chem. 2006;99(3):530-7.

62. Poureslami R, Raes K, Turchini GM, Huyghebaert G, De Smet S. Effect of Diet, Sex and Age on Fatty Acid Metabolism in Broiler Chickens: N-3 and N-6 Pufa. Br J Nutr. 2010;104(2):189-97.

63. Tu WC, Cook-Johnson RJ, James MJ, Muhlhausler BS, Gibson RA. Omega-3 Long Chain Fatty Acid Synthesis Is Regulated More by Substrate Levels Than Gene Expression. Prostaglandins Leukot Essent Fatty Acids. 2010;83(2):61-8.

64. Manor ML, Derksen TJ, Magnuson AD, Raza F, Lei XG. Inclusion of Dietary Defatted Microalgae Dose-Dependently Enriches Ω-3 Fatty Acids in Egg Yolk and Tissues of Laying Hens. J Nutr. 2019;149(6):942-50.

65. Lawlor JB, Gaudette N, Dickson T, House JD. Fatty Acid Profile and Sensory Characteristics of Table Eggs from Laying Hens Fed Diets Containing Microencapsulated Fish Oil. Anim Feed Sci Technol. 2010;156(3-4):97-103.

66. Kaewsutas M, Sarikaphuti A, Nararatwanchai T, Sittiprapaporn P, Patchanee P. The Effects of Dietary Microalgae (Schizochytrium Spp.) and Fish Oil in Layers on Docosahexaenoic Acid Omega-3 Enrichment of the Eggs. J Appl Anim Nutr. 2016;4.

67. Irawan A, Ningsih N, Hafizuddin, Rusli RK, Suprayogi WPS, Akhirini N, et al.

Supplementary N-3 Fatty Acids Sources on Performance and Formation of Omega-3 in Egg of Laying Hens: A Meta-Analysis. Poult Sci. 2022;101(1):101566.

68. Aguillon-Paez YJ, Romero LA, Diaz GJ. Effect of Full-Fat Sunflower or Flaxseed Seeds Dietary Inclusion on Performance, Egg Yolk Fatty Acid Profile and Egg Quality in Laying Hens. Anim Nutr. 2020;6(2):179-84.
69. Cufadar Y, Gocmen R, Kanbur G. The Effect of Replacing Soya Bean Oil with Glycerol in Diets on Performance, Egg Quality and Egg Fatty Acid Composition in Laying Hens. Animal. 2016;10(1):19-24.
70. Dong XF, Liu S, Tong JM. Comparative Effect of Dietary Soybean Oil, Fish Oil, and Coconut Oil on Performance, Egg Quality and Some Blood Parameters in Laying Hens. Poult Sci. 2018;97(7):2460-72.
71. Westbrook LA, Cherian G. Egg Quality, Fatty-Acid Composition and Gastrointestinal Morphology of Layer Hens Fed Whole Flaxseed with Enzyme Supplementation. Br Poult Sci. 2019;60(2):146-53.
72. Huang S, Baurhoo B, Mustafa A. Effects of Extruded Flaxseed on Layer Performance, Nutrient Retention and Yolk Fatty Acid Composition. Br Poult Sci. 2018;59(4):463-9.
73. Huang S, Baurhoo B, Mustafa A. Effects of Feeding Extruded Flaxseed on Layer Performance, Total Tract Nutrient Digestibility, and Fatty Acid Concentrations of Egg Yolk, Plasma and Liver. J Anim Physiol Anim Nutr (Berl). 2020;104(5):1365-74.
74. Kralik G, Kralik Z, Grcevic M, Galovic O, Hanzek D, Biazik E. Fatty Acid Profile of Eggs Produced by Laying Hens Fed Diets Containing Different Shares of Fish Oil. Poult Sci. 2021;100(10):101379.
75. Cherian G, Bautista-Ortega J, Goeger DE. Maternal Dietary N-3 Fatty Acids Alter Cardiac Ventricle Fatty Acid Composition, Prostaglandin and Thromboxane Production in Growing Chicks. Prostaglandins Leukot Essent Fatty Acids. 2009;80(5-6):297-303.
76. Hall JA, Jha S, Skinner MM, Cherian G. Maternal Dietary N-3 Fatty Acids Alter Immune Cell Fatty Acid Composition and Leukotriene Production in Growing Chicks. Prostaglandins Leukot Essent Fatty Acids. 2007;76(1):19-28.
77. Cherian G. Metabolic and Cardiovascular Diseases in Poultry: Role of Dietary Lipids. Poult Sci. 2007;86(5):1012-6.
78. Medzhitov R. Origin and Physiological Roles of Inflammation. Nature. 2008;454(7203):428-35.
79. Nathan C. Points of Control in Inflammation. Nature. 2002;420(6917):846-52.
80. Serhan CN, Levy BD. Resolvins in Inflammation: Emergence of the Pro-Resolving Superfamily of Mediators. J Clin Invest. 2018;128(7):2657-69.
81. Nathan C, Ding A. Nonresolving Inflammation. Cell. 2010;140(6):871-82.
82. Dennis EA, Norris PC. Erratum: Eicosanoid Storm in Infection and Inflammation. Nat Rev Immunol. 2015;15(11):724-.

83. Cha YI, Solnica-Krezel L, DuBois RN. Fishing for Prostanoids: Deciphering the Developmental Functions of Cyclooxygenase-Derived Prostaglandins. Dev Biol. 2006;289(2):263-72.

84. Dennis EA, Cao J, Hsu YH, Magrioti V, Kokotos G. Phospholipase A2 Enzymes: Physical Structure, Biological Function, Disease Implication, Chemical Inhibition, and Therapeutic Intervention. Chem Rev. 2011;111(10):6130-85.

85. Buczynski MW, Dumlao DS, Dennis EA. Thematic Review Series: Proteomics. An Integrated Omics Analysis of Eicosanoid Biology. J Lipid Res. 2009;50(6):1015-38.

86. Wang W, Zhu J, Lyu F, Panigrahy D, Ferrara KW, Hammock B, et al. Omega-3 Polyunsaturated Fatty Acids-Derived Lipid Metabolites on Angiogenesis, Inflammation and Cancer. Prostaglandins Other Lipid Mediat. 2014;113-115:13-20.

87. Sutherland M, Shankaranarayanan P, Schewe T, Nigam S. Evidence for the Presence of Phospholipid Hydroperoxide Glutathione Peroxidase in Human Platelets: Implications for Its Involvement in the Regulatory Network of the 12-Lipoxygenase Pathway of Arachidonic Acid Metabolism. Biochem J. 2001;353(Pt 1):91-100.

88. Tilley SL, Coffman TM, Koller BH. Mixed Messages: Modulation of Inflammation and Immune Responses by Prostaglandins and Thromboxanes. J Clin Invest. 2001;108(1):15-23.

89. Kroetz DL, Zeldin DC. Cytochrome P450 Pathways of Arachidonic Acid Metabolism. Curr Opin Lipidol. 2002;13(3):273-83.

90. Spector AA, Kim HY. Cytochrome P450 Epoxygenase Pathway of Polyunsaturated Fatty Acid Metabolism. Biochim Biophys Acta. 2015;1851(4):356-65.

91. Shahabi P, Siest G, Meyer UA, Visvikis-Siest S. Human Cytochrome P450 Epoxygenases: Variability in Expression and Role in Inflammation-Related Disorders. Pharmacol Ther. 2014;144(2):134-61.

92. Konkel A, Schunck WH. Role of Cytochrome P450 Enzymes in the Bioactivation of Polyunsaturated Fatty Acids. Biochim Biophys Acta. 2011;1814(1):210-22.

93. Kikut J, Komorniak N, Zietek M, Palma J, Szczuko M. Inflammation with the Participation of Arachidonic (Aa) and Linoleic Acid (La) Derivatives (Hetes and Hodes) Is Necessary in the Course of a Normal Reproductive Cycle and Pregnancy. J Reprod Immunol. 2020;141:103177.

94. Schuchardt JP, Schmidt S, Kressel G, Dong H, Willenberg I, Hammock BD, et al. Comparison of Free Serum Oxylipin Concentrations in Hyper- Vs. Normolipidemic Men. Prostaglandins Leukot Essent Fatty Acids. 2013;89(1):19-29.

95. Wall R, Ross RP, Fitzgerald GF, Stanton C. Fatty Acids from Fish: The Anti-Inflammatory Potential of Long-Chain Omega-3 Fatty Acids. Nutrition reviews. 2010;68(5):280-9.

96. Fer M, Dreano Y, Lucas D, Corcos L, Salaun JP, Berthou F, et al. Metabolism of Eicosapentaenoic and Docosahexaenoic Acids by Recombinant Human

Cytochromes P450. Arch Biochem Biophys. 2008;471(2):116-25.

97. Morisseau C, Inceoglu B, Schmelzer K, Tsai HJ, Jinks SL, Hegedus CM, et al. Naturally Occurring Monoepoxides of Eicosapentaenoic Acid and Docosahexaenoic Acid Are Bioactive Antihyperalgesic Lipids. J Lipid Res. 2010;51(12):3481-90.
98. Tejera N, Boeglin WE, Suzuki T, Schneider C. Cox-2-Dependent and -Independent Biosynthesis of Dihydroxy-Arachidonic Acids in Activated Human Leukocytes. J Lipid Res. 2012;53(1):87-94.
99. Bannenberg G, Serhan CN. Specialized Pro-Resolving Lipid Mediators in the Inflammatory Response: An Update. Biochim Biophys Acta. 2010;1801(12):1260-73.
100. Isobe Y, Kato T, Arita M. Emerging Roles of Eosinophils and Eosinophil-Derived Lipid Mediators in the Resolution of Inflammation. Front Immunol. 2012;3:270.
101. Deng B, Wang CW, Arnardottir HH, Li Y, Cheng CY, Dalli J, et al. Maresin Biosynthesis and Identification of Maresin 2, a New Anti-Inflammatory and Pro-Resolving Mediator from Human Macrophages. PLoS One. 2014;9(7):e102362.
102. Eilati E, Bahr JM, Hales DB. Long Term Consumption of Flaxseed Enriched Diet Decreased Ovarian Cancer Incidence and Prostaglandin E(2)in Hens. Gynecol Oncol. 2013;130(3):620-8.
103. Eilati E, Small CC, McGee SR, Kurrey NK, Hales DB. Anti-Inflammatory Effects of Fish Oil in Ovaries of Laying Hens Target Prostaglandin Pathways. Lipids Health Dis. 2013;12(1):152.
104. Liu G, An S, Yuan J, Guo Y, Liu D, Chen H, et al. Dietary Fish Oil and Zinc Reduced Plasma Prostaglandin E2 Content by Inhibiting Phospholipase A2 Production in Broilers. The Journal of Poultry Science. 2014;51(1):66-70.
105. Cherian G. Nutrition and Metabolism in Poultry: Role of Lipids in Early Diet. J Anim Sci Biotechnol. 2015;6(1):28.
106. Bautista-Ortega J, Goeger DE, Cherian G. Egg Yolk Omega-6 and Omega-3 Fatty Acids Modify Tissue Lipid Components, Antioxidant Status, and Ex Vivo Eicosanoid Production in Chick Cardiac Tissue. Poult Sci. 2009;88(6):1167-75.
107. Borish LC, Steinke JW. 2. Cytokines and Chemokines. J Allergy Clin Immunol. 2003;111(2 Suppl):S460-75.
108. Dinarello CA. Proinflammatory Cytokines. Chest. 2000;118(2):503-8.
109. O'Shea JJ, Ma A, Lipsky P. Cytokines and Autoimmunity. Nat Rev Immunol. 2002;2(1):37-45.
110. Dinarello CA. Historical Insights into Cytokines. Eur J Immunol. 2007;37 Suppl 1(Suppl 1):S34-45.
111. Scheller J, Chalaris A, Schmidt-Arras D, Rose-John S. The Pro- and Anti-Inflammatory Properties of the Cytokine Interleukin-6. Biochim Biophys Acta. 2011;1813(5):878-88.
112. Calder PC. Marine Omega-3 Fatty Acids and Inflammatory Processes: Effects,

Mechanisms and Clinical Relevance. Biochim Biophys Acta. 2015;1851(4):469-84.

113. Hayden MS, West AP, Ghosh S. Nf-Kappab and the Immune Response. Oncogene. 2006;25(51):6758-80.

114. Szanto A, Nagy L. The Many Faces of Ppargamma: Anti-Inflammatory by Any Means? Immunobiology. 2008;213(9-10):789-803.

115. Calder PC. Omega-3 Fatty Acids and Inflammatory Processes: From Molecules to Man. Biochem Soc Trans. 2017;45(5):1105-15.

116. Oh DY, Talukdar S, Bae EJ, Imamura T, Morinaga H, Fan W, et al. Gpr120 Is an Omega-3 Fatty Acid Receptor Mediating Potent Anti-Inflammatory and Insulin-Sensitizing Effects. Cell. 2010;142(5):687-98.

117. Sijben JW, Schrama JW, Parmentier HK, van der Poel JJ, Klasing KC. Effects of Dietary Polyunsaturated Fatty Acids on in Vivo Splenic Cytokine Mrna Expression in Layer Chicks Immunized with Salmonella Typhimurium Lipopolysaccharide. Poult Sci. 2001;80(8):1164-70.

118. Ibrahim D, El-Sayed R, Khater SI, Said EN, El-Mandrawy SAM. Changing Dietary N-6:N-3 Ratio Using Different Oil Sources Affects Performance, Behavior, Cytokines Mrna Expression and Meat Fatty Acid Profile of Broiler Chickens. Anim Nutr. 2018;4(1):44-51.

119. Maroufyan E, Kasim A, Ebrahimi M, Loh TC, Bejo MH, Zerihun H, et al. Omega-3 Polyunsaturated Fatty Acids Enrichment Alters Performance and Immune Response in Infectious Bursal Disease Challenged Broilers. Lipids Health Dis. 2012;11(1):15.

120. Yang X, Guo Y, He X, Yuan J, Yang Y, Wang Z. Growth Performance and Immune Responses in Chickens after Challenge with Lipopolysaccharide and Modulation by Dietary Different Oils. Animal. 2008;2(2):216-23.

121. Lowry SF. Human Endotoxemia: A Model for Mechanistic Insight and Therapeutic Targeting. Shock. 2005;24 Suppl 1(Supplement 1):94-100.

122. Moran AP, Prendergast MM, Appelmelk BJ. Molecular Mimicry of Host Structures by Bacterial Lipopolysaccharides and Its Contribution to Disease. FEMS Immunol Med Microbiol. 1996;16(2):105-15.

123. Bryant CE, Spring DR, Gangloff M, Gay NJ. The Molecular Basis of the Host Response to Lipopolysaccharide. Nat Rev Microbiol. 2010;8(1):8-14.

124. Palsson-McDermott EM, O'Neill LA. Signal Transduction by the Lipopolysaccharide Receptor, Toll-Like Receptor-4. Immunology. 2004;113(2):153-62.

125. Gioannini TL, Teghanemt A, Zhang D, Levis EN, Weiss JP. Monomeric Endotoxin:Protein Complexes Are Essential for Tlr4-Dependent Cell Activation. J Endotoxin Res. 2005;11(2):117-23.

126. Miyake K. Roles for Accessory Molecules in Microbial Recognition by Toll-Like Receptors. J Endotoxin Res. 2006;12(4):195-204.

127. Zielen S, Trischler J, Schubert R. Lipopolysaccharide Challenge: Immunological

Effects and Safety in Humans. Expert Rev Clin Immunol. 2015;11(3):409-18.

128. Ghosh S, May MJ, Kopp EB. Nf-Kappa B and Rel Proteins: Evolutionarily Conserved Mediators of Immune Responses. Annu Rev Immunol. 1998;16(1):225-60.

129. May MJ, Ghosh S. Signal Transduction through Nf-Kb. Immunology Today. 1998;19(2):80-8.

130. Ulevitch RJ, Tobias PS. Receptor-Dependent Mechanisms of Cell Stimulation by Bacterial Endotoxin. Annu Rev Immunol. 1995;13(1):437-57.

131. Ulevitch RJ, Tobias PS. Recognition of Gram-Negative Bacteria and Endotoxin by the Innate Immune System. Curr Opin Immunol. 1999;11(1):19-22.

132. Wright SD. Cd14 and Innate Recognition of Bacteria. J Immunol. 1995;155(1):6-8.

133. Gehad AE, Lillehoj HS, Hendricks GL, Mashaly MM. Initiation of Humoral Immunity. I. The Role of Cytokines and Hormones in the Initiation of Humoral Immunity Using T-Independent and T-Dependent Antigens. Developmental & Comparative Immunology. 2002;26(8):751-9.

134. Webel DM, Finck BN, Baker DH, Johnson RW. Time Course of Increased Plasma Cytokines, Cortisol, and Urea Nitrogen in Pigs Following Intraperitoneal Injection of Lipopolysaccharide. J Anim Sci. 1997;75(6):1514-20.

135. Vedder H, Schreiber W, Yassouridis A, Gudewill S, Galanos C, Pollmacher T. Dose-Dependence of Bacterial Lipopolysaccharide (Lps) Effects on Peak Response and Time Course of the Immune-Endocrine Host Response in Humans. Inflamm Res. 1999;48(2):67-74.

136. Kemna E, Pickkers P, Nemeth E, van der Hoeven H, Swinkels D. Time-Course Analysis of Hepcidin, Serum Iron, and Plasma Cytokine Levels in Humans Injected with Lps. Blood. 2005;106(5):1864-6.

137. Shini S, Kaiser P, Shini A, Bryden WL. Differential Alterations in Ultrastructural Morphology of Chicken Heterophils and Lymphocytes Induced by Corticosterone and Lipopolysaccharide. Vet Immunol Immunopathol. 2008;122(1-2):83-93.

138. Altieri A, Piyadasa H, Recksiedler B, Spicer V, Mookherjee N. Cytokines Il-17, Tnf and Ifn-Gamma Alter the Expression of Antimicrobial Peptides and Proteins Disparately: A Targeted Proteomics Analysis Using Somascan Technology. Vaccines (Basel). 2018;6(3):51.

139. Schuerwegh AJ, Dombrecht EJ, Stevens WJ, Van Offel JF, Bridts CH, De Clerck LS. Influence of Pro-Inflammatory (Il-1 Alpha, Il-6, Tnf-Alpha, Ifn-Gamma) and Anti-Inflammatory (Il-4) Cytokines on Chondrocyte Function. Osteoarthritis Cartilage. 2003;11(9):681-7.

140. Erf GF. Cell-Mediated Immunity in Poultry. Poult Sci. 2004;83(4):580-90.

141. Geng Y, Ma Q, Wang Z, Guo Y. Dietary Vitamin D(3) Supplementation Protects Laying Hens against Lipopolysaccharide-Induced Immunological Stress. Nutr Metab (Lond). 2018;15:58.

142. Kogut MH, Iqbal M, He H, Philbin V, Kaiser P, Smith A. Expression and Function of Toll-Like Receptors in Chicken Heterophils. Dev Comp Immunol. 2005;29(9):791-807.
143. Li Q, Verma IM. Erratum: Nf-Kb Regulation in the Immune System. Nat Rev Immunol. 2002;2(12):975-.
144. Liu SD, Song MH, Yun W, Lee JH, Kim HB, Cho JH. Effect of Carvacrol Essential Oils on Immune Response and Inflammation-Related Genes Expression in Broilers Challenged by Lipopolysaccharide. Poult Sci. 2019;98(5):2026-33.
145. Li Y, Zhang H, Chen YP, Yang MX, Zhang LL, Lu ZX, et al. Bacillus Amyloliquefaciens Supplementation Alleviates Immunological Stress in Lipopolysaccharide-Challenged Broilers at Early Age. Poult Sci. 2015;94(7):1504-11.
146. Xie H, Rath NC, Huff GR, Huff WE, Balog JM. Effects of Salmonella Typhimurium Lipopolysaccharide on Broiler Chickens. Poult Sci. 2000;79(1):33-40.
147. Oh S, Lillehoj HS, Lee Y, Bravo D, Lillehoj EP. Dietary Antibiotic Growth Promoters down-Regulate Intestinal Inflammatory Cytokine Expression in Chickens Challenged with Lps or Co-Infected with Eimeria Maxima and Clostridium Perfringens. Front Vet Sci. 2019;6:420.
148. Wu QJ, Wang YQ, Qi YX. Influence of Procyanidin Supplementation on the Immune Responses of Broilers Challenged with Lipopolysaccharide. Anim Sci J. 2017;88(7):983-90.
149. Wu QJ, Zhou YM, Wu YN, Zhang LL, Wang T. The Effects of Natural and Modified Clinoptilolite on Intestinal Barrier Function and Immune Response to Lps in Broiler Chickens. Vet Immunol Immunopathol. 2013;153(1-2):70-6.
150. Robinson BS, Arthur CM, Evavold B, Roback E, Kamili NA, Stowell CS, et al. The Sweet-Side of Leukocytes: Galectins as Master Regulators of Neutrophil Function. Front Immunol. 2019;10:1762.
151. Gehad AE, Lillehoj HS, Hendricks GL, 3rd, Mashaly MM. Initiation of Humoral Immunity. I. The Role of Cytokines and Hormones in the Initiation of Humoral Immunity Using T-Independent and T-Dependent Antigens. Dev Comp Immunol. 2002;26(8):751-9.
152. Gilani S, Howarth GS, Kitessa SM, Tran CD, Forder REA, Hughes RJ. Intestinal Permeability Induced by Lipopolysaccharide and Measured by Lactulose, Rhamnose and Mannitol Sugars in Chickens. Animal. 2017;11(7):1174-9.
153. Leshchinsky TV, Klasing KC. Divergence of the Inflammatory Response in Two Types of Chickens. Dev Comp Immunol. 2001;25(7):629-38.
154. Leshchinsky TV, Klasing KC. Profile of Chicken Cytokines Induced by Lipopolysaccharide Is Modulated by Dietary Alpha-Tocopheryl Acetate. Poult Sci. 2003;82(8):1266-73.
155. Hubrecht RC, Kirkwood J. The Ufaw Handbook on the Care and Management of

Laboratory and Other Research Animals. 8th ed. Newark: John Wiley & Sons, Incorporated, 2010.

156. Nakamura K, Mitarai Y, Yoshioka M, Koizumi N, Shibahara T, Nakajima Y. Serum Levels of Interleukin-6, Alpha1-Acid Glycoprotein, and Corticosterone in Two-Week-Old Chickens Inoculated with Escherichia Coli Lipopolysaccharide. Poult Sci. 1998;77(6):908-11.

157. Livak KJ, Schmittgen TD. Analysis of Relative Gene Expression Data Using Real-Time Quantitative Pcr and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.

158. Bai AP, Ouyang Q, Zhang W, Wang CH, Li SF. Probiotics Inhibit Tnf-Alpha-Induced Interleukin-8 Secretion of Ht29 Cells. World J Gastroenterol. 2004;10(3):455-7.

159. Echeverry H, Alizadeh M, Yitbarek A, Slominski B, Rodriguez-Lecompte JC. Yeast Cell Wall Polysaccharides Enhanced Expression of T Helper Type 1 and 2 Cytokines Profile in Chicken B Lymphocytes Exposed to Lps Challenge and Enzyme Treatment. Br Poult Sci. 2021;62(1):125-30.

160. Boshtam M, Asgary S, Kouhpayeh S, Shariati L, Khanahmad H. Aptamers against Pro- and Anti-Inflammatory Cytokines: A Review. Inflammation. 2017;40(1):340-9.

161. De Boever S, Beyaert R, Vandemaele F, Baert K, Duchateau L, Goddeeris B, et al. The Influence of Age and Repeated Lipopolysaccharide Administration on Body Temperature and the Concentration of Interleukin-6 and Igm Antibodies against Lipopolysaccharide in Broiler Chickens. Avian Pathol. 2008;37(1):39-44.

162. Cheng HW, Freire R, Pajor EA. Endotoxin Stress Responses in Chickens from Different Genetic Lines. 1. Sickness, Behavioral, and Physical Responses. Poult Sci. 2004;83(5):707-15.

163. Ottaway CA, Fong IW, da Silva B, Singer W, Karrass L. Integrative Aspects of a Human Model of Endotoxemia. Can J Physiol Pharmacol. 1998;76(5):473-8.

164. Oda S, Hirasawa H, Shiga H, Nakanishi K, Matsuda K, Nakamua M. Sequential Measurement of Il-6 Blood Levels in Patients with Systemic Inflammatory Response Syndrome (Sirs)/Sepsis. Cytokine. 2005;29(4):169-75.

165. Hong YH, Lillehoj HS, Lillehoj EP, Lee SH. Changes in Immune-Related Gene Expression and Intestinal Lymphocyte Subpopulations Following Eimeria Maxima Infection of Chickens. Vet Immunol Immunopathol. 2006;114(3-4):259-72.

166. Waititu SM, Yitbarek A, Matini E, Echeverry H, Kiarie E, Rodriguez-Lecompte JC, et al. Effect of Supplementing Direct-Fed Microbials on Broiler Performance, Nutrient Digestibilities, and Immune Responses. Poult Sci. 2014;93(3):625-35.

167. Tang Q, Chen W, Hendricks RL. Proinflammatory Functions of Il-2 in Herpes Simplex Virus Corneal Infection. J Immunol. 1997;158(3):1275-83.

168. Lowenthal JW, York JJ, O'Neil TE, Steven RA, Strom DG, Digby MR. Potential Use of Cytokine Therapy in Poultry. Vet Immunol Immunopathol. 1998;63(1-

2):191-8.

169. Nunes RA, Duarte MdS, Campos PHRF, de Oliveira LL, e Silva FF, Kreuz BS, et al. Active Vitamin D3-Glycoside Preserves Weight Gain and Modulates the Inflammatory Response in Broiler Chickens Challenged with Lipopolysaccharide. Anim Feed Sci Technol. 2020;270:114704.
170. Couper KN, Blount DG, Riley EM. Il-10: The Master Regulator of Immunity to Infection. J Immunol. 2008;180(9):5771-7.
171. Wang F, Liu J, Hu X, Zhong Y, Wen F, Tang X, et al. The Influence on Oxidative Stress Markers, Inflammatory Factors and Intestinal Injury-Related Molecules in Wahui Pigeon Induced by Lipopolysaccharide. PLoS One. 2021;16(5):e0251462.
172. Hammad S, Pu S, Jones PJ. Current Evidence Supporting the Link between Dietary Fatty Acids and Cardiovascular Disease. Lipids. 2016;51(5):507-17.
173. Punia S, Sandhu KS, Siroha AK, Dhull SB. Omega 3-Metabolism, Absorption, Bioavailability and Health Benefits–a Review. PharmaNutrition. 2019;10:100162.
174. Stupin A, Rasic L, Matic A, Stupin M, Kralik Z, Kralik G, et al. Omega-3 Polyunsaturated Fatty Acids-Enriched Hen Eggs Consumption Enhances Microvascular Reactivity in Young Healthy Individuals. Appl Physiol Nutr Metab. 2018;43(10):988-95.
175. Javed A, King AJ, Imran M, Jeoh T, Naseem S. Omega-3 Supplementation for Enhancement of Egg Functional Properties. J Food Process Preserv. 2019;43(8):n/a.
176. Feng J, Long S, Zhang HJ, Wu SG, Qi GH, Wang J. Comparative Effects of Dietary Microalgae Oil and Fish Oil on Fatty Acid Composition and Sensory Quality of Table Eggs. Poult Sci. 2020;99(3):1734-43.
177. Mariod AA, Mukhtar MAE, Salih ME, Herwan T. Effect of Addition of Fish Oil on the Performance Parameters of Laying Hens and the Fatty Acid Composition of Their Egg Yolk. American Journal of Food Science and Health. 2015;1(2):38-42.
178. Neijat M, Eck P, House JD. Impact of Dietary Precursor Ala Versus Preformed Dha on Fatty Acid Profiles of Eggs, Liver and Adipose Tissue and Expression of Genes Associated with Hepatic Lipid Metabolism in Laying Hens. Prostaglandins Leukot Essent Fatty Acids. 2017;119:1-17.
179. Keegan JD, Currie D, Knox A, Moran CA. Heterotrophic Aurantiochytrium Sp. Supplementation to Layer Diets Sustainably Increases the Omega-3 Concentration of Eggs. Br Poult Sci. 2019;60(5):570-8.
180. Pauls SD, Rodway LA, Winter T, Taylor CG, Zahradka P, Aukema HM. Anti-Inflammatory Effects of Alpha-Linolenic Acid in M1-Like Macrophages Are Associated with Enhanced Production of Oxylipins from Alpha-Linolenic and Linoleic Acid. J Nutr Biochem. 2018;57:121-9.
181. Caligiuri SPB, Parikh M, Stamenkovic A, Pierce GN, Aukema HM. Dietary Modulation of Oxylipins in Cardiovascular Disease and Aging. Am J Physiol Heart Circ Physiol. 2017;313(5):H903-H18.
182. Shearer GC, Walker RE. An Overview of the Biologic Effects of Omega-6

Oxylipins in Humans. Prostaglandins Leukot Essent Fatty Acids. 2018;137:26-38.

183. Caligiuri SP, Aukema HM, Ravandi A, Guzman R, Dibrov E, Pierce GN. Flaxseed Consumption Reduces Blood Pressure in Patients with Hypertension by Altering Circulating Oxylipins Via an Alpha-Linolenic Acid-Induced Inhibition of Soluble Epoxide Hydrolase. Hypertension. 2014;64(1):53-9.

184. Hercule HC, Schunck WH, Gross V, Seringer J, Leung FP, Weldon SM, et al. Interaction between P450 Eicosanoids and Nitric Oxide in the Control of Arterial Tone in Mice. Arterioscler Thromb Vasc Biol. 2009;29(1):54-60.

185. Lee CR, Imig JD, Edin ML, Foley J, DeGraff LM, Bradbury JA, et al. Endothelial Expression of Human Cytochrome P450 Epoxygenases Lowers Blood Pressure and Attenuates Hypertension-Induced Renal Injury in Mice. FASEB J. 2010;24(10):3770-81.

186. Kopkan L, Huskova Z, Sporkova A, Varcabova S, Honetschlagerova Z, Hwang SH, et al. Soluble Epoxide Hydrolase Inhibition Exhibits Antihypertensive Actions Independently of Nitric Oxide in Mice with Renovascular Hypertension. Kidney Blood Press Res. 2012;35(6):595-607.

187. Ncho CM, Berdos JI, Gupta V, Rahman A, Mekonnen KT, Bakhsh A. Abiotic Stressors in Poultry Production: A Comprehensive Review. J Anim Physiol Anim Nutr (Berl). 2025;109(1):30-50.

188. Abo-Al-Ela HG, El-Kassas S, El-Naggar K, Abdo SE, Jahejo AR, Al Wakeel RA. Stress and Immunity in Poultry: Light Management and Nanotechnology as Effective Immune Enhancers to Fight Stress. Cell Stress Chaperones. 2021;26(3):457-72.

189. Dohms JE, Metz A. Stress--Mechanisms of Immunosuppression. Vet Immunol Immunopathol. 1991;30(1):89-109.

190. Dietert RR, Golemboski KA, Austic RE. Environment-Immune Interactions. Poult Sci. 1994;73(7):1062-76.

191. Akbari Moghaddam Kakhki R, Kiarie EG. Effect of Escherichia Coli Lipopolysaccharide Challenge on Eggshell, Tibia, and Keel Bone Attributes in Isa Brown Hens Exposed to Dietary N-3 Fatty Acids Prior to Onset of Lay. Poult Sci. 2021;100(11):101431.

192. Lee SM, Kim HK, Lee HB, Kwon OD, Lee EB, Bok JD, et al. Effects of Flaxseed Supplementation on Omega-6 to Omega-3 Fatty Acid Ratio, Lipid Mediator Profile, Proinflammatory Cytokines and Stress Indices in Laying Hens. J Appl Anim Res. 2021;49(1):460-71.

193. Liu J, Zhao L, Zhao Z, Wu Y, Cao J, Cai H, et al. Rubber (Hevea Brasiliensis) Seed Oil Supplementation Attenuates Immunological Stress and Inflammatory Response in Lipopolysaccharide-Challenged Laying Hens. Poult Sci. 2022;101(9):102040.

194. Leng S, Winter T, Aukema HM. Dietary Ala, Epa and Dha Have Distinct Effects on Oxylipin Profiles in Female and Male Rat Kidney, Liver and Serum. J Nutr Biochem. 2018;57:228-37.

195. Mendonca AM, Cayer LGJ, Pauls SD, Winter T, Leng S, Taylor CG, et al. Distinct Effects of Dietary Ala, Epa and Dha on Rat Adipose Oxylipins Vary by Depot Location and Sex. Prostaglandins Leukot Essent Fatty Acids. 2018;129:13-24.
196. So J, Wu D, Lichtenstein AH, Tai AK, Matthan NR, Maddipati KR, et al. Epa and Dha Differentially Modulate Monocyte Inflammatory Response in Subjects with Chronic Inflammation in Part Via Plasma Specialized Pro-Resolving Lipid Mediators: A Randomized, Double-Blind, Crossover Study. Atherosclerosis. 2021;316:90-8.
197. da Cunha de Sa RDC, Simao JJ, Silva VSD, Farias TM, Cruz MM, Antraco VJ, et al. Fish Oil Enriched in Epa, but Not in Dha, Reverses the Metabolic Syndrome and Adipocyte Dysfunction Induced by a High-Fat Diet. Nutrients. 2021;13(3):1-18.
198. Care CCOA. Ccac Guidelines On: Euthanasia of Animals Used in Science. Ottawa, 2010.
199. Jing M, Li S, Zhao S, House JD. Splenic Gene Expression of Cytokines at Multiple Time Points Following Lipopolysaccharide Challenge in Layers. Am J Vet Res. 2024;85(5):1-7.
200. Folch J, Lees M, Sloane Stanley GH. A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues. J Biol Chem. 1957;226(1):497-509.
201. Leng S, Winter T, Aukema HM. Dietary La and Sex Effects on Oxylipin Profiles in Rat Kidney, Liver, and Serum Differ from Their Effects on Pufas. J Lipid Res. 2017;58(8):1702-12.
202. Monirujjaman M, Devassy JG, Yamaguchi T, Sidhu N, Kugita M, Gabbs M, et al. Distinct Oxylipin Alterations in Diverse Models of Cystic Kidney Diseases. Biochim Biophys Acta Mol Cell Biol Lipids. 2017;1862(12):1562-74.
203. Pfaffl MW. A New Mathematical Model for Relative Quantification in Real-Time Rt-Pcr. Nucleic Acids Res. 2001;29(9):e45.
204. Gabbs M, Zahradka P, Taylor CG, Aukema HM. Time Course and Sex Effects of Alpha-Linolenic Acid-Rich and Dha-Rich Supplements on Human Plasma Oxylipins: A Randomized Double-Blind Crossover Trial. J Nutr. 2021;151(3):513-22.
205. Devassy JG, Leng S, Gabbs M, Monirujjaman M, Aukema HM. Omega-3 Polyunsaturated Fatty Acids and Oxylipins in Neuroinflammation and Management of Alzheimer Disease. Adv Nutr. 2016;7(5):905-16.
206. Weylandt KH, Chiu CY, Gomolka B, Waechter SF, Wiedenmann B. Omega-3 Fatty Acids and Their Lipid Mediators: Towards an Understanding of Resolvin and Protectin Formation. Prostaglandins Other Lipid Mediat. 2012;97(3-4):73-82.
207. Greupner T, Kutzner L, Nolte F, Strangmann A, Kohrs H, Hahn A, et al. Effects of a 12-Week High-Alpha-Linolenic Acid Intervention on Epa and Dha Concentrations in Red Blood Cells and Plasma Oxylipin Pattern in Subjects with a Low Epa and Dha Status. Food Funct. 2018;9(3):1587-600.

208. Hampson AJ, Grimaldi M. 12-Hydroxyeicosatetrenoate (12-Hete) Attenuates Ampa Receptor-Mediated Neurotoxicity: Evidence for a G-Protein-Coupled Hete Receptor. J Neurosci. 2002;22(1):257-64.
209. Cambiaggi L, Chakravarty A, Noureddine N, Hersberger M. The Role of Alpha-Linolenic Acid and Its Oxylipins in Human Cardiovascular Diseases. Int J Mol Sci. 2023;24(7):6110.
210. Zahradka P, Neumann S, Aukema HM, Taylor CG. Adipocyte Lipid Storage and Adipokine Production Are Modulated by Lipoxygenase-Derived Oxylipins Generated from 18-Carbon Fatty Acids. Int J Biochem Cell Biol. 2017;88:23-30.
211. Shi Z, He Z, Wang DW. Cyp450 Epoxygenase Metabolites, Epoxyeicosatrienoic Acids, as Novel Anti-Inflammatory Mediators. Molecules. 2022;27(12):3873.
212. Borsini A, Nicolaou A, Camacho-Munoz D, Kendall AC, Di Benedetto MG, Giacobbe J, et al. Omega-3 Polyunsaturated Fatty Acids Protect against Inflammation through Production of Lox and Cyp450 Lipid Mediators: Relevance for Major Depression and for Human Hippocampal Neurogenesis. Mol Psychiatry. 2021;26(11):6773-88.
213. Yang T, Peng R, Guo Y, Shen L, Zhao S, Xu D. The Role of 14,15-Dihydroxyeicosatrienoic Acid Levels in Inflammation and Its Relationship to Lipoproteins. Lipids Health Dis. 2013;12(1):151.
214. Arnardottir H, Pawelzik SC, Sarajlic P, Quaranta A, Kolmert J, Religa D, et al. Immunomodulation by Intravenous Omega-3 Fatty Acid Treatment in Older Subjects Hospitalized for Covid-19: A Single-Blind Randomized Controlled Trial. Clin Transl Med. 2022;12(9):e895.
215. Yang J, Oh YT, Wan D, Watanabe RM, Hammock BD, Youn JH. Postprandial Effect to Decrease Soluble Epoxide Hydrolase Activity: Roles of Insulin and Gut Microbiota. J Nutr Biochem. 2017;49:8-14.
216. Farias NC, Borelli-Montigny GL, Fauaz G, Feres T, Borges AC, Paiva TB. Different Mechanism of Lps-Induced Vasodilation in Resistance and Conductance Arteries from Shr and Normotensive Rats. Br J Pharmacol. 2002;137(2):213-20.
217. Pleiner J, Mittermayer F, Schaller G, Marsik C, MacAllister RJ, Wolzt M. Inflammation-Induced Vasoconstrictor Hyporeactivity Is Caused by Oxidative Stress. J Am Coll Cardiol. 2003;42(9):1656-62.
218. Schuchardt JP, Ostermann AI, Stork L, Fritzsch S, Kohrs H, Greupner T, et al. Effect of Dha Supplementation on Oxylipin Levels in Plasma and Immune Cell Stimulated Blood. Prostaglandins Leukot Essent Fatty Acids. 2017;121:76-87.
219. Elhamouly M, Nii T, Isobe N, Yoshimura Y. Expression of Pro- and Anti-Inflammatory Cytokines and Chemokines during the Ovulatory Cycle and Effects of Aging on Their Expression in the Uterine Mucosa of Laying Hens. Cytokine. 2018;111:303-8.
220. Rattanasrisomporn J, Tantikositruj C, Thiptara A, Kitpipit W, Wichianrat I, Kayan A, et al. Pro-Inflammatory Cytokine Release from Chicken Peripheral Blood

Mononuclear Cells Stimulated with Lipopolysaccharide. Vet World. 2022;15(4):885-9.

221. Al-Khalaifah H, Al-Nasser A, Givens DI, Rymer C, Yaqoob P. Comparison of Different Dietary Sources of N-3 Polyunsaturated Fatty Acids on Immune Response in Broiler Chickens. Heliyon. 2020;6(1):e03326.

222. Cachaldora P, García-Rebollar P, Alvarez C, De Blas JC, Méndez J. Effect of Type and Level of Basal Fat and Level of Fish Oil Supplementation on Yolk Fat Composition and N-3 Fatty Acids Deposition Efficiency in Laying Hens. Anim Feed Sci and Technol. 2008;141(1-2):104-14.

223. Gao Z, Zhang J, Li F, Zheng J, Xu G. Effect of Oils in Feed on the Production Performance and Egg Quality of Laying Hens. Animals (Basel). 2021;11(12):3482.

224. Neijat M, Ojekudo O, House JD. Effect of Flaxseed Oil and Microalgae Dha on the Production Performance, Fatty Acids and Total Lipids of Egg Yolk and Plasma in Laying Hens. Prostaglandins Leukot Essent Fatty Acids. 2016;115:77-88.

225. Cachaldora P, Garcia-Rebollar P, Alvarez C, De Blas JC, Mendez J. Effect of Type and Level of Fish Oil Supplementation on Yolk Fat Composition and N-3 Fatty Acids Retention Efficiency in Laying Hens. Br Poult Sci. 2006;47(1):43-9.

226. Goldberg EM, Gakhar N, Ryland D, Aliani M, Gibson RA, House JD. Fatty Acid Profile and Sensory Characteristics of Table Eggs from Laying Hens Fed Hempseed and Hempseed Oil. J Food Sci. 2012;77(4):S153-60.

227. Rey C, Nadjar A, Buaud B, Vaysse C, Aubert A, Pallet V, et al. Resolvin D1 and E1 Promote Resolution of Inflammation in Microglial Cells in Vitro. Brain Behav Immun. 2016;55:249-59.

228. Orr SK, Palumbo S, Bosetti F, Mount HT, Kang JX, Greenwood CE, et al. Unesterified Docosahexaenoic Acid Is Protective in Neuroinflammation. J Neurochem. 2013;127(3):378-93.

229. Laye S, Nadjar A, Joffre C, Bazinet RP. Anti-Inflammatory Effects of Omega-3 Fatty Acids in the Brain: Physiological Mechanisms and Relevance to Pharmacology. Pharmacol Rev. 2018;70(1):12-38.

230. Serhan CN, Chiang N, Dalli J. The Resolution Code of Acute Inflammation: Novel Pro-Resolving Lipid Mediators in Resolution. Semin Immunol. 2015;27(3):200-15.

231. Zivkovic AM, Telis N, German JB, Hammock BD. Dietary Omega-3 Fatty Acids Aid in the Modulation of Inflammation and Metabolic Health. Calif Agric (Berkeley). 2011;65(3):106-11.

232. Bannenberg GL, Chiang N, Ariel A, Arita M, Tjonahen E, Gotlinger KH, et al. Molecular Circuits of Resolution: Formation and Actions of Resolvins and Protectins. J Immunol. 2005;174(7):4345-55.

233. Groeger AL, Cipollina C, Cole MP, Woodcock SR, Bonacci G, Rudolph TK, et al. Cyclooxygenase-2 Generates Anti-Inflammatory Mediators from Omega-3 Fatty Acids. Nat Chem Biol. 2010;6(6):433-41.

234. Calder PC. Dietary Modification of Inflammation with Lipids. Proc Nutr Soc.

2002;61(3):345-58.
235. Zhang G, Ghosh S. Molecular Mechanisms of Nf-Kappab Activation Induced by Bacterial Lipopolysaccharide through Toll-Like Receptors. J Endotoxin Res. 2000;6(6):453-7.
236. Lee J, Cheng V, Kiarie EG. Growth and Response to Escherichia Coli Lipopolysaccharide Challenge in Lohmann Lsl-Lite Pullets When Fed a Source of Omega-3 Fatty Acids and Yeast Bioactives from Hatch through to 16 Wk of Age. Poult Sci. 2023;102(10):102940.
237. Leeson S, Summers JD. Commercial Poultry Nutrition. 3rd ed. Guelph, Ont: University Books, 2005.
238. Deems R, Buczynski MW, Bowers-Gentry R, Harkewicz R, Dennis EA. Detection and Quantitation of Eicosanoids Via High Performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry. Methods Enzymol. United States: Elsevier Science & Technology, 2007:59-82.
239. Burdge GC, Calder PC. Conversion of Alpha-Linolenic Acid to Longer-Chain Polyunsaturated Fatty Acids in Human Adults. Reprod Nutr Dev. 2005;45(5):581-97.
240. Selvaraj RK, Cherian G. Dietaryn-3 Fatty Acids Reduce the Delayed Hypersensitivity Reaction and Antibody Production More Thann-6 Fatty Acids in Broiler Birds. European Journal of Lipid Science and Technology. 2004;106(1):3-10.
241. Meuronen T, Lankinen MA, Fauland A, Shimizu BI, de Mello VD, Laaksonen DE, et al. Intake of Camelina Sativa Oil and Fatty Fish Alter the Plasma Lipid Mediator Profile in Subjects with Impaired Glucose Metabolism - a Randomized Controlled Trial. Prostaglandins Leukot Essent Fatty Acids. 2020;159:102143.
242. Metherel AH, Chouinard-Watkins R, Trepanier MO, Lacombe RJS, Bazinet RP. Retroconversion Is a Minor Contributor to Increases in Eicosapentaenoic Acid Following Docosahexaenoic Acid Feeding as Determined by Compound Specific Isotope Analysis in Rat Liver. Nutr Metab (Lond). 2017;14(1):75.
243. Ulu A, Harris TR, Morisseau C, Miyabe C, Inoue H, Schuster G, et al. Anti-Inflammatory Effects of Omega-3 Polyunsaturated Fatty Acids and Soluble Epoxide Hydrolase Inhibitors in Angiotensin-Ii-Dependent Hypertension. J Cardiovasc Pharmacol. 2013;62(3):285-97.
244. Ishihara T, Yoshida M, Arita M. Omega-3 Fatty Acid-Derived Mediators That Control Inflammation and Tissue Homeostasis. Int Immunol. 2019;31(9):559-67.
245. Devassy JG, Yamaguchi T, Monirujjaman M, Gabbs M, Ravandi A, Zhou J, et al. Distinct Effects of Dietary Flax Compared to Fish Oil, Soy Protein Compared to Casein, and Sex on the Renal Oxylipin Profile in Models of Polycystic Kidney Disease. Prostaglandins Leukot Essent Fatty Acids. 2017;123:1-13.
246. Ibrahim NH, Jia Y, Devassy JG, Yamaguchi T, Aukema HM. Renal Cyclooxygenase and Lipoxygenase Products Are Altered in Polycystic Kidneys and

by Dietary Soy Protein and Fish Oil Treatment in the Han:Sprd-Cy Rat. Mol Nutr Food Res. 2014;58(4):768-81.

247. Janský L, Vybíral S, Pospíšilová D, Roth J, Dornand J, Zeisberger E, et al. Production of Systemic and Hypothalamic Cytokines during the Early Phase of Endotoxin Fever. 1995;62(1):55-61.

248. Demasi M, Caughey GE, James MJ, Cleland LG. Assay of Cyclooxygenase-1 and 2 in Human Monocytes. Inflamm Res. 2000;49(12):737-43.

249. Park BS, Lee JO. Recognition of Lipopolysaccharide Pattern by Tlr4 Complexes. Exp Mol Med. 2013;45(12):e66.

250. Hwang EH, Kim TH, Oh SM, Lee KB, Yang SJ, Park JH. Toll/Il-1 Domain-Containing Adaptor Inducing Ifn-Beta (Trif) Mediates Innate Immune Responses in Murine Peritoneal Mesothelial Cells through Tlr3 and Tlr4 Stimulation. Cytokine. 2016;77:127-34.

251. Rogero MM, Calder PC. Obesity, Inflammation, Toll-Like Receptor 4 and Fatty Acids. Nutrients. 2018;10(4):432.

252. Brenner RR. The Desaturation Step in the Animal Biosynthesis of Polyunsaturated Fatty Acids. Lipids. 1971;6(8):567-75.

253. Cherian G. Egg Quality and Yolk Polyunsaturated Fatty Acid Status in Relation to Broiler Breeder Hen Age and Dietary N-3 Oils. Poult Sci. 2008;87(6):1131-7.

254. González-Esquerra R, Leeson S. Alternatives for Enrichment of Eggs and Chicken Meat with Omega-3 Fatty Acids. Can J Anim Sci. 2001;81(3):295-305.

255. Kajla P, Sharma A, Sood DR. Flaxseed-a Potential Functional Food Source. J Food Sci Technol. 2015;52(4):1857-71.

256. Baucells MD, Crespo N, Barroeta AC, Lopez-Ferrer S, Grashorn MA. Incorporation of Different Polyunsaturated Fatty Acids into Eggs. Poult Sci. 2000;79(1):51-9.

257. Cherian G, Sim JS. Effect of Feeding Full Fat Flax and Canola Seeds to Laying Hens on the Fatty Acid Composition of Eggs, Embryos, and Newly Hatched Chicks. Poult Sci. 1991;70(4):917-22.

258. Silva EP, Malheiros EB, Sakomura NK, Venturini KS, Hauschild L, Dorigam JCP, et al. Lysine Requirements of Laying Hens. Livest Sci. 2015;173:69-77.

259. Widowski T, Torrey S. Rearing Young Birds for Adaptability. Advances in Poultry Welfare, 2018:49-76.

260. Ploegaert TC, De Vries Reilingh G, Nieuwland MG, Lammers A, Savelkoul HF, Parmentier HK. Intratracheally Administered Pathogen-Associated Molecular Patterns Affect Antibody Responses of Poultry. Poult Sci. 2007;86(8):1667-76.

261. Star L, Nieuwland MG, Kemp B, Parmentier HK. Effect of Single or Combined Climatic and Hygienic Stress on Natural and Specific Humoral Immune Competence in Four Layer Lines. Poult Sci. 2007;86(9):1894-903.

262. O'Donnell VB, Murphy RC. New Families of Bioactive Oxidized Phospholipids Generated by Immune Cells: Identification and Signaling Actions. Blood.

2012;120(10):1985-92.

263. Schebb NH, Ostermann AI, Yang J, Hammock BD, Hahn A, Schuchardt JP. Comparison of the Effects of Long-Chain Omega-3 Fatty Acid Supplementation on Plasma Levels of Free and Esterified Oxylipins. Prostaglandins Other Lipid Mediat. 2014;113-115:21-9.
264. Balvers MG, Verhoeckx KC, Bijlsma S, Rubingh CM, Meijerink J, Wortelboer HM, et al. Fish Oil and Inflammatory Status Alter the N-3 to N-6 Balance of the Endocannabinoid and Oxylipin Metabolomes in Mouse Plasma and Tissues. Metabolomics. 2012;8(6):1130-47.
265. Kang JX, Weylandt KH. Modulation of Inflammatory Cytokines by Omega-3 Fatty Acids. Subcell Biochem. 2008;49:133-43.
266. Dehkordi NG, Noorbakhshnia M, Ghaedi K, Esmaeili A, Dabaghi M. Omega-3 Fatty Acids Prevent Lps-Induced Passive Avoidance Learning and Memory and Camkii-Alpha Gene Expression Impairments in Hippocampus of Rat. Pharmacol Rep. 2015;67(2):370-5.
267. Shi Z, Ren H, Huang Z, Peng Y, He B, Yao X, et al. Fish Oil Prevents Lipopolysaccharide-Induced Depressive-Like Behavior by Inhibiting Neuroinflammation. Mol Neurobiol. 2017;54(9):7327-34.
268. Michaeli B, Berger MM, Revelly JP, Tappy L, Chiolero R. Effects of Fish Oil on the Neuro-Endocrine Responses to an Endotoxin Challenge in Healthy Volunteers. Clin Nutr. 2007;26(1):70-7.
269. Gutierrez S, Svahn SL, Johansson ME. Effects of Omega-3 Fatty Acids on Immune Cells. Int J Mol Sci. 2019;20(20):5028.