THE EFFECT OF INCREASING LEVELS OF OMEGA-3 FATTY ACIDS FROM EITHER FLAXSEED OIL OR PREFORMED DOCOSAHEXAENOIC (DHA) ON HEALTH INDICES OF LAYING HENS

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 received considerable attention, including in poultry nutrition. The latter has primarily focused on producing omega-3 enriched eggs and meat for human consumption. The current study, however, was designed to determine potential health benefits to laying hens receiving different sources of dietary omega-3 fatty acids, as assessed during an inflammatory challenge induced by lipopolysaccharide (LPS) administration. Our results indicated that dietary ALA (provided by flaxseed oil) or DHA (algal DHA biomass) and their increasing levels in diets increased the levels of both individual and total omega-3 PUFA and lowered the ratio of omega-3 to omega-6 in egg yolk, liver and plasma and reduced ARA-derived oxylipins, whereas they increased EPA-derived and certain DHA-derived oxylipins. Additionally, supplementation of increasing levels of omega-3 PUFA impart significant effects on the production of toll-like receptor (TLR) 4 in laying hens under acute conditions of LPS. Therefore, these findings could provide information regarding: 1) the effect of inclusion of either flaxseed oil, as a source of ALA, or preformed DHA in the diets of laying hens; and 2) the metabolic functions of oxylipins supplied with omega-3 fatty acids on the immune responses of laying hens exposed to LPS challenge.

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FOREWORD

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All manuscripts published or prepared for publication during my M.Sc. program have been listed as follows:

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2. **S Li.**, N. Mohamed., C. Dubois., H.M. Aukema and J.D. House. Effect of increasing dietary omega-3 fatty acids levels on fatty acid deposition and immune responses in laying hens. (In preparation for Poultry Science)

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LIST OF ABBREVIATIONS

AI	Adequate intake
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
EFA	Essential fatty acids
ELOVL2	ELOVL fatty acid elongase 2
ELOVL5	ELOVL fatty acid elongase 5
EOME	Epoxy-octadecenoic acid
EPA	Eicosapentaenoic acid
EpDPE	Epoxy-docosapentaenoic acid
EpETE	Epoxy-eicosatetraenoic acid
EpETrE	Epoxy-eicosatrienoic acid
EpODE	Epoxy-octadecadienoic acid

FA	Fatty acids
FADS1	Fatty acid desaturase 1
FADS2	Fatty acid desaturase 1
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
IκB	Inhibitory subunit of NF-κB
IL 1β	Interleukin 1β
IL 2	Interleukin 2
IL 6	Interleukin 6
IL 10	Interleukin 10
IL 12	Interleukin 12
IRAK	IL-1 receptor-associated kinase
LA	Linoleic acid
LBP	Lipopolysaccharide binding protein

LC-MS	Liquid chromatography mass spectrometry
LCPUFA	Long chain polyunsaturated fatty acids
LPS	Lipopolysaccharide
LOX	Lipoxygenase
LTs	Leukotrienes
MD-2	Myeloid differentiation protein 2
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor-kappa B
oxoODE	Oxo-octadecadienoic acid
oxoETE	Oxo-eicosatetraenoic acid
PGs	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
RA	Rheumatoid arthritis
RvD	Resolvin D
RvE	Resolvin E
sEH	Soluble epoxide hydrolase

TLR4	Toll-like receptor 4
TMUFA	Total monounsaturated fatty acids
TNF-α	Tumor necrosis factor-α
TRAF6	TNF receptor-associated factor 6
TSFA	Total saturated fatty acid
TXs	Thromboxanes

LIST OF APPENDICE

CHAPTER 1 GENERAL INTRODUCTION

Omega-3 polyunsaturated fatty acids (PUFA), including alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have received considerable attention over the past decades due to their benefits to humans and animals (Baker et al., 2016; Carragher et al., 2016; Kang et al., 2020). The predominant sources of omega-3 PUFA are fish oil (EPA and DHA) and plant oils such as flaxseed oil (ALA). Additionally, minor amounts of omega-3 PUFA are found in nuts and seeds, vegetables, certain fruits, and agricultural products, particularly egg yolk and meat (Kris-Etherton et al., 2000). The effects of omega-3 PUFA on human health are generally well-described. In 2002, the Food and Nutrition Board of the U.S. Institute of Medicine gave guidance on the adequate intake (AI) levels for total omega-3 fatty acids, being 1.6 and 1.1 g/day for adult males and females, respectively (IOM, 2005).

Achieving such levels can potentially reduce the risk of diseases, such as cardiovascular disease (Endo and Arita, 2016), psychiatric disease (Bozzatello et al., 2016) and some cancers (Fabian et al., 2015; Nabavi et al., 2015). Some research has also provided evidence that omega-3 PUFA may provide additional benefits to animals, including livestock raised for the production of foods of animal origin. A number of studies have focused on the development of optimal dietary treatments to increase the omega-3 fatty acid content of eggs, via the provision of flaxseed and hempseed products enriched in ALA, fish oil enriched in EPA and DHA and algal oil found mostly in DHA (Goldberg et al., 2012; Neijat et al., 2016). However, a defined requirement value for ALA has not been established for poultry, particularly laying hens. Furthermore, it has not been well

established as to whether and how the provision of omega-3 PUFA lead to health-protective responses in birds.

In addition to serving as a source of dietary fatty acids (FA), PUFA can also be oxidized into lipophilic signaling molecules known as oxylipins through three major enzymatic pathways, namely the cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) pathways (Orr et al., 2013; Rey et al., 2016; Layé et al., 2018; Schmöcker et al., 2018). Oxylipins modulate a variety of biological functions/responses including inflammation and immunity, and their regulatory effects differ based on the types of their derivatives (Calder, 2015), with the potential to provide either anti- or pro-inflammatory properties. For example, eicosanoids, the well-known oxylipins derived from arachidonic acid (ARA), are mainly linked to the stimulation of the immune system and act as pro-inflammatory mediators (Zivkovic et al., 2011); whereas, EPA-derived eicosanoids possess less biological potency and anti-inflammatory effects. Moreover, the newly discovered oxylipins, including resolvins, protectins and maresins derived from EPA and DHA, are more anti-inflammatory and inflammation resolving (Calder, 2015). Lipopolysaccharide (LPS) is the major component of the outer membrane of Gram-negative bacteria and has been widely used in inflammation models. It can stimulate the release of certain inflammatory cytokines and lead to an acute inflammatory response. LPS can also directly stimulate the secretion of eicosanoids in macrophages and B cells to induce inflammation (Park et al., 2009; Balistreri et al., 2011; Chang et al., 2001).

A number of studies have shown that dietary supplementation of EPA and/or DHA reduced the expression of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-a, interleukin (IL)-1β and IL-6, and increased anti-inflammatory cytokines (IL-10),

after LPS challenge in both cell culture and animal feeding models (Caughey et al., 1996; Meydani et al., 1991; Trebble et al., 2003). The level of mRNA expression of interferon (IFN)-γ in the spleen was lower in omega-3 PUFA-fed mice compared with that observed in mice consuming omega-6 PUFA-enriched and a low PUFA diet group (Fritsche et al., 1999). However, the mechanisms by which dietary fatty acids modulate cytokine production have not yet been fully elucidated. Particularly, there is limited information on the relationships between intake of omega-3 PUFA, oxylipin profiles and inflammatory responses in poultry, including laying hens. In this review, we will explore whether the type and dose of omega-3 PUFA in diets have the potential to strengthen immune responses and elicit health benefits in laying hens.

CHAPTER 2 LITERATURE REVIEW

2.1 Introduction to fatty acids (FA)

2.1.1 Metabolism of polyunsaturated fatty acids (PUFA)

FA are hydrocarbon chains with a methyl group at one end of the molecule and a carboxyl group at the opposite end. There are various classifications of FA based on many characteristics. They can be categorized into: a) saturated fatty acids (no double bonds), monounsaturated fatty acids (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 (LCFA, more than 12 carbon molecules), based on the chain length; c) non-essential fatty acids and essential fatty acids (EFA), based on essentiality for humans and animals; or d) omega-6 PUFA and omega-3 PUFA, based on the position of omega carbon (Neijat, 2014; Jha, 2004). Among them, PUFA, especially, omega-3 type, have been considerably studied over the past decades.

Omega-3 and omega-6 are two classes of EFA that must be obtained by animals from the diet. These FA, particularly the precursor FA LA and ALA, cannot be synthesized de novo in humans and animals due to the lack of Δ12-desaturases and Δ15-desaturases for EFA desaturation (Kang, 2003; Saini and Keum, 2018). The difference between omega-3 and omega-6 FA is position of the first double bond, counting from the methyl terminus of the acyl chain. EFA can be metabolized to long chain polyunsaturated fatty acids (LCPUFA, 20 to 22 carbons) (Simopoulos et al., 2001). ALA is the simplest omega-3 PUFA that converts to EPA and DHA, and the omega-6 PUFA are represented by LA, which is metabolized to ARA (Calder, 2012; Simopoulos, 2016). The conversion of LA and ALA

to the LCPUFA is illustrated in **Figure 2.1**, which shows the primary products of ARA, and EPA and DHA respectively during the process (Scorletti and Byrne, 2018).

The liver has a central location within the anatomy of the gastrointestinal tract and is one of the main metabolic organs for the processes of anabolism and catabolism (Scorletti and Byrne, 2013). The liver is the major site of lipid metabolism in both avian species (Leveille et al., 1975) and humans (Patel et al., 1975) due to its key roles in regulating several metabolic fates, such as desaturation and elongation reactions of LA and ALA to long- LCPUFA, β-oxidation and transportation to other tissues, including muscle or adipose tissues (Neijat, 2014). The key enzymes Δ5 and Δ6 desaturases are encoded by the genes fatty acid desaturase 1 (FADS1) and 2 (FADS2), respectively (Cho et al., 1999a, b), and the two elongase enzymes are derived from the genes ELOVL2 and ELOVL5 (Leonard et al., 2004; Wang et al., 2005). Particularly, Δ6 desaturase has been regarded as a major regulatory point in the conversion of plant-derived ALA to EPA or DHA in mammals (James et al., 2003). Gregory et al. (2013) recently characterized the chicken elongase enzymes and have provided evidence that chickens have enriched expression of ELOVL5, compared to other animals examined to date.

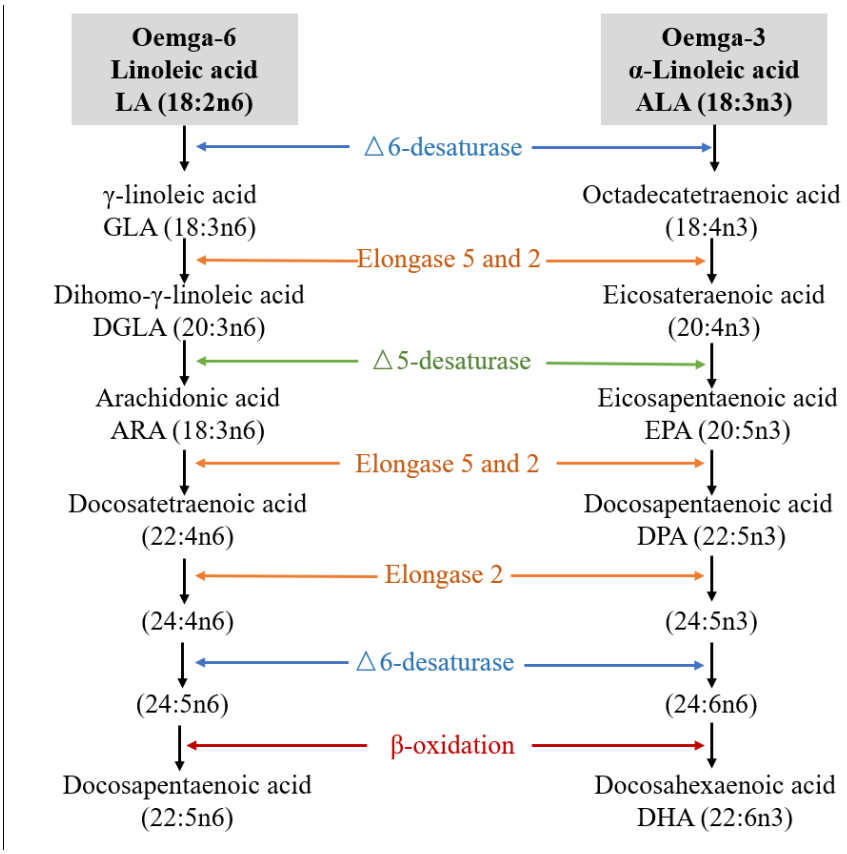


Figure 2.1 The general pathway for conversion of LA and ALA to LCPUFA in laying hens (Wood et al., 2015; Neijat, 2014).

The conversion of ALA to long-chain omega-3 PUFA (mainly EPA and DHA) is inefficient (Domenichiello et al., 2015). 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**. Another factor limiting conversion is that high amounts of ALA (60%-85%) undergo β-oxidation to provide energy, but for other FA (Huang et al., 2004), like ARA, only around 30% is catabolized (Barceló-Coblijn et al., 2009). EPA and DPA can also be reconverted from DHA due to limited peroxisomal β-oxidation (Calder and Yaqoob, 2009). The low conversion of ALA may lead to a reduced status for the omega-3 LCPUFA which are not beneficial to animals. Therefore, nutrition guidelines should be reconsidered in light of the dietary needs for omega-3 PUFA in animals including laying hens. Additionally, PUFA, as precursors, can be oxidized into highly active metabolites known as oxylipins, which will be discussed in greater detail in section 2.3 (Cha et al., 2006).

2.1.2 Dietary sources and intake of 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 flaxseed oil accounts for more than 50% of total fatty acids. Other sources of ALA include canola oil and soybean oil, making up approximately 10% of the FA profile (Burdge et al., 2006). Animal-based foods, such as chicken, beef, and lamb, can supply EPA and DHA, in modest amounts, but marine products are the richest sources of the omega-3 LCPUFA (Saini et al., 2018). The sources of omega-3 PUFA in selected plant and animal-based foods are briefly summarized in **Table 2.1**.

Table 2.1 Contents of omega-3 PUFA (g/100 g) in selected plant and animal-based foods (after Saini et al., 2018).

Food		Omega-3 PUFA (g/100 g)		
		ALA	EPA	DHA
Plant oil	Flaxseed, cold pressed	53.57	-	-
	Canola	9.14	-	-
	Soybean, salad or cooking	6.79	-	-
	Corn, salad or cooking	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
Fish	Caviar, black and red, granular	-	2.74	3.80
	Shad, American, raw	-	1.09	1.32
	Salmon, Chinook, raw	-	1.01	0.94
	Herring, Atlantic, kippered	-	0.97	1.18
	Salmon, Atlantic, farmed, raw	0.15	0.86	1.10

People, such as the Japanese, who consume more oily fish than North American populations, for example, have higher intakes of the omega-3 LCPUFA, approximating 5-6 g/day (Calder, 2012; Meyer, 2011). In contrast, the intake of omega-3 PUFA among European and North American populations is lower, approximating 0.15-0.25 g/day (Blasbalg et al., 2011). In Australia, the mean intake is estimated to be 0.19 g/day (Meyer et al., 2003). The recommended intake of ALA is 1.6 g/day and 1.1 g/day for men and women, respectively (≥0.5% total fat), while the ranges of an adequate intake of EPA and DHA are between 0.25 and 2 g/day (Elmadfa and Kornsteiner, 2009; Smit et al., 2009). The recommended dietary ratio of omega-6:omega-3 PUFA for human health is 1:1–2:1 (Simopoulos, 2006).

2.2 Nutrition guides of PUFA in poultry industry

EFA including LA and ALA must be supplied in chicken diets due to a lack of desaturases to form double bonds beyond Δ9 carbon (Cherian, 2015). Traditionally, there are imbalanced contents of omega-3 and omega-6 PUFA in hens' diet because the latter are predominantly found in corn and other traditional sources of dietary fat used in poultry diets. An estimated dietary requirement of LA for mature laying hens and pre-lay pullets is approximately 1.0% of the diet and the deficiency symptoms are well demonstrated (NRC, 1994). The natural sources of omega-3 PUFA are known to be limited in use in poultry feeding systems for economic reasons. There are two ways to incorporate omega-3 PUFA into poultry products. The first way is to utilize a high amount of DHA products without ALA such as fish oil and algae oil (Poorghasemi et al., 2013; Candela et al., 2011; Samman et al., 2009; Qi et al., 2010) and the second one is to apply economical ALA-enriched feeding regimens such as hempseed and flaxseed oil to poultry diets. Among these

ALA-rich ingredients, flaxseed oil is one of the most recommended ingredients in poultry diet to incorporate mainly ALA in poultry products (Cherian, 2008). In general, the inclusion of omega-3 PUFA in diets has been associated with certain health benefits for chickens (Swiatkiewicz et al., 2015), including enhancing the growth performance, egg production, fertility, immunity and bone parameters (Lee et al., 2019; Alagawany et al., 2019). According to previous studies of utilization of hempseed products in laying hen diets in our lab (Gakhar et al., 2012; Neijat et al., 2016; Goldberg et al., 2012), the average feed intake of hens was 100 g/day and the optimal range of omega-3 PUFA intake, especially the ALA, is 500-600 mg/day, because the DHA accumulation reaches to a steady status in egg yolk. It is noted that flaxseed oil can lead to a higher DHA deposition in egg yolk, compared with hempseed products (Gakhar et al., 2012), but incorporating either flaxseed oil or hempseed products had a similar efficiency of deposition of DHA into egg yolk (Baucells et al., 2000; Cherian et al., 1991; Scheideler et al., 1996). Therefore, dietary intake of 600 mg omega-3 PUFA can serve as a supplementation guide level for laying hen diets. More important, the steady status of DHA deposition in egg yolk can be considered as key criteria for ALA requirement of laying hens. In addition, several studies were conducted to evaluate the effect of dietary ALA versus preformed DHA on performance, egg quality, FA profiles in egg yolk, liver and plasma in laying hens (Neijat et al., 2014; Neijat et al., 2016; Neijat et al., 2017). These studies provided evidence that hen performance and eggshell quality were not influenced by dietary source and amount of omega-3 PUFA. Increasing levels of total omega-3 PUFA in the diets of hens supplied from either ALA or algal DHA increased the levels of the omega-3 PUFA (ALA, EPA, DPA and DHA in egg yolk, liver and plasma. Inclusion of omega-3 PUFA in diets of

poultry has been conducted over the past years, with the primary focus being the enrichment of poultry-based foods with omega-3 PUFA. However, specific requirements of ALA (omega-3 PUFA) for poultry have yet to be clearly defined.

2.2.1 **Applications of omega-3 PUFA in poultry**

Dietary intervention with omega-3 PUFA in poultry may directly influence their production of products, mainly meat and eggs, which are in demand by human consumers (Mousa et al., 2017). Fish and other types of seafood are generally considered to be major sources of the long-chain omega-3 PUFA, but they may not serve as primary foods in some countries, therefore, omega-3 PUFA-enriched poultry products may be good alternatives to meet the nutritional requirements of humans for the omega-3 PUFA. Studies have shown that manipulation of omega-3 PUFA in poultry diets significantly improved growth and productive performance, immune response and anti-oxidative properties, meat quality, and the quality and nutritional values of eggs (Alagawany et al., 2019). The following section will detail the beneficial effects of omega-3 fatty acids on the characteristics of poultry products including meat and eggs.

2.2.1.1 Enrichment of meat in broiler chickens

Inclusion of dietary omega-3 PUFA in poultry diets to improve the nutritional value of poultry meat is considered as a safe and efficient way to introduce additional omega-3 LCPUFA in humans (Zhang et al., 2010). Konieczka et al (2017) reported that the levels of EPA and DHA were increased in breast and thigh meat lipids in flaxseed- and rapeseed-fed groups compared to those consuming the basal diet. Moreover, the flaxseed-fed group reduced the ratio of omega-6 to omega-3 PUFA in breast meat, compared to the rapeseed and basal diet fed groups. The results also indicated that one-week and two-week of feeding

the experimental diets were adequate to enrich breast (33% FA/100 g) and thigh (15.5% FA/100 g) meat with omega-3 PUFA, respectively. Carragher et al. (2016) indicated that broilers fed with a high ALA diet containing 2.5% of flaxseed oil had an approximately 4-fold increase in omega-3 PUFA in breast meat without compromise of performance or increase in the fat content of breast meat, compared to the basal diet feeding. In addition, increasing dietary ALA in broiler diets from about 0.3 to 8% of total energy led to an increase between 4- and 9-fold in accumulation of EPA, DPA and DHA in breast and thigh meat compared to the broilers fed control diet (Kartikasari et al., 2012).

2.2.1.2 Improvement of quality and nutritional value of eggs

Appropriate application of feeding strategies can enhance the nutritional value and health benefits of eggs from laying hens (Dhama et al., 2014; Laudadio et al., 2015; Sujatha and Narahari, 2011), including improvement of the quality and quantity of eggs (Sihvo et al., 2014). Eggs are one of the best-selling animal products globally and may serve as important sources of omega-3 PUFA.

Numerous studies have shown that the level of omega-3 PUFA in eggs can be enriched, via modification of the laying hen diet. Eggs from hens consuming diets containing 23% Nannochloropsis oceanica microalgae (DNOM) had the highest concentrations of EPA (0.62 mg/g), DHA (4.8 mg/g) and total omega-3 PUFA (5.9 mg/g), and the lowest omega-6:omega-3 PUFA ratios (4.8 mg/g) in the yolk at the final week compared with control and lower concentrations of DNOM (2.86%, 5.75% and 11.5% of diets) (Manor et al., 2019). When feeding laying hens on diets containing 4% fish oil, 1% and 2% microalgae, the DHA content of egg yolk increased to 10.61±1.30 mg, 75.49±18.75 mg and 114.35±16.66 mg/egg, respectively (Kaewsutas et al., 2016). Previous studies

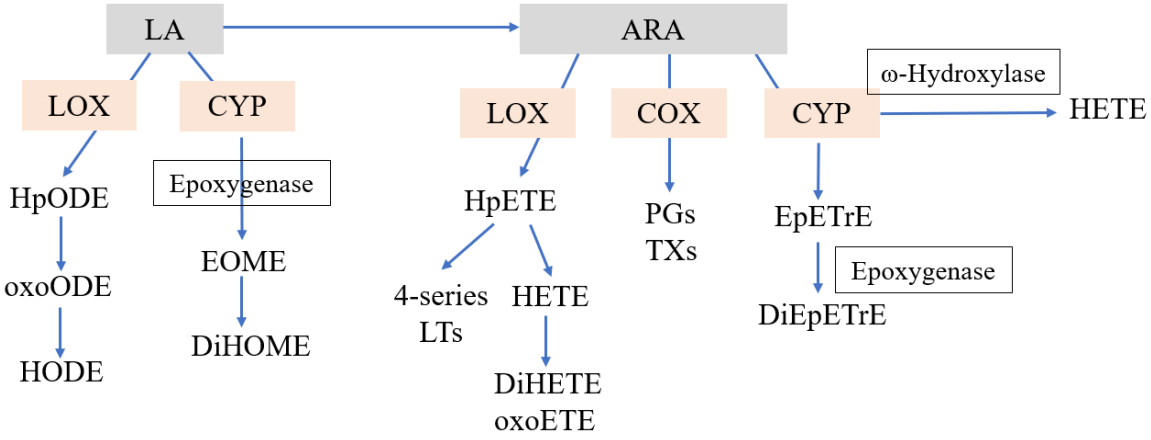
conducted in our lab showed that feeding ALA-rich hemp products to laying hens increased the content of egg yolk DHA (approximately 55 mg/egg) which however eventually reached a plateau with further increases in dietary ALA levels (Gakhar et al., 2012). Neijat et al (2016) demonstrated that DHA-fed hens incorporated 3-fold more DHA in eggs compared with ALA-fed hens (179±5.55 vs. 66.7±2.25 mg/ yolk, respectively) at the highest inclusion levels (0.6% of both flaxseed oil and algal DHA). Nonetheless, the precise mechanisms underlying limited DHA deposition remain incompletely elucidated.

In summary, supplementation of omega-3 PUFA to poultry diets mediates the total lipid profile and FA deposition in both meat and egg yolk, which facilitates the practice of fortification of animal foods with omega-3 PUFA. Despite numerous studies conducted on the enhancement of characteristics of animal products for human consumption, there is not a defined requirement value for ALA for poultry. Therefore, filling this gap will help improve birds' performance and well-being.

2.3 Inflammatory products of omega-3 PUFA

2.3.1 Formation of oxylipins

In addition to FA desaturation and chain elongation, PUFA can also be oxidized into highly active metabolites known as oxylipins. This metabolism involves one or more mono- or dioxygen-dependent reactions, which mediate a variety of physiological processes such as inflammation, immunity, reproduction, and development (Cha et al., 2006). There are three general types of oxylipins derived from PUFA (Figure 2), including octadecanoids from LA (18:2n–6) or ALA (18:3n–3), eicosanoids from ARA (20:4n–6) or EPA (20:5n–3), and docosanoids from DHA (22:6n–3) (Calder, 2006).


LA
ARA
LOX
CYP
LOX
COX
CYP
ω-Hydroxylase
HETE
HpODE
Epoxygenase
HpETE
PGs
TXs
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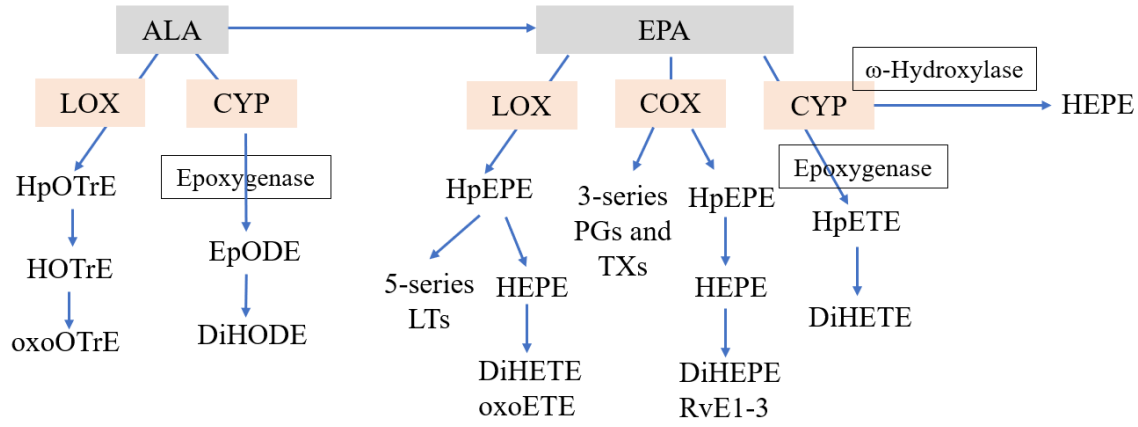
LTs
HETE
Epoxygenase
DiEpETrE
HODE
DiHOME
DiHETE
oxoETE


ALA
EPA
LOX
CYP
LOX
COX
CYP
ω-Hydroxylase
HEPE
HpOTrE
Epoxygenase
HpEPE
3-series
PGs and
TXs
HpEPE
Epoxygenase
HpETE
HOTrE
EpODE
5-series
LTs
HEPE
HEPE
DiHETE
oxoOTrE
DiHODE
DiHETE
oxoETE
DiHEPE
RvE1-3

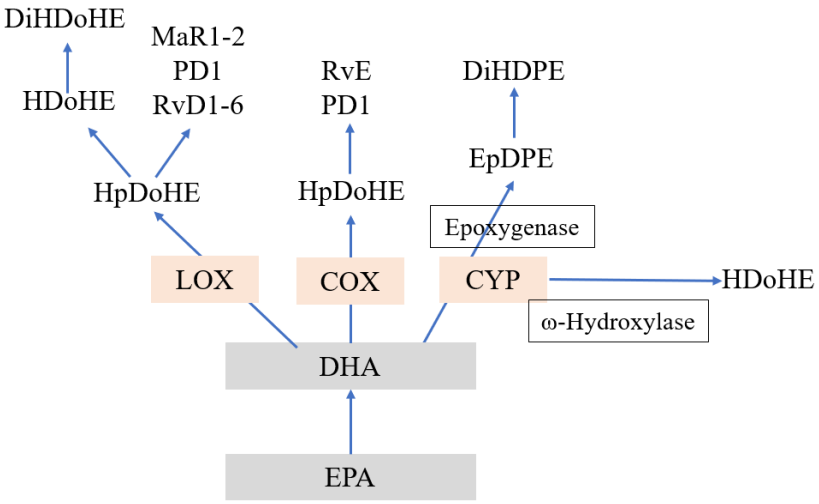

DiHDoHE
MaR1-2
PD1
RvD1-6
RvE
PD1
DiHDPE
HDoHE
EpDPE
HpDoHE
HpDoHE
Epoxygenase
LOX
COX
CYP
HDoHE
ω-Hydroxylase
DHA
EPA

Figure 2.2 Oxylipin synthesis pathways. ARA: arachidonic acid, DiHETE: dihydroxy-eicosatetraenoic acid, DiHOME: dihydroxy-octadecaenoic acid, EET: epoxy-eicosatrienoic acid, EpDPE: epoxydocosapentaenoic acid, EpODE: epoxy-octadecadienoic acid, EOME: epoxy-octadecenoic acid, HDoHE: hydroxy-docosahexaenoic acid, HETE: hydroxyeicosatetraenoic acid, HEPE: hydroxy-eicosapentaenoic acid; HODE: hydroxy-octadecadienoic acid, HOTrE: hydroxy-octadecatrienoic acid, HpETE: hydroperoxyeicosatetraenoic acid, LA, LTs: leukotrienes, LXs: lipoxins, oxoODE: oxo-octadecadienoic acid, oxoETE: oxo-eicosatetraenoic, PGs: prostaglandins; PD1: protectin 1, RvE: E-series resolvin, RvD: D-series resolvin, MaR: maresin, TXs: thromboxanes (Gabbs et al., 2015; Rey et al., 2019).

Oxylipin formation starts with cell activation. PUFA cannot spontaneously generate and liberate large amounts of free fatty acid forms as they are stored at the sn-2 position on the glycerol backbone of phospholipid membranes (Dennis et al., 2011). Phospholipase A_2 (PLA_2) is the primary enzyme that releases sn-2 position fatty acids into free forms (Buczynski et al., 2009). This enzyme is classified into five types, including cytosolic PLA_2 ($cPLA_2$), secreted PLA2 ($sPLA_2$), calcium-independent PLA_2, platelet-activating factor acetylhydrolase, and lysosomal PLA_2. The free-form PUFA are then oxidized to distinct classes of oxylipins such as eicosanoids and docosanoids by three main metabolic pathways, involving cyclooxygenases (COXs), lipoxygenases (LOXs) and cytochrome P450 (CYP) enzymes (Wang et al., 2014).

COXs, as the first oxylipin formation pathway enzyme, can convert PUFA into prostanoids (PGs and TXs) (Funk, 2001; Buczynski et al., 2009; Bos et al., 2004), and select hydroxy fatty acids, including 11-hydroxy-eicosatetraenoic acid (11-HETE) from ARA, 13-hydroxy-docosahexaenoic acid (13-HDoHE) from DHA, and 9-hydroxy-octadecadienoic acid (9-HODE) from LA (O'Neill et al., 1994; Thuresson et al., 2000; Serhan et al., 2002; Laneuville et al., 1995). LOX is the second enzyme of the oxylipin metabolic pathway, primarily catalyzing hydroperoxy and hydroxy fatty acids, such as 5-hydroperoxy-eicosatetraenoic acid (5-HpETE) and 5-HETE that are formed from ARA. Additionally, some hydroxy fatty acids (e.g., 5-HETE) are further metabolized to their ketos such as oxo-eicosatetraenoicacid (oxo-ETE) or dihydroxy derivatives such as 5,15-dihydroxy-eicosatetraenoic acid (5,15-DiHETE). The third enzyme involved in oxylipin metabolism is CYP that forms oxylipins with the activities of epoxygenase or ω-hydroxylase. For example, ARA, EPA, and DHA can be oxidized into epoxy-eicosatrienoic

acid (EpETrE), epoxy-eicosatetraenoic acid (EpETE), and epoxydocosapentaenoic acid (EpDPE) respectively by epoxygenase, which can further be rapidly converted to their dihydroxy fatty acids derivatives including dihydroxy-eicosatrienoic acid (DiHETrE), DiHETE, and dihydroxy-docosapentaenoic acid respectively, via soluble epoxide hydrolase (sEH). (Gabbs et al., 2015)

2.3.2 Functions of oxylipins

Oxylipins that are generated from omega-6 PUFA and omega-3 PUFA have been well documented with respect to their effects on inflammation (Fleming, 2007; Innes and Calder, 2018; Wall et al., 2010). The ARA-derived oxylipins PGs, TXs, LTs, and other oxidized derivatives are the main metabolic forms of eicosanoids (Calder, 2006). The biosynthesis of PGs via COX is significantly increased in the inflamed tissue (Smyth et al., 2009). PGE_2, PGI_2 and PGD_2 are considered as predominant pro-inflammatory PGs, which stimulate leukocyte infiltration via promoting blood flow as well as increasing vascular permeability and leukocyte infiltration (Hata and Breyer, 2004). Additionally, PGD_2, a key product of mast cells, leads to inflammation in allergic responses of the lung (Claar et al., 2015).

Generally speaking, omega-6 PUFA-derived oxylipins have more inflammatory, vasoconstrictory, and proliferative effects. However, public attention has increasingly focused on the state of omega-3 PUFA-derived oxylipins in both humans and animals, due to their anti-inflammatory and pro-resolving properties. These include eicosanoids, resolvins, protectins and maresins (Bazan, 2009; Serhan et al., 2011). One key mechanism underlying anti-inflammation is the suppression of the metabolism of ARA to generate eicosanoids (Tejera et al., 2012). This can be achieved in multiple ways, including the reduction of the release of ARA from membrane phospholipids, inhibition of the activities

of metabolic enzymes, and competition with ARA for enzymic conversions. Inclusion of fish oil to human diets has been reported to contribute to decreased production of PGE_2 (Trebble et al., 2003b), TXB_2 (Hall et al., 2011), LTB_4 (Wei et al., 2014), 5-hydroxyeicosatetraenoic acid (Galet et al., 2014) by inflammatory cells, and may therefore provide anti-inflammatory functions. For example, PGE_2 can induce IL-10 production and lead to the suppression of TNF-α in monocytes/macrophages to prevent serious tissue injury (Linke et al., 2017) and LTB_4 and its receptor LTB_4R1 can recruit and activate adipose tissue B2 cells to promote insulin resistance (Ying et al., 2017). Intake of EPA and DHA increases the generation of omega-3-series lipid mediators through LOX, COX and CYP enzymatic pathways which play a role in inhibiting inflammation (Wang et al., 2014). Dong et al (2018) reported that intake of fish oil decreased cerebral expression of the pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β, induced by pro-inflammatory stimuli especially LPS.

Studies found that the mediators generated from EPA seem to be less potent than those generated from ARA. For example, LTB_5 possesses 10 – 100-fold less activity, compared with LTB_4, leading to decreased inflammation and bronchoconstriction (Mickleborough et al., 2006; Lee et al., 1984). 15-HEPE has anti-cancer effects by inhibiting cancer cell growth and the production of ARA oxylipins (Vang et al., 2005). PGE_3 showed less affinity and activity in colorectal cancer cells due to binding with the EP4 receptor (Hawcroft et al., 2010), subsequently exhibiting less mitogenetic and inflammatory activity in fibroblasts and monocytes, compared with PGE_2 (Bagga et al., 2003; Wang et al., 2014). In addition, DHA-derived oxylipins such as 13- HDoHE and 17- HDoHE (metabolites from a series of HDoHE) inhibited TNF-a-induced cytokine production in human

microglial cells (Serhan et al., 2004; Weylandt et al., 2011). EpDPE is proven to be effective in anti-inflammatory, vasodilatory, and anticancer (Zhang et al., 2013; Jung et al., 2012), and inhibits angiogenesis and metastasis (Zhang et al., 2013).

Recently, a novel group of E-series and D-series oxylipins, derived from EPA and DHA respectively, has been discovered and elucidated. These include resolvins (produced from EPA and DHA), protectins and maresins (produced from DHA), and their structures, actions and mechanisms are well-documented (Bannenberg et al., 2010; Serhan et al., 2008; Serhan and Chiang, 2013). The biosynthesis of these new oxylipins is presented in Figure 2 and Figure 3 (Duvall et al., 2016).

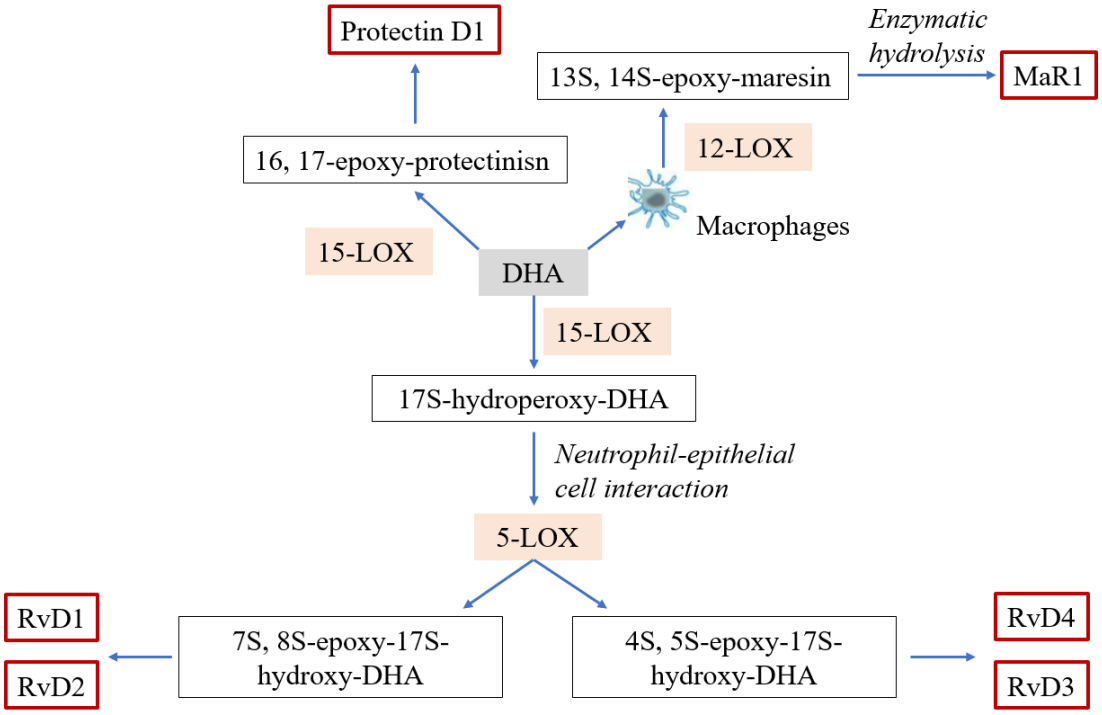


Figure 2.3 Biosynthesis of D-series resolvins, maresins, and protectins via DHA.

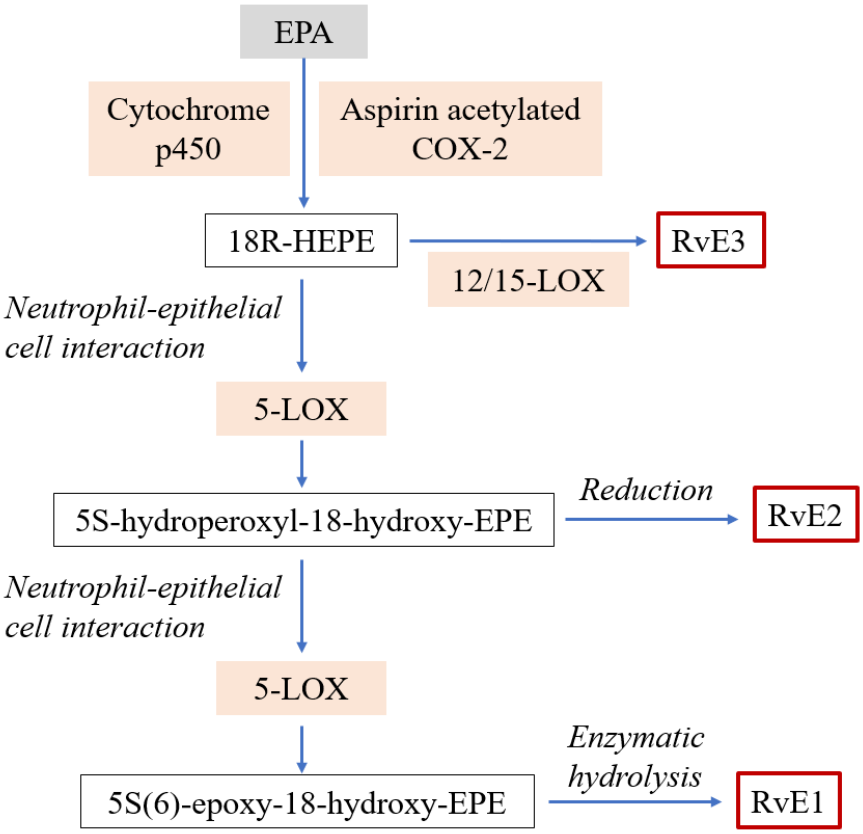


Figure 2.4 Biosynthesis of E-series resolvins via EPA.

Anti-inflammatory effects of these lipid mediators are gaining attention. Upon the activation of B cells, RvD1 increased the production of immunoglobulin (Ig) M and IgG, eliciting humoral-mediated immunity (Ramon et al., 2012); Protectin D1 inhibited the secretion of TNF-α and IFN-γ from human T cells *in vivo* and blocked T cell migration (Ariel et al., 2005, 2006); RvE1 was shown to promote the phagocytosis of apoptotic neutrophils from macrophages (Schwab et al., 2007). Examples of novel group of E-series and D-series oxylipins functions are summarized in **Table 2.2**.

Table 2.2 Examples of novel E-series and D-series oxylipin functions.

FA precursor	Enzymic pathway	Special pro-resolving mediators	Disease models/ Species	Functions	References
EPA	5-LOX	RvE1	Stromal keratitis/mice	Reduces the influx of CD4+ T cells and neutrophis, increases IL10 and decreases pro-inflammatory cytokines.	Rajasagi et al., 2011
			Atopic dermatitis/ mice	suppresses skin lesions via limiting leukocyte infiltration	Kim et al., 2012
	5-LOX	RvE2	Peritonitis/mice	Inhibits zymogen-induced polymorphonuclear leukocyte infiltration and increased the production of anti-inflammatory cytokine.	Tjonahen et al., 2006; Oh et al., 2012
	12/15-LOX	RvE3	Depression-like behavior/mice	Has antidepressant effects	Deyama et al., 2018
DHA	5-LOX	RvD1	Rheumatoid arthritis/mice	Suppresses angiopoiesis and decreased the expression of critical factor in RA progression, connective tissue growth factor.	Sun et al., 2020
			Chronic lung disease/human lung fibroblasts	Accelerates the resolution of lung inflammation, drives macrophage polarization from M1 to M2 phenotype.	Hsiao et al., 2013
	5-LOX	RvD2	Tissue injury/mice	Inhibits the inflammatory pain	Park et a., 2011

				via inhibiting transient receptor potential subtype vanilloid 1 and TRP ankyryn 1	
	5-LOX	RvD3	Acute lung injury/mice Spinal cord injury/mice	Limits the leukocyte Infiltration and edema in lung Promotes *in vitro* inflammatory resolution via an increase of IL-10 and a decrease of IL-6.	Dalli et al., 2013 Kim et al., 2020
	15-LOX	PD1	epimorphic regeneration/ Zebrafish larvae Epilepsy/mice	accelerates the switch of macrophage phenotypes and decreases expression of pro-inflammatory cytokines in LPS-activated human macrophages Promotes resolution of neuroinflammation and decreases production of IL-1β, TNF-a	Nguyen - Chi et al., 2020 Frigerio et al., 2018
	12-LOX	MaR1	Colitis/mice	reduced neutrophil migration and production of IL-1 β , TNF-a, IL-6	Marcon et al., 2013

Taken together, it can be concluded that links exist between the supply of FA (particularly omega-3 type), oxylipin profiles and the state of inflammation.

2.3.3 Oxylipin metabolism in avian species

There is strong scientific evidence that the metabolism of oxylipins can be modified by the dietary intake of omega-3 PUFA in avian species. Specifically, dietary omega-3 PUFA have been reported to decrease tissue ARA concentrations, inhibit cell membranes from releasing ARA and compete with ARA as a substrate of the COX and LOX enzymes with a consequence of decreasing production of pro-inflammatory eicosanoids (Belch and Hill, 2000; Green et al., 2005). Studies in laying hens showed that consumption of fish oil or flaxseed reduced the concentrations of PGE_2 and expression of COX-2 in ovaries and therefore reduced the incidence and severity of ovarian cancer (Eilati et al., 2013a, b). In addition, the administration of fish oil significantly decreased the contents of plasma PGE_2 and TXB_2 in broiler chickens (Liu et al., 2014). It has also been indicated that modulating maternal dietary omega-3 PUFA in the 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 PGE_2 (Cherian, 2011). Similarly, production of LTB_4 and ratio of LTB_4 to LTB_5 by thrombocytes from chicks that hatched from high ratio of omega-6 to omega-3 PUFA (15.0) eggs was higher than those chicks hatched from lower ratio eggs (Hall et al., 2007). The concentration of PGE_2 was greater whereas TXA_3 was lower in heart tissue of chicks hatched from high omega-3 PUFA enriched eggs, when the maternal diet contains 3.5% of sunflower or fish oil (Bautista-Ortega et al., 2009). 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 from either ALA (flaxseed oil) or DHA in laying hens, which needs to be further investigated.

2.3.4 Interactions between omega-3 PUFA oxylipins and immunity

It is generally considered that omega-3 PUFA reduce the production of molecules and substances related to inflammation, including inflammatory eicosanoids and cytokines. People who experience chronic diseases such as rheumatoid arthritis (RA) and inflammatory bowel disease, exhibited reduced production of eicosanoids including 2-series PGs and 4-series LTs that are derived from ARA when taking high doses of EPA and DHA (Calder, 2015). On the other hand, numerous studies both *in vitro* and *in vivo* have demonstrated that the inclusion of omega-3 PUFA to diets decreased secretion of the classic pro-inflammatory cytokines, including TNF-α, IL-1β and IL-6 (Labrousse et al., 2012; Orr et al., 2013; Dehkordi et al., 2015), and increased the production of the anti-inflammatory cytokine IL-10 (Calder, 2015) in response to lipopolysaccharide (LPS). Studies also indicated that DHA plays an anti-inflammatory role in the brain, which is mediated through microglia, the brain's innate immune cells (Pettit et al., 2013, Chang et al., 2015, Fourrier et al., 2017).

Several mechanisms have been proposed to explain the protective effects of omega-3 fatty acids against inflammation. Initially, researchers believed that omega-3 PUFA exert anti-inflammatory effects only via blocking the metabolism of pro-inflammatory eicosanoids from ARA (Kang and Weylandt, 2008). Recently, it has been reported that they also modify the expression of inflammatory cytokines (Kang and Weylandt, 2008). Nuclear factor-kappa B (NF-κB) is a widely expressed inducible transcription factor,

composed of homodimers and heterodimers. It mediates the synthesis and/or activities of several pro-inflammatory cytokines and enzymes that play critical roles in the pathogenesis of chronic inflammatory diseases. NF-κB heterodimers are released by the degradation of IκB, via the process of phosphorylation of cytoplasmic IκB by the IκB kinase (IKK) complex, and translocated to the nucleus binding to κB motifs in the promoters of pro-inflammatory genes, including TNF-a, IL-1β, IL-6 and cyclooxygenase (COX)-2, thereby leading to their induction (Hayden et al., 2006).

In addition, peroxisome proliferator-activated receptor-γ (PPAR-γ), another transcription factor, is involved in the anti-inflammatory effects of omega-3 PUFA. PPAR-γ potentially inhibits the translocation of NF-κB to the nucleus and subsequent activation. A number of studies have indicated that omega-3 PUFA, especially DHA, can activate PPAR-γ and reduce TNF-a and IL-6 after LPS challenge (Kong et al., 2010; Forman et al, 1997; Kliewer et al., 1997; Krey et al., 1997). Moreover, another anti-inflammatory action of DHA and EPA might be achieved through PPAR-γ targeted genes induced in dendritic cells (Zapata-Gonzalez et al., 2008). Collectively, omega-3 PUFA may influence the activation of PPAR-γ, an effect associated with suppression of NF-κB activation.

However, the results of the effect of dietary omega-3 PUFA intake on the level of IFN-γ are inconsistent among various species. Studies in humans found that consumption of omega-3 PUFA reduced the production of IFN-γ (Blok et al., 1996; Fritsche et al., 1999). However, mice in a *Listeria monocytogenes* infected group had significantly higher concentrations of IFN-γ in blood when receiving a fish oil-enriched diet compared with those fed diets low in omega-3 PUFA diets (Fritsche et al., 1997). Moreover, the inclusion of omega-3 PUFA to laying hen diets had no significant effect on mRNA expression of

IFN-γ (Sijben et al., 2001; Sijben et al., 2003). The underlying mechanisms impacting IFN-γ production are inconsistent among different species and thus it requires further development.

2.4 Lipopolysaccharide (LPS)

LPS, the major component of the cell wall of Gram-negative bacteria and a highly efficient pro-inflammatory substance, has been widely used to model bacterial infections experimentally in poultry and other animals (Yang et al., 2008). LPS are large molecules comprised of an outer polysaccharide region (commonly known as 'O' antigen, a linear or branched component of oligosaccharide residues), a unique polysaccharide core region consisting of short-chain sugars, and an inner fatty acid-rich region termed lipid A (Moran et al., 1996). In general, LPS signaling is sequentially activated by an acute-phase protein, LPS-binding protein (LBP), the cluster of differentiation 14 (CD14; either in a GPI-anchored or in a soluble secreted form), the lipid-binding accessory protein MD-2, and toll-like receptor4 (TLR4) (Bryant et al., 2010). LBP can recognize and form a high-affinity complex with the lipid A (Pålsson-McDermott et al., 2004) which then form a ternary complex with CD14. Thus, LBP and CD14 play an amplifier role in responding to LPS. Besides, the responses to LPS can also be stimulated by the downstream molecules including TLR4 and MD-2 (Gioannini et al., 2005; Miyake, 2006). TLR4 is indispensable in the signaling and requires MD-2 to respond efficiently to LPS (Shimazu et al., 1999; Miyake 2004). Subsequently, Myeloid differentiation primary response 88 (MyD88) is required to interact with IL-1R-associated kinase (IRAK)-1, -2 or –M, then generates the TNF receptor-associated factor 6 (TRAF6) and IKKα/β/γ complex, followed by phosphorylation and degradation of IκB and nuclear translocation of NF-κB of which

activation plays a key role in the regulation of inflammation and immune responses. NF-κB is then released and translocated into the nucleus and initiates the expression of targeted genes (Ghosh et al., 1998; May and Ghosh, 1998). Ultimately, LPS leads to the production of a variety of endogenous mediators, such as proinflammatory cytokines, adhesion molecules, acute phase proteins, nitric oxide and prostaglandins (Wright, 1995; Ulevitch and Tobias, 1995; Ulevitch and Tobias, 1999). **Figure 2.5** briefly illustrates the LPS-induced signaling pathways and activities.

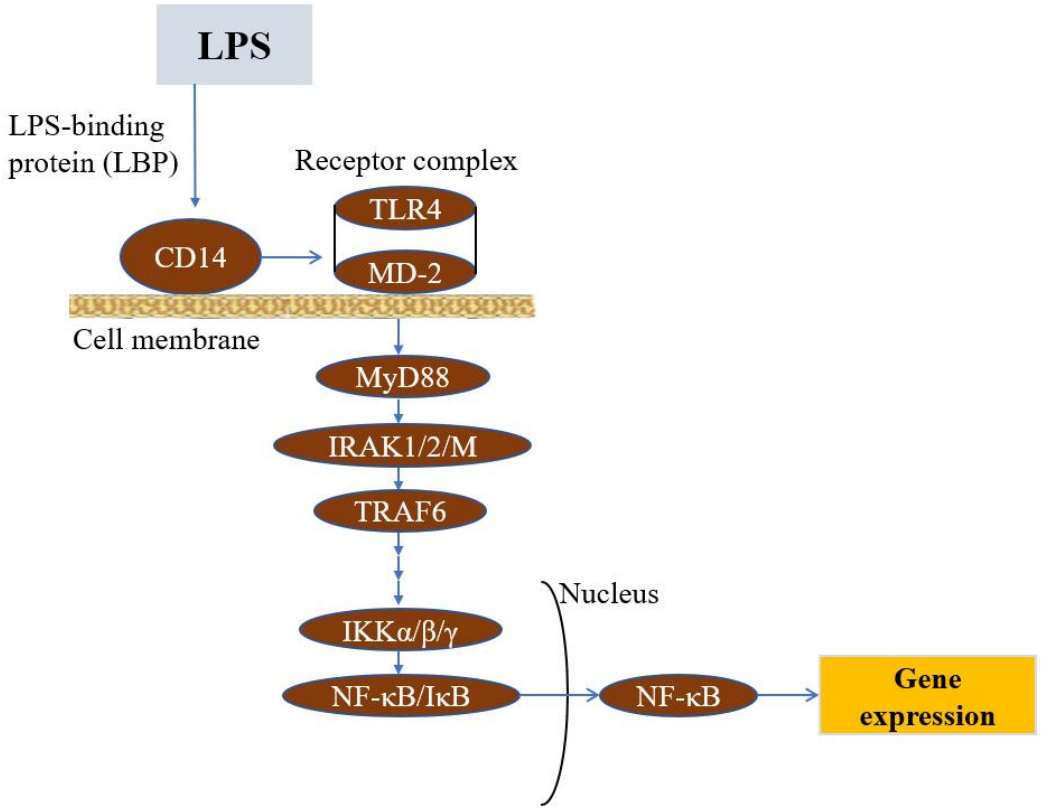


Figure 2.5 LPS-induced signaling pathways and the process of activation of NFκB, eventually induces gene expression (Zhang and Ghosh, 2000). CD 14: Cluster of differentiation 14; TLR4: Toll-like receptor 4; MD-2: Myeloid differentiation protein 2; MyD88: myeloid differentiation primary response 88; I κ B: inhibitory subunit of NF- κ B; NF-κB: Nuclear factor-kappa B; TRAF6: TNF receptor-associated factor 6; IRAK1/2/M: IL-1 receptor-associated kinase-1, 2, M; IKK α / β / γ : I κ B kinase.

LPS also stimulates host cells to produce a large amount of pro-inflammatory mediators, mainly TNF-α, IL-1α and IL-6, by activating several types of transcription factors (Zhang and Ghosh, 2000).

Evidence regarding the impact of dietary omega-3 PUFA on the production of oxylipins in avian species has been provided, however additional factors, including inflammation, are known to influence the metabolism of oxylipins (Maccarrone et al., 2001). For example, LPS can directly activate and induce monocytes and macrophages to produce eicosanoids, such as PGs, especially PGE_2 (Janský et al., 1995; Calder, 2002). Balvers et al. (2012a) also demonstrated that treatment of mice with LPS changed the profile of eicosanoids in the plasma, liver, ileum and adipose tissue. Willenberg et al. (2016) demonstrated that LPS induced sepsis led to a significant increase in plasma PGE_2, and kidney PGD_1, dihomo-$PGF_{2\alpha}$ and PGF_{1a} in murine species. Furthermore, the effect of inflammation on oxylipins can be mediated by dietary omega-3 PUFA. Increasing dietary intake of omega-3 PUFA resulted in elevated levels of omega-3 derived oxylipins and decreased synthesis of PGE_2, after LPS administration or other inflammatory stimuli (Calder and Yaqoob, 2009; Balvers et al., 2012b). Dumlao et al. (2012) reported that feeding fish oil inhibited Borrelia burgdorferi-induced production of PGE_2. Serum profiles of EPA and DHA derived oxylipins in asthmatics significantly shifted following dietary supplementation with omega-3 PUFA (Lundström et al., 2013). Caligiuri et al. (2014) recently showed that elevated levels of proinflammatory oxylipins in older subjects were normalized by flaxseed consumption. LPS induced a growth in omega-6 PUFA generated mediators, TxB_2 and 8-HETE in the brain of mice that fed by omega-3 PUFA deficient diet and this was blunted in the sufficient omega-3 PUFA-supplemented mice (Rey et al., 2019).

Collectively, these studies demonstrate that dietary omega-3 PUFA can positively modulate or correct changes in the synthesis of oxylipins after immune challenge, however, relevant situations in chickens including laying hens are poorly investigated. The potential exists for the implementation of nutritional regimens to reduce the severity of disease and to test or validate nutritional regimens that strengthen immune responses.

2.5 Conclusion

Overall, it can be concluded that dietary omega-3 PUFA can elicit protective immune responses through modulating the production of oxylipins and cytokines. Currently, whether the supply of different types and levels of omega-3 PUFA in diets can make a difference with regards to birds' productivity has been explored. Moreover, dietary omega-3 PUFA supplementation patterns yielded predictable responses in plasma, liver and egg yolk FA concentrations in response to increasing dietary ALA or DHA. However, whether this pattern generated from either ALA or DHA can be affected by LPS and relevant situations of omega-3 PUFA and immune response in laying hens have been inadequately investigated. Therefore, studies are warranted to provide data as to the omega-3 needs of laying hens which would potentially correct the oversight in current feeding and management guidelines that fail to state the importance of these nutrients. Additionally, a good understanding of the relationship between omega-3 PUFA status and immune status in response to LPS challenge will allow for the development of management strategies to enhance hen health and wellness, and to ensure optimal immunocompetence and potentially reduce the risk of diseases in laying hens.

CHAPTER 3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

Omega-3 PUFA can positively modulate changes in oxylipin synthesis and certain cytokines after immune challenge in laying hens thus leading to an increase in the anti-inflammatory response.

3.2 Objectives

The overall objective of this thesis was to investigate the immunomodulatory properties of increasing levels of omega-3 PUFA from either ALA or preformed DHA for improving the health status of laying hens. Specific objectives were:

1. To establish omega-3 PUFA requirements for optimal health and productivity in laying hens.
2. To identify whether either ALA or DHA in the diet modulate immunological responses in birds subjected to LPS challenge.

CHAPTER 4 MANUSCRIPT

EFFECT OF INCREASING DIETARY OMEGA-3 FATTY ACID LEVELS ON FATTY ACID DEPOSITION AND IMMUNE RESPONSES IN LAYING HENS

4.1 Abstract

The dietary provision of omega-3 polyunsaturated fatty acids (PUFA) to humans and animals, including poultry, has been reported to yield positive health benefits. However, in considering omega-3 fatty acid supply to poultry, studies have primarily focused on dietary levels needed to optimize the production of omega-3 enriched eggs and meat for human consumption. To date, no official estimate of the omega-3 PUFA requirement has been for poultry despite the acknowledgment of the essentiality of alpha-linolenic acid (ALA). In addition, the potential immunomodulatory properties of omega-3 PUFA, which can be beneficial for the health maintenance of birds, have not been thoroughly investigated. The study was designed to determine potential health benefits to laying hens receiving different sources of omega-3 PUFA, as assessed during an inflammatory challenge induced by lipopolysaccharide (LPS) administration. A total of 80 Lohmann LSL-Classic (white egg layer, 20 weeks of age) were randomly assigned to 1 of 8 treatment diets with 10 hens/treatment, provided 0.2%, 0.4%, 0.6%, or 0.8% level of total dietary omega-3 PUFA from either flaxseed oil (precursor of ALA) or algal DHA biomass for 8 weeks. At termination, hens from each treatment were injected with either saline (Sham; n=5) or *Escherichia coli* (*E. coli*) LPS (8 mg/kg i.v; n=5). Egg yolk, plasma, liver and spleen (post-CO_2 asphyxiation) were collected for subsequent analysis of fatty acid profiles, plasma biochemical constituents, oxylipins and mRNA expression of specific cytokines. Dietary

omega-3 supplementation patterns yielded predictable responses in plasma, liver and yolk fatty acid concentrations in response to increasing dietary ALA or DHA. Increases in total omega-3 PUFA intake led to decreases in ARA-derived oxylipins and increases in EPA- and certain DHA- derived oxylipins. The mRNA expressions of interferon (IFN)-γ and toll-like receptor (TLR) 4 in the spleen were significantly increased after the LPS challenge. Dietary omega-3 PUFA supplementation significantly reduced the mRNA expression of TLR4 in the DHA-fed group. In conclusion, dietary intake of omega-3 PUFA modulated fatty acid deposition in different tissues, the content of certain oxylipins and cytokine gene expression in the spleen of laying hens under LPS administration.

Keywords: Omega-3 PUFA, oxylipins, cytokines, lipopolysaccharide, laying hens

4.2 Introduction

Omega-3 polyunsaturated fatty acids (PUFA), including alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have received considerable attention due to their reported health benefits in humans (Baker et al., 2016; Carragher et al., 2016; Kang et al., 2020). These benefits include the resolution of inflammation (Im, 2012), and the prevention of cardiovascular (Endo and Arita, 2016), and psychiatric diseases (Bozzatello et al., 2016), some cancers (Fabian et al., 2015; Nabavi et al., 2015), and the protection of brain development (Innis, 2008). Several strategies have been proposed to increase the dietary intake of omega-3 PUFA by humans (Garg et al., 2006), including the guidance to increase fatty fish intake and the use of dietary supplements. Alternatively, the use of enriched food products, including hen eggs with omega-3 PUFA has been positioned as an effective strategy to supply humans with this class of fatty acids (FA). However, most of the previous research on omega-3 PUFA for laying hens has focused on levels needed to enrich eggs with these nutrients that are in demand by human consumers, via the provision of flaxseed, fish and algal oil and hemp to hen diets (Fraeye et al., 2012; Gakhar et al., 2012; Goldberg et al., 2012, 2013; Neijat et al., 2016). A defined requirement value for omega-3 PUFA has not been established for poultry, despite their general recognition as being essential fatty acids (NRC, 1994).

PUFA can exert anti-inflammatory functions mainly via modulating the synthesis of lipid mediators and the production of cytokines (Ventro et al., 2017. Zivkovic et al., 2011). PUFA can be oxidized into the lipophilic signaling molecules oxylipins, which includes eicosanoids and docosanoids, via the three major enzymatic pathways involving cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) enzymes (Orr

et al., 2013; Tourdot et al., 2014; Rey et al., 2016; Layé et al., 2018). Arachidonic acid (ARA), one of the omega-6 PUFA, can be metabolized to generate pro-inflammatory mediators known as the eicosanoids, which include prostaglandins (PGs), thromboxanes (TXs), and leukotrienes (LTs) (Zivkovic et al., 2011). Additionally, oxylipins converted from omega-3 PUFA, primarily EPA and DHA, have defined anti-inflammatory and pro-resolving benefits (Bazan, 2009; Serhan et al., 2011; Calder, 2015). Inclusion of increasing levels of omega-3 PUFA in the diet occurs partly at the expense of omega-6 PUFA, particularly ARA (Rees et al., 2006; Walker et al., 2015), leading to a suppression of eicosanoid synthesis and an increase in the production of anti-inflammatory mediators in different species subjected to a lipopolysaccharide (LPS) model of inflammation or other inflammatory stimuli (Tejera et al., 2012). Balvers et al. (2012a) demonstrated that treatment of mice with 3mg/kg LPS changed the profile of eicosanoids in the plasma, liver, ileum and adipose tissue after 2, 4,8, and 24-hour LPS injection. Increasing the dietary intake of omega-3 PUFA resulted in elevated levels of omega-3-derived oxylipins and inhibition in the synthesis of PGE_2, after LPS administration or other inflammatory stimuli (Calder, 2009; Balvers et al., 2012b; Dumlao et al., 2012). Caligiuri et al. (2014) recently showed that elevated levels of proinflammatory oxylipins in older subjects were normalized by flaxseed consumption. Collectively, these studies provide evidence that the inclusion of omega-3 PUFA in diets can positively modulate the synthesis of oxylipins after immune challenge. In addition, the production of cytokines could be subsequently affected by the synthesis of different types and/or amounts of lipid mediators, or via direct effects by the PUFA on the modulation of gene expression (Sijben et al., 2001; Kang and Weylandt, 2008). Numerous studies have confirmed that the inclusion of omega-3 PUFA

to diets decreased interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-a, in both humans and animals, after LPS challenge (Orr et al., 2013; Dehkordi et al., 2015; Shi et al., 2016). However, the effects on interferon (IFN)-γ are variable among different species. Of particular importance, there is a lack of data on the effects that dietary omega-3 PUFA may have on cytokine profiles in avian species, including laying hens (Sijben et al., 2001; Sijben et al., 2003).

Since there are limited reports on the relationship between intake of omega-3 PUFA, oxylipin profiles and cytokine and receptor expression under LPS treated in laying hens, therefore, this study aims to provide information about: 1) the effect of inclusion of either flaxseed oil, as a source of ALA, or algal DHA biomass in the diets on performance, FA profiles in egg yolk, plasma and liver of laying hens; 2) the metabolic functions of oxylipins in plasma via HPLC-MS/MS and their potential for having beneficial effects on laying hens; 3) The gene expression of toll-like receptor (TLR) 4 and IFN-γ supplied with graded level of omega-3 PUFA on the immune responses of laying hens under the LPS administration.

4.3 Materials and Methods

The experimental protocol including details of animal usage and care was reviewed and approved by the University of Manitoba Animal Care Protocol Management and Review Committee, and all the birds were fed and managed according to the recommendations established by Canadian Council on Animal Care (CCAC, 2009).

4.3.1 Chickens and Experimental Design

A total of 80 twenty-week-old Lohmann LSL-Classic (white egg layer, with average BW of 1.57 kg) were obtained from (Steinbach Hatchery & Feed Ltd., Steinbach, MB, Canada) and accommodated in individual cages (38 × 52 cm to provide 1,976 cm^2 area/space per bird) with independent feeders, nipple waterer, and perch. Water and feed were supplied *ad libitum* and light was provided for 15 hours throughout the whole experimental period. All birds were raised, fed, and managed in the Poultry Metabolism Unit, University of Manitoba. Prior to the start of the official experimental protocol, all chickens were allowed a 2-week adaption period within the metabolism room and received commercial layer diets essentially devoid of omega-3 PUFA. Following the adaptation period, chickens were weighed and randomly assigned to 1 of 8 treatment diets (n=10 replicate cages per treatment). Treatments were included at 0.2%, 0.4%, 0.6%, or 0.8% total omega-3 PUFA, provided as either ALA (ALA, provided by flaxseed oil), obtained from Dyets, Inc. (Bethlehem, PA, USA) or algal DHA biomass (DHA), a source of a dried whole-cell algae product (DHAgold^{TM} S17-B, DSM Nutritional Products, North America, Parsippany, NJ, USA). The dietary fomulations of 0.2%, 0.4%, 0.6%, and 0.8% of ALA and DHA were achieved by adding 0.33%, 0.76%, 1.18% and 1.60% of flaxseed oil and adding 0.93%, 2.14%, 3.35% and 4.53% agal DHA biomass in per kg of diet, respectively. Vitamin E was provided at levels of 150 IU/kg of diet per treatment during the diet formulation and mixing to avoid lipid peroxidation. The study was designed as a 2 × 8 factorial arrangement of main factors involving diets (8 treatments) and immunological challenge (injection with LPS or saline). All diets were formulated to meet or exceed NRC requirements (NRC, 1994) for hens consuming 100 g of feed per day formulated to contain

2700 kcal/kg metabolizable energy and 17% crude protein. The ingredients and nutrient composition of the experimental diets are shown in **Table 4.1**.

Table 4.1 Ingredients and nutrient composition of experimental diets for laying hens containing increasing levels of total omega-3 fatty acids.

	ALA diets				DHA diets			
Total omega-3 fatty acids (% of diet)	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80
Ingredients (%)								
Corn	59.05	59.04	58.97	57.04	58.63	58.00	57.36	56.28
Soybean meal	21.54	21.54	21.55	22.40	21.39	21.21	21.03	20.95
Wheat	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
VM premix[1]	1.50	1.50	1.50	1.50	1.50	1.50	1.50	1.50
Flaxseed oil	0.33	0.76	1.18	1.60	-	-	-	-
DHAgold^{TM} S17-B	-	-	-	-	0.93	2.14	3.35	4.53
Corn oil	0.83	0.41	-	-	0.85	0.44	0.03	-
Biophos	1.32	1.32	1.32	1.71	1.32	1.33	1.33	1.34
Limestone	9.84	9.85	9.89	10.17	9.79	9.79	9.79	9.79
Salt	0.35	0.35	0.35	0.36	0.34	0.34	0.34	0.34
DL-methionine	0.143	0.143	0.143	0.141	0.145	0.147	0.149	0.151
L-lysine HCL	0.093	0.093	0.093	0.071	0.099	0.106	0.113	0.119
Calculated nutrients								
AMEn (Poultry; kcal/kg)	2800	2800	2,800	2,800	2790	2774	2759	2765
Crude fat (%)	3.5	3.5	3.5	3.9	3.5	3.5	3.5	3.9
Crude protein (%)	16.8	16.8	16.8	17.0	16.8	16.8	16.8	16.8
Crude fibre (%)	2.21	2.21	2.21	2.2	2.19	2.17	2.15	2.13
Calcium (%)	3.82	3.82	3.83	4.00	3.80	3.80	3.80	3.80
Phosphorus (%)	0.59	0.59	0.59	0.67	0.60	0.61	0.62	0.63
Analyzed nutrients								
AMEn (Poultry; kcal/kg)	2796	2815	2809	2807	2832	2792	2767	2841
Crude fat (%)	3.74	3.90	3.70	4.05	3.70	3.59	3.68	4.09
Crude protein (%)	16.81	17.05	17.19	17.03	16.96	16.79	17.23	16.81
Crude fibre (%)	2.61	2.85	3.06	2.54	2.65	2.99	3.78	2.54
Calcium (%)	4.68	4.40	4.44	4.69	4.28	4.58	4.56	4.62
Phosphorus (%)	0.61	0.62	0.62	0.69	0.60	0.64	0.62	0.61
Calculated fatty acids (%, by wt)								
18:2n-6 (LA)	1.60	1.43	1.27	1.31	1.55	1.32	1.09	1.06
18:3n-3 (ALA)	0.20	0.40	0.60	0.80	0.04	0.04	0.03	0.03
22:6n-3 (DHA)	-	-	-	-	0.16	0.36	0.57	0.77
Total omega-3[2]	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80
Analyzed fatty acid (%, by wt)								
18:2n-6 (LA)	1.50	1.82	1.45	1.61	1.81	1.67	1.43	1.40
18:3n-3 (ALA)	0.16	0.29	0.67	0.93	0.17	0.08	0.08	0.08

22:6n-3 (DHA)	0.10	0.13	0.01	0.00	0.11	0.52	0.80	0.99
Total omega-3[2]	0.26	0.43	0.68	0.93	0.29	0.62	0.92	1.12

[1]Provided per kilogram of diet, vitamin-mineral premix contained: 11,000 IU of vitamin A; 3000 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, 1000 mg of choline chloride, 125 mg antioxidant (ethoxyquin), 66 mg of manganese oxide, 70 mg of zinc oxide, 80 mg of ferrous sulfate, 10 mg of copper sulfate, 0.3 mg of sodium selenite, 0.4 mg of calcium iodate, 0.67 mg of sodium chloride.

[2]Total omega-3 fatty acids = sum of ALA, EPA (20:5n-3), DPA (22:5n-3) and DHA.

4.3.2 Sample Collection

Daily feed intake, body weight (BW) and egg weight were recorded to calculate the egg production rate and feed conversion ratio. During the final week of study, eggs were collected specifically for yolk fatty analysis, and separated yolks frozen at -20℃ for subsequent FA analysis. At termination, 10 hens from each treatment were assigned equally and randomly to receive an intravenous injection of either *Escherichia coli* 8 mg/kg of BW LPS (serotype 0111:B4, Sigma Aldrich Inc., St. Louis, MO; n=5) or sterile saline (n=5), respectively. The LPS treatment was based on methods described by Gehad et al. (2002) and Shini et al. (2008). Feed was withdrawn after injection. At 4-hours post-injection, a total of 10 mL plasma sample was collected from the wing vein and were divided into 4 aliquots (approximately 2.5 mL each) into four heparinized Vacutainer tubes that were kept on ice during collection for the subsequent analysis of biochemical constituents, FA and oxylipins. The whole blood samples used for FA and oxylipin analysis were centrifuged at 2000 × g for 20 min at 4°C, and the plasma were stored at −80◦C until analyzed. All birds were euthanized by CO_2 asphyxiation. The liver and spleen were quickly removed, wrapped in aluminum foil, and immediately snap-frozen in liquid nitrogen prior to storage at -80℃ in advance of further analyses.

4.3.3 Fatty Acid Extraction and Analysis

For the analysis of FA, total lipids from plasma, liver and egg yolk samples, as well as from the experimental diets, were extracted and esterified following the procedure of Folch et al (1957). Egg yolk (1g), liver samples (1g) and experimental diets (finely ground 1g) and plasma (100uL) pre-conditioned with chloroform/methanol (2:1, by volume) were used for extraction of fatty acids, using C13:0 as internal standard to quantify fatty acids.

For extraction, aliquots of a known volume (to contain 40–50 mg lipid) were dried under nitrogen, and methylated using 3 mL of Boron trifluoride (BF_3) by heating for 2 h at 80 °C. The final fatty acids of samples were extracted into iso-octane, carefully transferred to gas chromatography (GC) vials, and the FA profiles of the methyl esters 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 temperature started from 70 °C for 2 min, followed by raising to 180 °C at 30 °C/min and being stable for 1 min; then continued to increase to 200 °C at 10 °C/min for 2 min; increased to 220 °C at 2 °C/min for 10 min and eventually reached to 240 °C at 20 °C/min for 5 min. Samples were run for 36.67 min in total and were run with a 20:1 split ratio (1 μl injection). A flow rate of 1.3 mL/min Hydrogen was used as the carrier gas and the retention time of each fatty acid were compared to the standard samples (Lipid standards, Nu-Chek Prep, Inc., Elysian, MN, USA) and the final composition of each test fatty acid can be determined. The FA content of samples total lipid was calculated as concentration = [(peak area of a given fatty acid × concentration of internal standard (mg/mL)/peak area of internal standard) ×dilution factor of extracted lipid] divided by sample weight (g) or volume (mL).

4.3.4 Biochemical Constituents in Plasma

The blood samples were immediately analyzed for plasma metabolites using an automated analyzer (Cell-Dyn 3500 system, Abbott Laboratories, Abbott Park, IL) at the Manitoba Veterinary Services Laboratory (Winnipeg, Manitoba, Canada).

4.3.5 Oxylipin Extraction in Plasma

Plasma samples (200 µL) were pre-conditioned with 1 mL of water (pH 3), 10 µL of antioxidant solution, 100 µL of deuterated internal standard and be acidified to pH 3 for oxylipin extraction. All samples were applied to Strata-X SPE (Phenomenex, 33u, 60 mg/3mL) columns and later eluted with methanol for subsequent drying under nitrogen and water bath (37°C). After re-constituting in 100 µL of water-acetonitrile-formic acid (70:30:0.02, v/v/v LC-MS grade), the oxylipin profiles of the plasma were determined using high-performance liquid chromatography mass spectrometry (HPLC-MS/MS; QTRAP 6500; Sciex, ON, Canada), based on the method of Leng et al (2017). Quantification of oxylipins was determined by using the stable isotope dilution method (Hall and Murphy, 1998) and expressed as nanograms per milliliter of plasma.

4.3.6 Total RNA Extraction and Reverse Transcription

Total RNA was extracted from spleen samples (10 ± 0.5 g) using the RNeasy Mini kit (Qiagen Canada Inc., Mississauga, ON, Canada) and eliminate the possibility of genomic DNA contamination by a DNase set kit (Qiagen Canada Inc., Mississauga, ON, Canada) following the manufacturer's protocol. One µg of RNA was used for cDNA synthesis using a Superscript® VILO™ cDNA Synthesis Kit (Invitrogen Canada Inc., Burlington, ON, Canada), following the protocol of the manufacturer. The cDNA samples were stored at –20°C for quantitative real-time PCR analysis.

4.3.7 Quantitative Real-Time PCR and analysis

Quantitative real-time PCR was performed using a OneStep Real-Time Detection System (Applied Biosystems, Canada). Primer sequences for β-actin, TLR4, and IFN-γ were obtained from Primer-Blast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/)

(Table 4.2). All samples were run in duplicates with 2 µL of cDNA solution with 10 µL of detector, SYBR Green, 6 µL of nuclease-free water and 1 µL each of forward and reverse primer (calculation based on the final concentration 500 nM of the primers) to form a final volume of 20 µL reaction. Controls without cDNA were also conducted. The quantitative RT-PCR cycle parameters involved a 3-min holding stage of 95 °C, and 40 cycles of denaturation at 95 °C for 10 s, followed by an annealing/extension at 61 °C for 30 s, and finally a one 2-segment cycle of product melting stage of 95 °C for 10 s and 60 °C for 5 s. 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 (Livak and Schmittgen, 2001). Real-time PCR efficiencies were acquired according to formula: E = 10−1/slope of standard curve (Pfaffl, 2001). The expressed PCR efficiency of all primers used in this project were between 95% and 100%.

Table 4.2 Toll-like receptor and cytokine primer sequences[1]

Genes	Primer sequences (5' to 3')	Amplicon length (bp)	GenBank access
TLR4	F: CCAAACACCACCCTGGACTTG R: TGTATGGATGTGGCACCTTGAA	120	NM_001030693
IFN-γ	F: GCTGACGGTGGACCTATTATTGT R: ACGCCATCAGGAAGGTTGTT	188	NM_205149
β- Actin	F: CAACACAGTGCTGTCTGGTGGTA R: ATCGTACTCCTGCTTGCTGATCC	205	NM_205518

[1] F = forward; R = reverse.

4.3.8 Statistical analysis

The experiment was designed as a completely randomized design with dietary treatment and experimental unit (laying hens with 10 replicates per treatment) regarded as the fixed and the random effects, respectively. Data for performance, including body weight gain, egg weight, feed intake and rate of production were analyzed using the MIXED procedure of SAS as repeated measures. Least squares means (LSM), adjusted using Tukey's test, were compared for significant differences ($P<0.05$). Data for plasma biochemistry, FA, oxylipins and gene expression were analyzed by the MIXED procedure of SAS. The Shapiro-Wilk test was used for assessing normal distribution. All data were compared for significant differences, using a P value of 0.05, via Tukey's significant difference test.

4.4 Results

4.4.1 Hen Performance

No significant effects of the type and amount of dietary omega-3 PUFA were observed on performance (feed intake, BW, egg weight, rate of production and feed conversion rate; **Table 4.3**). Collectively, over the whole study, laying hens consumed 105.1g/day of diets presented with final body weights of 1.64 kg, produced 54.7 g of egg, averaged 96.6% egg production, and had an average feed conversion ratio of 1.93. Although there was a significant main effect of week on feed intake, with decreases during week 2 and week 3 compared to the initial dietary intake in week 1, there was a stable increase in BW and egg weight throughout the entire feeding period. The egg production rate was significantly reduced in week 2 and week 3 as well, likely reflecting the concurrent decreases in feed

intake. The rates increased thereafter. Additionally, significant interaction effects of diet and week were noted for feed intake, BW and egg weight (**Table 4.3).**

Table 4.3 Performance of hens (from 20 to 28 weeks of age) consuming diets containing increasing levels of total omega-3 from either ALA or DHA[1].

	Feed intake (g/hen/day)	BW (kg)	Egg production (%)	Egg weight (g/egg)	FCR (g/g)[2]
Diet effect (total omega-3 in diet, %)					
ALA (flaxseed oil)					
0.20	104.3	1.63	96.6	55.9	1.87
0.40	107.7	1.62	96.4	55.7	1.94
0.60	105.4	1.61	97.1	55.3	1.91
0.80	100.0	1.58	95.5	54.8	1.84
DHA (Algal DHA biomass)					
0.20	105.4	1.66	97.7	53.3	1.98
0.40	105.0	1.66	97.1	55.9	1.89
0.60	107.0	1.67	97.0	54.0	1.99
0.80	106.0	1.65	95.2	52.4	2.03
SEM	2.75	0.031	1.25	0.92	0.049
Week effect					
Wk 1	106.7^{a}	1.60^{e}	96.8^{abc}	51.7^{e}	2.07^{a}
Wk 2	103.3^{bc}	1.61^{e}	91.5^{c}	52.2^{e}	2.00^{a}
Wk 3	100.4^{c}	1.61^{de}	93.3^{bc}	53.6^{d}	1.88^{b}
Wk 4	103.1^{bc}	1.62^{cd}	97.7^{ab}	54.7^{c}	1.89^{b}
Wk 5	105.3^{ab}	1.63^{bc}	98.0^{ab}	55.1^{c}	1.92^{b}
Wk 6	106.1^{ab}	1.65^{ab}	97.6^{ab}	56.0^{b}	1.90^{b}
Wk 7	107.1^{a}	1.66^{a}	98.7^{a}	56.5^{ab}	1.90^{b}
Wk 8	106.5^{ab}	1.67^{a}	99.1^{a}	57.0^{a}	1.88^{b}
SEM	1.20	0.011	0.90	0.36	0.022
P values					
Diet	0.3151	0.1445	0.8711	0.056	0.115
Week	< 0.001	< 0.001	0.001	< 0.001	< 0.001
Diet × week	< 0.001	< 0.001	0.2122	0.014	< 0.001

[1]Data are presented as least squares means and their SEM (n = 10). Values with different superscripts within a column are significantly different at $P < 0.05$.

[2]g of feed consumed per g of egg produced

4.4.2 Biochemical Constituents in Plasma

There were no significant diet effects or interaction effects of diet × challenge on plasma biochemistry, but significant main effects of LPS challenge were observed, with increases in Chloride and A/G ratio, and decreases in Calcium, Phosphorus, Total protein, Albumin as well as Globulin concentrations (**Table 4.4**).

Table 4.4 Plasma biochemistry of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	Diet effect (total omega-3 in diet, %)									Challenge effect			*P* values		
	ALA				DHA				SEM	Saline	LPS	SEM	Diet	Challenge	Diet × challenge
	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80							
Sodium (mmol/L)	145	147	145	144	146	147	147	148	1.0	146	147	0.5	0.189	0.118	0.679
Potassium (mmol/L)	3.99	4.14	4.27	3.81	4.21	4.68	3.87	3.94	0.239	4.15	4.07	0.138	0.607	0.692	0.792
Chloride (mmol/L)	121	123	121	121	121	123	122	122	1.0	119[b]	125[a]	0.5	0.336	< 0.001	0.492
Calcium (mmol/L)	4.94	5.42	5.18	5.26	4.99	5.35	5.45	4.71	0.268	5.78[a]	4.55[b]	0.134	0.492	< 0.001	0.824
Phosphorus (mmol/L)	1.23	1.38	1.25	1.15	1.23	1.39	1.39	1.19	0.107	1.53[a]	1.02[b]	0.053	0.194	< 0.001	0.380
ALKP (U/L)[2]	209	178	173	164	173	214	198	206	21.3	184	195	10.7	0.592	0.464	0.990
AST (U/L)[2]	237	180	236	181	241	254	205	332	39.9	204	262	22.0	0.211	0.067	0.363
CK (U/L)[2]	1165	1829	928	1136	999	1440	1382	1003	262.2	1480[a]	990[b]	148.8	0.648	0.026	0.666
Glucose (mmol/L)	13.68	13.02	13.63	13.92	12.99	12.49	12.41	12.05	0.420	12.89	13.16	0.214	0.086	0.367	0.312

Cholesterol (mmol/L)	2.01	2.33	2.04	2.14	1.95	2.21	2.41	1.66	0.250	2.10	2.09	0.126	0.445	0.844	0.686
Total protein (g/L)	38.5	40.6	41.2	38.7	39.8	42.1	41.7	36.5	1.87	46.3[a]	33.5[b]	0.94	0.411	< 0.001	0.714
Albumin (g/L)	15.7	16.6	16.4	15.6	16.1	16.6	16.6	15.3	0.62	18.5[a]	13.7[b]	0.31	0.680	< 0.001	0.675
Globulin (g/L)	23.0	24.1	24.7	24.4	23.6	25.5	24.9	21.3	1.31	28.1[a]	19.8[b]	0.65	0.413	< 0.001	0.593
A/G ratio	0.69	0.69	0.67	0.69	0.68	0.66	0.69	0.74	0.019	0.67[b]	0.71[a]	0.010	0.183	< 0.01	0.429
Uric acid (μmol/L)	199	182	188	178	241	208	212	224	22.0	205	203	11.0	0.468	0.907	0.652

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2] ALKP: alkaline phosphatase; AST: aspartate aminotransferase; CK: creatine kinase.

4.4.3 Egg Yolk, Plasma and Liver Fatty Acid Composition

In egg yolk, total saturated fatty acid (TSFA; myristic (14:0); palmitic (16:0) and stearic (18:0) acids) contents in the total lipid of egg yolk did not vary significantly as a function of increasing levels of either ALA or algal DHA biomass (**Table 4.5**). Although the addition of either ALA or DHA to the diets of hens had no significant effects on the level of total monounsaturated fatty acids (TMUFA; palmitoleic (16:1) and oleic (18:1) acids) individually, the increase in the amount of total omega-3 PUFA feeding via both diets led to a significant decrease in the level of TMUFA (**Table 4.5**). Omega-6 and omega-3 PUFA levels in the total lipid of egg yolk, particularly the LCPUFA, exhibited a highly significant ($P < 0.0001$) effects due to the dietary treatments, with in increasing levels of total omega-3 PUFA observed when using either ALA or DHA. Eggs produced by ALA-fed hens deposited an overall 10-fold ALA higher than DHA-fed hens. Additionally, the amount of EPA in egg yolk was significantly increased with the incremental level of total omega-3 PUFA ($P < 0.001$). It is noted that the accumulation of DHA in egg yolk reached a plateau at 0.4% of ALA with 79.5 mg/yolk, which indicated a low conversion efficiency from ALA to DHA. The algal DHA biomass treatment, with the bulk of the total omega-3 PUFA supplied by DHA, increased the level of DHA in egg yolk by 3-fold over that of highest ALA-fed hens (**Table 4.5**). With the increasing level of total omega-3 PUFA in diets, total omega-3 PUFA increased ($P < 0.001$) whereas the total omega-6 PUFA level

reduced ($P < 0.001$) in egg yolk, leading to a decrease in the ratio of omega-6 to omega-3 PUFA in both the ALA and DHA treatment groups ($P < 0.001$) (**Table 4.5**).

Similar significant dietary effects were also observed in the FA composition of plasma (**Table 4.6**) and liver samples (**Table 4.7**) of laying chickens, with specific differences observed between the ALA-fed hens and those receiving the DHA treatment group. Plasma and livers from chickens fed either ALA or DHA decreased the omega-6/omega-3 ratio to similar levels and achieved a steady state of accumulation of DHA and total omega-3 PUFA at 0.4% of ALA and 0.6% of agal DHA biomass, respectively, in diets.

With respect to the inflammatory challenge model, LPS injection elicited specific effects on the deposition of FA in both plasma and liver. LPS caused a significant reduction in liver EPA ($P < 0.05$) and in the ratio of omega-6 to omega-3 PUFA in plasma ($P < 0.05$) Furthermore, there was a significant interaction effect (Diet × Challenge) on plasma EPA and DHA (**Table 4.6**), and for liver LA, ALA and total omega-6 PUFA (**Table 4.7**).

Table 4.5 Fatty acid composition of egg yolk (mg/yolk) as a function of increasing levels of total omega-3 PUFA using either ALA or DHA in diets of hens fed from 20 to 28 weeks of age[1]

	Total omega-3 in diet (%)									
	ALA				DHA				SEM	*P* values[3]
	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80		
TSFA[2]	1579	1505	1454	1336	1492	1468	1530	1502	59.0	0.267
TMUFA[2]	1931^{a}	1863^{ab}	1793^{ab}	1611^{ab}	1765^{ab}	1587^{ab}	1630^{ab}	1523^{b}	81.7	< 0.01
LA (18:2n-6)	598^{a}	575^{ab}	493^{bc}	476^{bc}	501^{bc}	465^{c}	412^{cd}	374^{d}	21.0	< 0.001
GLA (18:3n-6)	5.18^{a}	4.30^{b}	3.56^{bcd}	3.24^{cd}	3.78^{bc}	3.01^{d}	2.22^{e}	2.18^{e}	0.170	< 0.001
ALA (18:3n-3)	38.9^{c}	82.6^{b}	112.8^{a}	147.5^{a}	11.8^{d}	14.0^{d}	15.0^{d}	13.7^{d}	3.24	< 0.001
ARA (20:4n-6)	85.6^{a}	64.2^{b}	52.2^{c}	44.8^{cde}	66.4^{b}	48.6^{cd}	44.0^{de}	40.1^{e}	1.80	< 0.001
EPA (20:5n-3)	1.23^{f}	3.21^{d}	5.15^{c}	5.87^{bc}	1.95^{e}	5.11^{c}	7.53^{ab}	9.86^{a}	0.315	< 0.001
DPA (22:5n-3)	4.34^{de}	5.41^{cd}	7.61^{ab}	8.16^{a}	2.69^{f}	3.81^{e}	5.59^{bcd}	6.72^{abc}	0.337	< 0.001
DHA (22:6n-3)	65.2^{e}	79.5^{d}	77.0^{d}	78.4^{d}	112.1^{c}	146.6^{b}	198.3^{a}	231.5^{a}	4.25	< 0.001
Total *n*-6[2]	689^{a}	642^{ab}	549^{bcd}	525^{cd}	571^{bc}	517^{cd}	458de	416^{e}	21.7	< 0.001
Total *n*-3[2]	109.7^{e}	170.7^{d}	202.6^{c}	239.9^{ab}	128.6^{e}	169.5^{d}	226.4^{bc}	261.8^{a}	7.29	< 0.001
Ratio *n*-6/*n*-3	6.28^{a}	3.76^{c}	2.71^{d}	2.20^{e}	4.45^{b}	3.08^{d}	2.04^{ef}	1.59^{f}	0.104	< 0.001

[1]Data are presented as least squares means and their SEM (n = 10). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2]TSFA (total saturated fatty acids) = sum of myristic (14:0), palmitic (16:0) and stearic (18:0); TMUFA (total monounsaturated fatty acids) = sum of palmitoleic (16:1) and oleic (18:1); total *n*-6 = sum of LA, GLA and ARA; total *n*-3 = sum of ALA, EPA, DPA and DHA.

Table 4.6 Plasma fatty acid composition (mg/mL) of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	Diet effect (total omega-3 in diet, %)									Challenge effect			*P* values		
	ALA				DHA				SEM	Saline	LPS	SEM	Diet	Challenge	Diet × Challenge
	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80							
TSFA[2]	6.953	7.712	6.584	6.963	6.456	8.236	8.329	5.351	0.874	7.237	6.909	0.473	0.301	0.628	0.446
TMUFA[2]	9.123^{ab}	9.892^{a}	8.744^{ab}	9.082^{ab}	7.976^{ab}	9.824^{a}	9.671^{ab}	5.606^{b}	1.170	8.662	8.817	0.625	0.087	0.601	0.429
LA (18:2n-6)	2.678^{a}	3.138^{a}	2.404^{ab}	2.703^{ab}	2.226^{ab}	2.830^{a}	2.536^{ab}	1.580^{b}	0.28	2.486	2.538	0.16	0.00	0.65	0.32
GLA (18:3n-6)	0.022^{ab}	0.024^{a}	0.018^{ab}	0.018^{abc}	0.017^{ab}	0.017^{ab}	0.014^{bc}	0.009^{c}	0.002	0.018	0.017	0.001	<.0001	0.35	0.90
ALA (18:3n-3)	0.144^{b}	0.392^{a}	0.433^{a}	0.670^{a}	0.053^{c}	0.083^{bc}	0.100^{bc}	0.062^{c}	0.04	0.262	0.222	0.03	<.0001	0.41	0.81
ARA (20:4n-6)	0.409^{a}	0.397^{a}	0.308^{a}	0.286^{a}	0.315^{a}	0.308^{a}	0.292^{a}	0.161^{b}	0.03	0.305	0.314	0.02	<.0001	0.60	0.51
EPA (20:5n-3)	0.009^{c}	0.019^{b}	0.026^{ab}	0.030^{ab}	0.010^{c}	0.030^{ab}	0.047^{a}	0.035^{ab}	0.003	0.029	0.023	0.002	<.0001	0.052	0.01
DPA (22:5n-3)	0.017^{bc}	0.028^{ab}	0.034^{a}	0.039^{a}	0.011^{c}	0.022^{ab}	0.043^{a}	0.024^{ab}	0.004	0.027	0.028	0.002	<.0001	0.72	0.26
DHA (22:6n-3)	0.333^{c}	0.432^{c}	0.373^{c}	0.397^{c}	0.465^{bc}	0.820^{ab}	1.051^{a}	0.900^{a}	0.07	0.635	0.558	0.04	<.0001	0.28	0.04
Total *n*-6[2]	3.110^{a}	3.560^{a}	2.730^{ab}	3.007^{ab}	2.558^{ab}	3.155^{a}	2.842^{ab}	1.750^{b}	0.312	2.809	2.868	0.174	0.002	0.81	0.43
Total *n*-3[2]	0.503^{c}	0.871^{ab}	0.868^{ab}	1.135^{a}	0.539^{bc}	0.954^{a}	1.241^{a}	1.022^{a}	0.114	0.953	0.831	0.067	< 0.001	0.213	0.265
n-6/*n*-3	6.637^{a}	4.100^{bc}	3.182^{cd}	2.739^{de}	4.731^{b}	3.381^{cd}	2.485^{e}	1.796^{f}	0.190	3.349^{b}	3.914^{a}	0.110	< 0.001	0.001	0.645

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2]TSFA (total saturated fatty acids) = sum of myristic (14:0), palmitic (16:0) and stearic (18:0); TMUFA (total monounsaturated fatty acids) = sum of palmitoleic (16:1) and oleic (18:1); total *n*-6 = sum of LA, GLA and ARA; total *n*-3 = sum of ALA, EPA, DPA and DHA.

Table 4.7 Liver fatty acid composition (mg/g) of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	Diet effect (total omega-3 in diet, %)									Challenge effect			*P* values		
	ALA				DHA				SEM	Saline	LPS	SEM	Diet[3]	Challenge	Diet × challenge
	0.20	0.40	0.60	0.80	0.20	0.40	0.60	0.80							
TSFA[2]	32.1	25.1	31.4	21.9	46.0	35.3	40.9	36.9	5.58	35.7	31.7	2.80	0.071	0.317	0.413
TMUFA[2]	40.6	28.5	38.2	25.7	51.4	37.2	44.7	37.3	7.10	39.0	36.9	3.55	0.249	0.670	0.437
LA (18:2n-6)	10.75	8.38	9.01	7.80	12.02	10.13	10.10	9.42	1.051	10.17	9.23	0.547	0.056	0.322	< 0.05
GLA (18:3n-6)	0.070	0.069	0.066	0.047	0.104	0.063	0.048	0.049	0.0095	0.068	0.061	0.0058	0.052	0.375	0.648
ALA (18:3n-3)	0.66^{bc}	0.89^{ab}	1.38^{a}	1.56^{a}	0.25^{d}	0.28^{cd}	0.32^{cd}	0.31^{cd}	0.7049	0.81^{a}	0.60^{b}	0.088	< 0.001	0.11	0.032
ARA (20:4n-6)	1.87^{a}	1.69^{ab}	1.59^{ab}	1.41^{bcd}	1.52^{ab}	1.48^{bc}	1.19^{cd}	1.09^{d}	0.077	1.48	1.48	0.37	< 0.001	0.896	0.472
EPA (20:5n-3)	0.024^{d}	0.061^{c}	0.112^{ab}	0.138^{a}	0.032^{d}	0.070^{c}	0.107^{ab}	0.116^{ab}	0.0067	0.088^{a}	0.077^{b}	0.0038	< 0.001	0.034	0.243
DPA (22:5n-3)	0.047^{de}	0.072^{bc}	0.101^{a}	0.117^{a}	0.036^{e}	0.053^{cd}	0.075^{b}	0.087^{ab}	0.0057	0.075	0.072	0.0028	< 0.001	0.441	0.565
DHA (22:6n-3)	1.335^{c}	1.412^{c}	1.418^{c}	1.383^{c}	1.895^{b}	2.419^{b}	3.782^{a}	5.537^{a}	0.1691	2.47	2.329	0.1092	< 0.001	0.376	0.063
Total *n*-6[2]	12.50^{ab}	10.14^{ab}	10.66^{ab}	9.26^{b}	13.65^{a}	11.67^{ab}	11.34^{ab}	10.56^{ab}	1.010	11.67	10.78	0.506	< 0.05	0.308	< 0.05
Total *n*-3[2]	1.96^{d}	2.43^{cd}	3.01^{c}	3.19^{bc}	2.22^{d}	2.82^{cd}	4.28^{ab}	6.05^{a}	0.236	3.41	3.08	0.131	< 0.001	0.080	0.077
Ratio *n*-6/*n*-3	6.33^{a}	4.15^{b}	3.53^{bc}	2.89^{bc}	6.31^{a}	4.47^{ab}	2.76^{c}	1.81^{d}	0.390	4.01	4.05	0.195	< 0.001	0.441	0.413

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

[2]TSFA (total saturated fatty acids) = sum of myristic (14:0), palmitic (16:0) and stearic (18:0); TMUFA (total monounsaturated fatty acids) = sum of palmitoleic (16:1) and oleic (18:1); total *n*-6 = sum of LA, GLA and ARA; total *n*-3 = sum of ALA, EPA, DPA and DHA.

4.4.4 Plasma Oxylipin Composition

We measured the oxylipins originating from omega-6 (mainly ARA) and omega-3 PUFA (ALA, EPA and DHA) in the plasma of laying hens supplied with increasing level of omega-3 PUFA from either ALA or DHA 4h after saline or LPS injection. The LC-MS/MS detected LTs, Tx and PG (LTB_4, TxB_2, PGF_{2a}, PGE_2, PGD_2, dhk PGF_{2a}), hydroxy FA (5-HETE, 8-HETE, 11-HETE, 15-HETE, 17-HETE, 18-HETE, 20-HETE, 15-oxoETE, 12-HHTrE) and dihydroxy FA (8,9 DiHETrE, 11,12 DiHETrE, 14,15 DiHETrE), all derived from ARA; ALA derived keto FA, 9 oxo OTrE, and epoxygenated FA, 12, 13-EpODE; EPA derived hydroxy FA (8-HEPE, 9-HEPE, 12-HEPE, 15-HEPE, 18-HEPE), dihydroxy FA (14, 15 diHETE) and PGD_3; DHA derived hydroxy FA (7-HDoHE, 10-HDoHE, 11-HDoHE, 13-HDoHE, 14-HDoHE, 16-HDoHE, 17-HDoHE and 20-HDoHE), dihydroxy FA (16,17 DiHDoPE, 19,20 DiHDoPE) and epoxy FA (19,20 EpDPE) in plasma.

A significant effect of dietary omega-3 PUFA was found for ARA-derived PGF_{2a} (**Table 4.8a**) and 5-HETE (**Table 4.8b**) *($P < 0.05$)*; ALA-derived 12,13 EpODE and 9 oxoOTrE (**Table 4.9**; *$P<0.05$*); EPA-derived 9-, 12-, 18-HEPE (**Table 4.10**; *$P < 0.05$*) and DHA-derived 16,17 DiHDoPE, 19,20 DiHDoPE, 19,20 EpDPE, 10-, 11-, 14-, 16- and 20-HDoHE (**Table 4.11**; *$P < 0.05$*). In general, the level of ARA-derived oxylipins decreased with the increasing level of omega-3 PUFA in the diet and was particularly reduced in the DHA-fed groups, among these, PGF_{2a} and 5-HETE decreased significantly (*$P<0.05$*). Moreover, ALA-derived oxylipins significantly increased with graded ALA levels in the

diet and also was significantly higher than that in the DHA-fed group (**Table 4.9**; *P<0.05*). 9-, 12-, 18-HEPE generated from EPA and 7-,11-, 13-, 14-, 16-, 17-HDoHE exhibited significant increases as the total omega-3 PUFA increased (Table 4.9; $P < 0.05$), however, certain oxylipins generated from DHA, such as 16, 17- and 19,20-DiHDoPE decreased significantly only in the ALA-fed group (**Table 4.11**).

The acute inflammatory challenge elicited via an infusion of LPS significantly influenced the majority of detected oxylipins that derived from ARA ($P < 0.05$) and EPA derived 9-HEPE ($P < 0.05$). However, there were no differences between ALA- and DHA-derived oxylipins following LPS treatment. LPS significantly induced the EPA-derived 9-HEPE and the ARA-derived oxylipins, including PGD_2, PGE_2, PGF_{2a}, dhk PGF_{2a}, 8,9 DiHETrE, 11,12 DiHETrE, 14,15 DiHETrE, 5-HETE, 8-HETE, 17-HETE, 18-HETE and 20-HETE increased. However, LTB_4 derived from ARA reduced after LPS treatment ($P < 0.05$). No significant interaction effects (Diet × Challenge) were noted for ARA-, ALA-, EPA- and DHA-derived oxylipins.

Table 4.8a ARA-derived oxylipins (ng/mL) via COX pathway in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	12-HHTrE	dhk PGF2a	PGD_2	PGE_2	PGF2a	TXB_2
Diet effect (total omega-3 in diet, %)						
ALA						
(Flaxseed oil)						
0.20	0.0717	0.0075	0.0433	0.0089	0.0064	0.0207
0.40	0.0525	0.0087	0.0301	0.0061	0.0040	0.0208
0.60	0.1254	0.0080	0.0224	0.0054	0.0034	0.0213
0.80	0.0194	0.0091	0.0199	0.0043	0.0045	0.0132
DHA						
(Algal DHA biomass)						
0.20	0.0840	0.0156	0.0420	0.0057	0.0045	0.0100
0.40	1.2731	0.0104	0.0413	0.0062	0.0042	0.2668
0.60	0.0741	0.0042	0.0246	0.0046	0.0021	0.0157
0.80	0.0338	0.0068	0.0163	0.0044	0.0009	0.0073
SEM	0.1704	0.0026	0.0102	0.0017	0.0012	0.0370
Challenge effect						
LPS	0.3513	0.0112^{a}	0.0426^{a}	0.0079^{a}	0.0059^{a}	0.0780
Saline	0.0834	0.0064^{b}	0.0176^{b}	0.0035^{b}	0.0016^{b}	0.0160
SEM	0.2027	0.0013	0.0104	0.0010	0.0007	0.0446
P values						
Diet	0.106	0.122	0.368	0.820	<0.001	0.530
Challenge	0.377	<0.01	<0.01	<0.01	<0.01	0.354
Diet × Challenge	0.135	0.403	0.331	0.659	0.153	0.771

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

Table 4.8b ARA-derived oxylipins (ng/mL) via LOX pathway in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	11-HETE	15-HETE	15-oxoHETE	5-HETE	8-HETE	LTB_4
Diet effect (total omega-3 in diet, %)						
ALA						
(Flaxseed oil)						
0.20	0.0160	0.0182	0.0058	0.0341[a]	0.0225	0.0007
0.40	0.0145	0.0099	0.0036	0.0304[ab]	0.0091	0.0007
0.60	0.0129	0.0141	0.0039	0.0333[ab]	0.0133	0.0007
0.80	0.0099	0.0161	0.0023	0.0174[ab]	0.0183	0.0004
DHA						
(Algal DHA biomass)						
0.20	0.0127	0.0176	0.0086	0.0380[a]	0.0271	0.0017
0.40	0.0437	0.0337	0.0037	0.0451[ab]	0.0297	0.0019
0.60	0.0137	0.0069	0.0064	0.0346[ab]	0.0179	0.0009
0.80	0.0091	0.0064	0.0025	0.0153[b]	0.0177	0.0009
SEM	0.0053	0.0057	0.0018	0.0070	0.0061	0.0004
Challenge effect						
LPS	0.0224	0.0180	0.0049	0.0372[a]	0.0238[a]	0.0006[b]
Saline	0.0108	0.0127	0.0043	0.0248[b]	0.0151[b]	0.0014[a]
SEM	0.0054	0.0038	0.0009	0.0037	0.0057	0.0002
P values						
Diet	0.167	0.191	0.228	<0.05	0.283	0.113
Challenge	0.162	0.339	0.645	<0.05	<0.05	<0.01
Diet × Challenge	0.179	0.777	0.161	0.435	0.109	0.437

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

Table 4.8c ARA-derived oxylipins (ng/mL) via CYP pathway in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	11,12 DiHETrE	14,15 DiHETrE	8,9 DiHETrE	17-HETE	18-HETE	20-HETE
Diet effect (total omega-3 in diet, %)						
ALA						
(Flaxseed oil)						
0.20	0.0159	0.0336	0.0084	0.0060	0.0017	0.0633
0.40	0.0101	0.0258	0.0043	0.0057	0.0023	0.0563
0.60	0.0078	0.0221	0.0044	0.0034	0.0017	0.0488
0.80	0.0070	0.0215	0.0033	0.0034	0.0020	0.0402
DHA						
(Algal DHA biomass)						
0.20	0.0124	0.0331	0.0053	0.0051	0.0019	0.0458
0.40	0.0086	0.0266	0.0053	0.0050	0.0014	0.0402
0.60	0.0128	0.0278	0.0068	0.0040	0.0023	0.0528
0.80	0.0058	0.0205	0.0040	0.0049	0.0022	0.0378
SEM	0.0031	0.0037	0.0017	0.0012	0.0005	0.0076
Challenge effect						
LPS	0.0147[a]	0.0324[a]	0.0080[a]	0.0062[a]	0.0025[a]	0.0670[a]
Saline	0.0055[b]	0.0204[b]	0.0025[b]	0.0032[b]	0.0014[b]	0.0294[b]
SEM	0.0018	0.0019	0.0017	0.0006	0.0003	0.0052
P values						
Diet	0.365	0.137	0.597	0.370	0.919	0.531
Challenge	<0.01	<.001	<.001	<0.01	<0.01	<.001
Diet × Challenge	0.402	0.850	0.739	0.859	0.290	0.785

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

Table 4.9 ALA-derived oxylipins (ng/mL) in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1].

		Diet effect (total omega-3 in diet, %)								Challenge effect				*P* values		
		ALA				DHA				SEM	LPS	Saline	SEM	Diet	Challenge	Diet × Challenge
		0.2	0.4	0.6	0.8	0.2	0.4	0.6	0.8							
CYP-e	12,13 EpODE	0.0013^{abc}	0.0014^{ab}	0.0027^{a}	0.0035^{abc}	0.0005^{abc}	0.0006^{c}	0.0004^{abc}	0.0004^{bc}	0.0004	0.0018	0.0013	0.0002	<.0001	0.083	0.721
LOX	9 oxoOTrE	0.0624^{ab}	0.0996^{bc}	0.1143^{b}	0.1889^{b}	0.0275^{ac}	0.0206^{ac}	0.0274^{a}	0.0172^{a}	0.0173	0.1083	0.0540	0.0125	0.002	0.135	0.579

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

Table 4.10 EPA-derived oxylipins (ng/mL) in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	PGD_3 (COX)	14,15 diHETE (CYP-e)	18-HEPE (CYP-h)	12-HEPE (LOX)	15-HEPE (LOX)	8-HEPE (LOX)	9-HEPE (LOX)
Diet effect (total omega-3 in diet, %)							
ALA							
(Flaxseed oil)							
0.20	0.0013	0.0624	0.0101	0.0027	0.0037	0.0033	0.0049^{c}
0.40	0.0014	0.0738	0.0097	0.0054	0.0045	0.0144	0.0100^{ab}
0.60	0.0028	0.0674	0.0108	0.0051	0.0058	0.0048	0.0166^{ab}
0.80	0.0025	0.0993	0.0167	0.0070	0.0066	0.0240	0.0217^{ab}
DHA							
(Algal DHA biomass)							
0.20	0.0026	0.0959	0.0240	0.0105	0.0077	0.0175	0.0034^{c}
0.40	0.0023	0.1251	0.0370	0.0210	0.0140	0.0234	0.0222^{ab}
0.60	0.0035	0.2079	0.0672	0.0454	0.0127	0.0281	0.0414^{ab}
0.80	0.0045	0.2265	0.0645	0.1049	0.0075	0.0294	0.0882^{a}
SEM	0.0011	0.0308	0.0034	0.0107	0.0024	0.0099	0.0112
Challenge effect							
LPS	0.0027	0.1323	0.0279	0.0182	0.0093	0.0245	0.0393^{a}
Saline	0.0023	0.1058	0.0321	0.0323	0.0063	0.0118	0.0127^{b}
SEM	0.0006	0.0200	0.0074	0.0104	0.0014	0.0056	0.0078
P values							
Diet	0.657	0.120	<0.047	<0.017	0.198	0.170	<0.05
Challenge	0.685	0.362	0.688	0.363	0.124	0.118	<0.05
Diet × Challenge	0.818	0.759	0.883	0.881	0.704	0.559	0.461

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

Table 4.11 DHA-derived oxylipins (ng/mL) in plasma of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge[1]

	16,17 DiHDPE (CYP-e)	19,20 DiHDPE (CYP-e)	19,20 EpDPE (CYP-e)	20-HDoHE (LOX)	10-HDoHE (LOX)	11-HDoHE (LOX)	13-HDoHE (LOX)	14-HDoHE (LOX)	16-HDoHE (LOX)	17-HDoHE (LOX)	7-HDoHE (LOX)
Diet effect (total omega-3 in diet, %)											
ALA											
(ALA Flaxseed oil)											
0.20	0.0111^{a}	0.0535^{bc}	0.0007^{ab}	0.0215^{abc}	0.0104^{ab}	0.0055	0.0085	0.0221^{b}	0.0100^{b}	0.0298	0.0401
0.40	0.0084^{bc}	0.0452^{c}	0.0017^{ab}	0.0182^{bc}	0.0070^{b}	0.0022	0.0039	0.0150^{b}	0.0083^{b}	0.0233	0.0120
0.60	0.0077^{c}	0.0403^{cd}	0.0011^{ab}	0.0162^{c}	0.0090^{b}	0.0027	0.0165	0.0163^{b}	0.0116^{b}	0.0200	0.0247
0.80	0.0075^{c}	0.0390^{d}	0.0002^{b}	0.0170^{bc}	0.0068^{b}	0.0048	0.0113	0.0178^{b}	0.0112^{b}	0.0149	0.0179
DHA											
(Algal DHA biomass)											
0.20	0.0168^{ab}	0.0830^{bc}	0.0019^{ab}	0.0253^{abc}	0.0182^{a}	0.0105	0.0179	0.0263^{ab}	0.0243^{a}	0.0484	0.0355
0.40	0.0255^{ab}	0.1208^{ab}	0.0025^{ab}	0.0485^{ab}	0.0349^{ab}	0.0216	0.0611	0.0536^{a}	0.0443^{a}	0.1234	0.0521
0.60	0.0402^{a}	0.1849^{a}	0.0084^{a}	0.0451^{abc}	0.0208^{ab}	0.0204	0.0288	0.0791^{a}	0.0250^{a}	0.0879	0.0518
0.80	0.0427^{a}	0.1848^{a}	0.0081^{a}	0.0529^{a}	0.0374^{ab}	0.0148	0.0420	0.0863^{a}	0.0244^{a}	0.0529	0.0635
SEM	0.0044	0.0183	0.0014	0.0056	0.0047	0.0037	0.0110	0.0070	0.0054	0.0210	0.0189
Challenge effect											
LPS	0.0222	0.0126	0.0020	0.0328	0.0213	0.0118	0.0311	0.0394	0.0210	0.0539	0.0352
Saline	0.0178	0.0853	0.0041	0.0284	0.0148	0.0088	0.0165	0.0395	0.0188	0.0463	0.0392
SEM	0.0029	0.0116	0.0010	0.0031	0.0028	0.0022	0.0073	0.0043	0.0032	0.0122	0.0104
P values											
Diet	<.001	<.001	<0.05	<0.01	<0.01	<0.021	0.051	<.001	<0.01	0.177	0.490
Challenge	0.303	0.304	0.165	0.327	0.111	0.330	0.173	0.992	0.625	0.662	0.786

Diet × Challenge	0.850	0.881	0.327	0.576	0.512	0.633	0.193	0.535	0.577	0.734	0.423

[1]Data are presented as least squares means and their SEM (n = 5). Values with different superscripts within a row are significantly different at $P < 0.05$.

4.4.5 Relative mRNA Expression

Dietary omega-3 PUFA supplementation had a significant effect on the expression of TLR4 in the spleen (**Figure 4.2(A);** $P < 0.001$). Supplementation with increasing levels of DHA reduced the expression of TLR4. Especially, there was a significant decrease in the group receiving 0.8% DHA group ($P < 0.001$). In addition, LPS injection induced a significantly higher expression of both TFN-γ (**Figure 4.1(B)**) and TLR4 (**Figure 4.2(B)**) in the spleen ($P < 0.001$). No significant diet × challenge interaction effects were observed for TFN-γ and TLR4 expression.

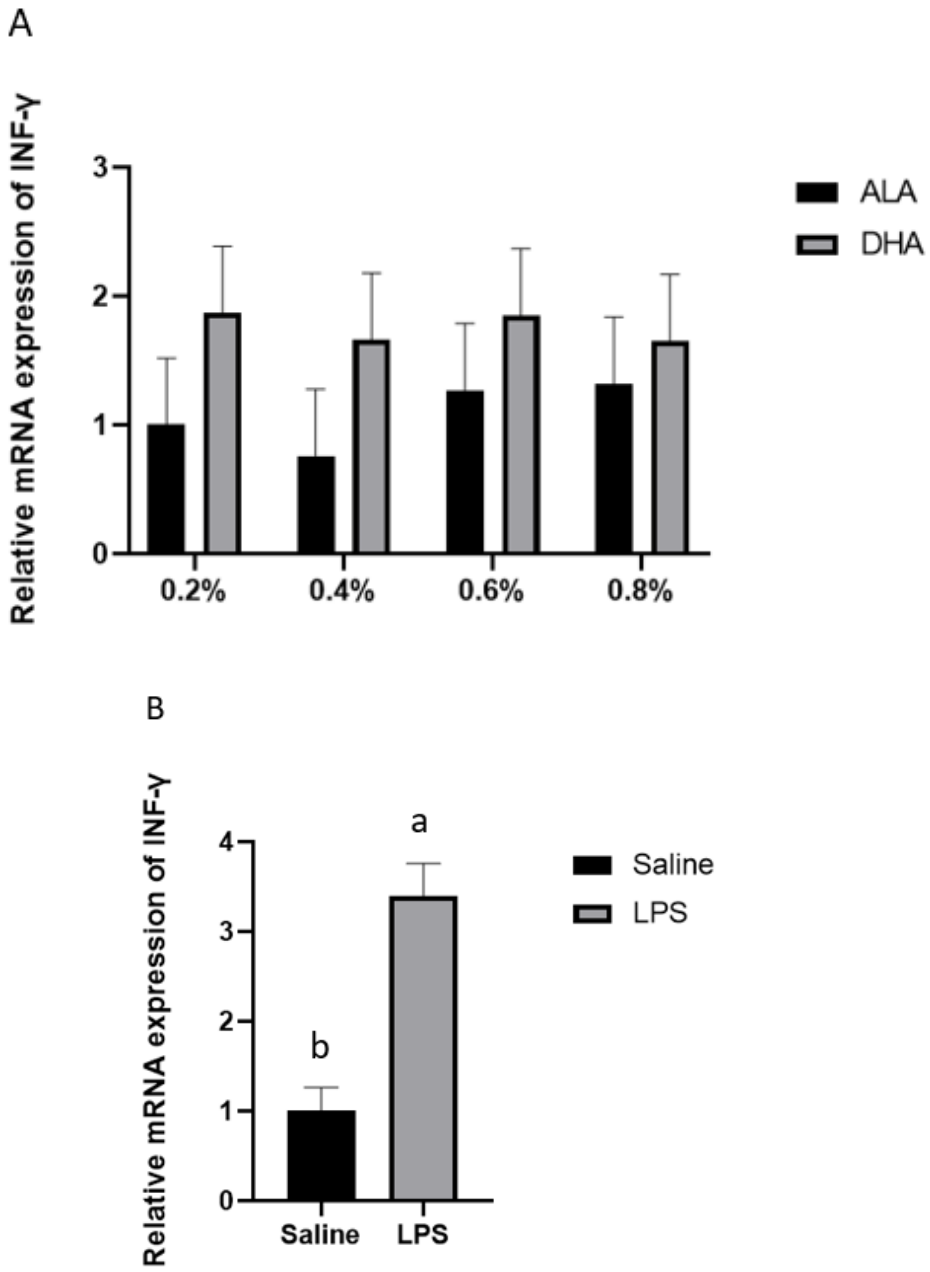


Figure 4.1 Diet effect (A) and challenge effect (B) on the relative mRNA expression of *INF-γ* in the spleen of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge.

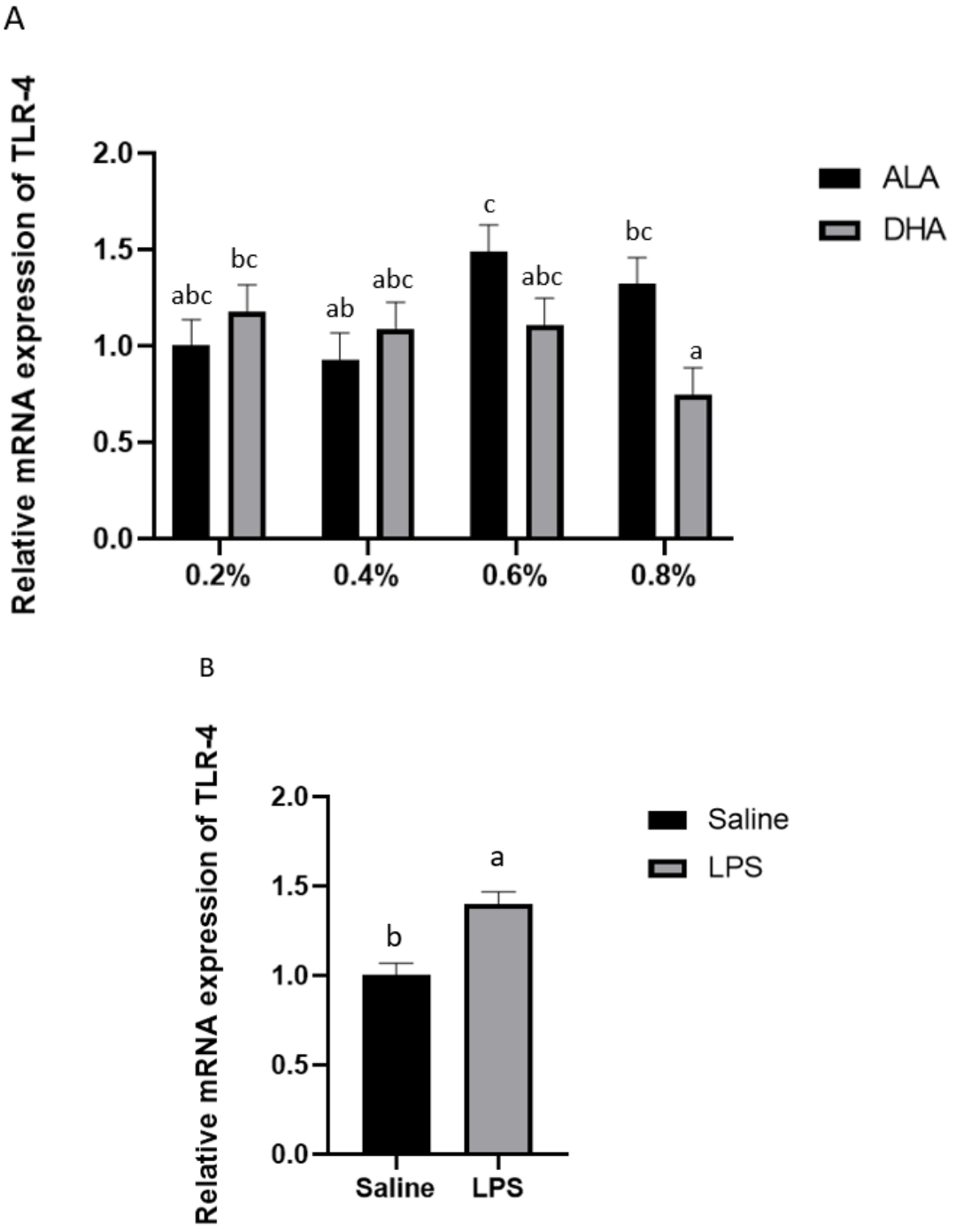


Figure 4.2 Diet effect (A) and challenge effect (B) on relative mRNA expression of *TLR4* in the spleen of laying hens fed diets containing increasing levels of total omega-3 from either ALA or DHA following LPS challenge.

4.5 Discussion and conclusion

The present study was designed to: 1) define omega-3 PUFA requirements for optimal health and performance; 2) identify whether ALA (provided by flaxseed oil) or DHA (algal DHA biomass) and their increasing levels in diet can make a difference with regards to birds' health and productivity; and 3) determine the optimum quantity (level) of ALA or DHA to develop immunomodulatory effects in birds in order to overcome an immune challenge elicited by LPS. The increasing levels (0.2%, 0.4%, 0.6%, 0.8% by weight in the diet) of either ALA or DHA were added to the basal diets of laying hens, the latter formulated to be low in total omega-3 PUFA.

Hen performance, the concentration of other lipids of egg yolk, plasma and liver, plasma oxylipins and biochemistry, and mRNA expression of cytokines in spleen were assessed. No significant differences were observed between treatments with respect to hen performance. However, the current results indicated that the inclusion of increasing levels of total omega-3 PUFA in the diet, provided as either the ALA or DHA, significantly increased the levels of both the individual as well as the total omega-3 whereas decreased omega-6 PUFA and the omega-6 to omega-3 PUFA ratio in egg yolk, plasma and liver in laying hens. The accumulation of DHA in egg yolk reaches a plateau at 0.4% of ALA in the diet, although no significant differences were noted in liver and plasma in the ALA group. These results agree with previous studies (Neijat, et al., 2016; Selvaraj and Cherian, 2004; Hall et al., 2007a; Gonzalez et al., 2011). Neijat et al. (2016, 2017) reported that

individual as well as total omega-3 PUFA in the yolk, plasma and liver increased ($P < 0.001$) linearly as a function of total omega-3 PUFA intake and the deposition of DHA in egg yolk and liver reached to a stable status at 0.4% of ALA containing in the diet. Therefore, 0.4% of ALA by diet weight (**Table 4.1**) would be an estimated requirement in laying hens' diet. The *de novo* synthesis of fatty acids and lipid metabolism occurs primarily in the liver, positioning this organ as a key mediator of peripheral tissue fatty acid composition (Gonzalez et al., 2011; Calder, 2012).

The current results specifically demonstrated that algal DHA biomass tended to reach higher accumulation than treatments with the primary ALA precursor, likely due to the presumed low conversion efficiency of ALA to EPA and DHA in vertebrates. Previous human studies indicated that the efficiency of conversion of ALA to EPA to DPA and eventually to DHA was noticed to be approximately 0.20%, 3.65% and 37%, respectively (Pawlosky et al., 2001; Plourde and Cunnane, 2007). One main reason for these efficiency rates is that there is a competition between LA and ALA for the desaturase and elongase enzymes during conversion to final products (Wood et al., 2015). Another factor limiting conversion is that high amounts of ALA undergo β-oxidation to provide energy (60%-85%), but for other FA, like ARA, only around 30% is catabolized (Barceló-Coblijn et al., 2009). Thus, the conversion of ALA to EPA and DHA in vertebrates is known to be inefficient and subsequently leads to a low deposition of ALA in poultry tissues and products. Whereas exogenous (dietary) DHA can be incorporated into the egg yolk without

any conversion steps, thus enabling a more efficient deposition of DHA. Importantly, our results indicated that the nutritional value of eggs was improved with the decreased ratios of omega-6 to omega-3 of 1.80/1– 2.74/1 in the eggs derived from hens consuming 0.8% of total omega-3 fatty acid in the diet, which is close to the recommended levels for human consumptions (with ratios of omega-6 to omega-3 of 1/1 or 2/1) (Simopoulos, 2008).

Although no diet effects were observed on plasma biochemistry, LPS significantly decreased the level of calcium (Ca) and phosphorus (P) ($P<0.001$). The current results indicated that LPS challenge can have an impact on the metabolism of both Ca and P. This can be potentially explained by the fact that, with either acute or chronic responses induced by LPS, neuroendocrine and physiological processes can be altered. This may lead to decreased intake and absorption of nutrients, including vitamins, minerals and trace elements, leading to a reduction of these nutrients in plasma, to the point where, particularly under chronic conditions, growth performance in laying hens is compromised (Nie et al., 2018). Moreover, total protein, albumin, globulin and A/G ratio were also significantly reduced after LPS administration. Recruitment of proteins during the acute phase response has been shown to be substantial in avian species, therefore, the decrease of total protein, albumin, globulin after 4h PLI in the current research may be due to the fact that peripheral protein reserves were drawn upon to support the synthesis of acute-phase proteins in response to the acute effects of LPS (Munyaka et al., 2012).

The current study provides a comprehensive and novel description of the plasma oxylipin profile, especially those derived from ARA, ALA, EPA and DHA, in laying hens and demonstrates how it is affected by dietary omega-3 PUFA and LPS challenge. Oxylipins are synthesized by the oxidation of PUFA in an activation-dependent manner. Upon cellular activation, phospholipase A2 (PLA_2) hydrolyzes PUFA from the lipid membrane generating free PUFA. The free PUFA are then oxidized to distinct classes of oxylipins such as eicosanoids and docosanoids derived exclusively from LCPUFA, via three major enzymatic pathways, cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 (CYP) (Tourdot et al., 2014). Advances in targeted lipidomics approaches, including LC-MS/MS methods, have been widely used to report the formation of potent anti-inflammatory mediators derived from the omega-3 PUFA (mainly ALA, EPA and DHA) in recent studies (Devassy et al., 2016; Caligiuri et al., 2016; Leng et al., 2017). Increasing the level of total omega-3 PUFA (either ALA or DHA) markedly changed the plasma oxylipin profiles. In this study, increasing levels of intake of dietary omega-3 PUFA led to decrease levels of ARA-derived oxylipins, including PGs, TXB_2, LTB_4, HETEs and DiHETrE, which might afford metabolic and physiologic protection to hens, as has been observed in previous studies (Liu et al., 2014) and these results were also shown in other species, including mice (Ferdouse et al., 2019; Rey et al., 2019). However, the latter relationships in chickens, including laying hens, have received limited attention. PGs, as well as TXs, are produced from ARA via the COX pathway, and yield more

inflammatory, vasoconstrictory, and proliferative effects compared to oxylipins that derived from omega-3 PUFA, such as increased vascular permeability, increased vascular dilation, stimulated migration and proliferation of smooth muscle cell (Gabbs et al., 2015; Kang and Weylandt, 2008). Nevertheless, EPA can form 3-series PGs and TXs (e.g., PGD_3) through LOX pathways with less pro-inflammatory or even opposite effects as compared to their counterparts generated from ARA (Simopoulos, 2002; Calder, 2006). LTB_4, a potent inflammatory lipid mediator (Serhan et al., 1995), is derived from ARA via the LOX pathway and it contributes to the pathogenesis of rheumatoid arthritis in human (Alten et al., 2004) and mouse models (Kim et al., 2006; Shao et al., 2006). ARA also yields hydroxy FA oxylipins via the LOX and COX pathways, resulting in HETEs and these are further converted to oxo-ETE via dehydrogenase activity or DiHETE by further COX activity (as shown in **Table 4.9a**). The products, particularly 5-HETE and 15-HETE, are related to chronic diseases such as inflammation (Uderhardt and Kronke, 2012), obesity (Martínez-Clemente et al., 2011), cardiovascular disease (Poeckel and Funk, 2010) and cancer (Menna et al., 2010). Additionally, in the current results, compared with the ALA fed group, feeding DHA to hens did not impact the overall plasma ARA oxylipin profiles. These results were not consistent with those of previous studies, potentially due to species differences (Devassy et al., 2017; Ferdouse et al., 2019).

There is little functional information available on ALA-derived oxylipins in laying hens. 12, 13-EpODE and 9 oxo OTrE were synthesized via LOX and CYP-e pathway

respectively and were detected in this study. The significantly higher levels of ALA oxylipins in the ALA-fed group compared with the DHA-fed group indicated that oxylipin production can be predicted from their precursor PUFA (Leng et al., 2018).

Our current study noted that EPA-derived oxylipins, including HEPEs and PGD_3, were elevated due to increasing levels of total omega-3 PUFA supplementation (either precursor of ALA or preformed DHA). Furthermore, the results showed a higher level of EPA increased the formation of 3-series prostaglandins via COX and LOX pathways, and these have less pro-inflammatory or even positive effects as compared to their counterparts derived from ARA (Simopoulos, 2002; Calder,2006). Significant diet effects were observed for 9-HEPE, 12-HEPE and 18-HEPE. EPA can produce hydroperoxy FA, which can be further converted to hydroxy FA (e.g., 9-HEPE and 12-HEPE) via the LOX enzymatic pathway (Kulkarni and Srinivasan, 1986). Moreover, hydroxy FA from EPA with hydroxy groups on the 18 to 20-carbon positions can also be formed via ω-hydroxylase activity of the CYP pathway (e.g., 18-HEPE), and the majority of these possess anti-inflammatory effects (Isobe et al., 2012). DHA can yield lipid mediators with purported beneficial effects, for example, HDoHE via LOX, including the 10-, 11-, 13-, 14-, 16-, 17- HDoHE species, or via CYP ω-hydroxylase activity resulting 20-HDoHE (Serhan et al., 2002; González-Périz et al., 2006). Oxylipins can be produced from DHA via CYP epoxygenase activity yielding epoxy FA (e.g., 19,20 EpDPE), and further metabolized to dihydroxy-docosahexaenoic acid (DiHDoHE) (Gabbs et al., 2015). Most of

the previous research focused on the dietary omega-3 PUFA in relation to specific oxylipins in mammalian species and in this study, we offered data on an avian species, specifically the laying hen.

Current results in laying hens provide evidence that oxylipins produced from DHA were elevated with the increasing level of algal DHA biomass, whereas DiHDPE, 19,20 EpDPE and 20-HDoHE were observed to decrease in the ALA fed group. This result was consistent with previous research conducted on participants who were diagnosed as hypertensive and this observation may explain by the fact that other oxylipins may have contributed as well (Caligiuri et al., 2014). ARA can generate the EpETrE, with further metabolism to DiHETrE by the enzyme soluble epoxide hydrolase (sEH) (Sudhahar et al., 2010). sEH also converts the protoxins, epoxyoctadecenoic acids, to the dihydroxyoctadecenoic acids, generally regarded as potent cytotoxic and proinflammatory metabolites (Moghaddam et al., 1997; Zheng et al., 2001) and 19,20- DiHDPE is also thought to be an sEH product. DHA derived HDoHE, mainly 13- and 17-HDoHE, decreased TNF-a-induced cytokine production and LPS-induced TNF-a secretion respectively (Serhan et al., 2002; Weylandt et al., 2011). Collectively, the combination of higher levels of EPA and DHA oxylipins and lower levels of ARA oxylipins via the inclusion of total omega-3 PUFA to diet would be considered to have an overall protective effect on the immune functions of laying hens.

Numerous studies confirmed that the dietary intake of omega-3 PUFA can modulate the synthesis of oxylipins, and inflammation is known to influence the metabolism of oxylipins. Additionally, omega-3 PUFA can reduce the production of LPS-induced pro-inflammatory cytokines. LPS is the main component of the cell wall of Gram-negative bacteria such as *Escherichia coli* (*E. coli*), and a highly efficient pro-inflammatory substance (Masashi and Ken-ichi, 2002; Wang et al., 2007). It has been widely used in numerous studies to model bacterial infection experimentally in poultry and other animals (Sunwoo et al., 1996; Abbas et al., 2000; Yang et al., 2008). In this study, laying hens received an intravenous injection of *E. coli* equivalent to 8 mg/kg of BW LPS. Since chickens are more resistant to endotoxin from gram-negative bacteria (Gehad et al, 2002), the 8 mg/kg of BW of LPS can be used to induce the acute phase response after 4 h post-injection (Munyaka et al., 2012; Munyaka et al., 2013; Jing et al., 2014). In addition, LPS can induce monocytes or macrophages to release pro-inflammatory cytokines, such as TNF-α, IL-1, IL-6 and IL-8, via the interaction with the TLR4 receptor present on these cells (Rietschel et al., 1998). Based on previous research, LPS induces the release of pro-inflammatory cytokines including IFN-γ, IL-1, IL-6, except TNF-α, which can reach a significantly higher level at 3-4 h post LPS injection (PLI) both in mRNA expression level in spleen and protein expression levels in the plasma of laying hens (Nakamura et al., 1998; Gehad et al, 2002; Leshchinsky and Klasing, 2001; Leshchinsky and Klasing, 2003). Therefore, in this study, all the samples were collected at 4h PLI.

In the current study, LPS markedly increased ARA-derived oxylipins **(Table 4.8a-c**). LPS can be a useful inflammatory substrate because it has been shown to directly activate and induce monocytes and macrophages to produce eicosanoids, such as PGE_2 (Janský et al., 1995; Calder, 2002). LPS also led to a decrease in EPA-derived oxylipins, notably, LPS significantly reduced 9-HEPE. It was observed that LPS decreased EPA in liver, thus, the decreased EPA in liver may be the result of enhanced usage for the synthesis of EPA-derived oxylipins. Moreover, LPS can lead to a more pronounced increase in oxylipins and may be better suited to investigate effects on the ARA cascade (Willenberg et al., 2016), however data with respect to the effect of omega-3 PUFA on oxylipins under the acute response of LPS in the avian species, especially the laying hens, are limited. Thus, inhibition of the generation of ARA-derived pro-inflammatory mediators via competition with ARA for the COX and LOX enzymes may represent the primary mechanism underlying the anti-inflammatory effect of omega-3 PUFA (James et al., 2000).

Certain receptors, including TLR4, are proteins involved in the recognition of LPS by cells of the innate immune system (Park and Lee, 2013). Detection of LPS by the receptors on the surface of innate immune cells leads to the production of cytokines by the activated cells that perform as immunomodulatory agents and mediate innate immune response (Kogut et al., 2005). The TLR4 has also been shown to recognize LPS of gram-negative bacteria such as *E. coli* and *Salmonella* in laying hens in a time- and tissue-dependent manner. In mammals, LBP mediates the transfer of LPS to CD14, further delivers the LPS

to myeloid differentiation protein-2 and eventually transfers the LPS to a TLR4 molecule (Beutler, 2000), however, there are no clear orthologues to human LBP investigated in the chicken genome. Hens can still be sensitive to LPS through the TLR4 pathway and subsequently induce NF-κB mediated inflammatory response (Tan et al., 2014). In this study, we evaluated the expression of TLR4 and IFN-γ in the spleen of laying hens 4 h after an i.v. LPS injection. The relative expression of TLR4 in the spleen was significantly reduced in birds consuming diets with 0.8% of DHA, due potentially to the fact that TLR4 can be inhibited by omega-3 PUFA, particularly DHA (Rogero and Calder, 2018; Hwang et al., 2016). One of the mechanisms whereby EPA and DHA modulate the inflammatory response is in their capabilities to bind to peroxisome proliferator-activated receptors (PPARs), which leads to further inhibition of the activation of NF-κB (Li and Glass, 2004; Calder, 2017). EPA and DHA have anti-inflammatory effects on this signaling pathway mainly due to diminished nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, further leading to lower TLR4 recruitment for lipid rafts and TLR4 dimerization (Hwang et al., 2016). In addition, the lower NADPH oxidase activity also reduces the generation of reactive oxygen species that is essential for the activation of the TLR4 signaling pathway (Rogero and Calder, 2018). The relationship between flaxseed oil (precursor of ALA) and TLR4 expression level is unclear, which may be due to the fact that the deposition of total omega-3 PUFA in plasma and liver in those four treatments is much lower compared to the treatments providing preformed. Studies in mammals suggest

that the dietary intake of omega-3 PUFA decreases the production of proinflammatory cytokines, including TNF-a, IL-1β, IL-6, and T-cell-derived IL-2, IL-12 and IFN-γ (Blok et al., 1996; Fritsche et al., 1999; Upadhaya et al., 2015). An increase in the production of IFN-γ protein was also observed in mice that were fed fish oil-enriched diets, high in EPA and DHA (Fritsche et al., 1997). However, the current results related to IFN-γ mRNA expression level in the spleen of layers were not significantly influenced by the diet, which seems inconsistent with previous research in mammals. It has been reported that eicosanoids (e.g., PGE_2) inhibit the production of IFN-γ (Betz and Fox, 1991; Sijben et al., 2003) and no significant diet effects were noted on the results of ARA-derived eicosanoids (**Table 4.8a-c**). These apparent inconsistencies may be due to species differences (Blok et al., 1996).

In conclusion, ALA (provided from flaxseed oil) or algal DHA biomass (DHA) and their increasing levels in the diet may not make a significant difference with regards to performance, but the dietary intake of omega-3 PUFA can significantly influence the production of oxylipins. It can also decrease the omega-6 to omega-3 ratio of yolks, plasma and liver-derived from either ALA or DHA. Steady-state accumulations were achieved at 0.40% of total omega-3 PUFA, especially for ALA, with algal DHA biomass yielding significantly higher individual and total omega-3 PUFA value than ALA. Increasing the total omega-3 PUFA decreased ARA-derived oxylipins whereas it increased EPA-and DHA-derived oxylipins. The production of TLR4 and IFN-γ were not significantly

modulated by diet in laying hens under acute conditions of LPS. However, other factors including the expression of cytokines at various time points may influence the ability of birds to mount inflammatory responses. Additionally, the mechanism(s) whereby omega-3 PUFA mediate the biosynthesis of oxylipins needs further investigation.

CHAPTER 5 GENERAL DISCUSSION AND CONCLUSION

5.3 General discussion

Essential fatty acids, especially omega-3 PUFA, are widely utilized in poultry feeding systems (Alagawany et al., 2019). They were reported to improve the health and productivity of birds (Cherian, 2015; Lee et al., 2019) due to their ability to mediate numerous biological and physiological processes (Simopoulos, 2011; Simopoulos, 2016; Jump, 2002). In addition, an adequate intake of omega-3 PUFA in human diets may protect against nutrition-related chronic health diseases (McGlory et al., 2019; Abdelhamid et al., 2020). Currently, enrichment of poultry products with omega-3 PUFA via their inclusion in poultry diets are considered effective strategies to add omega-3 PUFA to the human diet (Neijat et al., 2016; Neijat et al., 2017). Omega-3 PUFA are abundant in vegetable oils (Kassis et al., 2010) and oils are main energy sources for poultry. Dietary oils have multiple functions, including their ability to improve diet palatability and enhance the utilization of the consumed energy (Poorghasemi et al., 2013). Flaxseed oil, a commercially available source of ALA for livestock and poultry diets, contains 53% ALA (Raghuwanshi et al., 2019), and has widely been recognized to modify the omega-3 PUFA content in poultry products (Gonzalez-Esquerra and Leeson, 2001). In this study, in order to maintain the energy balance, corn oil was reduced with the graded increases in ALA in the diet. In a previous study, our lab used hempseed (7% of ALA) and hempseed oil (18% of ALA) as a model of ALA in diet, with these treatments yielding a relatively low conversion

efficiency of ALA to DHA in egg yolk of 3.5% and 12.5%, respectively. When flaxseed oil was used as a source of ALA, the conversion ratio of ALA increased to 11% for DHA and 29% for total omega-3 PUFA (Neijat, 2014). To date, feeding flaxseed and fish oil or algal oil to hens has been considered effective approaches to enrich the omega-3 PUFA content in eggs.

In this study, the first objective was to utilize ALA and algal DHA biomass as potential sources of dietary ALA and DHA, respectively, as a means to assist in the estimation of the requirements of these nutrients and identify whether ALA or DHA in diet can make a difference with regards to birds' productivity. The results from this study indicated that different sources of omega-3 PUFA had no effect on performance, however, the deposition of individual and total omega-3 PUFA can be significantly modified by dietary omega-3 PUFA intake. In addition, the trends of fatty acid deposition were similar in egg yolk, plasma and liver. Our current study also provided an estimated requirement of ALA in laying hens of 0.4% ALA by weight of diet based on reaching to a plateau of DHA deposition in egg yolk. A number of studies have confirmed that the conversion of ALA to DHA is known to be inefficient (Williams and Burdge, 2006; Neijat et al., 2016), potentially due to the need to maintain stearidonic acid levels and overcome the enzymatic competition with omega-6 PUFA when ALA acts as a substrate for DHA synthesis (Neijat et al., 2017). In the experimental diet, the content of EPA in algal oil biomass is higher than that of EPA in ALA. However, in this study, the ALA group showed the same level

of EPA in liver and plasma compared with the algal DHA biomass group at each percentage of omega-3 PUFA. It is confirmed that, regardless of the source of omega-3 PUFA, the liver retains a putative level of EPA. Hence, similar hepatic and plasma EPA content in both groups indicated that the conversion of ALA to EPA in ALA enriched diet was effective, potentially because Δ6-desaturase made an efficient utilize of ALA (Gregory et al., 2011). Besides, a high level of EPA in algal DHA biomass may rapidly transform into DPA and DHA, which suggests that the liver maintains a relatively defined content of EPA, thus, algal DHA biomass skips the EPA deposition, and was directly and efficiently incorporated as DHA into the egg yolk. (Lawlor et al., 2010; Šefer et al., 2011).

In the current study, in terms of determining the optimum quantity and type of omega-3 PUFA for the expression of optimal immunomodulatory effects in order to overcome an immune challenge in laying hens, the profile of oxylipin biosynthesis, and the expression of receptors and cytokines in lymphoid tissues were measured. In general, SFA is mainly esterified at the sn-1 position and some MUFA, most PUFA, especially the LCPUFA, occupy the sn-2 position (Neijat et al., 2020). PLA_2 exists in FA located at the sn-2 positions (Diez et al., 1994). The distribution pattern of FA (primary sn-1 and sn-2) is considered to be associated with their physiological role in the body. For example, ARA, present as the most prevalent omega-6 LCPUFA, mainly produces the highly pro-inflammatory PGs and TXs via the COX enzymatic pathway. PGE_2, PGF_2 and PGD_2 represent the common PGs generated from immune cells. In this study, PGE_2, $PGF_{2\alpha}$ and

PGD_2 were detected and were significantly induced by LPS, indicating that PUFA from the lipid membrane were released by PLA_2 and further successfully converted to eicosanoids. Hence, a relationship between the position of FA located on the glycerol backbone of membrane-bound lipids and enzymes that mediated oxylipins synthesizes may exist. Consequently, the effect of specific FA' position on oxylipin synthesis via specific phospholipases and the enzymes can be further investigated. ARA was also reported to yield LTs by the LOX pathway and LTB_4 was detected. Although no significant diet effects were observed, PGs (PGE_2, $PGF_{2\alpha}$ and PGD_2) and LTB_4 were decreased with the increasing level of omega-3 PUFA. The latter may be achieved in multiple ways including the reduction of ARA released from membrane phospholipids, the inhibition of metabolizing enzyme activities, and the competition with ARA for enzymatic conversions with the subsequent decreasing of the pro-inflammatory eicosanoid production (Belch and Hill, 2000; Green et al., 2005). EPA and DHA-derived eicosanoids and docosanoids, including the new families resolvins (E-series and D-series), protectins and maresins, play protective, anti-inflammatory and pro-resolving roles in many inflammatory disease models (Bannenberg et al., 2005; Groeger et al., 2010). Although they were not observed in plasma, in our current study, diet had a significant effect on 18-HEPE derived from EPA via the COX pathway, which is the precursor of resolvin E. This data suggests that supplementation with graded levels of omega-3 PUFA can potentially enhance the anti-inflammatory and protective functions in poultry. Additionally, DHA-derived lipid

mediators have protective effects and were examined, and results support the inhibition of the activity of NF-κB and leukocyte migration, and induction of COX-2 (Hong et al., 2003). COX-2 is the rate-limiting enzyme in the biosynthesis of PGs and TXs. Omega-3 PUFA suppress the activities of COX-2 via: 1) competing for desaturase enzymes that convert LA to ARA with a consequence of decreasing incorporation of ARA into membrane phospholipids; 2) competing with ARA for access to the active site of COX-2; 3) the role that PGE_3 derived from EPA plays as a competitive inhibitor of PGE_2 receptors (DiNicolantonio et al., 2014). Therefore, increased levels of DHA and EPA can compete with ARA for the COX and LOX enzymes to inhibit the production of ARA-derived pro-inflammatory mediators (James et al., 2000). In the future, the expression of COX and LOX can be measured to support these observations.

In addition to modulating the profile of lipid mediators involved in inflammatory processes, omega-3 PUFA have effects on the production of pro-inflammatory cytokines (Kang and Weylandt, 2008). Numerous studies have revealed that omega-3 PUFA, especially EPA and DHA, can alleviate the effects of classic pro-inflammatory cytokines, such as TNF-α, interleukin IL-1β and IL-6, both *in vivo* and *in vitro*, in response to LPS (Orr et al., 2013; Dehkordi et al., 2015; Shi et al., 2016). The actions of omega-3 PUFA mediate the expression of cytokines, firstly, via their involvement with PPAR transcription factors, including PPARα and PPARγ isoforms. PPARα regulates several enzymes of β-oxidation and lipoprotein metabolism (Schoonjans et al., 1996), whereas PPARγ is mainly

expressed in adipose tissues and inflammatory cells to participate in the process of anti-inflammatory functions (Szanto et al., 2008). DHA can induce the activation of PPARγ and a number of its target genes, subsequently related to the lower production of the pro-inflammatory cytokines TNF-a and IL-6 under endotoxin stimulation (Kong et al., 2010; Zapata-Gonzalez et al., 2008). Omega-3 PUFA are also associated with the activation of NF-κB via inhibiting the phosphorylation of subunit IκB (Lee et al., 2001; Novak et al., 2003). An interaction effect exists between PPARγ and NF-κB. Activated PPARγ interacts physically with NF-κB, leading to the prevention of NF-κB translocation to the nucleus (Berghe et al., 2003). Therefore, omega-3 PUFA can inhibit the upregulation of NF-κB target pro-inflammatory genes via binding to PPARγ. Furthermore, G-protein coupled surface receptors (GPR) are involved in the mechanisms, especially GPR120, involved in the binding with LCPUFA. However, reports of the effect of omega-3 PUFA on IFN-γ are lacking and/or inconsistent with respect to differences between species (Blok et al., 1996; Fritsche et al., 1997; Fritsche et al., 1999). In the current study, no significant diet effect was noted on the gene expression of IFN-γ. Fritsche et al (1999) indicated that mice fed with omega-3 PUFA-enriched diets had higher circulating IL-12 and IFN-γ levels and splenic mRNA expression of IFN-γ compared with those mice consuming an omega-6 PUFA-enriched and low PUFA-containing diet. One possibility that exists is that there is a shift from a T-helper (Th)-1 type to a Th-2 type of immune response. Th-1 cells are known to produce proinflammatory cytokines, such as, IL-2 and IFN-γ and Th-2 cells can

generate anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 that improve human immune functions (Chen et al., 2000). PGE_2 has been shown to inhibit the production of IL-2 and IFN-γ in mice (Betz and Fox, 1991). Therefore, PGE_2 may potentially regulate the level of Th-1 cytokines affected by omega-3 PUFA. However, the underlying mechanisms whereby omega-3 PUFA modulate the production of cytokines have not been fully elucidated.

5.4 General conclusion

Overall, omega-3 PUFA, particularly the inclusion of exogenous DHA, play protective and anti-inflammatory roles by regulating oxylipin biosynthesis and the production of receptors and cytokines under LPS administration. Increasing levels of ALA and DHA may not make a significant difference with regards to the productivity of laying hens, however, an optimal level of omega-3 PUFA can be defined as 0.4% ALA by weight of diet due to the steady-state of DHA accumulation in egg yolk, plasma and liver. Inclusion of omega-3 PUFA in laying hen diets can be an acceptable strategy in terms of improving their overall health. Understanding the relationship between dietary omega-3 PUFA intake and their impact on immunological responses may yield new strategies to reduce the risk of diseases impacting the poultry industry.

CHAPTER 6 FUTURE DIRECTIONS

Future directions include:

1. To define requirements of omega-3 PUFA for optimal health and performance in different ages (e.g., pullets) and numbers of laying hens based on the data derived in the current project.
2. To conduct time course blood collections that will be applied to determine the changes or peaks of key cytokines.
3. To investigate a relationship between the position of FAs located on the glycerol backbone of membrane-bound lipids and enzymes that mediated oxylipin synthesis.
4. To determine the optimum quantity and type of omega-3 PUFA (ALA or DHA) for the expression of optimal immunomodulatory effects in order to overcome an immune challenge in pullets, based on measures of oxylipin profiles, antibody levels (titers) and expression of receptors and cytokines in lymphoid tissues.

CHAPTER 7 LITERATURE CITED

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Appendix 1. Animal care approval form.

UNIVERSITY OF MANITOBA | Research Ethics and Compliance
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17 September 2018

TO: Dr. J. House, Food and Human Nutritional Sciences

FROM: Dr. L. Connor, Chair, Fort Garry Campus Animal Care Committee

RE: Your protocol entitled **"Defining the optimal omega-3 fatty acid intake for pullets and laying hens to support health and productivity"**

Please be advised that your Animal Use Protocol form was reviewed by the Fort Garry Campus Animal Care Committee (FG ACC) at its meeting of **August 23 2018**. The committee recommended **APPROVAL** of your protocol **SUBJECT TO A SATISFACTORY RESPONSE TO THE QUERY NOTED BELOW.**

Protocol Reference Number: **F18-025 (AC11383)**
Animals approved for use:

Number	Species	Common Name	Sex	Age or Weight
80	Chickens - layers		Female only	20 weeks
320	Chickens - layers		Female only	Day old

Protocol approval is valid from: **August 30 2018 to August 29 2019**
Category of Invasiveness: **D**

As indicated above, your protocol has been approved, and as such, you are authorized to begin the work described. However, the Committee requires your written response **(which includes a revised protocol, applicable revised schedules and itemized answers)** on or before **September 24 2018** to questions 2-6 below under which this approval is subject:

1) The committee would like to commend you for submitting a very well written protocol.
2) Block 3: Will facility staff be assisting with any of the procedures? If yes, please indicate which facility the staff will be sourced from and which technical services/research interventions will be performed.
3) Block 6: Please clarify the fate of the birds that are not euthanized for sampling.
4) Block 7: Please clarify the source of the hens. Are the current hens in the poultry unit used?
5) Block 7: No spare birds are being requested. Are you not anticipating possible failure for treatment allocation?
6) Schedule 4 section 5: Please provide a monitoring sheet.

If your written response is not received by the date noted above, the protocol will be suspended and no animal experimentation will be allowed to continue until further clarification by you is provided.

Please direct your response to Ms Tracy VanOsch, Co-ordinator, Animal Care, tracy.vanosch@umanitoba.ca.

The protocol reference number must be used when ordering animals. It is understood that these animals will be used only as described in your protocol. Failure to follow this protocol will result in the termination of your ability to use animals.

The protocol must be kept current. Minor modifications to the protocol must be submitted in the form of an amendment. Major changes would necessitate preparation and submission of a new protocol. Failure to renew this protocol prior to the expiry date will result in the termination of your ability to continue ordering animals.

On behalf of the Fort Garry Campus Animal Care Committee, I would like to extend our best wishes for the successful completion of your research.

LC/tvo

copy: Veterinary Services
Dr. E. McGeough, Chair, LAUC, Faculty of Agricultural and Food Sciences
Dr. T. Gilson, Glenlea Research Station