A Dissertation

for the Degree of Doctor of Philosophy

Research on physiological effects of omega-3 and omega-6 fatty acid balance on animal inflammation and stress

오메가-3 와 오메가-6 지방산 균형이 가축 염증과 스트레스에 미치는 생리적 영향에 관한 연구

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농 학 박 사 학 위 논 문

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Summary

Although inflammation is an essential biological process protecting the host from harmful stimuli, chronic inflammatory response causes injury and cell death. People today are exposed to many causes of chronic inflammation such as an unbalanced diet and stress, which are closely related to increased prevalence of diseases such as diabetes, obesity and cancer. Since livestock animals are also in a similar environment, their performance may also be adversely affected.

In this context, nutraceuticals, which are food ingredients that can prevent or treat diseases, warrant attention. As they provide additive medical or physiological benefits beyond purely nutritional functions, numerous studies to identify bioactive materials and develop products in isolated form have been carried out. In the same context, the usage of nutraceuticals in livestock feed is also being attempted. Since many nutraceutical ingredients have anti-inflammatory or anti-oxidant properties, they can be effective in controlling inflammation and reducing oxidative stress. In particular, omega-3 fatty acids can potentially control livestock animal health state, as polyunsaturated fatty acids (PUFAs) are representative nutrient components that influence the inflammatory process. Also, its content in today's diet for both people and livestock is lacking compared to omega-6 fatty acids, and this unbalance can threaten host inflammatory homeostasis, thereby provoking various diseases. Meanwhile, probiotics such as *Lactobacillus* and *Bacillus* can be applied as nutraceuticals to

improve animal health because they have functions such as anti-pathogenic effects, fermentation, gut barrier reinforcement, and immunomodulatory effects.

To evaluate the health promoting effects of nutraceuticals, biological markers indicating inflammation and stress should be monitored. However, many studies have focused only on investigating changes in the performance of livestock, and the physiological changes caused by nutraceuticals have not been adequately researched. In this study, flaxseed and probiotics were selected as nutraceuticals to investigate various physiological changes including inflammation, stress state, and gut microbiota in laying hens. First, the effects of flaxseed on laying hen PUFA, lipid mediator profile, inflammatory indices, and stress indices were investigated. Next, the effects of a combination of flaxseed and two probiotics (*Lactobacillus plantarum*, Lp; *Bacillus licheniformis*, T2) were investigated with regard to the same traits. Moreover, the effects of overcrowding stress on laying hen physiology also were evaluated to determine whether flaxseed and the aforementioned two probiotics can alleviate overcrowding stress effects.

In study 1, the physiological effects of alpha-linolenic acid (ALA) were investigated *in vitro* and *in vivo*. In the *in vitro* study, ALA was treated in murine macrophage cell line RAW264.7 stimulated by a lipopolysaccharide. Several gene expressions of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1beta (IL-1beta) were decreased. Also, gene expression of the lipid mediator-producing enzyme

cyclooxygenase-2 was decreased by ALA treatment. In the *in vivo* study, the commercial flaxseed product Lintex 170, a representative material rich in ALA, was fed to 33-week-old laying hens for four weeks. With flaxseed feeding, the ratios of omega-6 to omega-3 in both egg and serum were decreased, which influenced omega-3 derived lipid mediator enrichment. This resulted in changes of the host inflammatory and stress state.

In study 2, the physiological effects of the combination of flaxseed and probiotics on 52-week-old laying hens were investigated. Lp and T2 were selected as probiotic materials based on production yield and target gene identification among three *Lactobacillus* and two *Bacillus* species, respectively. Specific changes by flaxseed and probiotics were investigated. The effects of flaxseed were similar to those in study 1 in terms of lowering the ratio of omega-6 to omega-3 in eggs, decreasing pro-inflammatory indices such as gene expression of pro-inflammatory cytokines in liver samples. Meanwhile, the specific effects from a combination of flaxseed and Lp were increment of c9, t11-conjugated linoleic acid (c9, t11-CLA) content in egg samples. Also, there were some boosting effects on inflammatory indices and stress indices, which resulted in improved laying performance. On the other hand, the combination of flaxseed and T2 had some negative effects on laying performance. The difference in laying performance depending on the type of probiotics could be linked to the results of gut microbiota metagenome prediction. As there were some significant changes in gut microbiota of laying hens by flaxseed and probiotics, their metagenome also had different

predicted parts depending on the group. For example, the group fed flaxseed and Lp was predicted to have lower phosphotransferase system activity than the other groups, which could be beneficial to host nutrient utilization and laying performance. In the case of the group fed flaxseed and T2, the group showed increased activity of energy metabolism compared to other groups, which could lead to inefficiency in energy utilization of the host and decreased laying performance.

In study 3, the physiological effects of the combination of flaxseed and probiotics on overcrowded 52-week-old laying hens were investigated. To this end, the effects of overcrowding stress on the laying hen inflammatory and stress state and the effects on laying hen gut microbiota were first examined with a commercial basal diet. The effects of flaxseed and probiotics on overcrowded 52-week-old laying hens were then investigated for the same traits. The overall physiologic parameters such as inflammatory index, stress index, and laying performance traits were aggravated by overcrowding stress and confirmed to be recovered by feeding flaxseed and probiotics. Also, the effects of flaxseed and probiotics on gut microbiota of each group and their predicted metagenome were examined. Among the groups, the gut microbiota of the group fed flaxseed and T2 confirmed the activation of amino acid-related metabolism, which may be related to the improvement of laying performance.

In this study, the physiological effects of nutraceuticals (flaxseed and probiotics) and overcrowding stress on laying hens were investigated. Based on results from study 1, it can be inferred that the lipid mediator profile as well as the ratio of omega-6 to omega-3 was altered by feeding flaxseed and this affects the inflammatory, stress indices, and laying performance of the laying hens. It could be concluded that a balanced intake of PUFAs by reinforcing omega-3 fatty acid in livestock diet might benefit the host physiology, especially under stress conditions. Moreover, probiotics such as *Lactobacillus* and *Bacillus* can have additive effects on anti-inflammatory and anti-stress effects to the host fed flaxseed via their byproducts such as c9, t11-CLA or amino acid metabolism-related activators. To date, more studies are needed to clarify the inflammatory or stress state of livestock animals in terms of whether chronic inflammation and stress actually affects their physiology. Also, repeated experiments should be conducted to demonstrate that some feed additives can help maintain the health of the host by controlling inflammation and stress conditions. Nevertheless, these results suggest that the welfare of livestock in harsh environments can be improved through nutritional management, which shows the possibility of a novel category of feed additive termed livestock welfare feed additives.

Keywords: Omega-6 to omega-3 fatty acid balance, nutraceutical, inflammation, stress, animal welfare

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List of Abbreviations

AGP: Antibiotic growth promoter

ALA: Alpha-linolenic acid

ANOVA: Analysis of variance

APC: Antigen presenting cell

CLA: Conjugated linoleic acid

COX: Cyclooxygenase

CYP450: Cytochrome P450

DHA: Docosahexaenoic acid

ELISA: Enzyme-linked immunosorbent assay

EPA: Eicosapentaenoic acid

EpETE: Epoxyeicosatetraenoic acid

GC: Gas chromatography

HDoHE: Hydroxydocosahexaenoic acid

H:L ratio: Heterophil : Lymphocyte ratio

HOTrE: Hydroxyoctadecatrienoic acid

HPA: Hypothalamic-pituitary-adrenal

IL: Interleukin

KEGG: Kyoto encyclopedia of genes and genomes

LAB: Lactic acid bacteria

LOX: Lipoxygenase

LPS: Lipopolysaccharide

LT: Leukotriene

LX: Lipoxin

NSAID: Non-steroidal anti-inflammatory drug

NGS: Next-generation sequencing

NSTI: Nearest sequenced taxon index

OTU: Operational taxonomic unit

PCA: Principal component analysis

PCoA: Principal coordinate analysis

PG: Prostaglandin

PICRUSt: Phylogenetic investigation of communities by reconstruction of unobserved States

PLS-DA: Partial least squares –discriminant analysis

PUFA: Polyunsaturated fatty acid

QIIME: Quantitative insights into microbial ecology

qRT-PCR: Quantitative real-time reverse transcription PCR

SCFA: Short-chain fatty acid

SPM: Specialized pro-resolving mediators

SRA: Sequence read archive

TNF: Tumor necrosis factor

UPLC-MS/MS: Ultra-performance liquid chromatography tandem mass spectrometry

VIP: Variable importance in projection

Introduction

Recently, chronic inflammation has been pinpointed as the cause of various diseases such as diabetes, obesity, cardiovascular disease, and cancer (Monteiro and Azevedo, 2010). These diseases occur when resolution fails, which can be caused by a deficiency of pro-resolving mediators or the occurrence of sustained stress. In particular, lipid mediators and specialized pro-resolving mediators (SPMs), inflammatory controlling materials synthesized from polyunsaturated fatty acids (PUFAs), are directly related to the deficiency due to a PUFA-unbalanced state in the modern diet. Also, the increased frequency of exposure to various stress inducers is threatening human health. Likewise, in the livestock industry, problems regarding PUFA-unbalanced diet and stressful environment also exist. Therefore, it is very likely that the problem of chronic inflammation is widespread.

Meanwhile, the livestock industry has recently focused on developing nutraceuticals as feed additives as antibiotic alternatives. The customer demand for safe livestock products such as antibiotic-free products has increased. Also, consumers have pointed out that the farming environment of livestock products is poor and threatening animal health, which results in a changing consuming trend toward choosing healthy-grown livestock products (Verbeke and Viaene, 2000). Thus, it is important to develop nutraceuticals that can satisfy both product livestock safety and animal health.

The method to determine animal health state is a crucial step to initiate the developing process. Therefore, some researchers have attempted to quantify livestock animal welfare using various biological markers and to develop solutions to improve the health state in terms of housing management and nutrition reinforcement. As facility improvements are mostly limited by conventional factory livestock farming, nutritional management is important in maintaining the health of livestock in the modern industry.

In other words, it is necessary to develop functional feed additives using nutraceuticals to improve animal welfare. Since the main threat factor of livestock health is the stress caused by overcrowded raising and unsanitary housing, which provokes chronic inflammation, the feed additives may have anti-inflammatory and anti-stress functions.

Flaxseed is one of the representative nutraceuticals commonly used in humans and animals due to its abundant omega-3 fatty acids, especially alpha-linolenic acid (ALA). As omega-3 fatty acids are precursors of various lipid mediators and specialized pro-resolving mediators (SPM) involved in the resolution of inflammation, supplying dietary flaxseed can be a solution to chronic inflammation in both humans and livestock animals.

Probiotics are also used as nutraceuticals. They are microorganisms that have various benefits regarding the host gut microbiome. Among the various probiotics, *Lactobacillus* and *Bacillus* have been widely used in the livestock industry as feed

additives. Both probiotics are known to have antimicrobial properties for pathogens by producing antimicrobial molecules such as bacteriocin, which can improve livestock performance. Furthermore, they can enrich the host gut microbiome via their encoded genes lacking in the host genome. This can contribute not only to maintaining the health of livestock but also to improving the quality and functionality of livestock products.

The aim of this study was to evaluate the physiological effects of flaxseed and probiotics on the inflammation and stress state of laying hens. Therefore, the effect of flaxseed was first ascertained in aspects of physiological traits and performance before examining the effects of a combination of two probiotics (Lp, T2) in both normal and overcrowded state (Figure 1). First, the physiological effects of alpha-linolenic acid and flaxseed were investigated in study 1. To accomplish this goal, gene expressions regarding inflammatory state were first analyzed in murine macrophage cell line RAW264.7 with alpha-linolenic acid treatment. After confirming bioactive effects of alpha-linolenic acid, a feed experiment feeding 0.9, 1.8, or 3.6 % (w/w) flaxseed to 33-week-old laying hens was carried out with analysis of PUFA profiles, lipid mediator profiles, inflammatory cytokines, and stress indices for four weeks. Next, studies on screening probiotics and physiological effects when laying hens were fed flaxseed were carried out in study 2. To accomplish this goal, three *lactobacillus* and two *bacillus* species were assessed by their production yield and encoded gene regarding fatty acid profile

modulation. After selecting probiotic candidates, they were fed to 52-week-old laying hen with 1.8% (w/w) flaxseed for four weeks. By measuring the PUFA profile, inflammatory cytokines, stress indices, and gut microbiota, their effects on laying hen physiology were assessed. Finally, after various physiological adverse effects of an overcrowding stress condition on host inflammation and stress indices were identified, it was observed that they could be alleviated by feeding flaxseed and probiotics according to the same traits in study 3 (Figure 2).

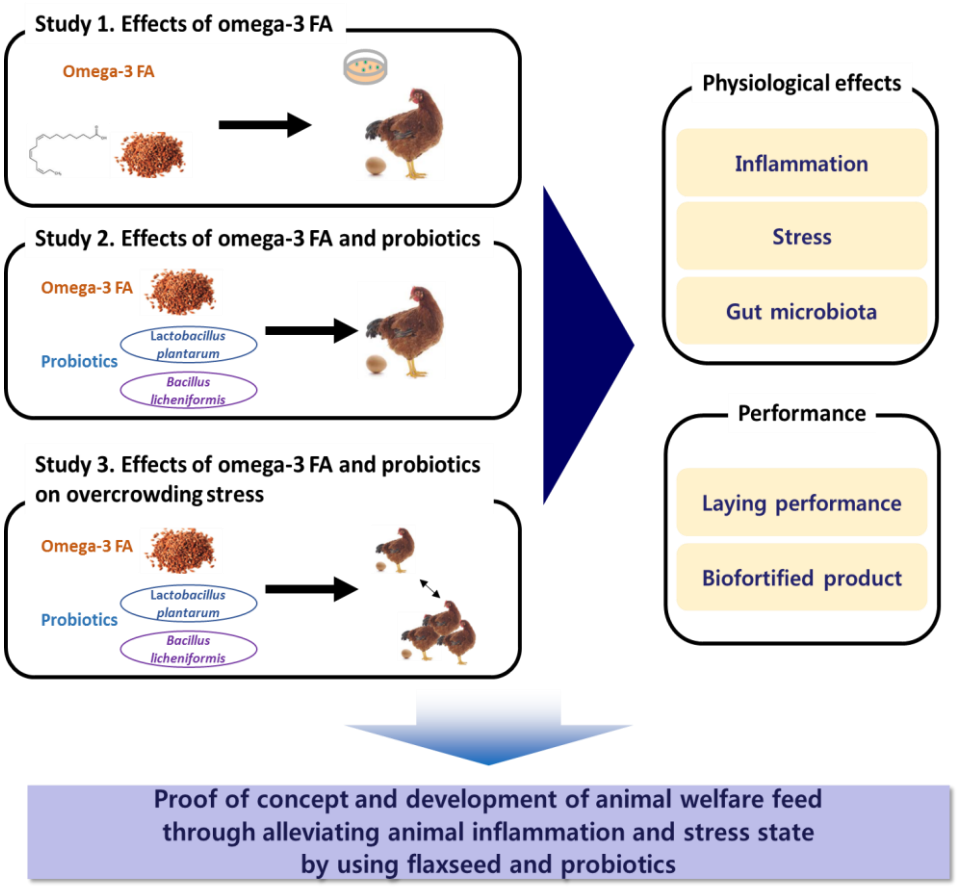

Figure 1. Overview of the research.

The research consists of studies 1, 2, and 3. In study 1, the physiological effects of alpha-linolenic acid and flaxseed were investigated. In study 2, the physiological effects of flaxseed and probiotics on laying hens were investigated. In the study 3, physiological effects of flaxseed and probiotics under an overcrowding condition were investigated.

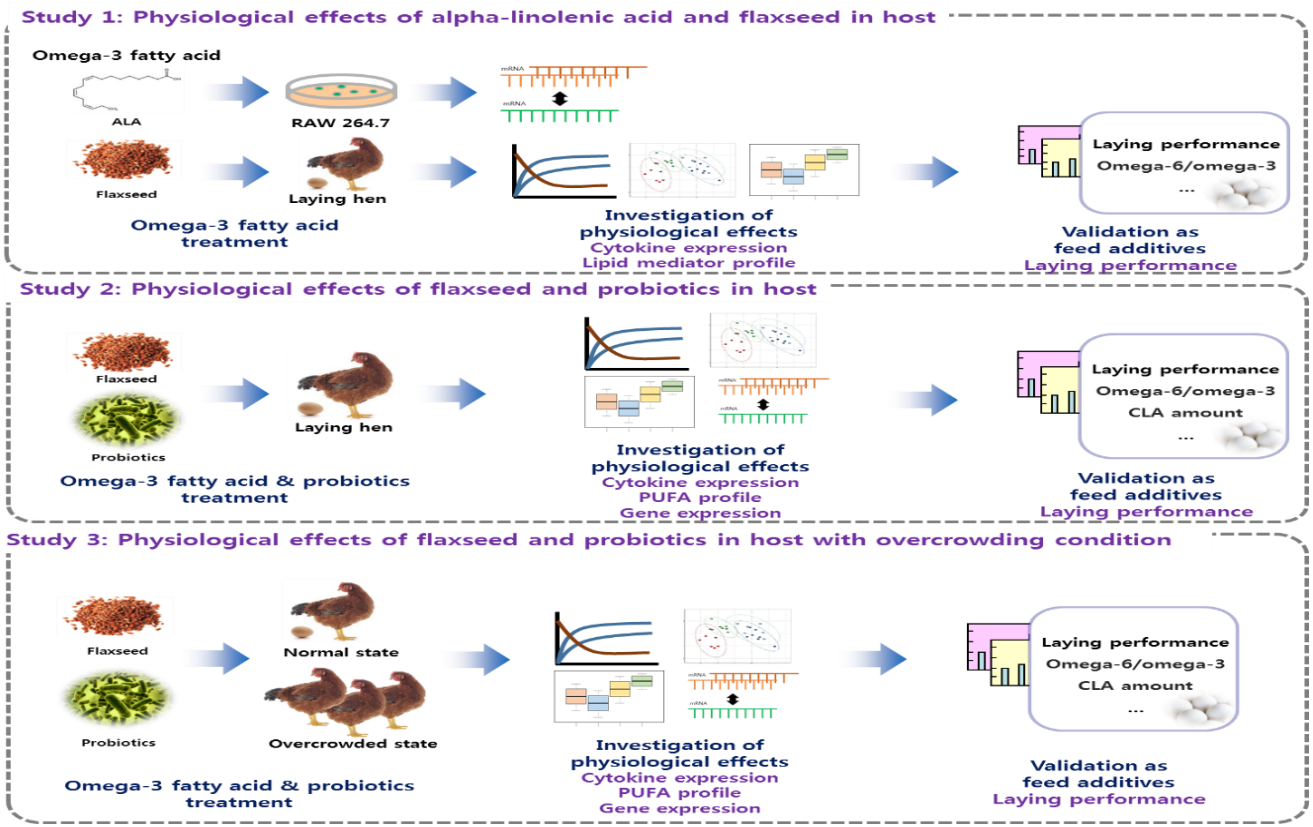

Figure 2. Experimental flow chart of the research.

Review of Literature

1. Inflammation

1) Inflammatory pathway

Inflammation is a complex biological response of body tissues to harmful stimuli such as stress and toxins (Ferrero-Miliani *et al.*, 2007). There are four main components involved in the process of inflammation: inducers, sensors, mediators and target site (Medzhitov, 2010) (Figure 3). When exogenous infection or tissue damage occurs, vasculature within and around the inflamed site responds by increasing blood flow and enhancing vascular permeability mediated by vasoactive amines (histamine and serotonin), which results in edema or redness. Neutrophils are then extravasated through the following stages: 1) margination: the vasodilation induced by damage slows the blood flow at the inflamed site so that neutrophils can move toward the periphery of the vessels, next to the walls of vascular endothelial cells; 2) rolling: marginated neutrophils have interactions with endothelial cells in low affinity mediated by selectin and then 'roll' along the vascular wall; 3) adhesion: by rolling, the neutrophils slow down, allowing higher affinity interactions between the integrins of the neutrophils and endothelial InterCellular Adhesion Molecules (ICAMS), which can completely stop the neutrophils; 4) extravasation: the bound neutrophils are extravasated by being squeezed between endothelial cells.

The newly extravasated neutrophil recognize the damage by sensors (e.g., toll-like receptors or nucleotide-binding oligomerization-domain protein-like receptors), which results in secretion of pro-inflammatory mediators. Also, some chemo-attractants are also released to activate leukocyte recruitment and anti-microbial activities (Serhan *et al.*, 2008). As a result, the extravasation of neutrophils and release of its mediator lead to a great number of neutrophils being recruited into the site of challenge and causing more pro-inflammatory mediators such as cytokines to be secreted at the site. This promotes monocyte infiltration and harmful stimuli thereby can be removed (Jin *et al.*, 2010). The that neutrophils formed phagolysosomes by fusing with lysosomal granules which have bacterial kill mechanisms and a debris clearance system using degradative enzymes and production of reactive oxygen species (ROS) (Mayer-Scholl *et al.*, 2004).

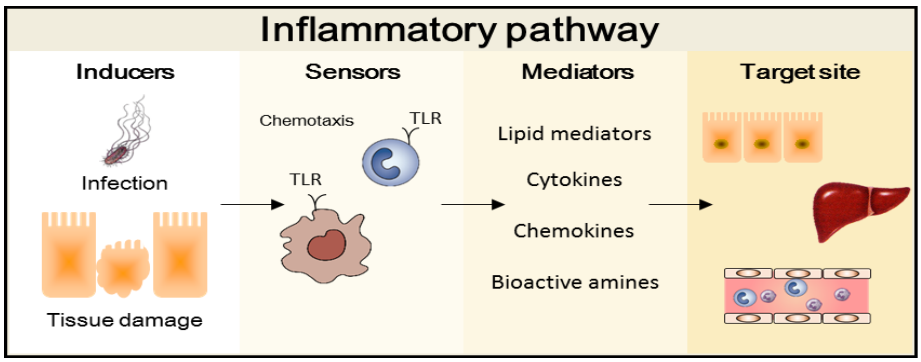

Figure 3. Components of inflammatory pathway.

There are four components in inflammatory process: inducers, sensors, mediators, and target site. When inducers exist in host, it can be detected by sensors and then, several metabolites mediated its occurrence to whole host system, so that host undergoes inflammatory process in that target site.

After performing their action, recruited neutrophils undergo apoptosis by macrophage ingestion. Clearance of apoptotic neutrophils causes the inflamed site to change from a pro- to an anti-inflammatory environment. For example, macrophage phenotype changes pro-inflammatory M1 into anti-inflammatory M2, which results in macrophage egress favoring termination of the inflammatory state and a return to homeostasis by switching the profiles of both lipid mediators and cytokines (Porcheray *et al.*, 2005). This series of biological processes is called resolution (Serhan *et al.*, 2008) (Figure 4). In addition, macrophages travel to local lymph nodes with degraded phagocytosed debris and act as antigen presenting cells (APCs), inducing an adaptive immune response against the infecting microbe. During the onset of inflammation and its resolution, various mediators are involved in this process such as cytokines and lipid mediators; pro-inflammatory mediators are mainly activated at the onset of inflammation whereas anti-inflammatory or pro-resolving mediators mainly operate in the phase of resolution.

2) Chronic inflammation

Although acute inflammation is self-limited and plays an important role in defense from harmful stimuli, it can lead to lethal outcomes such as septic shock when it fails to be resolved. This phenomenon is called chronic inflammation, and results in aberrant tissue remodeling and physiological dysfunction (Feehan and Gilroy, 2019) (Figure 4). Moreover, it is highly linked to several chronic diseases including diabetes (Calle and Fernandez, 2012), atherosclerosis (Ferrucci and Fabbri, 2018), rheumatoid arthritis (Arida *et al.*, 2018), cancer (Fox and Wang, 2007), inflammatory bowel disease (Grone, 2019), and multiple sclerosis (Frischer *et al.*, 2009). As of now, the therapeutic approach to those diseases is a 'switch-off' strategy mainly using nonsteroidal anti-inflammatory drugs (NSAIDs) and anti-cytokine therapies such as tumor necrosis factor (TNF) inhibition. However, side effects such as gastrointestinal ulcers, diarrhea, vomiting, heart attack and kidney disease were reported in the case of NSAIDs (Traversa *et al.*, 1995;Bally *et al.*, 2017;Lanas and Chan, 2017) and skin and joint damage sarcoidosis in the case of anti-TNF therapy (Berg, 2004;Vigne *et al.*, 2013;Drvarov *et al.*, 2015). Thus, many researchers have focused on developing novel therapeutic agents to treat these diseases.

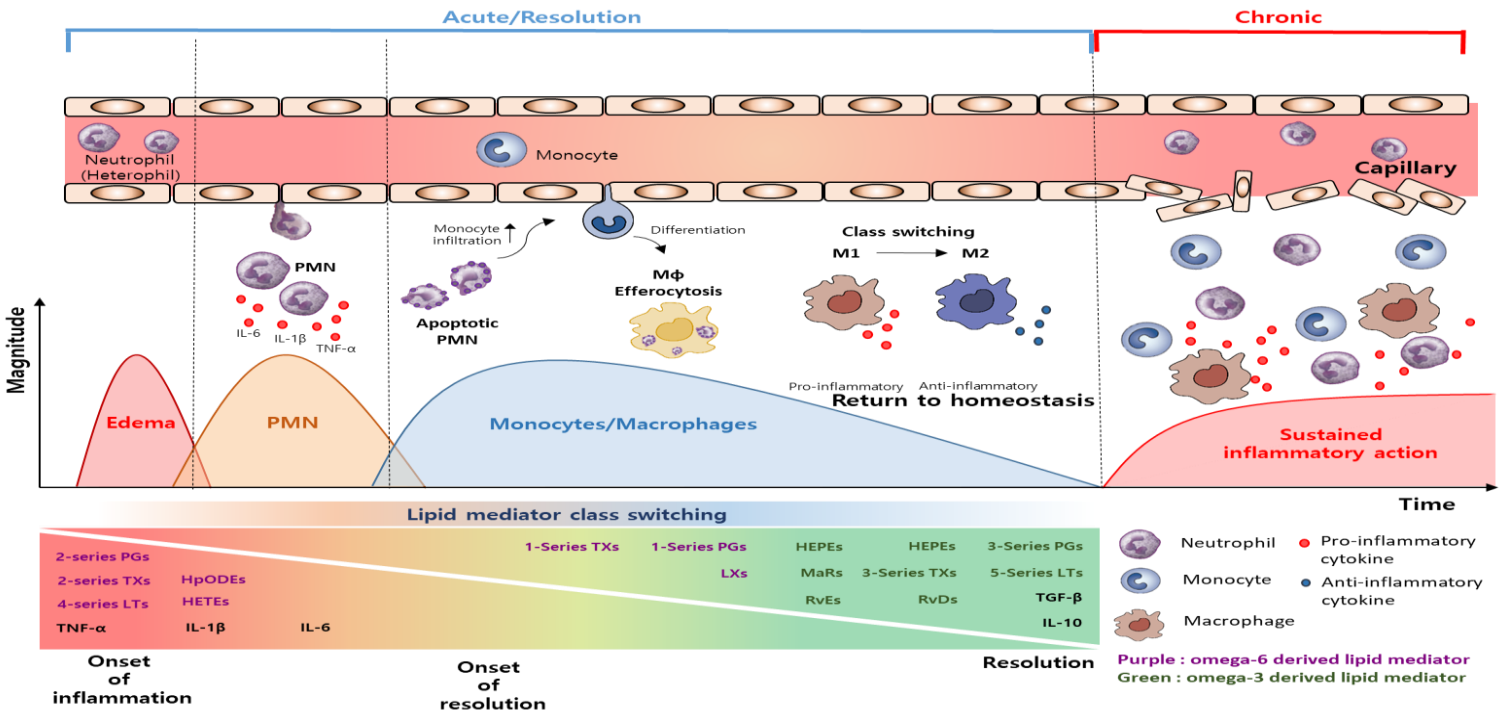

Figure 4. Overall inflammatory process.

Inflammation occurs with the changes their levels of various cytokines and lipid mediators. When their levels fail to recover normal state by resolution, they can be sustained inflammatory action, which is called chronic inflammation.

3) Crosstalk with stress

Stress is an organism's complex response to various stressors such as environmental condition. This complicated response was first studied by Hans Selye in 1936 and it impacts the fields of endocrinology, complementary medicine, social psychology and also animal breeding. In general, when individuals are stressed, they experience changes in their performance. An optimum level of stress leads to increased performance, and this stress is called 'eustress'. However, when the stress is beyond what the individual can afford, it induces a sudden drop in performance, which is called 'distress' (Figure 5).

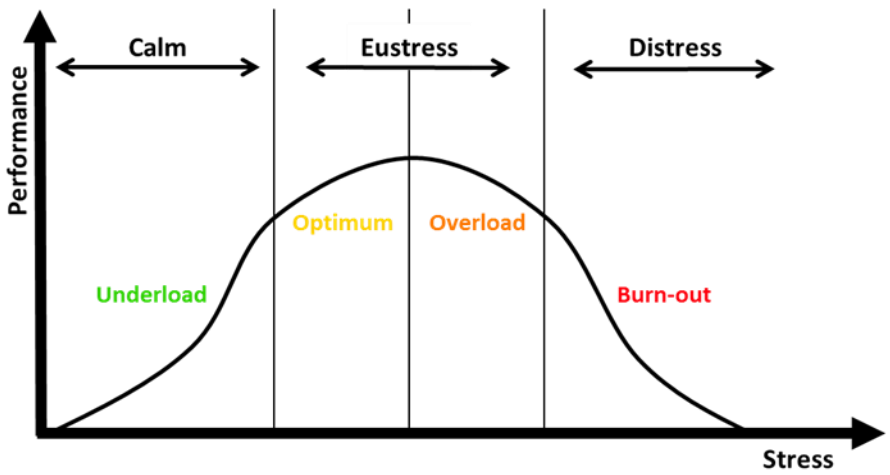

Figure 5. The relationship between stress and performance.

In eustress condition, stress affect host performance in positive way, whereas excessive stress provokes distress condition, lowering its performance.

To cope with the stresses of their lives, human beings as well as other animals have evolved certain mechanisms. The major systems are the autonomic nervous system and hypothalamic-pituitary-adrenal (HPA) axis (Tsigos and Chrousos, 2002). When acute stress occurs, an individual's fight-or-flight response may be activated through the sympathetic nervous system using energy to react stress. Second, the HPA axis releases cortisol or corticosterone to react to stress, which influences various body functions in metabolic and immunological aspects (Figure 6). Moreover, stress is one of the major influencing factors in the susceptibility of host pathogenic substances. In particular, the relationship between stress and inflammation has recently been elucidated. For example, oxidative stress acts to activate pro-inflammatory transcription factors including NF-κB and AP-1 (Reuter *et al.*, 2010). Therefore, the persistence of excessive oxidative stress can cause inflammation to the detriment of the host.

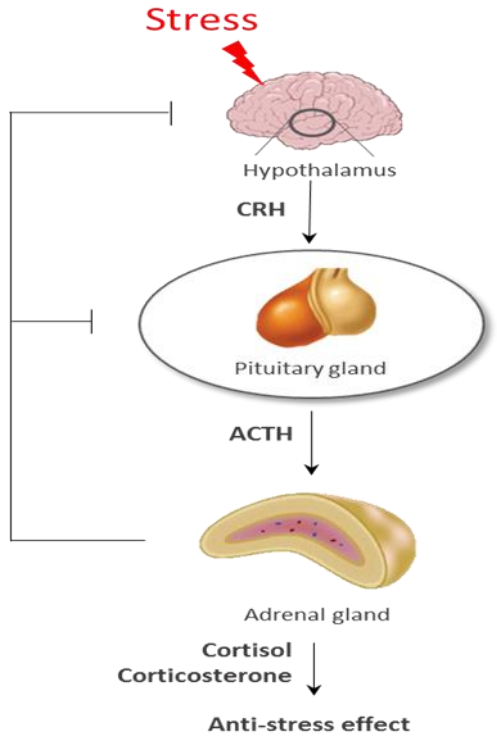

Figure 6. HPA axis responding to stress.

When stress occurs, corticotropin-releasing hormone (CRH) was upregulated in hypothalamus. Then, the upregulated hormone induced pituitary gland, so that upregulating level of adrenocorticotrophic hormone (ACTH). This change activated adrenal gland to release cortisol or corticosterone so that enduring external stress stimulus. This biological process is mediated by hypothalamic-pituitary-adrenal (HPA) axis.

While raising livestock, they also encounter a variety of stressors. These affect production, reproduction and health of livestock. For example, high ambient temperature exceeding the animal's capacity for enduring heat can have a critical effect on animals (Bernabucci *et al.*, 2010). Overloaded heat not only increases livestock mortality rates but also has adverse effects on their performance such as

decreased body weight or milk synthesis (Belhadj Slimen *et al.*, 2016). It has been found that chronic physiological stress can be a risk factor of inflammatory disease such as inflammatory bowel disease (Mawdsley and Rampton, 2005). The same tendency has been revealed in studies of other animals, such as mouse, strengthening the link between stress and inflammation in gastrointestinal dysfunction (Yisireyili *et al.*, 2018).

Conversely, inflammation can also cause stress. Normally, inflammatory cytokines activate the HPA axis increasing glucocorticoids, which inhibit cytokine synthesis (Auphan *et al.*, 1995;Ito *et al.*, 2006). However, when chronic inflammation occurs, inflammatory cytokines cause excessive activation of the HPA axis and chronic glucocorticoid hyperactivity, resulting in glucocorticoid resistance in the body (Figure 7). Thus, glucocorticoid functions in a normal state such as suppression of HPA axis activation and inhibition of pro-inflammatory cytokine production of immune cells are destroyed. Since the pro-inflammatory cytokines induce oxidative stress through leukocyte infiltration and activation of NF-kB, they function as stressors (Huang *et al.*, 2015).

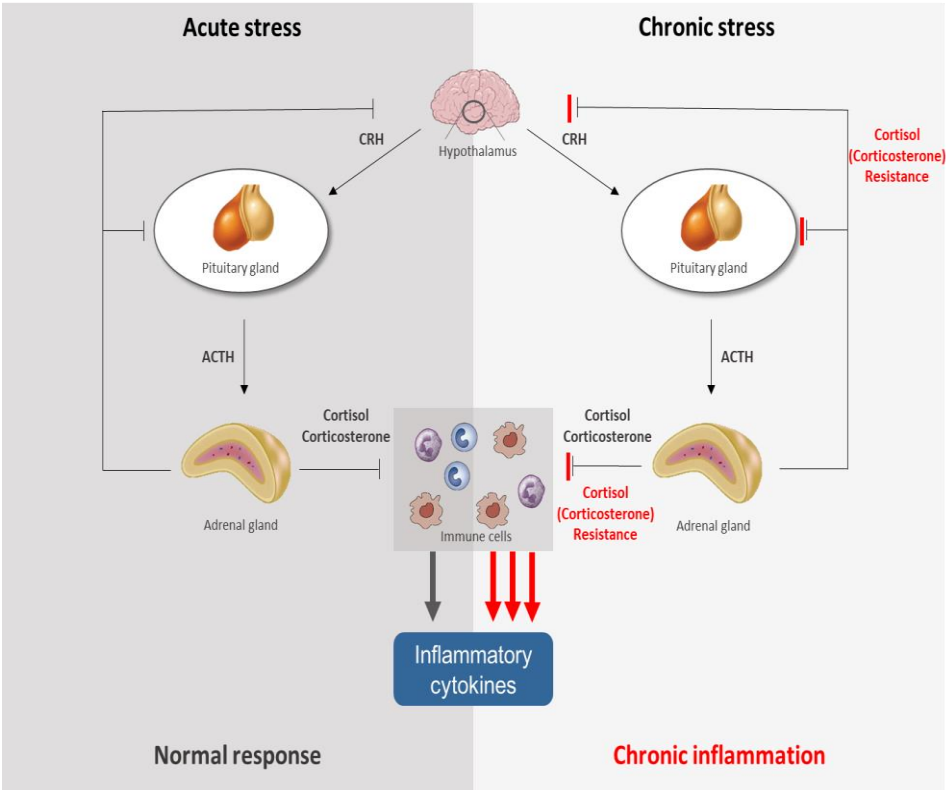

Figure 7. Crosstalk between inflammation and stress.

In normal condition, host can endure against stress by HPA axis, inhibiting pro-inflammatory cytokines. However, chronic stress induce cortisol (or corticosterone) resistance so that pro-inflammatory cytokine sustained its action provoking chronic inflammation.

Therefore, inflammation is closely related to a stress state in the body. This crosstalk between them is mainly connected by the HPA axis and inflammatory cytokines. Stress stimulates the HPA axis, thereby elevating cortisol or corticosterone levels in the blood. This results in suppression of pro-inflammatory cytokines in immune cells to overcome the stress condition. However, when chronic inflammation occurs, it causes corticoid hormone resistance, which results in failure to inhibit the pro-inflammatory cytokine production of immune cells.

Although extensive studies of chronic inflammation in humans are ongoing, only a limited number of studies have been performed in livestock such as laying hens. As chronic inflammation in humans is closely related to omega imbalance, this may also apply to livestock since the omega-6 to omega-3 fatty acid ratio in animal feed is also distorted, as in the human diet, and this could have adverse effects on their performance. The issue of chronic inflammation in livestock thus should be clarified and related evidence is needed. However, there has been insufficient study on this topic to date.

2. Nutraceuticals

1) General concept of nutraceuticals and their application as feed additives

Nutraceuticals are defined as food or food ingredients for preventing and treating diseases (Chauhan *et al.*, 2013). They delay, prevent, or treat diseases such as chronic inflammation and are used as alternatives to the products of the pharmaceutical industry. Contrary to pharmaceuticals, nutraceuticals are widely available and their effectiveness is minimally monitored. They originate from plants, animals and microbes and their representative modes of action are anti-inflammatory, anti-oxidant (Tapas *et al.*, 2008), anti-cancer (Sarkar *et al.*, 2010), osteogenetic (Pandey *et al.*, 2018) and positive influence on blood lipid profile (Das *et al.*, 2012) while some nutraceuticals have multiple functions (Table 1). For example, omega-3 PUFAs are known to be anti-inflammatory and have a positive influence on the blood lipid profile (Badimon *et al.*, 2010). Also, CLA has multiple functions regarding anti-cancer, anti-oxidant, and osteogenetic effects (Bhattacharya *et al.*, 2006).

Meanwhile, nutraceuticals is a field of interest in the livestock industry where production cost reductions are important. The production cost can be reduced when preventing disease by using nutraceuticals because they can reduce the use of veterinary medicines. In addition, as application of nutraceuticals can improve

the feed efficiency of livestock animals, many researchers have carried out studies to screen novel nutraceuticals and determine their effects on livestock performance. Traditionally, the livestock industry has applied antibiotics as feed additives, which are called antibiotic growth promoters (AGPs) (Dibner and Richards, 2005). Antibiotics are any medicines that kill or inhibit bacterial growth (Soares *et al.*, 2012). However, many livestock producers have misused and overused AGPs, leading to the issue of antibiotic residues in livestock products, which is threatening the credibility of livestock products (Cully, 2014). Therefore, there have been many trials to find livestock nutraceuticals that can be used as antibiotic alternatives, such as natural compounds or probiotics.

Table 1. Type of nutraceutical.

Type	Nutraceutical	Mode of action	Source
Probiotics	*Lactobacillus* etc.	Anti-pathogenic Anti-inflammatory	Yogurt, Kefir, Kimchi, Natto
Amino acids	N-acetylcysteine	Anti-oxidant	Cysteine, glycine supplement
Carotenoids	β-Carotene	Anti-oxidant	Yellow or orange fruits
	Lutein	Anti-oxidant	Green leafy vegetable Egg
	Zeaxanthin	Anti-oxidant	
	Lycopene	Anti-oxidant	Tomato, watermelon
Fatty acids	Omega-3 FA	Anti-inflammatory Positive influence on blood lipid profile	Flaxseed, nut, cold-water fish
	CLA	Anti-inflammatory Anti-obesity	Meat, Dairy product
Minerals	Copper	Cofactor in enzymatic reactions	Nut, meat, grains
	Selenium	Anti-oxidant	Meat, seafood
	Zinc	Anti-oxidant	Red meat, seafood
Vitamins	Water-soluble	Anti-oxidant	Kiwifruit, broccoli
	Fat-soluble	Anti-oxidant	Peanuts, walnuts
Polyphenols	Curcumin	Anti-cancer	Curry, turmeric spice
	Resveratrol	Anti-inflammatory Anti-diabetic	Grape, blueberry, raspberry
	EGCG	Anti-oxidant	Green tea leaves

2) Omega-3 fatty acid

(1) Physiological importance of omega-6 and omega-3 fatty acid balance

Omega-3 fatty acids are a kind of PUFAs with omega-6 fatty acids. They have been reported as regulator of host homeostasis and inflammatory processes independently and their derivatives termed lipid mediator (Zarate *et al.*, 2017). The difference between omega-3 and omega-6 fatty acids lies in their chemical structure. The position of the first double bond from the methyl group differs. Each type of fatty acid is as follows: 1) omega-3 fatty acid: alpha-linolenic acid (ALA, C18:3n-3), eicosatetraenoic acid (ETA, C20:4n-3), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3); 2) omega-6 fatty acid: linoleic acid (LA, C18:2n-6), gamma-linolenic acid (GLA, C18:3n-6), dihomo-gamma-linolenic acid (DHGLA, C20:3n-6), arachidonic acid (AA, C20:4n-6) and docosapentaenoic acid (n-6 DPA, C22:5n-6) (Figure 8A).

Among them, linoleic acid (LA), an omega-6 fatty acid and alpha-linolenic acid (ALA), an omega-3 fatty acid are essential fatty acids, which must be obtained from diet, and the omega-3 fatty acids cannot be converted to omega-6 fatty acids and vice versa in mammals and poultry. Dietary PUFA may affect host performance due to their alteration of serum PUFA, lipid mediator profile and cytokine expression level (Figure 8B). They undergo bioconversion through

common elongases (Elo) and desaturases (Des) when entering the host body, which results in conversion of LA to AA and ALA to EPA and DHA (Figure 9).

(A)

Polyunsatruated fatty acids(PUFAs)		
Carbon chain	**Omega-3**	**Omega-6**
C18	**ALA (C18:3n-3)**	**LA (C18:2n-6)**, GLA (C18:3n-6)
C20	ETA (C20:4n-3), EPA (C20:5n-3)	DHGLA (C20:3n-6), AA (C20:4n-6)
C22	DHA (C22:6n-3)	n-6 DPA (C22:5n-6)

Red: essential fatty acid

(B)

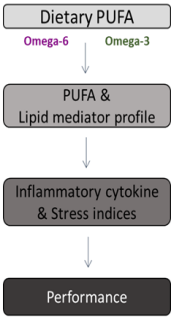

Figure 8. Overview of types of PUFAs and their physiological effects on livestock performance.

(A) Types of PUFAs.
(B) Physiological effects of PUFAs.

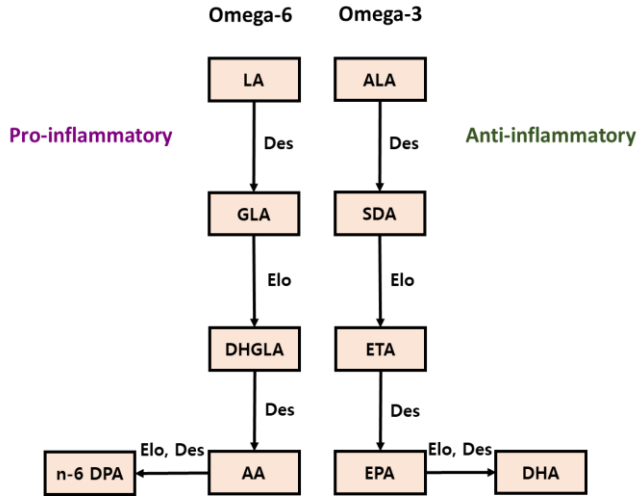

Figure 9. Bioconversion pathway of PUFAs.

Both omega-6 and omega-3 fatty acids undergo bioconversion process via common enzymes such as desaturases (Des) and elongases (Elo). However, omega-6 derived products mainly have pro-inflammatory properties whereas omega-3 derived products have anti-inflammatory.

Distinct from omega-6 fatty acids, omega-3 fatty acids were commonly reported to have anti-inflammatory effects. They can act directly or throughout biotransformation into lipid mediators. In the case of a direct effect, Oh *et al.* suggested that the effect was mediated by G protein-coupled receptor 120 (GPR 120), which inhibited production of pro-inflammatory cytokines (Oh *et al.*, 2010). Otherwise, they can be transformed in the form of lipid mediators, which act as anti-inflammatory and pro-resolving mediators (Groeger *et al.*, 2010). It has also been confirmed that omega-3 fatty acids may play significant roles against inflammatory diseases, such as central nervous system diseases (Wysoczanski *et*

al., 2016), diabetes (Zarate *et al.*, 2017), obesity (Kaliannan *et al.*, 2016) and cancer (Fuentes *et al.*, 2018).

Therefore, the ratio of omega-6 to omega-3 fatty acid is important to host inflammation state. Actually, many researchers have pointed to sudden changes the ratio in the diet as a cause of chronic inflammation. This is strongly linked to the mass production of vegetable oils such as corn and soybean oils, which are rich only omega-6 fatty acid. Actually, a recent study reported the ratio of omega-6 to omega-3 has been increased from 1:1 to 20:1 compared to ancient society (Simopoulus *et al.*, 2016). Previous researchers revealed that changes in this ratio from altered dietary patterns led to inflammation (Calder, 2012;Patterson *et al.*, 2012) and oxidative stress (Yang *et al.*, 2016). Also, some studies reported on the relationship between the imbalance of this ratio and chronic inflammation-related diseases (Simopoulos, 2002;Oddy *et al.*, 2004;Simopoulos, 2016). Meanwhile, many studies have suggested that the ideal ratio of omega-6 to omega-3 is between 1:1 and 1:4 (Grimm *et al.*, 1994;Siddiqui *et al.*, 2007). Therefore, the current ratio is likely to be excessive (Figure 10). As omega-6 and omega-3 fatty acids play crucial roles in the inflammatory response in the body, omega imbalance due to an excessive increase in omega-6 fatty acids is strongly associated with increased chronic inflammation in the body and an increased incidence of diseases.

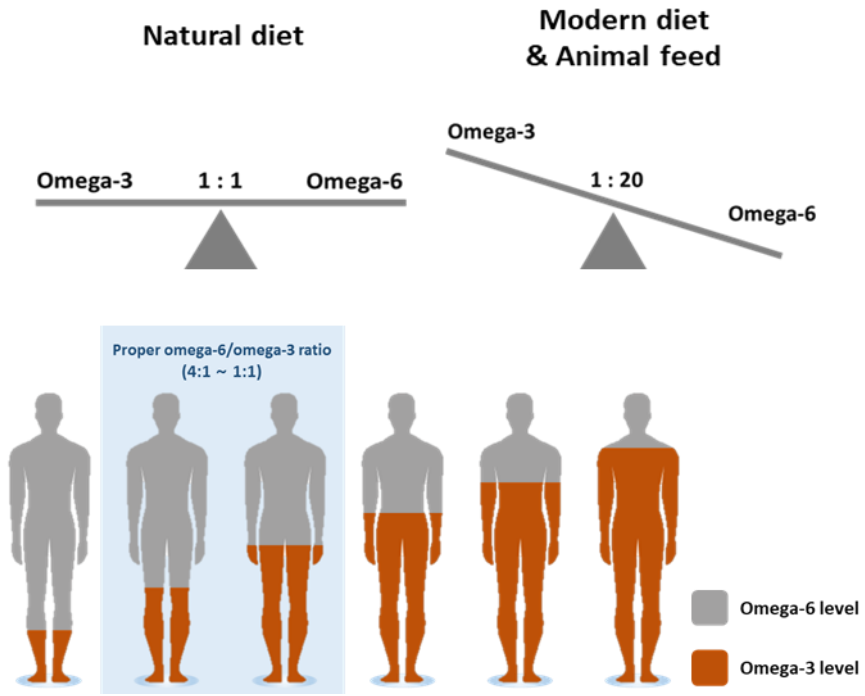

Figure 10. Unbalanced modern diet and ideal omega-6/omega-3 balance.

In diet, the ratio of omega-6 to omega-3 fatty acid is changed from 1:1 to 20:1, so that its ratio is under unbalanced state in modern diet and animal feed. In various human clinical studies, the idealistic ratio of omega-6 to omega-3 fatty acid is around 1:1 to 4:1.

(2) Lipid mediators and specialized pro-resolving mediators

Lipid mediators play crucial roles in the onset of inflammation and its resolution. They are produced through three sequential enzymatic steps. First, some inflammatory stimuli such as stress or lipopolysaccharide (LPS) induce expression of phospholipase A_2 (PLA_2), releasing PUFAs from the cell membrane that were incorporated by diet. Free PUFAs are then transformed to a parallel series of lipid mediators by cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome p450 (CYP450) enzymes and then exported extracellularly and act as ligands of GPCRs (Greene *et al.*, 2011) (Figure 11). As omega-3 fatty acids have one more double bond than omega-6 fatty acids in their chemical structure, omega-3-derived lipid mediators also have one more double bond than their omega-6 counterparts, which affects the nomenclature of lipid mediators. Lipid mediators are of particular interest recently because they play important roles in the onset and resolution of inflammation, and are nutritionally controllable depending on the amount of their precursors such as omega-6 and omega-3 fatty acids that is ingested (Serhan *et al.*, 2014).

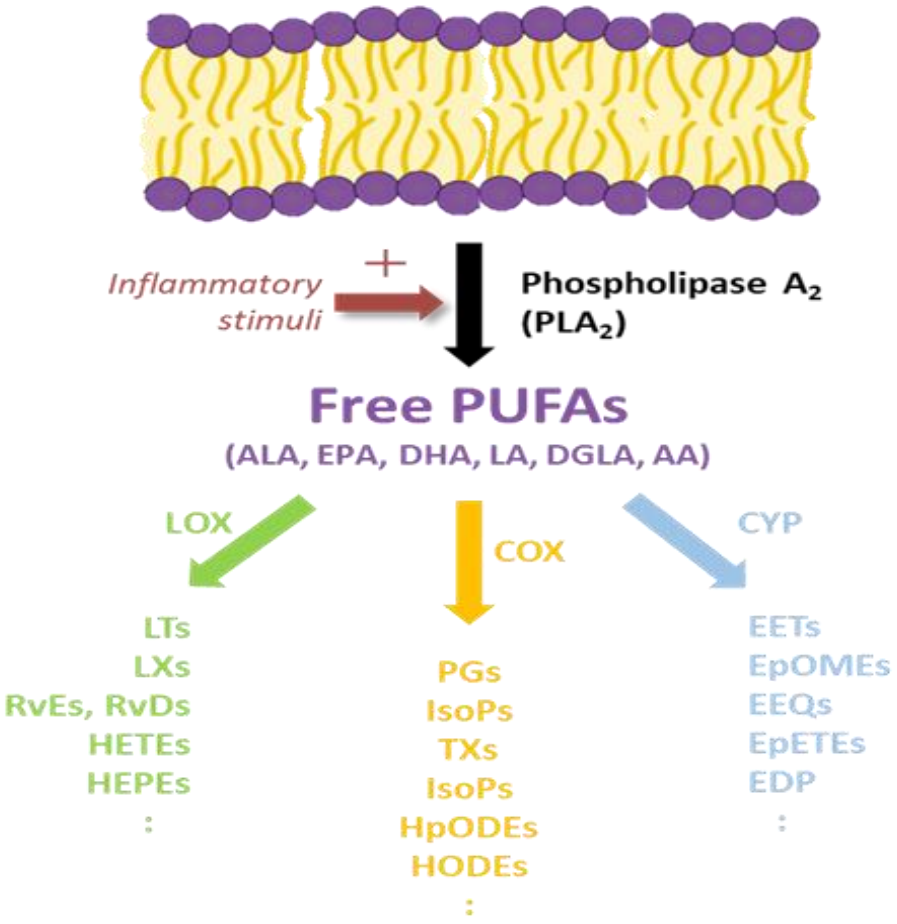

Figure 11. Lipid mediators derived from cell membrane PUFAs.

When inflammatory stimuli induced phospholipase A_2 (PLA_2), PUFAs were released from cell membranes. Then, free PUFAs transformed to lipid mediators by various enzymes such as lipoxygenase (LOX), cyclooxygenase (COX), and cytochrome P450 (CYP).

Contrary to omega-6-derived lipid mediators, the main target of NSAIDs, omega-3-derived mediators had not been noticed in the past because they were thought to be simply competitive substrates of omega-6 fatty acids that decrease omega-6-derived lipid mediator production. However, recent studies have identified omega-3-derived lipid mediators and suggested their distinct biological importance (Pazderka *et al.*, 2018). It is now recognized that there is a novel class of lipid mediators that function for resolution, and they are collectively called specialized pro-resolving lipid mediators (SPMs). These include lipoxins, resolvins, maresins, and protectins (Duffney *et al.*, 2018). Their mode of action is to mediate GPCRs, akin to PUFAs (Chiang and Serhan, 2017). The overall biotransformation pathway of PUFAs and its derivatives is illustrated in Figure 12.

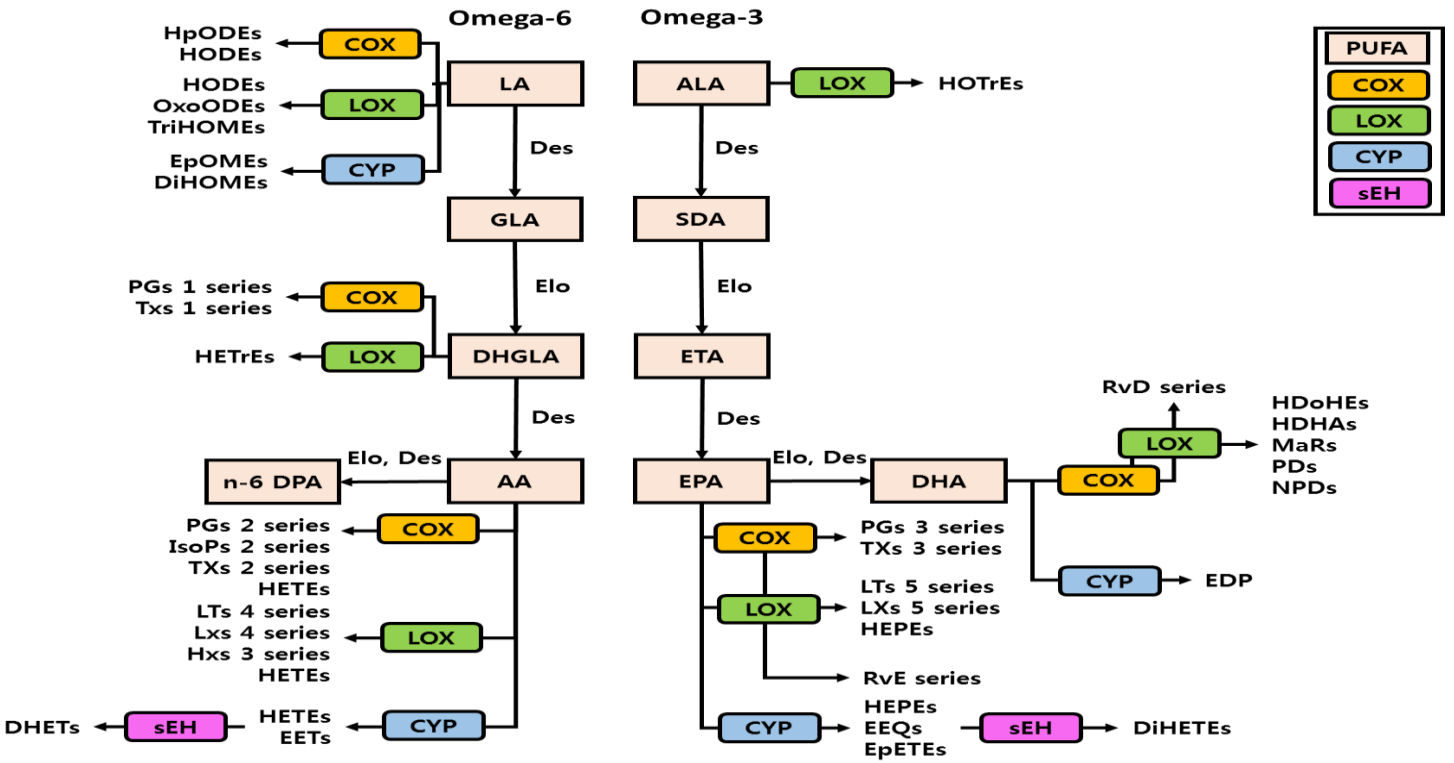

Figure 12. Biosynthesis pathway of lipid mediators.

PUFAs generate lipid mediators via various common enzymes.

In general, inflammation is resolved by “class switching”, where intracellular pro-inflammatory factors turn into anti-inflammatory and pro-resolving factors. In the case of lipid mediators, cells downregulate pro-inflammatory lipid mediator-producing enzymes such as prostaglandins and leukotrienes, while upregulating SPM-producing enzymes (Basil and Levy, 2016). The distinguished characteristic of SPMs compared to existing corticosteroids and non-steroidal anti-inflammatory drugs (NSAIDs) is that they do not suppress the immune response but provoke resolution. Also, their biosafety is expected to be high, because they exert effects in a nanomolar to picomolar range and are synthesized endogenously. Thus, their therapeutic potent as novel drugs for chronic inflammation is promising.

(3) Flaxseed

Flaxseed has been widely used as a dietary supplement of omega-3 fatty acids for humans and animals. Contrary to other vegetable oils which are abundant in omega-6 fatty acids, flaxseed oil contains abundant amounts of ALA, accounting for approximately 53% of total fatty acids (Moallem, 2018) (Figure 13). However, whole flaxseed is known to contain anti-nutritional factors such as mucilage, cyanogenic glycosides, phytic acid, and trypsin inhibitors (Rodriguez, *et al.*, 2001), which could induce adverse effects on digestion and absorption and even diarrhea (Gonzalez-Esquerra and Leeson, 2000). One of the common treatments available to reduce these side effects of flaxseed is extrusion (Bean and Leeson, 2002).

The beneficial effects of dietary flaxseed against cardiovascular diseases such as hypertension (Caligiuri *et al.*, 2014), platelet aggregation (Nandish *et al.*, 2018), atherosclerosis (Han *et al.*, 2018b) and several cancers such as breast (Zanoaga *et al.*, 2018) and colon (DeLuca *et al.*, 2018) have been reported.

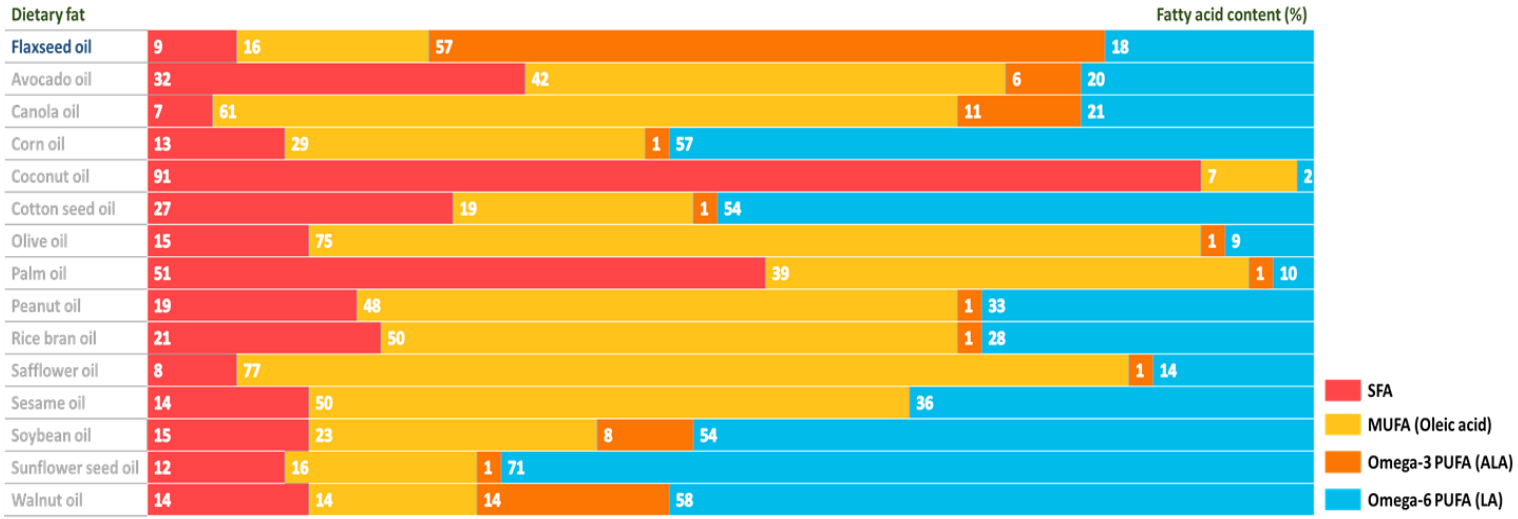

Figure 13. Fatty acid composition of vegetable oils.

Most vegetable oils contain excessive omega-6 PUFA contents compared to omega-3 content. However, flaxseed oil contains abundant omega-3 fatty acid, ALA contrary to others.

3) Probiotics

(1) Probiotics as nutraceuticals

Despite their strong effect on improving of livestock performance, antibiotics have gradually been banned due to antibiotic resistance and dysbiosis in livestock gut microbiota. However, the post-antibiotic banning period induced a decrease in livestock productivity. Thus, developing effective and safe alternatives to antibiotics has become an important issue in the livestock industry.

As antibiotics act mainly on the gut of livestock, nutraceuticals such as probiotics and prebiotics that are able to affect the gut were mainly studied as alternatives. The most representative candidate is probiotics which are living microorganisms that have potential to be beneficial to the host (Neri *et al.*, 2019). Their modes of action can be classified into four areas as follows: 1) anti-pathogenic effect; 2) fermentation; 3) gut barrier reinforcement; and 4) immuno-modulation (Figure 14).

Actually, the administration of probiotics to livestock animals can suppress pathogen mediated by lactic acid and bacteriocin (Prabhurajeshwar and Chandrakanth, 2017). Also, the enzymes encoded by probiotics may synthesize beneficial compounds such as vitamins (LeBlanc *et al.*, 2013) and conjugated linoleic acid (CLA) (Kishino *et al.*, 2013). Probiotics-producing short chain fatty acids (SCFAs) reinforce the gut barrier by strengthening the tight junction

(Venegas *et al.*, 2019) or thickening the mucus layer (Martin *et al.*, 2019). Moreover, it was reported that probiotics modulate the host immune system (Moens *et al.*, 2019). Furthermore, some traits can be used to evaluate each probiotic candidate such as toxicity, pathogenicity, and acid and bile tolerance, and lactic acid bacteria (LAB), bacilli and yeast were mostly used as probiotics.

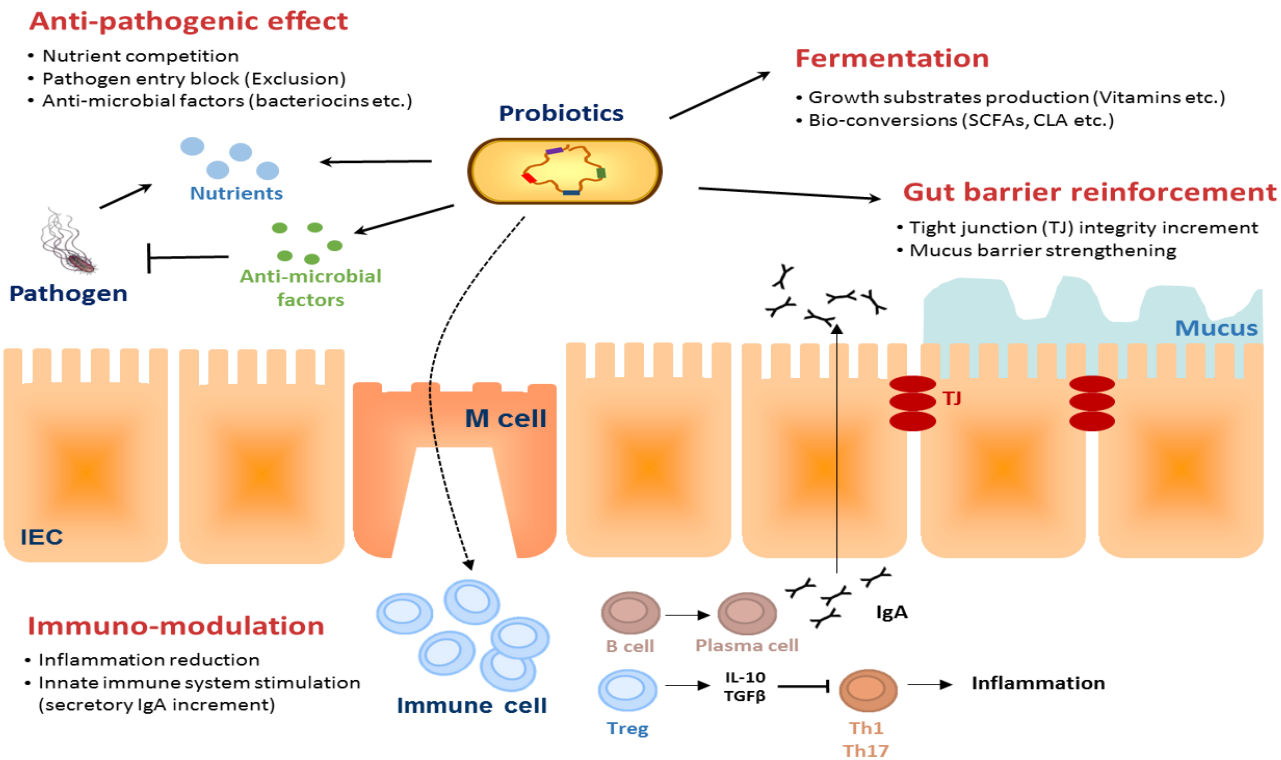

Figure 14. Biological effects and mechanism of probiotics.

Probiotics affect host in several mode of actions: anti-pathogenic effect, fermentation, gut barrier reinforcement, and immune-modulation.

(2) *Lactobacillus plantarum*

Among LABs, *Lactobacillus plantarum* has been widely studied as probiotics for their ability to produce antimicrobial substances such as lactic acid and a bacteriocin called plantaricin (Man and Xiang, 2019). Moreover, it has been recently reported that some strains in *Lactobacillus plantarum* are able to synthesize CLA from LA using their encoded enzyme (Kishino et al., 2002;Ando et al., 2004;Ares-Yebra et al., 2019). A gut barrier-enhancing effect was also observed with some strains of *Lactobacillus plantarum* (Anderson *et al.*, 2010a;Anderson *et al.*, 2010b). There was also a report that *Lactobacillus plantarum* Lp91 had an immunomodulatory effect, thereby improving health in an animal disease model (Duary *et al.*, 2012). Lastly, the administration of *Lactobacillus plantarum* led to improvement in livestock performance of various animal models (Lee *et al.*, 2012;Peng *et al.*, 2016)

(3) *Bacillus licheniformis*

Bacillus licheniformis is a species of *Bacillus*. Like other species, *Bacillus licheniformis* can produce endospores under harsh conditions. This can be advantageous when developing feed additives because the spore form can endure the feed mixing step with high temperature and pressure. Moreover, among *Bacillus* species, *Bacillus licheniformis* is GRAS with *Bacillus subtilis* contrary to other species (Beric *et al.*, 2014).

Also, it has an anti-microbial property with secretion of a bacteriocin called licheniocin, which has stability in a wider range of pH and temperature than *Lactobacillus*-origin bacteriocin (Abriouel *et al.*, 2011). It encodes keratinase, enabling degradation of feather into digestible protein (Cheng *et al.*, 1995;Korkmaz *et al.*, 2004). Moreover, it exerts an immunomodulatory effect when administered to livestock (Gobi *et al.*, 2018;Lin *et al.*, 2019). The effects on livestock performance were also observed (Deng *et al.*, 2012).

3. Biological markers indicating animal welfare

In recent years, many issues related to animal welfare in the livestock industry have become prevalent. While the producer's greatest interest in the past was to drive the maximum production of livestock products at the lowest cost, now it is no longer possible to ignore consumer demand for livestock products produced in well-kept animal welfare environments.

The concept of animal welfare was first officially articulated in the publication of the Brambell report issued by the British government in 1965. Also, this novel concept was established as a science after adopting a scientific approach focused on the effects of various factors under controlled conditions (Millman *et al.*, 2004). A large amount of research has since been carried out on this issue in various fields of interest such as developing welfare assessment methods and biological implications of animal welfare investigation. Therefore, studies on animal welfare are usually multidisciplinary, considering physical, psychological, immunological, and behavioral aspects simultaneously and linked together (Ferdowsian and Beck, 2011).

Two important factors in animal welfare are 'suffering' and 'need' (Millman *et al.*, 2004). In other words, it is necessary to know how animals feel and know what they want. For this, it is necessary to be able to identify how they feel through scientific indicators, and study how to improve those indicators. Traditionally, the

state of livestock animals was only determined by livestock performance, other biological markers were tested to identify livestock state such as inflammation or stress.

1) Inflammatory index

Most studies of inflammation mainly monitored inflammatory cytokines. Cytokines are soluble proteins secreted by both immune and parenchymal cells that act in autocrine or paracrine ways (Altan-Bonnet and Mukherjee, 2019). They modulate the host physiology, especially regarding the immune system including inflammation (Huang *et al.*, 2019). Among various cytokines, tumor necrosis factor-alpha (TNF-alpha), interleukin-1 (IL-1), and interleukin-6 (IL-6) act as promoters of inflammation and are called pro-inflammatory cytokines (Dinarello, 2000). TNF-alpha and IL-1 are recognized as master regulators of inflammation (Rhodus *et al.*, 2005). They are abundant at the inflamed site due to secretion by both immune cells and parenchymal cells at the site. Also, they provoke the induction of expression of other inflammatory cytokines and mediators. IL-6 is recognized as a pro-inflammatory marker despite its anti-inflammatory properties because it is stimulated by TNF-alpha and IL-1beta. In fact, a two- to three-fold increase in the plasma concentration of IL-6 was observed in elderly humans who were under systemic chronic inflammation (Forsey *et al.*, 2003). The levels of circulating pro-inflammatory cytokines are also elevated in several inflammatory

diseases such as Crohn's disease (Gabay, 2006;Szczeklik *et al.*, 2012) and obesity (Borges *et al.*, 2018). Moreover, the expression level of COX-2, which is inducible by inflammatory stimuli, is also considered an inflammatory marker in various disease models (Mohammed *et al.*, 2004;Yu *et al.*, 2006).

2) Stress index

As HPA axis activation by stress releases glucocorticoids, it is commonly used to determine the stress state (Sapolsky *et al.*, 2000). In particular, in poultry, the serum level of corticosterone was reported to be more responsive to stress stimuli than that of cortisol (Siegel, 1995), which is widely used as a biological marker (Puvadolpirod and Thaxton, 2000).

The ratio of neutrophil:lymphocyte is also a representative marker to detect a stress state. This value was first calculated by observed an increased number of lymphocytes and a decreased number of neutrophils as the glucocorticoid concentration increased (Sayers, 1950). Also, the value was increased by feeding glucocorticoids to livestock (Widowski *et al.*, 1989). In poultry, it is called the heterophil:lymphocyte ratio (H:L ratio) because heterophils replace the roles of the mammalian neutrophils (Gross and Siegel, 1983). Similar to studies using the neutrophil:lymphocyte ratio, the H:L ratio was also commonly used as a biological marker of stress state. Moreover, this value is thought to be a more reliable marker

than cortisol or corticosterone level because it fluctuates less by external stimuli (Mcfarlane and Curtis, 1989).

Hsp70 is a kind of heat shock protein. Heat shock proteins are proteins that help protein folding and its correction. Interestingly, they are abundant especially under stress conditions such as heat, osmotic pressure, and harsh pH condition (Zugel and Kaufmann, 1999). By this property, heat shock proteins were widely studied to determine biological markers of a stress state (Delmas *et al.*, 1996;Afsal *et al.*, 2019). Also, HSP70, a well-established intracellular stress chaperone, is expressed in chicken feather and is responsive to heat stress enabling the gene expression of HSP70 used as a stress marker (Greene *et al.*, 2019)

3) Gut microbiota

Gut microbiota is a complex community of microorganism species that live in the gastrointestinal tract of human and animals. It was recently discovered that gut microbiota takes part in bidirectional communication between the gut and host organs (Sochocka *et al.*, 2019). In particular, the development of high-throughput sequencing technology enables identification of microbial community alteration and distinguished bacteria between normal and various disease states, which was impossible by traditional culture-based analysis (Delzenne and Cani, 2011;Clarke *et al.*, 2014;Kasai *et al.*, 2015). Moreover, prediction of the metagenome based on

the microbial community population or actual metagenome data was also applied to gut microbiota analysis.

Meanwhile, it has been reported that inflammation and stress also affect gut microbiota. For example, a chronic inflammation condition such as aging alters gut microbiota in both human and livestock animals (Biagi *et al.*, 2011). Likewise, some changes were observed in a model of inflammatory bowel disease (Sartor, 2008). The potential role of gut microbiota on stress response was studied with a germ free murine model (Crumeyrolle-Arias *et al.*, 2014). Also, different activation of the HPA axis was observed between normal and germ free models (Mayer *et al.*, 2015).

As gut microbiota is a complex system, multidisciplinary research with other omics data such as genomic, transcriptomic, proteomic and metabolomic data has been conducted to understand its precise effect on host physiology (Figure 15). This can help to identify novel biological markers to detect inflammatory and stress states.

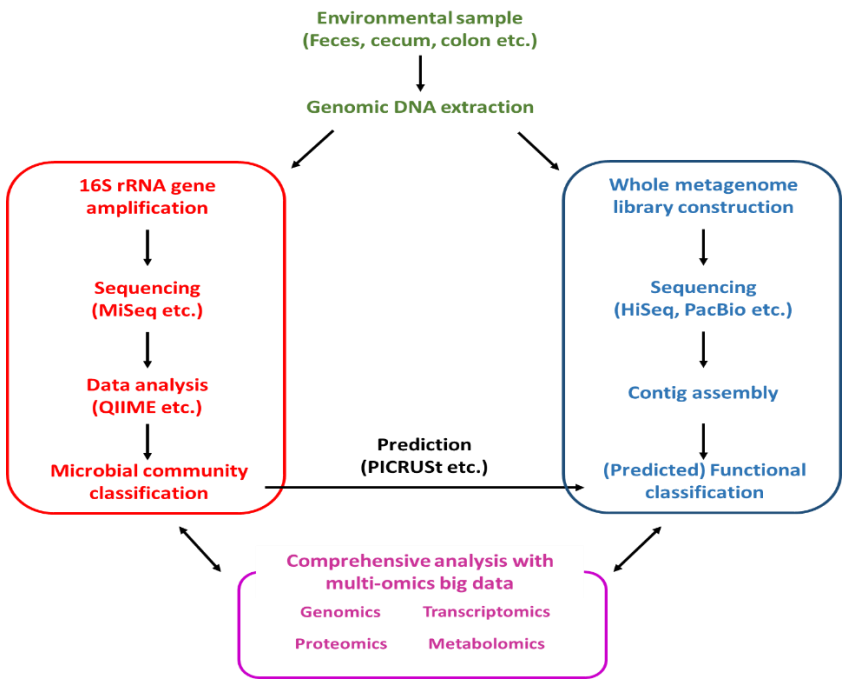

Figure 15. General procedure of microbiome study using high-throughput sequencing technology and bioinformatic tools.

To study microbiome of environment, genomic DNAs were obtained from environmental samples to sequence identifying marker (e.g. 16S rRNA gene) or whole metagenome. Microbial community and its function can be analyzed with other multi-omics data to interpret its meaning.

Study 1. Physiological effects of alpha-linolenic acid and flaxseed on host

1. Introduction

Polyunsaturated fatty acids (PUFAs) are fatty acids having more than two double bonds in their molecular structures. They are largely divided into omega-6 and omega-3 fatty acid by the position of the first double bond. As some of them are essential fatty acids and also have been reported as regulators of inflammatory process either themselves or derivatives such as lipid mediator, their balanced intake is essential to maintain host health (Zarate *et al.*, 2017).

Especially, many researches have attracted in the balance of omega-6 and omega-3 fatty acids because they play crucial but distinct roles regarding inflammatory process balance (Patterson *et al.*, 2012). Most of omega-6 fatty acids and their derivatives have pro-inflammatory effects whereas omega-3 fatty acids and their derivatives have anti-inflammatory and pro-resolving (Seki *et al.*, 2009) . Meanwhile, common diet for human and animals contain mostly excessive levels of omega-6 fatty acids but low omega-3 fatty acids so that the ratio of omega-6 to omega-3 ratio has increased to approximately 20:1, which is out of balance (Simopoulos, 2016).

Flaxseed is a representative omega-3 fatty acid abundant material especially ALA. It accounts for about 50% of total fatty acids in the flaxseed (Moallem, 2018). In addition, it is used as an actual feed additive by going through the removal process of the anti-nutritional factors by a method such as extrusion (Rodriguez *et al.*, 2001). There were many studies feeding flaxseed to livestock animals, which resulting in omega-3 fatty acids-fortified livestock products such as egg (Antruejo *et al.*, 2011) and milk (Zachut *et al.*, 2010).

However, there was few study to investigate physiological effects of flaxseed on livestock animals. Since the lowering ratio of omega-6 to omega-3 has been confirmed in livestock products by the addition of flaxseed, it is need to be determined if this lowering also occurs in the host inner body and if so, whether there is a change in the lipid mediator profile. Moreover, these changes may also lead to changes in the inflammatory and stress states, so it should be checked.

The aim of the study 1 was to investigate the physiological effects of ALA and flaxseed on host (Table 2). In study 1-1, the physiological effects of ALA regarding inflammatory cytokines and lipid mediator-related genes were monitored by levels of various gene expression. Then, in study 1-2, 33-week-old laying hen were fed basal diet with 0, 0.9, 1.8, 3.6 % (w/w) of commercial flaxseed product (Lintex 170) for 4 weeks to investigate the physiological effects. To analyze the changes in the PUFA and lipid mediator profiles, laying hen serum samples were analyzed with various chromatography assays. Furthermore,

various inflammatory and stress indices were monitored by same samples. Study 1 is the first report to investigate the lipid mediator profile and its alteration by omega-3-rich material.

Table 2. Summarization of the study 1.

Study	Category	Item
<Study 1-1> Physiological effect of alpha-linolenic acid *in vitro*	Experimental design	Cell (RAW264.7)
		Sampling (mRNA)
	Gene expression	mRNA level (Pro-inflammatory cytokines, lipid mediator synthesis)
<Study 1-2> Validation of flaxseed as feed additives *in vivo*	Experimental design	Animal (laying hen)
		Sampling (Egg, blood)
		Feed information
	Effects of flaxseed on fatty acid profile	Egg and serum omega-6/omega-3 ratio
	Effects of flaxseed on lipid mediator profile	Multi-variant analysis
		Relative concentrations of lipid mediators
		Overall diagram of alteration
	Inflammatory and stress indices	Inflammatory indices (serum cytokines)
		Stress indices (corticosterone, H/L ratio)
	Laying performance	Performance
		Egg quality

2. Materials and Methods

1) *In vitro* murine macrophage cell line (RAW 264.7) experiment

(1) Cell line and cell culture

Murine macrophage cell line RAW264.7 were used *in vitro*. After thawing a stock stored at liquid nitrogen tank, it cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM) (Gibco®, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 100 μmol/L penicillin and 100 μmol/L streptomycin changed into fresh medium every day until confluent. Culture condition was at 37℃ with 5% CO_2.

(2) Gene expression of pro-inflammatory cytokines and lipid mediator-related genes

RAW264.7 cells were plated in 6-well flat-bottom culture plates and pre-incubated 2 hr at a dose of 0.1 μg/mL lipopolysaccharide (LPS) treatment. After that, 100 μM ALA was treated for 24 hr. Total RNA was extracted from the cells using the TRIzol reagent (Invitrogen, USA), chloroform (Sigma-Aldrich, USA), isopropanol (Sigma-Aldrich, USA), ethanol (Sigma-Aldrich, USA) and diethyl pyrocarbonate-treated (DEPC-treated) water. Briefly, the media was aspirated

followed by washing cells with PBS 1ml. Then, PBS was aspirated to treat 1ml TRIzol so that detach cells from plates by cell scrappers. The mix of TRIzol and cell lysate were transferred to 1.5 ml tube and 200 μl chloroform was added to induce mRNA separation in upper layer. After transferring upper layer into a new tube, 1.2 ml isopropanol was treated to precipitate mRNA and stored at -20℃ overnight. Next, the isopropanol was removed and 1ml 75% ethanol (diluted with DEPC-treated water) was treated followed by centrifugation at 9,500 rpm for 5 min. The RNA pellet was collected by being removed ethanol and air-drying for 10 min. After resuspending RNA pellet to DEPC-treated water, the concentration was quantified by Nanodrop 2000 (Thermo Scientific, USA) at 260 nm. All mRNA samples were used to synthesize cDNA samples using ToYoBO cDNA synthesis kit (Toyobo, Japan). The gene expression was determined by qRT-PCR and the $2^{-\Delta\Delta Ct}$ method with magnetic induction cycler (Biomolecular systems, Australia) by mixing cDNA and QuantiSpeed SYBR No-ROX kit (PhileKorea, Republic of Korea). The primers used in this study are listed in Table 3.

Table 3. The primer sequences for detecting genes of pro-inflammatory cytokine and lipid mediator synthesis by quantitative real-time PCR.

Primers	Sequence (5'→ 3')	Reference
β-actin	F : ACGGCCAGGTCATCACTATTG R : AGGGGCCGGACTCATCGTA	Gao *et al.*, 2018
cox2	F : TTCAACACACTCTATCACTGGC R : AGAAGCGTTTGCGGTACTCAT	Gao *et al.*, 2019
5-lox	F : ATGCCCTCCTACACTGTCAC R : CCACTCCATCCATCTATACT	Gronert *et al.*, 2005
pgds	F : GGGAATCCCAAGAGACCCAG R : GCTCTGAGCAAATGGCTGC	Baker *et al.*, 2001
tnf-α	F : CCCTCACACTCAGATCATCTTCT R : GCTACGACGTGGGCTACAG	Zhao *et al.*, 2011
il-1β	F : TCAAATCTCGCAGCAGCACA R : CCAGCAGGTTATCATCATCA	Cheng *et al.*, 2015
il-6	F : GAGTGGCTAAGGACCAAGACC R : AACGCACTAGGTTTGCCGA	Ji *et al.*, 2014
mcp-1	F: GCTCAGCCAGATGCAGTTAA R : TCTTGAGCTTGGTGACAAAAACT	Liu *et al.*, 2016

2) *In vivo* laying hen feed experiment

(1) Animals and treatment

The experiment using laying hens (n = 200, 33-week-old, *Lohmann Brown-Lite*) was performed at Seoul National University animal farm (PyeongChang, Republic of Korea). The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-180219-1). The hens were divided randomly into four groups with five hens per cage (48 cm x 45 cm x 45 cm, of width x depth x height) and ten cages per group. Fifty hens were assigned to each of the following diet treatments for 4 weeks: 1) commercial basal diet (**C**); 2) basal diet with 0.9% (w/w) of flaxseed (**T1**); 3) basal diet with 1.8% (w/w) of flaxseed (**T2**); 4) basal diet with 3.6% (w/w) of flaxseed (**T3**). The nutrient compositions of experimental diets are calculated in Table 4. The ratio of omega-6 to omega-3 of each feed was confirmed to 21.8 (C), 8.1 (T1), 5.2 (T2), 2.9 (T3), respectively, analyzed by comparing with fatty acid methyl ester 37 (FAME 37) (Sigma-Aldrich, USA) standard peak pattern (Figure 16). Feed was offered 600 g per cage daily and fresh water was offered *ad libitum* during the experimental period. Flaxseed was supplemented as mixing commercial product termed Lintex170 into basal diet. Lintex 170 was developed by Seoul National University and HANYOU BNF Co., Ltd (Seoul, Republic of Korea) and manufactured by HANYOU BNF Co., Ltd. The product

consists of 85% extruded flaxseed and 15% starch source so that 17% of the product is ALA.

Table 4. Calculated nutrients of experimental diet of the study 1 *in vivo.*

Items	Group			
	C	**T1**	**T2**	**T3**
Calculated nutrients content (% of total feed)				
Metabolizable energy, Mcal/kg	2.70	2.72	2.75	2.79
Crude protein	16.50	16.54	16.57	16.65
Fat (ether extract)	2.50	2.79	3.07	3.65
Crude fiber	8.00	8.25	8.49	8.98
Crude ash	20.00	19.87	19.74	19.47
Calcium	3.50	3.47	3.44	3.37
Phosphorous	1.20	1.19	1.18	1.16
Methionine+Cysteine	0.60	0.59	0.59	0.58

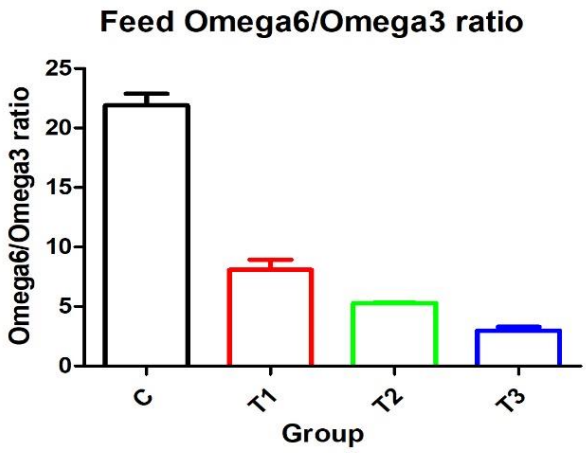

Figure 16. The ratio of omega-6 to omega-3 fatty acid in experimental diets.

The ratios are calculated by gas chromatography with FAME 37 mixture with 5 replicates.

(2) Reagents

The reagents used to analyze fatty acid methyl ester by gas chromatography, which are fatty acid methyl ester standard (Supelco® 37 Component FAME Mix, FAME 37), internal standard (tridecanoic acid, C13:0), potassium hydroxide and sulfuric acid were purchased from Sigma-Aldrich (Sigma-Aldrich, USA).

The lipid mediator standards used to detect serum lipid mediator profile by were as follows: 14, 15-epoxyeicosatrienoic acid (EET)-d11, 5(S)hydroxyeicosatetraenoic acid (HETE)-d8, leukotriene B_4 (LTB_4)-D4, prostaglandin D_2 (PGD_2)-d4 and arachidonic acid (AA)-d8 (Cayman Chemical, USA). HPLC-grade water, acetonitrile, isopropyl alcohol and hexane were purchased from J.T. Baker (Avantor Performance Material, USA). A Strata-x-33-μm polymerized solid reverse-phase extraction column (Cat no.8B-S100-UBJ) was purchased from Phenomenex (Phenomenex, USA).

The enzyme-linked immunosorbent assay (ELISA) kits to measure serum pro-inflammatory cytokines and corticosterone were purchased from CUSABIO (CUSABIO, Wuhan, China).

(3) Samples and data collection

Performance was assessed for each cage and the number of eggs and their weights were recorded every day. Abnormal eggs, such as broken, cracked or shell-less eggs, were also noted. Feed intake was measured every week, and then weekly feed conversion ratio [feed consumed (g) / egg mass (g)] was calculated. Egg samples [10 eggs/ (group × week)] were collected every week and randomly selected to analyze albumen height, Haugh unit, eggshell thickness and egg fatty acid profile. Albumen height was measured with a micrometer. Then, Haugh unit was calculated with albumen height and egg weight using the following formula: Haugh unit = $100 \times \log (H + 7.57 - 1.7 \times W^{0.37})$, where H = albumen height (mm) and W = egg weight (g) (Eisen, *et al.*, 1962). The value of eggshell thickness was determined by averaging measurements taken at 3 different locations on the egg (air cell, equator, and sharp end) by using a dial pipe gage. Also, 1 g of egg yolk was separated and collected in a glass tube to analyze egg fatty acid profile.

Blood samples were collected from 10 randomly selected hens per group at weeks 0, 2 and 4. They were collected in 2 tubes: BD Vacutainer SST™ Ⅱ Advance Tubes (Becton Dickinson, France) for sampling serum and V-Tube™ EDTA K3 Tubes (AB MEDICAL, Republic of Korea) for measuring complete blood cell count. Blood samples were centrifuged at 6,000 rpm for 10 min, and

supernatant serum was transferred to 1.5 ml plastic tubes and stored at -20°C until use. Blood samples for complete blood cell count were stored at 4°C until use.

The fatty acid profile of feed, egg and serum samples were analyzed by gas chromatography (GC) with 5 replicates. 1 g of ground feed and egg yolk and 500 µL of serum were used. Fatty acid was extracted and methylated in one tube using the direct methylation method (O'Fallon, *et al.*, 2007) with some modifications. Briefly, 0.5 mg of tridecanoic acid was added to samples in 15 ml glass tubes, followed by addition of 5.3 mL of methanol and 700 µL of 10 N KOH. Tubes were incubated in a water bath at 55°C for an hour and a half with brief vortexing every 20 min. Then, tubes were chilled at RT, and 580 µL of 24 N H_2SO_4 was added. Incubation and chilling steps were repeated as before. Finally, 3 ml of hexane was added, and the sample was vortexed for 5 min and centrifuged at 3,000 rpm for 5 min. Then, the upper phase was transferred to a GC vial (Agilent, USA) and analyzed with a gas chromatography-flame ionization detector (GC-FID, Agilent 7890B, USA) with SP-2560 (100 m x 0.25 mm, L x I.D; 0.2 µm, d_f, Sigma-Aldrich , USA). FAME 37 was used as reference for peak identification. The running condition of GC-FID followed the FAME 37 manual (oven temperature: 140°C, 5 min; ramp: 240°C at 4°C/min and hold for 28 min; injector and detector temperature: 260°C; split ratio: 1:30; injection volume: 1 µL).

Lipid mediators in serum were separated by the solid-phase extraction method (Kortz, *et al.*, 2014) using a Strata-x-33-µm polymerized solid reverse-phase

extraction column. Firstly, the columns were activated with 3.5 mL of methanol followed by the equilibration step with same amount of water. Samples were prepared by adding 10 ng of each deuterated internal standard mix to 1 mL of serum with 200 μL of methanol and 800 μL of distilled water. Samples were then loaded on columns and washed with 3.5 mL of 10% methanol in distilled water. Finally, samples were eluted with 1mL of methanol. These samples were concentrated using a Speed-Vac concentrator (Labconco, USA), resuspended in 90 μL of solvent A (70:30:0.02, water:acetonitrile:acetic acid, *v*:*v*:*v*) and stored at -80°C until use.

The ultra-performance liquid chromatography (UPLC) analyses and tandem mass spectrometry (MS/MS) analyses followed previously reported methods (Lee *et al.*, 2016a). The Skyline software package (MacCoss Laboratory, USA) was used to determine the peak area of each lipid from raw data. The extracted peak areas were normalized by internal standard. Partial least squares discriminant analysis **(PLS-DA)** plot of quantified lipid mediators and variable importance in projection **(VIP)** plot of 15 lipid mediators (VIP scores Top 15) were performed on the MetaboAnalyst web site (Chong, *et al.*, 2018)

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

In case of H:L ratio, the complete blood cell count method was used. First, 10 μL blood samples in EDTA K3 tubes were collected to make the blood smears on the slide glasses. Then, the smears were stained with Wright's stain. Then, we identified white blood cells, such as heterophils, lymphocytes, eosinophils, and monocytes with a compound microscope (Axio Scope.A1, Carl Zeiss, Germany) and calculated the ratio of H:L.

The statistical analysis were conducted by using SAS 9.3. The significant differences were determined by one-way ANOVA with post hoc Tukey HSD tests (*p-$value$<0.05; **p-$value$<0.01; ***p-$value$<0.001).

3. Results

1*) In vitro* evaluation of ALA

(1) Gene expression

To investigate effect of ALA regarding inflammation, LPS was treated to RAW264.7 followed by ALA treatment. Then, after incubating for 24 hr, various gene expression of lipid mediator synthesis-related genes (Cycloxygenase-2; ***cox2,*** 5-lipoxygeanse; ***5-lox***, prostaglandin D_2 synthase; ***pgds***) and pro-inflammatory cytokines (tumor necrosis factor-alpha; ***tnf-α***, interleukin-1beta; ***il-1β***, interleukin-6; ***il-6***, monocyte chemoattractant protein-1; ***mcp-1***) were monitored.

In case of lipid mediator synthesis-related genes, LPS stimulation significantly induced *cox2* expression whereas inhibiting *5-lox* expression while ALA mainly inhibited *cox2* and *pgds* under LPS-stimulated condition (Figure 17A-C). Likewise, LPS significantly induced gene expression of all 4 pro-inflammatory cytokines, which were alleviated by ALA treatment (Figure 17D-G).

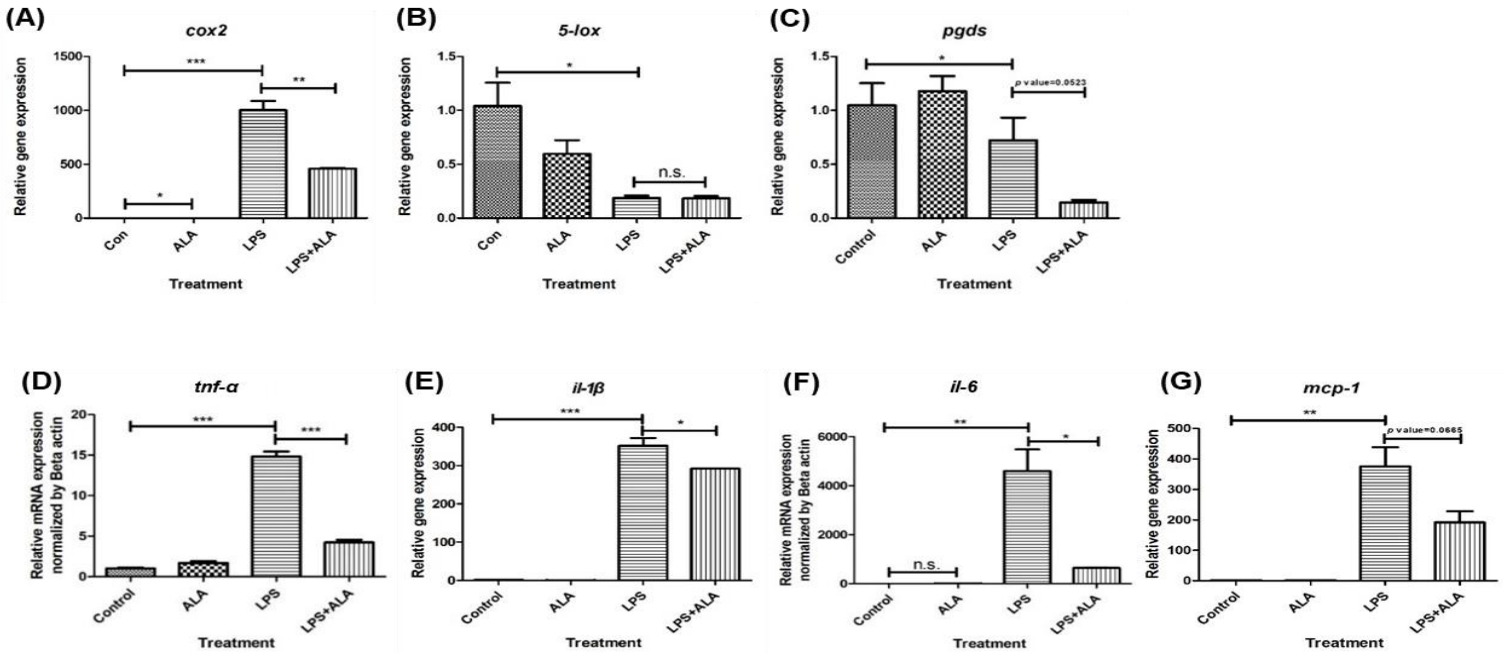

Figure 17. Analysis of gene expression of pro-inflammatory cytokines and lipid mediator synthesis in RAW264.7 treated with LPS and ALA.

(A) The gene expression of lipid mediator synthesis related genes *cox2*, (B) *5-lox*, (C) *pgds* and (D) pro-inflammatory cytokine *tnf-α*, (E) *il-1β* , (C) *il-6*, (D) *mcp-1* relative to β-actin was quantified by qRT-PCR.

2) *In vivo* evaluation of flaxseed

(1) Fatty acid profile of laying hen when fed flaxseed

Supplementing flaxseed for 4 weeks improved omega-6 to omega-3 ratios in both egg (Figure 18A) and serum (Figure 18B) samples. In both samples, the omega-6 to omega-3 ratio decreased with increasing flaxseed concentration. The average ratios in eggs at week 4 were 9.81 (T1), 6.87 (T2) and 4.16 (T3) compared to 25.85 (C). Likewise, the average ratios in serum samples were 8.33 (T1), 5.66 (T2), and 4.08 (T3) compared to 19.23 (C). The content of each PUFA component was also similar between egg and serum samples (Figure 19). The detailed fatty acid composition of 4 groups were listed in Table 5.

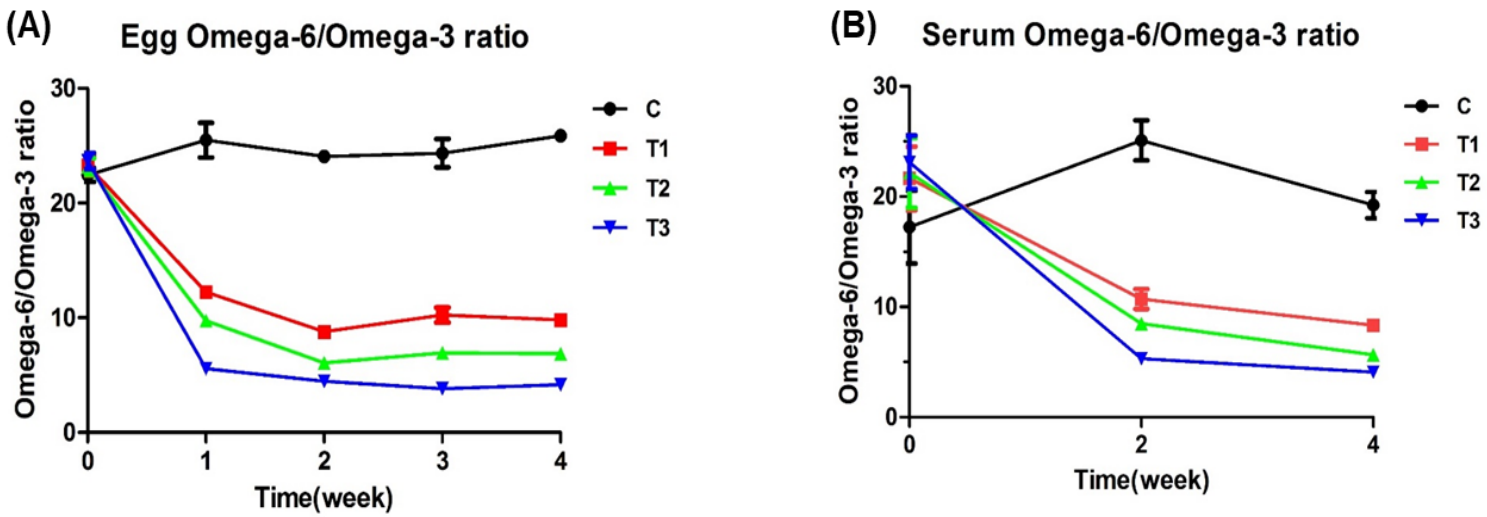

Figure 18. Changes in omega-6 to omega-3 fatty acid ratios during the experiment.

(A) The ratios in egg samples during weeks 0, 1, 2, 3, 4 and (B) the ratios in serum samples during weeks 0, 2, and 4.
The ratios were calculated by data of gas chromatography standardized with FAME 37 mix. Each group was 5 replicates.

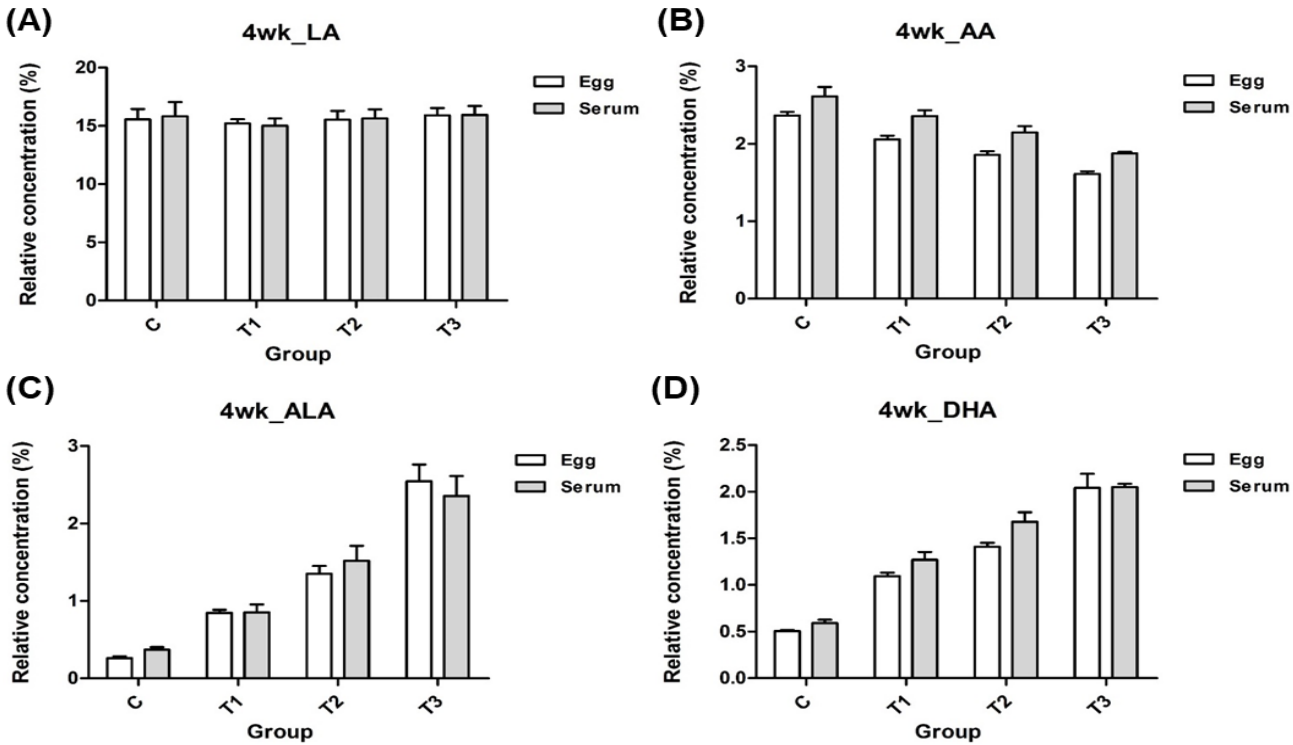

Figure 19. The relative concentrations of PUFAs in week 4 egg and serum samples.

(A) The relative concentrations of LA, (B) AA, (C) ALA, (D) DHA in week 4 egg and serum samples.

Table 5. Relative fatty acid concentration among groups on week 4 egg samples.

Fatty acid (%)	C	T1	T2	T3	*p-value*
Myristic acid (C14:0)	0.43±0.04^{b}	0.44±0.04^{b}	0.41±0.04^{ab}	0.36±0.02^{a}	0.010**
Myristoleic acid (C14:1)	0.10±0.01^{a}	0.12±0.02^{a}	0.10±0.02^{a}	0.09±0.01^{a}	0.116
Pentadecanoic acid (C15:0)	0.07±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.942
Palmitic acid (C16:0, PA)	27.47±0.47^{b}	27.04±0.79^{b}	26.36±0.93^{ab}	25.08±0.76^{a}	<0.001***
Palmitoleic acid (C16:1)	3.68±0.22^{a}	4.09±0.74^{a}	3.64±0.43^{a}	3.49±0.43^{a}	0.290
Margaric acid (C17:0)	0.17±0.02^{a}	0.16±0.01^{a}	0.17±0.02^{a}	0.18±0.02^{a}	0.698
Margaroleic acid (C17:1)	0.13±0.02^{a}	0.14±01.01^{a}	0.14±0.02^{a}	0.15±0.01^{a}	0.184
Stearic acid (C18:0, SA)	7.83±0.46^{a}	7.50±0.31^{a}	7.61±0.58^{a}	7.51±0.19^{a}	0.574
Elaidic acid (C18:1n9t)	0.21±0.01^{a}	0.2±0.02^{a}	0.14±0.08^{a}	0.18±0.00^{a}	0.134
Oleic acid (C18:1n9c, OA)	38.98±1.76^{a}	38.76±1.96^{a}	39.00±2.34^{a}	38.6±1.46^{a}	0.984
Linolelaidic acid (C18:2n6t)	1.39±0.06^{a}	1.45±0.13^{a}	1.38±0.07^{a}	1.31±0.14^{a}	0.304
Linoleic acid (C18:2n-6c, LA)	15.56±1.98^{a}	15.21±0.83^{a}	15.53±1.70^{a}	15.91±1.37^{a}	0.913
Arachidic acid (C20:0)	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.176
Gamma-linolenic acid (C18:3n6, GLA)	0.15±0.02^{b}	0.13±0.02^{ab}	0.12±0.02^{ab}	0.12±0.01^{a}	0.050*
Gondoic acid (C20:1)	0.20±0.02^{a}	0.19±0.01^{a}	0.20±0.02^{a}	0.21±0.03^{a}	0.743
α-linolenic acid (C18:3n-3, ALA)	0.26±0.05^{a}	0.85±0.09^{b}	1.35±0.23^{c}	2.55±0.48^{d}	<0.001***
Heneicosanoic acid (C21:0)	0.06±0.00^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.07±0.01^{a}	0.677
Eicosadienoic acid (C20:2)	0.15±0.02^{a}	0.16±0.01^{a}	0.16±0.03^{a}	0.16±0.02^{a}	0.809
Behenic acid (C22:0)	0.06±0.01^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	<0.001***
Dihomogamma-linolenic acid (C20:3n-6, DHGLA)	0.18±0.02^{a}	0.18±0.02^{a}	0.17±0.02^{a}	0.16±0.01^{a}	0.357
Erucic acid (C22:1n9)	0.02±0.00^{b}	0.02±0.00^{b}	0.02±0.00^{ab}	0.01±0.00^{a}	0.002**
Tricosanoic acid (C23:0)	0.00±0.00^{a}	0.03±0.00^{b}	0.03±0.00^{b}	0.06±0.01^{c}	<0.001***
Arachidonic acid (C20:4)	2.37±0.10^{d}	2.06±0.10^{c}	1.86±0.11^{b}	1.61±0.08^{a}	<0.001***
Docosadienoic acid (C22:2n6)	0.00±0.00^{a}	0.01±0.00^{b}	0.01±0.00^{b}	0.02±0.00^{c}	<0.001***
Eicosapentaenoic acid(C20:5n-3, EPA)	0.00±0.00^{a}	0.02±0.01^{b}	0.03±0.01^{c}	0.06±0.01^{d}	<0.001***
Docosahexaenoic acid(C22:6n-3, DHA)	0.50±0.03^{a}	1.09±0.08^{b}	1.41±0.09^{b}	2.04±0.34^{c}	<0.001***

[1] Each value represents the mean±SD of five replicate eggs.

[a-d] Means in a row analyzed using one-way ANOVA and post hoc TUKEY's test (**p-value*<0.05 ***p-value*<0.01 ****p-value*<0.001).

(2) Serum lipid mediator profile of laying hen when fed flaxseed

To investigate and compare lipid mediator profile among flaxseed-fed groups, week 4 serum samples were used for UPLC-MS/MS based multiple-reaction monitoring (MRM) method. Analytical condition optimization and performance validation of lipid mediator profile followed previously described methods (Lee *et al.*, 2016a). A total of 95 lipid mediators were identified in serum samples with 5 replicates for each group (Table 6). Statistical analyses were conducted after normalization by deuterated internal standards.

Table 6. Lipid mediator profile and its relative concentration per group.

Lipid_mediator	C	T1	T2	T3	*p-value*
LXA5	0.42±0.24[a]	0.26±0.1[a]	0.49±0.16[a]	0.36±0.09[a]	0.1540
LTB4 or 6t LTB4 or 12epi LTB4 or 6t,12epi LTB4	0.04±0.06[a]	0.05±0.04[a]	0.09±0.02[a]	0.04±0.02[a]	0.1452
20oh LTB4	0.75±0.43[a]	0.52±0.72[a]	0.63±0.45[a]	0.66±1.25[a]	0.9749
6R-LXA4 or 6S-LXA4	0.13±0.08[a]	0.14±0.07[a]	0.19±0.07[a]	0.16±0.04[a]	0.5581
14R-LXA4	0.15±0.06[a]	0.12±0.04[a]	0.17±0.13[a]	0.13±0.07[a]	0.8014
LXB4	0.04±0.03[a]	0.03±0.03[a]	0.04±0.03[a]	0.05±0.04[a]	0.7343
LTD4 or 11t LTD4 or EXD4	0.07±0.07[a]	0.06±0.04[a]	0.05±0.02[a]	0.07±0.05[a]	0.8572
Resolvin D3	1.14±1.04[a]	0.65±0.47[a]	1.01±0.92[a]	1.19±0.58[a]	0.7077
Resolvin D2	0.18±0.08[a]	0.17±0.07[a]	0.17±0.06[a]	0.18±0.05[a]	0.9649
Resolvin D1	0.03±0.02[a]	0.03±0.01[a]	0.03±0.01[a]	0.03±0.02[a]	0.8907

Resolvin E1	9.82±6.8[a]	7.63±5.36[a]	7.66±8.21[a]	4.93±5.14[a]	0.7049
6k-PGF1a	1580.6±1274.53[a]	3803.5±4221.66[a]	4063.19±5094.02[a]	3775.24±4837.33[a]	0.7622
TXB1	8.45±7.86[a]	4.95±6.93[a]	18.66±22.42[a]	6.14±9.08[a]	0.3693
TXB2	1098.34±1122.8[a]	757.89±1042.19[a]	1895.01±2266.57[a]	583.61±1208.85[a]	0.5348
PGF2a or 11-beta PGF2a or 8-iso PGF2a III	0.65±0.46[a]	0.52±0.31[a]	0.84±0.84[a]	0.97±0.88[a]	0.7281
PGF1a	1.7±2.35[a]	1.75±0.79[a]	2.59±2.11[a]	3.23±2.91[a]	0.6480
Delta-17 6k-PGF1a	4.1±3.37[a]	2.85±1.29[a]	4.39±2.69[a]	6.26±3.65[a]	0.3497
PGF3a	2.8±1.95[a]	5.34±2.34[a]	6.34±1.71[a]	11.85±4.76[b]	0.0014**
6,15 dk-,dh-PGF1a	6.14±7[a]	5.2±7.45[a]	9.79±10.96[a]	4.98±6.87[a]	0.7778
15k PGF2a	0.7±0.43[a]	1.18±0.61[a]	1.79±1.57[a]	1.66±1.39[a]	0.4191
dh PGF2a	1.21±0.39[a]	1.02±0.66[a]	0.86±0.49[a]	0.76±0.2[a]	0.4737
19oh PGF2a or 20oh PGF2a	1417.75±1909.85[a]	1960.31±2298.16[a]	1807.08±2306.57[a]	1789.84±2378.4[a]	0.9832

2,3 dinor 11beta PGF2a	6.77±5.17[a]	18.76±22.97[a]	3.87±3.45[a]	15.9±13.24[a]	0.2874
tetranor-PGFM	6.21±8.66[a]	1.35±0.97[a]	2.76±3.58[a]	7.07±6.54[a]	0.3649
PGE1 or PGD1	28.19±29.36[a]	21.3±15.09[a]	25.04±14.95[a]	84.41±93.02[a]	0.1885
PGE2 or 11beta PGE2 or PGD2	1150.81±1127[a]	846.97±617.51[a]	908.72±587.48[a]	2418.29±1943.66[a]	0.1739
PGE3 or PGD3	1561.95±581.19[a]	2210.06±830.9[a]	1558.75±527.67[a]	2404.61±1526.06[a]	0.3944
dihomo PGE2 or dihomo PGD2	36.16±48.43[a]	32.19±16.71[a]	52.66±41.48[a]	167.39±152.51[a]	0.0626
15k PGE2	0.76±0.56[a]	0.78±0.4[a]	1.92±1.42[a]	2.74±2.21[a]	0.0957
tetranor-PGEM	8.33±7.15[a]	8.74±3.8[a]	11.98±10.36[a]	23.93±18.3[a]	0.1402
PGK2	21.36±25.11[a]	13.12±5.14[a]	15.29±10.48[a]	22.22±12.43[a]	0.7301
dihomo PGJ2 or dihomo 15d PGJ2	2.38±2.56[a]	1.76±1.5[a]	3.44±3.99[a]	1.87±2.21[a]	0.7571
PGA2 or PGB2 or PGJ2 or 15d-PGD2	2.3±3.04[a]	1.66±0.9[a]	3.74±5.93[a]	7.36±10.71[a]	0.5044
15d-PGA2 or 15d-PGJ2	4.96±4.68[a]	2.09±1.07[a]	1.18±1.63[a]	2.88±3.13[a]	0.2607
dhk PGE2 or dhk PGD2	2.84±2.58[a]	2.48±4[a]	5.09±6.7[a]	7.37±11.18[a]	0.6657

5-iso PGF2a VI	1±0.97[a]	1.02±0.77[a]	1.41±0.86[a]	2.23±1.00[a]	0.1521
PD1 or 15t PD1 or 10S,17S-DiHDoHE	1.06±1.25[a]	0.75±0.66[a]	1.34±1.77[a]	1.7±1.25[a]	0.6901
12-oxoETE	1.17±0.73[a]	1.56±0.72[a]	0.94±0.36[a]	1.19±0.08[a]	0.3744
15-oxoETE	6.93±5.51[a]	7.14±9.03[a]	2.31±2.39[a]	2.2±0.96[a]	0.3126
9-oxoODE	0.8±0.85[a]	1.24±0.84[a]	0.32±0.16[a]	0.43±0.21[a]	0.1139
13-oxoODE	6.37±8.29[a]	6.22±3.56[a]	2.14±0.92[a]	2.32±0.74[a]	0.2995
15-oxoEDE	0.88±0.83[a]	1.17±1.34[a]	0.49±0.21[a]	0.39±0.15[a]	0.4047
5,6 EET	0.6±0.25[a]	0.8±0.66[a]	0.29±0.22[a]	0.56±0.26[a]	0.2767
HXB3	1.92±1.53[a]	2.88±4.94[a]	0.78±0.58[a]	1±1.46[a]	0.6072
14,15 EpETE	1.05±1.21[a]	0.82±0.66[a]	0.37±0.28[a]	0.44±0.20[a]	0.4095
16,17 EpDPE	1.12±0.9[a]	1.51±1.02[a]	0.75±0.29[a]	1.11±0.74[a]	0.5299
5,6 diHETE	0.06±0.05[a]	0.1±0.12[a]	0.14±0.16[a]	0.07±0.03[a]	0.6474
5,15 diHETE	0.04±0.02[a]	0.09±0.06[a]	0.08±0.09[a]	0.04±0.03[a]	0.3432

12-HHT	6.31±6.3[a]	4.94±6.13[a]	6.75±5.43[a]	0.81±1.31[a]	0.2927
11-HETE or 11,12 EET	52.43±63.41[a]	35.72±46.21[a]	36.96±28.68[a]	9.71±11.74[a]	0.4714
9-HETE	0.39±0.43[a]	0.28±0.36[a]	0.31±0.23[a]	0.17±0.25[a]	0.7601
9-HEPE	0.24±0.14[a]	1.01±0.6[b]	0.26±0.35[a]	0.35±0.25[ab]	0.0146*
8-HDoHE	0.04±0.02[a]	0.09±0.06[ab]	0.15±0.04[b]	0.13±0.07[ab]	0.0286*
5-HETE	0.49±0.14[a]	0.52±0.27[a]	0.48±0.1[a]	0.41±0.15[a]	0.8003
5-HEPE	0.13±0.09[a]	0.2±0.12[ab]	0.39±0.2[b]	0.23±0.07[ab]	0.0389
7-HDoHE	0.23±0.13[a]	0.44±0.3[a]	0.43±0.14[a]	0.9±0.32[b]	0.0032**
4-HDoHE	0.08±0.06[a]	0.17±0.1[ab]	0.2±0.07[ab]	0.26±0.05[b]	0.0088**
9-HOTrE	0.05±0.01[a]	0.15±0.07[ab]	0.24±0.08[b]	0.28±0.11[b]	0.0011**
5-HETrE	0.28±0.1[a]	0.29±0.17[a]	0.27±0.06[a]	0.29±0.15[a]	0.9956
15-HEPE	0.06±0.05[a]	0.03±0.02[a]	0.03±0.02[a]	0.08±0.12[a]	0.6012
8-HETE or 8,9 EET	0.17±0.06[a]	0.19±0.07[a]	0.46±0.24[a]	0.28±0.28[a]	0.0965

8-HEPE	0.55±0.32[a]	0.27±0.14[a]	0.8±0.51[a]	0.28±0.13[a]	0.0507
10-HDoHE	0.05±0.02[a]	0.05±0.03[a]	0.11±0.09[a]	0.06±0.08[a]	0.3375
8-HEtrE	0.29±0.12[a]	0.4±0.17[a]	0.48±0.25[a]	0.39±0.12[a]	0.4367
11-HDoHE	0.07±0.05[a]	0.16±0.09[a]	0.1±0.04[a]	0.08±0.05[a]	0.1491
5,6 DHET	0.1±0.04[a]	0.1±0.04[a]	0.18±0.1[a]	0.14±0.08[a]	0.3051
8,9 DHET	0.28±0.13[a]	0.37±0.25[a]	0.7±0.36[a]	0.54±0.41[a]	0.1759
11,12 DHET	0.14±0.07[a]	0.12±0.06[a]	0.29±0.14[a]	0.25±0.12[a]	0.0553
14,15 DHET	0.63±0.19[a]	0.81±0.37[a]	1.29±0.6[a]	0.92±0.59[a]	0.1910
tetranor 12-HETE	0.03±0.02[a]	0.03±0.03[a]	0.09±0.08[a]	0.05±0.03[a]	0.1469
11-HEPE	0.09±0.03[a]	0.11±0.11[a]	0.14±0.04[a]	0.09±0.04[a]	0.5258
13-HDoHE	0.05±0.06[a]	0.05±0.05[a]	0.12±0.08[a]	0.23±0.37[a]	0.4413
16-HDoHE	0.07±0.02[a]	0.16±0.06[ab]	0.42±0.29[b]	0.25±0.1[ab]	0.0179*
15-HETE or 14,15 EET	3.05±3.76[a]	2.35±3.03[a]	2.18±1.57[a]	0.75±0.63[a]	0.5577

17-HDoHE	0.14±0.09[a]	0.2±0.15[a]	0.32±0.17[a]	0.22±0.29[a]	0.5288
15-HETrE	1.22±1.48[a]	1.2±1.59[a]	1.04±0.77[a]	1.17±1.75[a]	0.9970
12-HETE	0.21±0.06[a]	0.16±0.03[a]	0.23±0.07[a]	0.12±0.06[a]	0.0490*
14-HDoHE	0.02±0.01[a]	0.05±0.03[ab]	0.13±0.1[b]	0.09±0.03[ab]	0.0255*
20-HETE	0.06±0.05[a]	0.07±0.04[a]	0.09±0.08[a]	0.06±0.05[a]	0.8128
19-HETE	0.1±0.08[a]	0.05±0.05[a]	0.08±0.07[a]	0.13±0.2[a]	0.7373
18-HETE	0.07±0.04[a]	0.07±0.03[a]	0.07±0.01[a]	0.06±0.03[a]	0.9161
17-HETE	0.02±0.01[a]	0.02±0.02[a]	0.04±0.03[a]	0.04±0.02[a]	0.2666
16-HETE	0.24±0.06[a]	0.41±0.28[a]	0.43±0.18[a]	0.41±0.05[a]	0.3064
17,18 EpETE	0.1±0.05[a]	0.2±0.11[ab]	0.23±0.07[ab]	0.25±0.08[b]	0.0477*
9-HODE or 9,10 EpOME	3.35±1.85[a]	3.78±2.2[a]	4.99±1.83[a]	2.65±0.92[a]	0.2404
13-HODE or 12,13 EpOME	6.26±2.75[a]	9.31±3.72[a]	8.9±2.24[a]	6.29±1.99[a]	0.1923
13-HOTrE	0.07±0.04[a]	0.2±0.08[ab]	0.27±0.17[b]	0.36±0.08[b]	0.0037**

13-HOTre-g	0.17±0.07[a]	0.31±0.2[a]	0.4±0.06[a]	0.3±0.14[a]	0.0824
19,20 DiHDPA	0.04±0.03[a]	0.11±0.08[a]	0.13±0.04[a]	0.14±0.12[a]	0.1967
9,10 diHOME	2.85±0.23[a]	3.35±1.87[a]	3.66±2.09[a]	3.09±1.27[a]	0.8565
12,13 diHOME	2.9±1.07[a]	3.38±1.82[a]	3.44±1.59[a]	2.58±1.09[a]	0.7488
ADA	94.74±109.99[a]	241.38±436.47[a]	17.6±18.05[a]	90.86±117.76[a]	0.5059
EPA	17.83±17.22[a]	66.94±42.36[a]	58.09±49.8[a]	388.4±308.04[b]	0.0064**
DHA	201.35±79.16[a]	308.7±93.29[a]	2351.6±1707.75[a]	5419.42±2695.39[b]	0.0003***
AA	1214.31±570.39[b]	328.89±194.05[a]	464.54±305.99[a]	536.34±263.57[a]	0.0064**

Data shown as the mean± standard deviation (SD). One-way ANOVA with Tukey's post hoc test was used (**p-value*< 0.05, ***p-value* < 0.01, ****p-value*< 0.001). Within a row, different superscript letters indicate significant difference (*p-value*<0.05).

As there were multiple factors in lipid mediator profile, partial least square discriminant analysis (PLS-DA) was performed to observe the dissimilarities of the lipid mediators profile among groups (Figure 20A). PLS-DA plot analysis revealed clustering among groups according to flaxseed concentration in the corresponding PLS-DA score plot including Component 1 (19.4%) and Component 2 (11.7%). Also, the lipid mediators that differentially regulated among groups were identified by the variable importance in projection (VIP) plot (Figure 21B). The individual lipid mediator species such as hydroxyoctadecatrienoic acid (HOTrE), DHA, prostaglandin F3 alpha (PGF3α), hydroxydocosahexaenoic acid (HDoHE), and epoxyeicosatetraenoic acid (EpETE) were mainly increased depending on the flaxseed concentration.

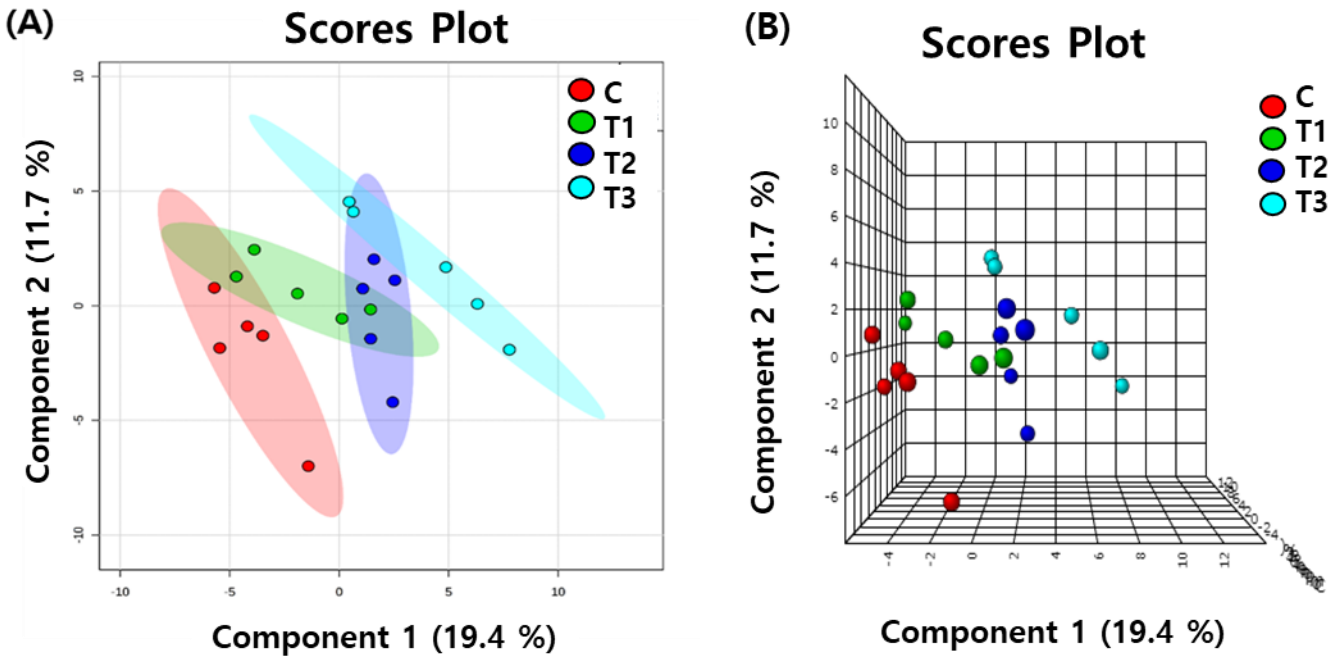

Figure 20. PLS-DA score plot based on laying hen serum lipid mediator concentration measured by UPLC-MS/MS.

(A) 2D scores plot (B) 3D scores plot.

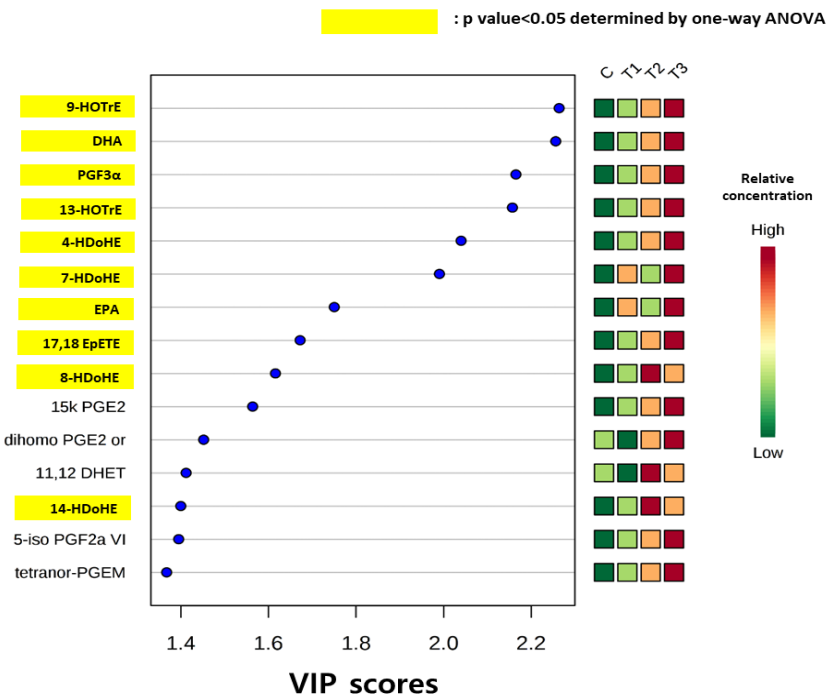

Figure 21. Variable importance in projection (VIP) plot of 15 lipid mediators (VIP scores Top 15) that were differently regulated among group based on laying hen serum lipid mediator concentration measured by UPLC-MS/MS.

Lipid mediators with yellow box indicate items with p value of 0.05 or less determined by one-way ANOVA with Tukey's post hoc test.

Next, one-way ANOVA and post-hoc TUKEY test of each PUFAs and lipid meidators were performed to identify differences among groups. In the case of PUFAs, arachidonic acid (AA), an omega-6 PUFA was downregulated in flaxseed-fed groups (T1, T2 and T3) compared to the C group (Figure 22A) while EPA and DHA, omega-3 PUFAs were upregulated in the T3 group (Figure 22B-C). Similar to PUFA, the AA-derived lipid mediator 12-hydroxyeicosatetraenoic acid (12-HETE) had a tendency to downregulate with flaxseed supplementation (*p-value*: 0.049) (Figure 23) whereas several lipid mediators derived from omega-3 fatty acids ALA (9-HOTrE, *p-value*: 0.0011; 13-HOTrE, *p-value*: 0.0037) (Figure 24), EPA (PGF-3α, *p-value*: 0.0014; 5-HEPE, *p-value*: 0.0389; 17,18-EpETE, *p-value*: 0.0477; 9-HEPE, *p-value*: 0.0146) (Figure 25) and DHA (4-HDoHE, *p-value*: 0.0088; 14-HDoHE, *p-value*: 0.0255; 7-HDoHE, *p-value*: 0.0032; 8-HDoHE, *p-value*: 0.0286; 16-HDoHE, *p-value*: 0.0179) (Figure 26) were clearly upregulated by dietary supplementation of flaxseed. To summarize, flaxseed alter not only PUFA but also lipid mediator profile in the way of upregulating omega-3 fatty acid and omega-3 derived lipid mediators in serum Figure 27.

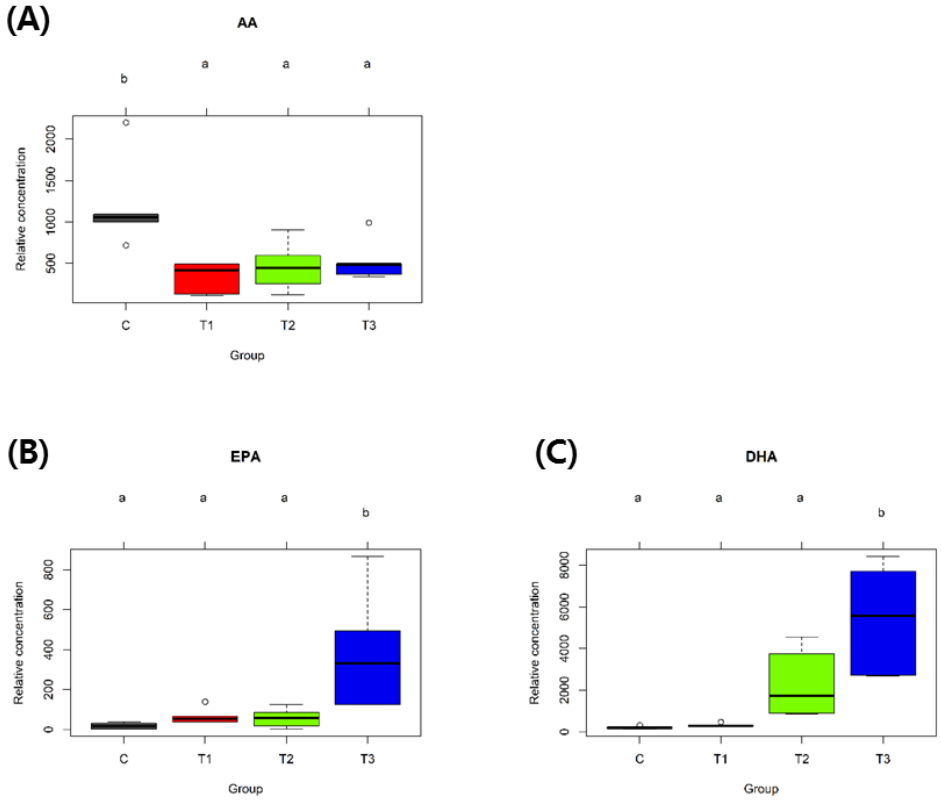

Figure 22. Relative concentration of PUFA in laying hen serum.

(A) Arachidonic acid, (B) Eicosapentaenoic acid, (C) Docosahexaenoic acid. Different superscript letters indicate statistical significance ($P < 0.05$).

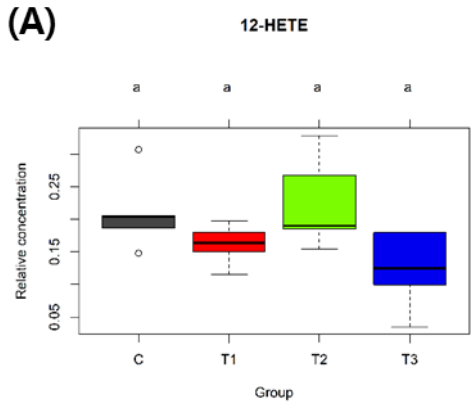

Figure 23. Relative concentration of AA-derived lipid mediator in laying hen serum.

(A) 12-HETE. Different superscript letters indicate statistical significance (P< 0.05).

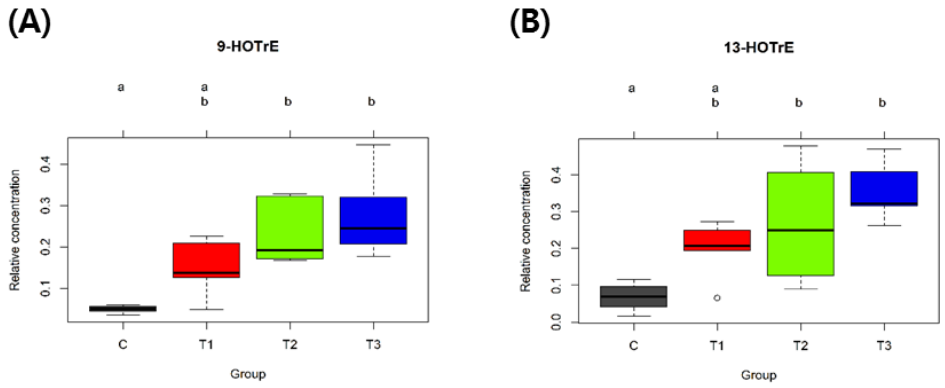

Figure 24. Relative concentration of ALA-derived lipid mediator in laying hen serum.

(A) 9-HOTrE, (B) 13-HOTrE. Different superscript letters indicate statistical significance (P< 0.05).

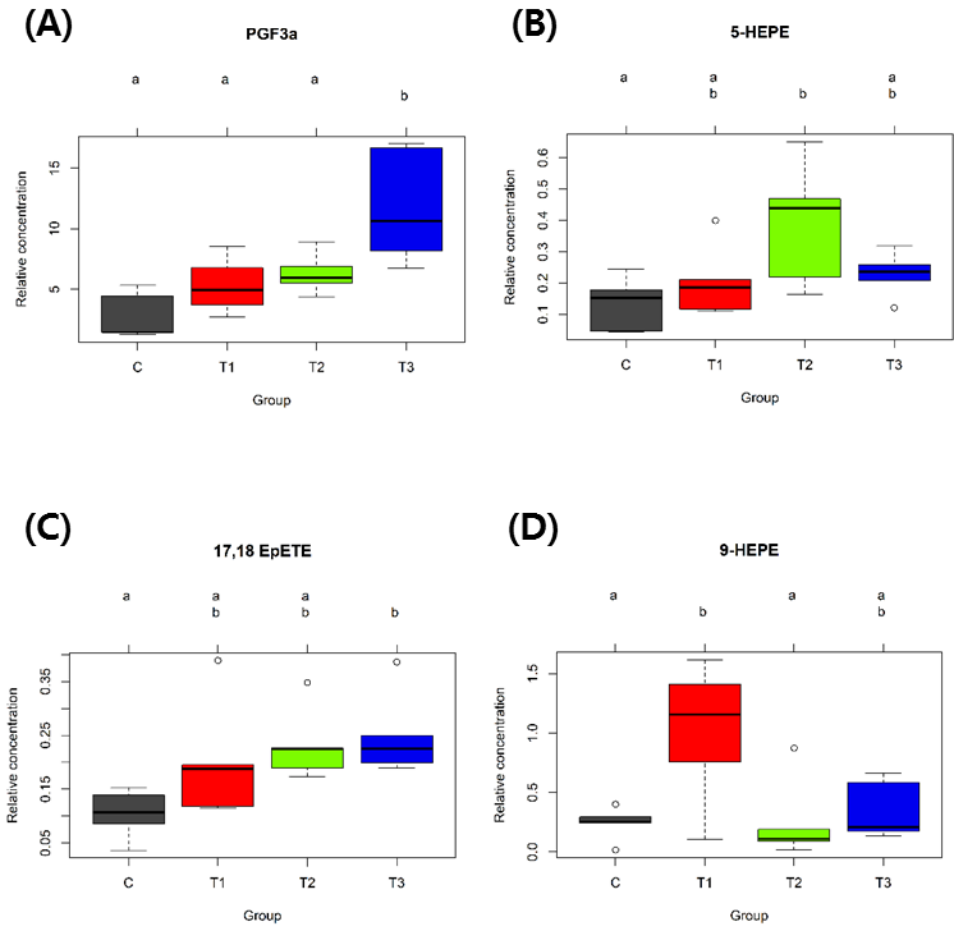

Figure 25. Relative concentration of EPA-derived lipid mediator in laying hen serum.

(A) PGF3α, (B) 5-HEPE, (C) 17,18-EpETE, (D) 9-HEPE. Different superscript letters indicate statistical significance ($P < 0.05$).

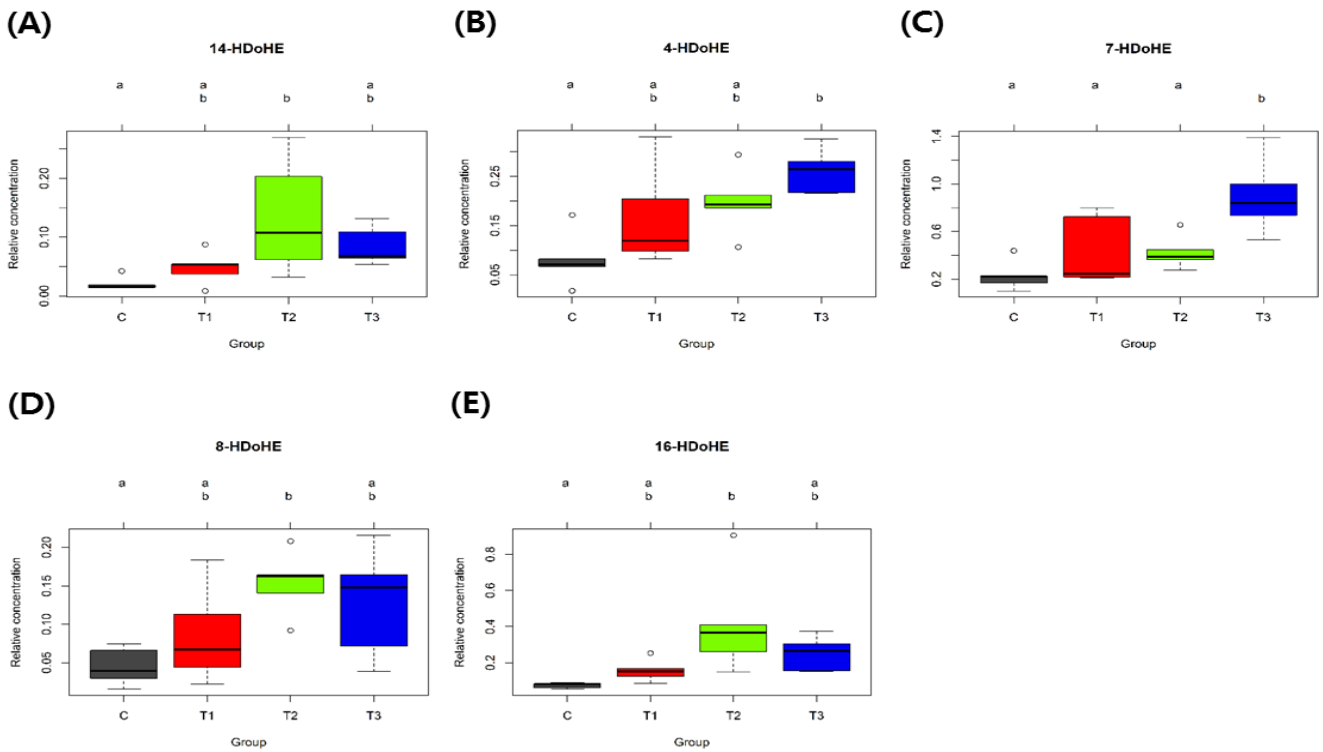

Figure 26. Relative concentration of DHA-derived lipid mediator in laying hen serum.

(A) 14-HDoHE, (B) 4-HDoHE, (C) 7-HDoHE, (D) 8-HDoHE, (E) 16-HDoHE. Different superscript letters indicate statistical significance (P< 0.05).

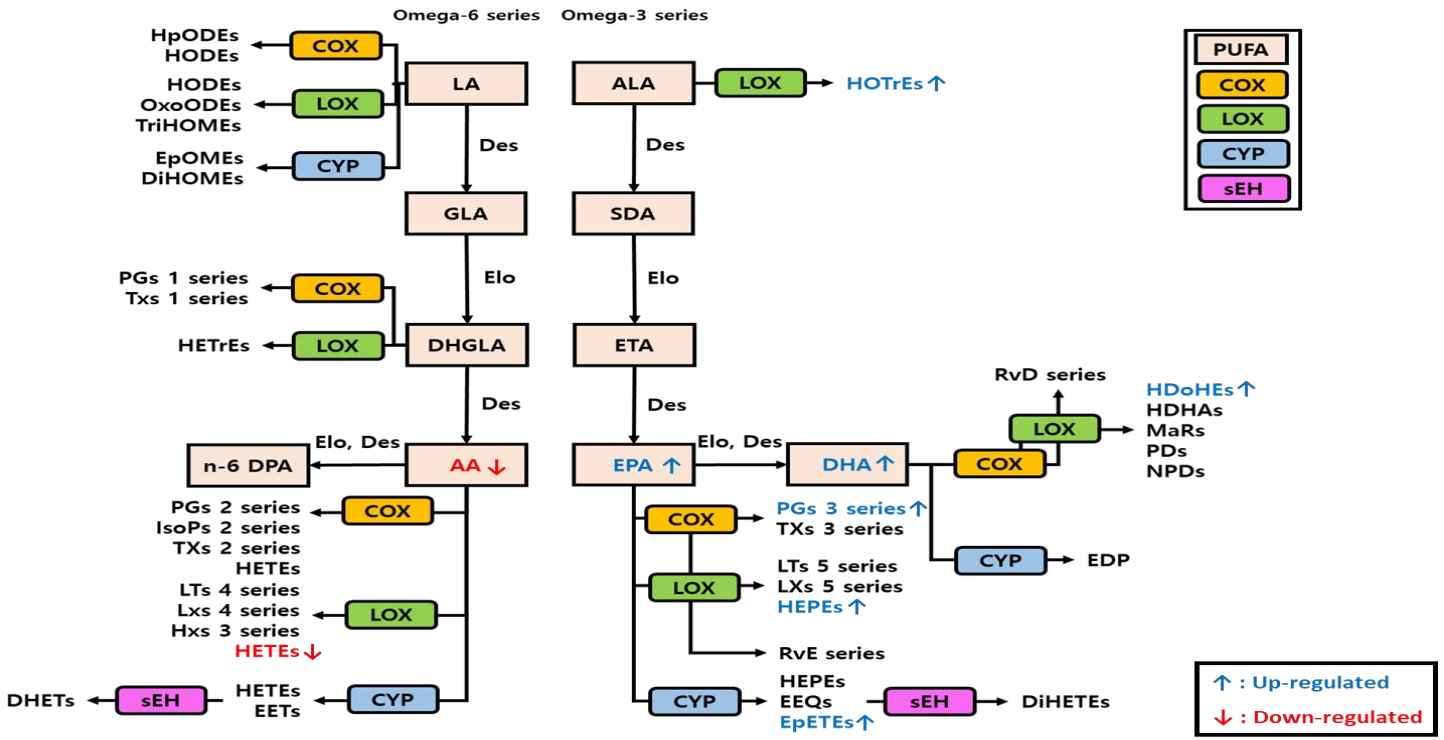

Figure 27. Overall changes in lipid mediators in laying hen serum when fed flaxseed.

Arrows with color indicates up-regulated lipid mediators (blue north arrow) and down-regulated lipid mediators (red south arrow).

(3) Serum pro-inflammatory cytokine level

Serum levels of pro-inflammatory cytokine were monitored to evaluate the effect of dietary flaxseed-induced changes in lipid mediators profile on inflammatory processes. No significant differences were observed in levels of TNF-alpha, IL-1beta, or IL-6 in serum samples, although levels of TNF-alpha (Figure 28A) and IL1beta (Figure 28B) tended to decrease in T2 and T3 groups and levels of IL-6 level tended to decrease in the T3 group (Figure 28C).

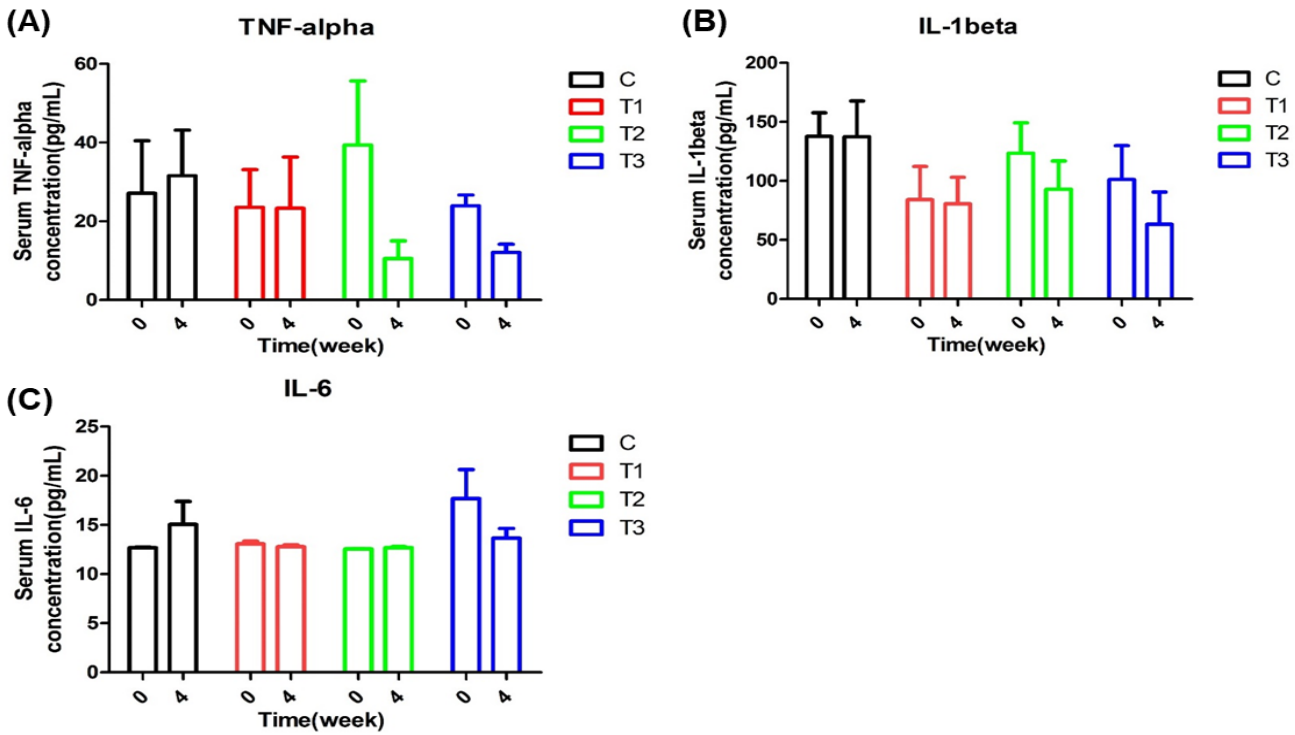

Figure 28 Pro-inflammatory cytokine levels in 0, 4 week laying hen serum.

(A) TNF-alpha (B) IL-1beta (C) IL-6.

(4) Stress indices

To investigate the flaxseed effect on stress state, two stress indices, serum corticosterone level and H:L ratio, were examined. During week 4, the T3 group showed a significant reduction in corticosterone level compared to week 0 (*p-value* = 0.0463) (Figure 29). Similarly, the T3 group also showed significantly decreased H:L ratios compared to week 0 (*p-value*: 0.0205) (Figure 30). The results clearly showed decreased stress indices with at least 3.6% (w/w) flaxseed supplementation.

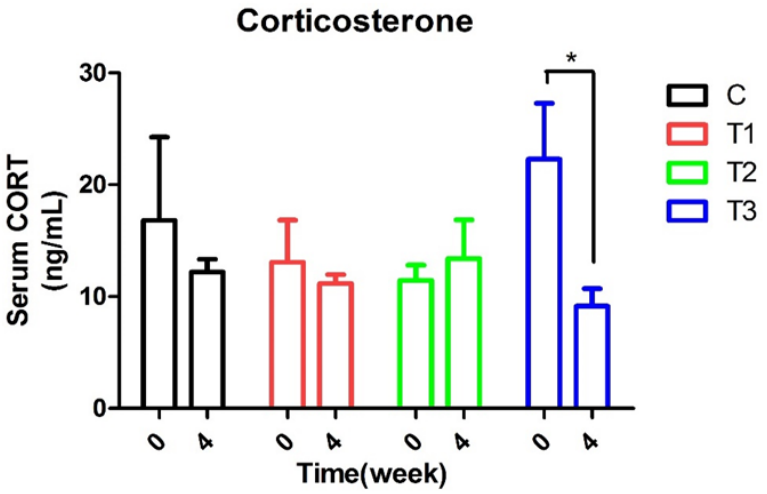

Figure 29 Serum corticosterone concentration level in 0, 4 week laying hen serum.

The levels of corticosterone in serum samples were measured with 5 replicates per group.

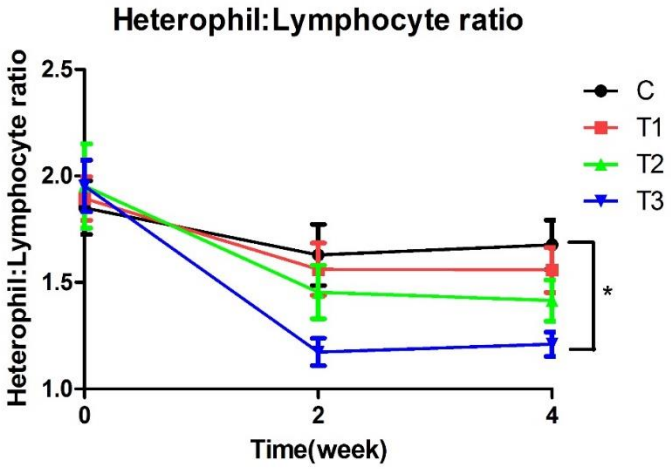

Figure 30. The ratios of heterophil : lymphocyte in 0, 2, 4 week laying hen serum.

The ratios of heterophil to lymphocyte ratio were calculated using data of complete blood cell count of 5 replicates per group.

(5) Laying performance and egg quality when fed flaxseed

By flaxseed supplementation, all treatment groups (T1, T2 and T3) significantly improved in hen-day egg production and egg mass production especially since the second week (week 2, week 3, week 4) compared to the C group (hen-day egg production *p-value*s: 0.003, 0.029 and <0.001; egg mass production *p-value*s: 0.001, 0.021 and <0.001, respectively). Hen-day egg production was the highest in the T1 group during the second week but was highest in the T3 group during the third and fourth weeks. Egg mass production was greatest in the T3 group during the fourth week. Additionally, average egg weight was elevated in all treatment groups during the overall period. Overall, dietary supplementation of flaxseed appeared showing positive effects on improvement of the performance with the laying hens (Table 7).

Meanwhile, the indices of egg quality such as albumen height, Haugh unit and eggshell thickness showed no predominant improvement by flaxseed supplementation for 4 weeks except significant differences in week 2 values of albumen height (*p-value*: 0.026) and Haugh unit (*p-value*: 0.027) between T3 group and C group (Table 8).

Table 7. Effects of flaxseed in diet on performance of laying hens during 33-37 weeks of age[1].

Items	Group				*p-value*
	C	T1	T2	T3	
Week 1					
Daily feed intake (g day^{-1} hen^{-1})	115.59±2.29^{a}	118.61±1.10^{a}	117.11±2.09^{a}	117.07±1.18^{a}	0.684
Average egg weight (g/egg)	55.59±0.82^{a}	57.10±0.19^{ab}	58.21±0.34^{b}	57.37±0.18^{ab}	0.005**
Feed conversion ratio (g feed/g egg mass)	2.08±0.03^{a}	2.08±0.01^{a}	2.01±0.01^{a}	2.04±0.01^{a}	0.034*
Hen-day egg production (%)	61.42±6.32^{a}	75.43±7.77^{a}	72.29±7.10^{a}	69.71±6.67^{a}	0.540
Week 2					
Daily feed intake (g day^{-1} hen^{-1})	119.53±0.44^{a}	119.97±0.01^{a}	119.94±0.03^{a}	119.95±0.01^{a}	0.434
Average egg weight (g/egg)	57.67±0.40^{a}	58.95±0.35^{bc}	59.64±0.15^{b}	58.10±0.15^{ac}	<0.001***
Feed conversion ratio (g feed/g egg mass)	2.07±0.01^{a}	2.04±0.01^{ac}	2.01±0.01^{bc}	2.06±0.01^{a}	<0.001***
Hen-day egg production (%)	81.14±1.30^{a}	90.57±0.72^{b}	86.86±1.84^{a}	84.57±2.21^{a}	0.003**
Week 3					
Daily feed intake (g day^{-1} hen^{-1})	119.96±0.02^{a}	119.95±0.03^{a}	119.99±0.01^{a}	119.94±0.02^{a}	0.390
Average egg weight (g/egg)	58.01±1.49^{a}	59.85±0.09^{ab}	61.19±0.11^{b}	59.67±0.18^{ab}	0.050
Feed conversion ratio (g feed/g egg mass)	2.08±0.06^{a}	2.00±0.00^{a}	1.96±0.00^{a}	2.01±0.01^{a}	0.090
Hen-day egg production (%)	91.43±1.56^{a}	93.14±0.59^{a}	95.71±1.41^{a}	96.57±1.21^{b}	0.029*
Week 4					
Daily feed intake (g day^{-1} hen^{-1})	119.89±0.07^{a}	119.71±0.24^{a}	119.96±0.02^{a}	119.94±0.02^{a}	0.520
Average egg weight (g/egg)	58.83±1.08^{a}	60.52±0.20^{ab}	61.73±0.25^{b}	60.17±0.16^{ab}	0.014*
Feed conversion ratio (g feed/g egg mass)	2.04±0.04^{a}	1.98±0.01^{ab}	1.94±0.01^{b}	1.99±0.01^{ab}	0.026*
Hen-day egg production (%)	95.43±0.57^{a}	92.57±0.84^{b}	96.00±0.62^{a}	99.43±0.37^{c}	<0.001***
Week 0 to 4					
Daily feed intake (g day^{-1} hen^{-1})	118.74±0.63^{a}	119.56±0.28^{a}	119.25±0.54^{a}	119.22±0.35^{a}	0.673
Average egg weight (g/egg)	57.52±0.54^{a}	59.10±0.27^{bc}	60.20±0.29^{b}	58.83±0.23^{c}	<0.001***
Feed conversion ratio (g feed/g egg mass)	2.07±0.02^{a}	2.02±0.01^{bc}	1.98±0.01^{b}	2.03±0.01^{ac}	<0.001***
Hen-day egg production (%)	82.36±2.98^{a}	87.93±2.32^{a}	87.71±2.56^{a}	87.57±2.81^{a}	0.391

[1] Each value represents the mean±SD of ten replicate cages (five birds per cage).

[a-c] Means in a row without a common superscript letter differ as analyzed by one-way ANOVA and the TUKEY test.(*p-value<0.05, ** p-value<0.01, ***p-value<0.001).

Table 8. Effects of flaxseed in diet on egg quality of laying hens during 33-37 weeks of age[1].

Items	Time, week	Group				*p-value*
		C	T1	T2	T3	
Albumen height, mm	**0**	11.57±0.72	12.38±0.66	12.36±1.34	11.98±1.40	0.309
	1	10.53±1.27	10.47±1.34	11.37±1.26	11.24±1.68	0.356
	2	9.46±1.01[a]	9.67±0.75[ab]	10.10±1.11[ab]	10.74±0.92[b]	0.026*
	3	10.24±0.82	10.09±1.13	10.67±1.30	10.72±1.16	0.509
	4	10.71±1.10	10.14±1.34	10.25±1.36	10.83±1.25	0.549
Haugh unit	**0**	106.66±2.89	109.01±1.98	109.00±5.10	107.85±5.12	0.513
	1	101.83±6.05	101.61±5.97	105.27±5.23	104.84±6.36	0.374
	2	96.17±4.94[a]	97.44±3.48[ab]	99.23±4.55[ab]	101.92±3.92[b]	0.027*
	3	100.06±3.91	99.39±5.43	101.79±5.57	101.95±4.64	0.579
	4	101.85±4.84	99.26±5.58	99.52±5.74	102.12±5.02	0.499
Egg shell thickness, mm	**0**	0.40±0.02	0.40±0.03	0.40±0.03	0.39±0.02	0.918
	1	0.40±0.02	0.42±0.03	0.40±0.02	0.40±0.02	0.172
	2	0.41±0.03	0.40±0.02	0.40±0.01	0.41±0.01	0.698
	3	0.40±0.02	0.41±0.04	0.40±0.02	0.41±0.02	0.610
	4	0.40±0.03	0.40±0.02	0.40±0.02	0.41±0.01	0.595

[1]Each value represents the mean±SD of ten replicates with fifty hen per replicate.

[a,b] Values within the same row with different superscript letters significantly differ($p<0.05$).

4. Discussion

In study 1, omega-3 fatty acid, especially ALA was evaluated as feed additive candidate to affect host health. In study 1-1 *in vitro* study, ALA was treated to LPS-induced RAW264.7 to investigate effect on gene expression regarding lipid mediator synthesis and pro-inflammatory cytokines. By that, it was suggested that ALA might have anti-inflammatory effects owing to inhibiting lipid mediator synthesis and pro-inflammatory cytokines, which is similar to several previous other studies. For example, Erdinest *et al.* reported that the treating ALA to human corneal epithelial cells downregulated expression of various pro-inflammatory cytokines mainly mediated by reduction of I-κBα (Erdinest *et al.*, 2012) and similar results were observed in human (Zhao *et al.*, 2004). In contrast, several studies have suggested that ALA showed no significant anti-inflammatory effects (Nordstrom *et al.*, 1995;Geleijnse *et al.*, 2010). Although the anti-inflammatory effect of ALA is controversial as of now, other omega-3 fatty acids EPA and DHA have been reported to be more clearly anti-inflammatory (Gorjao *et al.*, 2009;Swanson *et al.*, 2012). In case of laying hen, their conversion efficiency of ALA to DHA is higher than other livestock or human, so that ALA can be effective feed additive to control animal inflammatory state.

In study 1-2, to determine effect of ALA on laying hen, *in vivo* feed experiment was performed with flaxseed supplemented to basal diet to reinforce ALA contents in the diet so that rebalancing the ratio of omega-6 to omega-3. As a

result, it clearly downregulated the ratio of eggs and sera. In case of egg, similar results that enriching ALA and DHA content in egg yolk were reported by several previous researchers (Petit, 2002;Bean and Leeson, 2003;Anjum *et al.*, 2013;Lee *et al.*, 2016b). For example, Lee *et al*., reported that containing 0.5% (w/w), 1% (w/w) of flaxseed oil in experimental diets enriched omega-3 fatty acid contents so that the omega-6 to omega-3 ratio reached approximately 3.81 and 2.53 respectively. These results were consistent with the results of this study. Actually, this biological phenomenon has been studied since 1990s, so this is applied to produce omega-3 fatty acid bio-fortified eggs.

However, the study of the omega-3 fatty acid supplementation to livestock animals is still limited so far even though it started from 1990s. A large amount of research have been only focused on producing omega-3 fatty acid bio-fortified livestock products. In recent years, consumers have considered not only their health but also livestock welfare when choosing livestock products. Therefore, it is need to observe and evaluate the physiological changes of livestock animals during housing period. In case of feed additive administration, it can be critical to guarantee the safety and efficacy of feed additive.

As PUFAs have biological roles by itself or through biotransformation to lipid mediators controlling inflammation, changing their content in the diet can affect host physiology. Thus, contrary to previous studies, this study focused on physiological changes of omega-3 fatty acid supplementation particularly focused

on profiles of serum PUFA, lipid mediators, serum levels of pro-inflammatory cytokines, serum corticosterone, and H:L ratio. First, the ratio of omega-6 to omega-3 in serum was confirmed its clear decrement just like the ratio of egg. Moreover, about 95 lipid mediators were monitored by UPLC-MS/MS. Interestingly, it was confirmed that 11 lipid mediators which significant upregulating were originated from the omega-3 fatty acids while only 1 lipid mediator was originated from the arachidonic acid, an omega-6 fatty acid, which showed downregulation by the dietary flaxseed supplementation. That is to say, omega-3-derived lipid mediators were mainly increased. It has been reported that omega-3-derived lipid mediators mainly have anti-inflammatory effects. For example, ALA-derived lipid mediators, 9-HOTrE and 13-HOTrE which were upregulated by flaxseed supplementation in this study, have been known to exert anti-inflammatory effects by inactivating nod-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome (Yang *et al.*, 2017;Zahradka *et al.*, 2017). Moreover, all EPA-derived lipid mediators (PGF3α, 5-HEPE, 9-HEPE and 17, 18-EpETE) and DHA-derived lipid mediators (14-HDoHE, 4-HDoHE, 7-HDoHE, 8-HDoHE and 16-HDoHE) have been reported to anti-inflammatory lipid metabolites through countering pro-inflammation signals in other animals such as mouse, rat and monkey (Yang *et al.*, 2014;Devassy *et al.*, 2016;Onodera *et al.*, 2017;Wang *et al.*, 2017;Nagatake *et al.*, 2018). Also, 5-HEPE was recognized as a potent inducer of regulatory T cells which play a pivotal role in regulating excessive immune responses (Onodera *et al.*, 2017). On the other hand, 12-HETE,

an AA-derived lipid mediator downregulated by flaxseed supplementation in our study has been reported for its pro-inflammatory action (Zhang *et al.*, 2018).

These changes in the ratio and profile may be due to changes in the ratio of intaken PUFA. These findings are consistent with the hypothesis that dietary PUFA can affect host physiology especially serum PUFA and lipid mediator profiles. As PUFA and its derivatives can contribute to inflammation, their effects on host inflammatory state were monitored with serum pro-inflammatory cytokines. Furthermore, stress has crosstalk with inflammation so that host stress state indices such as H:L ratio and serum corticosterone level were also checked. As a result, there was significant difference in stress indices contrary to inflammatory indices. It was able to assume that stress indices would be improved through alteration of lipid mediator profile. However, more studies are needed to clarify the relationship between change in host lipid mediator profile and host inflammatory state.

Also, laying performance was improved by dietary flaxseed. It was assumed that the increased levels of omega-3 derived anti-inflammatory lipid mediators and reduced levels of stress indices, the groups of flaxseed-fed hens showed improvement in overall laying performance. Clearly, there was limitation regarding gross difference in metabolizable energy or crude protein among experimental diets. As Lintex170 contain high level of metabolizable energy due to high content of crude fat, the higher flaxseed addition amount makes the diet

higher in metabolizable energy. These differences may affect laying performance. However, some previous study were suggested that there was no effect in laying performance after extra calorie supplementation by adding lipid resources (Atteh and Leeson, 1985;Grobas *et al.*, 1999;Grobas *et al.*, 2001). Therefore, this study implied that the flaxseed supplementation to the laying hens could alleviate inflammation and stress states by modulating the lipid mediators profile in a way of upregulating anti-inflammatory lipid mediators originated from omega-3 fatty acids, which also improved overall laying performance even though more studies with iso-calorie and iso-nutrient diet should be performed.

In the study 1, the physiological effects of ALA and flaxseed were analyzed in laying hen, and several changes such as lipid mediator profile alteration, changes in stress indices and improved laying performance were confirmed (Table 9). Although, there are many issues to clarify such as the chronic inflammation issue in livestock and the relationship between lipid mediator profile and stress state, these results may suggest the insights into understanding the relationship between dietary PUFA and host physiology. Also, this study suggested the possibility that feed additive can be developed to control livestock inflammation and stress state. In particular, extruded form of flaxseed can be a good candidate to control them at reasonable costs. Compared to flaxseed oil, whole flaxseed or extruded flaxseed are relatively inexpensive because they do not undergo extraction and purification processes (Oomah, 2001). Therefore, they have an advantageous aspect to use as feed additive where price competitiveness is important. However, whole flaxseed

has some limitation to be used as feed additive because it contains anti-nutritional factors such as mucilage and toxigenic compounds such as cyanogenic glycosides. Meanwhile, their contents in flaxseed can be downregulating by extrusion (Wu et al., 2008). Taken together, extruded flaxseed is likely to be reasonable materials to be used as feed additive enriching omega-3 fatty acid and controlling host inflammatory homeostasis.

Table 9. Summary of study 1.

Contents	Results
***In vitro* ALA treatment**	Pro-inflammatory cytokine mRNA level ↓ Lipid mediator synthesis mRNA level ↓
***In vivo* laying hen feed study (flaxseed)**	Omega-6/omega-3 ratio ↓ (egg, serum) Omega-3 derived lipid mediator ↑ Pro-inflammatory cytokine ↓ Stress indices ↓ (corticosterone, H/L ratio) Laying performance ↑ (Average egg weight, feed conversion ratio)

Study 2. Physiological effects of flaxseed and probiotics in laying hen

1. Introduction

Gut microbiota is a complex of microorganisms that lives in host gastrointestinal tracts (Tremaroli and Backhed, 2012). Recently, its interaction with host and effects on host health has been elucidated which is called symbiosis (Aziz *et al.*, 2013). Moreover, as high-throughput sequencing technology such as NGS has been developed, its symbiosis are revealed as mutualism, which means the relationship benefits each other. Actually, several researchers reported that damaged gut microbiota (dysbiosis) provokes various diseases such as obesity, cardiovascular disease and cancer (Scanlan *et al.*, 2008;Jose and Raj, 2015;Rezasoltani *et al.*, 2017). Thus, many researches have attracted to gut microbiota as novel target for maintaining or improving host health. To modulate gut microbiota in a good way, probiotics are widely used to both human and livestock.

Probiotics are living microorganisms providing health benefits when consumed, generally by improving or restoring the gut microbiota (Sanders, 2008). They are regarded as nutraceutical by their various benefits such as suppressing pathogen, reinforcing gut barrier, modulating immune system and fermenting secondary

metabolites. Therefore, many researchers are studied its possibility to develop as novel drugs or novel feed additives. Although various probiotics are used to analyze its effect on livestock performance (Jung *et al.*, 2008;Cao *et al.*, 2013;Forte *et al.*, 2016;Zhang *et al.*, 2016), however, only a limited number of studies are investigated its physiological effects. As each probiotic strain has distinct genetic information even in same species, its effects on gut microbiota and host physiology may differ. Also, identification of genes regarding performance or host health is essential to select appropriate probiotic candidate to use feed additive. In this regard, understanding the physiological effects of feeding probiotics is important work in livestock industry.

The aim of the study 2 was to investigate the physiological effects of flaxseed and probiotics on laying hen under a commercial environment. To accomplish this goal, *Lactobacillus plantarum* 177 (Lp) and *Bacillus licheniformis* T2 (T2) were screened among 3 lactobacilli species and 2 baclli, which were fed to 52-week-old laying hen with flaxseed (Table 10). First, the changes in the PUFA profile and c9,t11-CLA content in egg was analyzed. To monitor host inflammatory state, pro-inflammatory cytokine levels were measured using sera samples and gene expression of inflammatory cytokines and lipid mediator synthesis were analyzed with liver sample. Also, serum corticosterone, liver HSP70 gene expression and H:L ratio were investigated to detect host stress state. Moreover, the laying hen ceca microbiota were compared among groups. First, microbial communities were

analyzed and compared among groups. Next, predicted metagenome were assessed by their treatment.

Table 10. Summarization of the study 2.

Study	Category	Item
<Study 2> *In vivo* validation of flaxseed and probiotics as feed additives	Experimental design	Animal (laying hen)
		Sampling (egg, blood, liver, cecum)
		Feed information
		Probiotics production for feed experiment
	Effects on PUFAs	Egg omega-6/omega-3 ratio
		Multi-variant analysis
		Relative concentrations of fatty acid profile
		Egg c9,t11-CLA amount
	Inflammatory and stress indices	Inflammatory indices (serum cytokine, liver mRNA)
		Stress indices (corticosterone, H/L ratio, HSP70 mRNA)
	Gut microbiota	Composition of OTUs (Richness, Diversity, Relative abundance)
		Metagenome prediction
	Laying performance	Performance
		Egg quality

2. Materials and Methods

1) Probiotics screening

To select probiotic fed laying hen, 3 lactobacilli (*Lactobacillus salivarius* KLW001, **Ls**; *Lactobacillus reuteri* KLR3004, **Lr**; *Lactobacillus plantarum* WT 177, **Lp**) and 4 bacilli (*Bacillus polyxyma* T1, **T1**; *Bacillus licheniformis* T2, **T2**; *Bacillus thurigiensis* T3, **T3**; *Bacillus subtilis* T4, **T4**) were listed as candidates, which were previously isolated by lab (Table 11). In case of bacilli, only 2 species (T2, T4) were considered to GRAS so that analyzed. There were two criteria for screening: production yield and target gene identification.

Table 11. Probiotics used in this study.

Strain	Isolation host
Lactobacillus salivarius KLW001	Pig
Lactobacillus reuteri KLR3004	Pig
Lactobacillus plantarum WT 177	Pig
Bacillus polyxyma T1	Pig
Bacillus licheniformis T2	Pig
Bacillus thurigiensis T3	Pig
Bacillus subtilis T4	Pig (Isolated from commercial product)

Production yield was confirmed by colony forming units (CFU) counting. Briefly, each probiotic stock were thawed and seed-cultured in broth incubated for 12 hr. Then, its bacterial culture were inoculated in fresh broth and incubated for 24 hr. 1ml bacterial culture were 10-fold-serial-diluted ($10^0 \sim 10^{-7}$) and dropped into the agar plate. After incubating at 37°C, colonies were counted and compared among candidates.

In lactobacilli study, target genes were CLA-related genes (CLA enone reductase, ***cla-er***; CLA isomerase, ***cla-dc***; CLA dehydrogenase, ***cla-dh***; CLA hydratase***, cla-hy***), which convert LA into CLA. Bacilli were studied about fatty acid desaturase genes (delta-12 desaturase, ***des***; delta-5 desaturase, ***fad6***; delta-6 desaturase, ***d6d***), which desaturase PUFA. The detection of genes were confirmed by PCR with genomic DNA samples and its expression were confirmed by qRT-

PCR with mRNA samples after treating PUFA for 2 hr. The genomic DNA from probiotics were extracted according to Reyes-Escogido *et al.* (Reyes-Escogido *et al.*, 2010) with some modifications. Briefly, bacteria were inoculated in incubating broth (Lactobacilli: MRS, bacilli: TSB) and incubated at 37°C for 12 h. About 500 µl of bacterial culture were transferred to a new 1.5 ml tube followed by centrifugation at 8,000 g for 1 min. After removing supernatant, cell pellets were washed with 1 ml TE buffer (10 mM Tris base, 1 mM EDTA, pH 8.0) and centrifuged again. Then, the cells were resuspended in 200 µl TE buffer followed by step of microwaving at 625 W for 1 min, cooling at RT for 30 sec and re-microwaving for 1 min. The lysates were vortexed and centrifuged, and 100 µl supernatant was transferred to a new tube. Then, the genomic DNA was measured its concentration by Nanodrop 2000 and used to perform PCR. The PCR mixture consisted of 50ng of the genomic DNA was added to 20 µl of PCR master mix containing 10 µl of i-Taq 2x PCR master mix solution (Intron Biotechnology, Korea). PCR cycle was as follows: 95°C for 3min; 40cycles of 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec; 72°C for 5 min. The primers used to PCR were listed in Table 12 (CLA-related genes of Lp) and Table 13 (Fatty acid desaturase genes of T2), respectively. In case of primers for CLA-related genes, they were designed by primer BLAST with reference genes (***cla-er***, GenBank accession no. NC_004567, region: 61378-62031; ***cla-dc***, GenBank accession no. NC_004567, region: 60505-61350; ***cla-dh***, GenBank accession no. NC_004567, region: 59613-60473; ***cla-hy***, GenBank accession no. NC_004567, region: 122436-124130)

encoded in *Lactobacillus plantarum* WCFS1 reference genome. The primers for fatty acid desaturase genes were designed by primer BLAST with reference genes (***des***, GenBank accession no. NZ_CP023729, region: 2694250-2695308; ***fad6***, GenBank accession no. NZ_CP023729, region: 571832-572860 encoded in *Bacillus licheniformis* strain ATCC 9789; ***d6d***, GenBank accession no. AP012495, region: 6127406-6128485 encoded in *Bacillus subtilis* BEST7613) encoded in bacilli genomes.

The mRNA samples were extracted after treating 2 μM PUFA using the TRIzol® Max™ Bacterial RNA Isolation Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. After extracting mRNA samples, cDNA synthesis and qRT-PCR were performed as described in study 1-1 normalized by *rpsE*. The primers used in qRT-PCR were same with those used in PCR.

Table 12. The primer sequences for quantitative real-time PCR of *Lactobacillus plantarum* WT177 CLA-related genes.

Target gene	Sequence (5'→3')	Length (mer)	Tm (℃)	%GC	Product size
rpsE	F : GCGTCGTATTGACCCAAGC	19	57.1	58	(Reference) Wecke *et al.*, 2011
	R : TACCAGTACCGAATCCTACG	20	55.4	50	
cla-er	F : GCGCCATCGGCATGTAATTT	20	59.97	50	149
	R : TGGGTGTCACCGGCAATAAA	20	59.89	50	
cla-dc	F : GATGGGCGCTTACCCGATTA	20	59.97	55	235
	R : CTGCCCCGCAAATTGTTTCA	20	59.97	50	
cla-dh	F : CGGATCGGGGTATGAAGCTC	20	60.04	60	225
	R : ATTCCCAAATTCGGCCAGGT	20	59.96	50	
cla-hy	F : TTGGTGCCGGGCTATCAAAT	20	60.03	50	291
	R : TATCTTCAGCCGCCGTCATC	20	59.97	55	

Table 13. The primer sequences for quantitative real-time PCR of *Bacillus licheniformis* T2 fatty acid desaturase genes.

Target gene	Sequence (5'→3')	Length (mer)	Tm (℃)	%GC	Product size
rpsE	F : GCGTCGTATTGACCCAAGC	19	57.1	58	(Reference) Wecke *et al.*, 2011
	R : TACCAGTACCGAATCCTACG	20	55.4	50	
des	F : GATCGGCGTTTGGCTGTTTT	20	60.04	50	220
	R : GTGCTGTTCATGTGCGGTTT	20	59.97	50	
fad6	F : CGTAAAGGGGCAAAACGCAA	20	55.4	50	102
	R : GTTTTGCCAGCCAATAGCCC	20	57.4	55	
d6d	F : CTGGTTGTTTTCCGCTTGGG	20	59.97	55	162
	R : CGGTTGATGTGGGGATTGGA	20	60.03	55	

2) Animals and treatment

The experiment using laying hens (n = 75, 52-week-old, *Lohmann Brown-Lite*) was conducted at Seoul National University animal farm (PyeongChang, Republic of Korea). The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-180219-1). The hens were divided randomly into five groups with five hens per cage (48 cm x 45 cm x 45 cm, of width x depth x height) and three cages per group. Fifteen hens were assigned to each of the following diet treatments for 4 weeks: 1) commercial basal diet **(5C)**; 2) basal diet with 1.8% (w/w) of Lintex 170 **(5F)**; 3) basal diet with 1.8% (w/w) of Lintex 170 and 0.1% (w/w) Lp **(5FL)**; 4) basal diet with 1.8% (w/w) of Lintex 170 and 0.1% (w/w) T2 (**5FB**); 5) basal diet with 1.8% (w/w) of Lintex 170, 0.05% (w/w) Lp, and 0.05% (w/w) T2 (**5FBL**) (Table 14). Commercial basal diet used in study 2 was same commercial product used in study 1 and its formulation was described in study 1. Feed was offered 600 g per cage daily and fresh water was offered *ad libitum* during the experimental period.

Table 14. Feed information of experimental groups used in study 2.

Group	Feed
5C	**Commercial basal diet (C)**
5F	**C + 1.8% (w/w) Lintex 170**
5FL	**C + 1.8% (w/w) Lintex 170 + 0.1% (w/w) Lp**
5FB	**C + 1.8% (w/w) Lintex 170 + 0.1% (w/w) T2**
5FBL	**C + 1.8% (w/w) Lintex 170 + 0.05% (w/w) Lp + 0.05% (w/w) T2**

3) Samples and data collection

The method of collecting egg, blood samples during the 4-week-experiment were same as described in study 1. Egg quality (albumen height, Haugh unit, eggshell thickness) and laying performance (daily feed intake, average egg weight, feed conversion ratio, hen-day egg production) were investigated in the same way of study 1.

The PUFA profile measured by GC were only performed with 1 g of egg yolk as described in study 1. To measure c9, t11-CLA amount in egg samples, analytical standard of conjugated (9Z, 11E)-Linoleic acid (Sigma-Aldrich, USA) were methylated and analyzed in the same manner of method using yolk samples described in study 1. By checking its retention time of peak, c9, t11-CLA content in egg samples were measured.

Blood were collected from 6 randomly selected hens per group at weeks 0, 2 and 4 and divided into 2 tubes mentioned in study 1 (BD Vacutainer SST™ Ⅱ Advance Tubes (Becton Dickinson, Le Pont de Claix, France) to collect serum and V-Tube™ EDTA K3 Tubes (AB MEDICAL, Republic of Korea) to analyze complete blood cell count with whole blood sample). After centrifugation at 6,000 rpm for 10 min, serum was transferred to 1.5 ml plastic tubes and stored at -20°C until use. The whole blood samples for complete blood cell count were stored at 4°C until use.

On end of week 4, the 5 selected birds per group were euthanized by CO_2 asphyxiation and the ceca and liver samples were collected. All the samples (ceca, liver) were quenched using liquid nitrogen and kept in a freezer at -70 °C for further analysis. Liver samples were used to extract mRNA by vortexing with 1ml TRIzol reagent. The subsequent process to extract mRNA was described in study 1-1. Using liver mRNA samples, cDNA samples were synthesized to investigate gene expression of inflammatory-related cytokines (***tnf-α, il-1β, il-6, cox-2***) and stress-related gene (***hsp70***) as markers of inflammation and stress state by qRT-PCR normalized with β-actin. The primer sequences to detect each genes were listed in Table 15 (inflammatory-related genes) and Table 16 (stress-related genes) respectively.

Table 15. The primer sequences for quantitative real-time PCR of chicken inflammatory-related genes.

Primers	Sequence (5'→ 3')	Reference
β-actin	F : GAGAAATTGTGCGTGACATCA R : CCTGAACCTCTCATTGCCA	Lian *et al.*, 2010
tnf-α	F : GCCCTTCCTGTAACCAGATG R : ACACGACAGCCAAGTCAACG	Zhao *et al.*, 2017
il-1β	F : CAGCAGCCTCAGCGAAGAG R : CTGTGGTGTGCTCAGAATCCA	Zhao *et al.*, 2017
il-6	F : AAATCCCTCCTCGCCAATCT R : CCCTCACGGTCTTCTCCATAAA	Zhao *et al.*, 2017
cox-2	F : TGTCCTTTCACTGCTTTCCAT R : TTCCATTGCTGTGTTTGAGGT	Zhao *et al.*, 2017

Table 16. The primer sequences for quantitative real-time PCR of chicken stress-related genes.

Primers	Sequence (5'→ 3')	Reference
β-actin	F : GAGAAATTGTGCGTGACATCA R : CCTGAACCTCTCATTGCCA	Lian *et al.*, 2010
hsp70	F : ATTCTTGCGTGGGTGTCTTC R : GATGGTGTTGGTGGGGTTC	Cedraz *et al.*, 2017

The fatty acid profile of feed, egg and serum samples were analyzed by gas chromatography (GC) with 5 replicates by the direct methylation method as described in study 1. 1 g of ground feed and egg yolk and 500 μL of serum were used.

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

In case of H:L ratio, the complete blood cell count method was used with whole blood sample of week 0, 2, 4. The detailed method was mentioned in study 1.

The statistical analysis were mainly conducted to one-way ANOVA with Tukey's HSD post hoc test to investigate significant differences among the groups. It was performed using R statistical package v.3.0.3 combined with home-made perl script to manage large amounts of data. (*p-$value$<0.05; **p-$value$<0.01; ***p-$value$<0.001).

4) Gut microbiome analysis

(1) DNA extraction and amplification of 16S rRNA gene

At the end of experiment, cecal samples were collected from each laying hen and stored at -70 ℃ until DNA extraction was performed. DNA was extracted from 200 mg of each fecal sample using a Accuprep®Stool DNA extraction kit (Bioneer, Republic of Korea) according to the manufacturer's protocol and measured each concentration of genomic DNA by Nanodrop 2000. The genomic DNA samples were stored at -20 ℃ until further analysis. The V4 region of the bacterial 16S rRNA gene was amplified from the total extracted genomic DNA using Takara Ex-taq polymerase (Takara Bio, Japan) and universal primers (forward: 5'-GTGCCAGCMGCCGCGGTAA-3' and reverse: 5'-GGACTACHVGGGTWTCTAAT-3'). The amplification program consisted as follows: 1 cycle of 94 ℃ for 3 min, 40 cycles of 94 ℃ for 45 sec, 55 ℃ for 1 min, and 72 ℃ for 1.5 min, and 1 cycle of 72 ℃ for 10 min. The amplicons were separated by agarose gel electrophoresis (100V, 45min) and purified using a QIAquick Gel Extraction Kit (Qiagen, USA).

(2) Sequencing

The NEBNext Ultra DNA Library Prep Kit for Illumina (New England Biolabs, USA) was used to constructing DNA libraries with some modifications of the manufacturer's instructions. Briefly, the size selection steps for the adaptor-ligated DNAs and the cleanup steps were replaced by PCR product purification using a QIAquick PCR Purification Kit. The adaptor and index primers were added to the amplicons using the NEBNext Multiplex Oligos for Illumina Kit (New England Biolabs, USA). The construction of the DNA libraries was confirmed by agarose gel electrophoresis, and the libraries were purified using a QIAquick Gel Extraction Kit. The components of the libraries were then sequenced using an Illumina MiSeq 2 × 250 bp paired-end sequncing platform (Macrogen, Republic of Korea). The 16S rRNA gene sequences determined in this study were deposited in the NCBI SRA database with accession number SRP197263 for the study 2 and study 3 experiments.

(3) Microbial community analysis

The raw sequence reads were trimmed by their quality and demultiplexed using several software and in-house perl scripts. Briefly, the raw sequence reads were checked their quality by FastQC v0.11.8 and trimmed by FASTX-Toolkit v0.0.13 software before poor quality region, which is after 245 bp. Then, trimmed paired-end reads were merged using FLASH v1.2.11 (parameter settings: -m 50 –M 205) (Magoc and Salzberg, 2011) and demultiplexed. The microbial communities were analyzed using QIIME v1.9.1 software (Caporaso *et al.*, 2010b). The OTU tables were normalized to 3,000 reads per sample by single rarefaction and further analyzed. The remaining sequences were clustered into OTUs by subsampled open-reference OTU picking at 97% identity with the GreenGenes 13_8 database as the reference (DeSantis *et al.*, 2006). The OTU picking method was usearch61 (Edgar, 2010), and the value of parameter percent subsample was 0.1. The representative sequences were aligned using PyNAST (Caporaso *et al.*, 2010a). The representative sequences were taxonomically assigned using the uclust consensus taxonomy assigner.

The microbial diversity of the samples (alpha diversity) was determined using the observed OTUs as richness index. It was calculated from 3,007 sequence reads through rarefaction with 10 iterations. PCoA was performed based on weighted and unweighted UniFrac distances, and the effect of flaxseed and probiotics on the microbial community was evaluated. The abundance of microbial taxa was

expressed as a percentage of total 16S rRNA gene sequences. To analyze the effects of flaxseed and probiotics on gut microbiota of laying hen, the OTU table was divided by treatment using split_otu_table.py script in QIIME. One-way ANOVA and post hoc Tukey's HSD test for multiple mean comparisons were used to find significant differences in alpha diversity and microbial taxa among groups using the R statistical package v3.0.3 (R Foundation for Statistical Computing, Austria), and *p-value*<0.05 was considered to be significant..

(4) Prediction of the functions of the microbial communities

To predict the gut metagenome of laying hen, PICRUSt v1.0.0 was used with obtained 16S rRNA gene sequence (Langille *et al.*, 2013). The OTUs unmatched with the GreenGene database were removed from the OTU table. The resulting BIOM files were normalized according to known/predicted 16S rRNA gene copy numbers, and the metagenomes were predicted using pre-calculated KEGG orthologs. The predicted metagenomes were sorted into a specified level in a hierarchy using the KEGG pathway metadata. Eukaryotic and unclassified functional categories were eliminated from the analysis.

3. Results

1) Probiotic screening

In study 2, probiotics were screened to feed laying hen combination with flaxseed. To use probiotics as feed additives, their production yield should be considered to efficient mass production so that lactobacilli and bacilli were evaluated by their production yield respectively. Then, selected probiotics were analyzed whether respective target genes were encoded and expressed.

(1) Lactobacilli screening

① Production yield

The production yield of 3 lactobacilli (Lsa, Lr, Lp) were evaluated by CFU counting after seed culture and 24 hr incubation with PUFA as described above (Figure 31). After seed culture, the viable cell counts were significantly differed among groups (*p-value* : 0.010) and Lp was the most produced (Figure 31A). Although there was no significant difference when probiotics were incubated for 24 hr with PUFA treatment, Lp was selected to analyze target gene identification (Figure 31B).

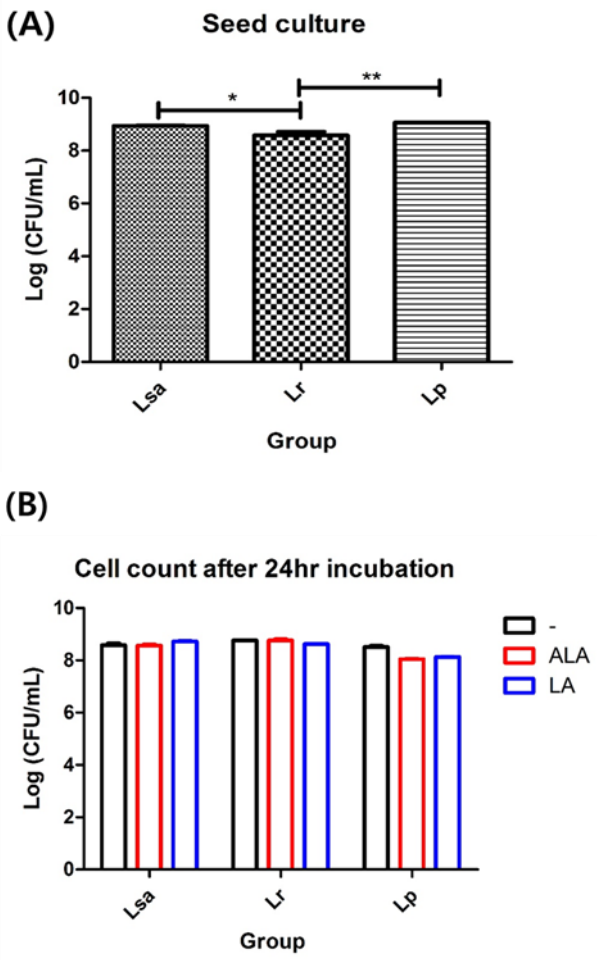

Figure 31. Production yield of 3 *Lactobacillus* candidates.

(A) 3 candidates were cultured in MRS broth for 24hr and viable cell count were measured by CFU.
(B) Viable cell count were measured by CFU after incubating in MRS with PUFAs for 24 hr.

② Target gene identification

The target genes to identify using Lp were CLA-related genes (***cla-er, cla-dc, cla-dh, cla-hy***), which convert LA to CLA encoded enzymes CLA-ER, CLA-DC, CLA-DH, CLA-HY respectively. To detect these 4 genes, the primers were designed by primer BLAST based on *Lactobacillus plantarum* WCFS1 reference genome (Figure 32). By amplifying PCR with primers, Lp was confirmed that all 4 CLA-related genes were encoded (Figure 33). Then, their expression level when treated PUFA were investigated by qRT-PCR, which are upregulated when treating especially LA which is precursor of CLA (Figure 34). In conclusion, Lp encoded 4 CLA-related genes which were all activated. Thus, Lp was selected to use as *Lactobacillus* in laying hen feed experiment.

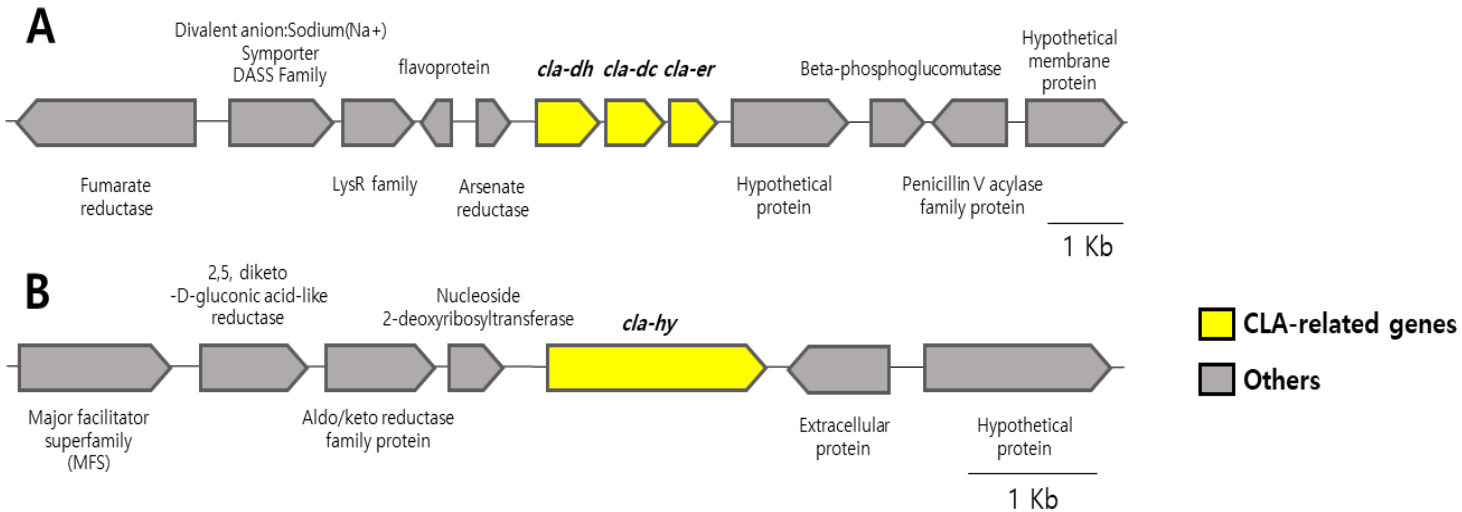

Figure 32. Flanking region of CLA-related genes.

(A) *cla-dh*, *cla-dc*, *cla-er* (B) *cla-hy*.

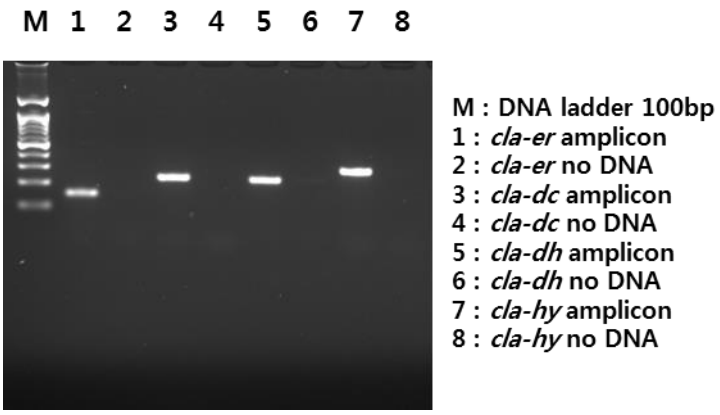

Figure 33. Gel loading pics for *cla-er, cla-dc, cla-dh, cla-hy* in Lp

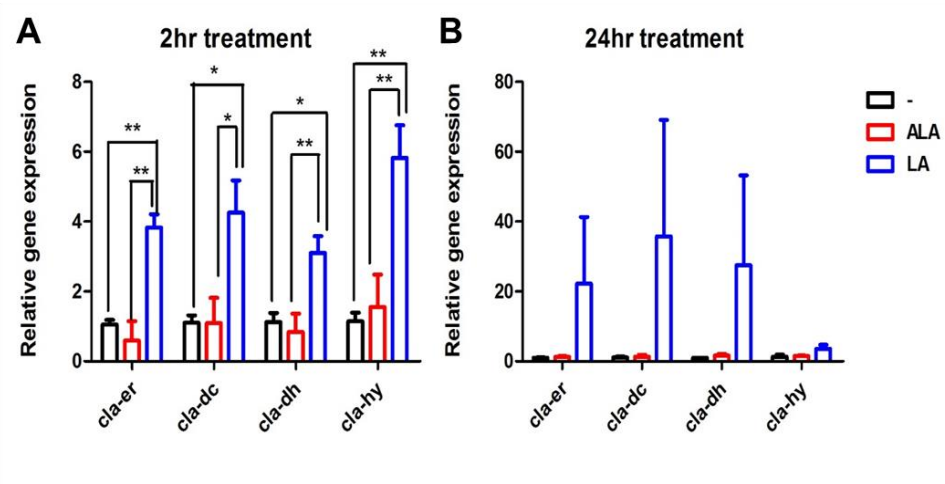

Figure 34. qRT results of *cla-er, cla-dc, cla-dh, cla-hy* genes in *Lactobacillus plantarum* 177 (Lp) when treated ALA, LA 100uM (A) 2hr (B) 24hr.

(2) Bacilli screening

① Production yield

The production yield of 2 bacilli (T2, T4) were evaluated by CFU counting after seed culture, mass production and post-lyophilized condition. The media used during seed culture and mass culture were TSB and BSM4 respectively. After mass production, post-lyophilized powder was produced by mixing lyophilized cell powder with zeolite. During all procedures, T2 had significantly higher viable cell count than T4 (Figure 35). Therefore, T2 was selected to analyze target gene identification.

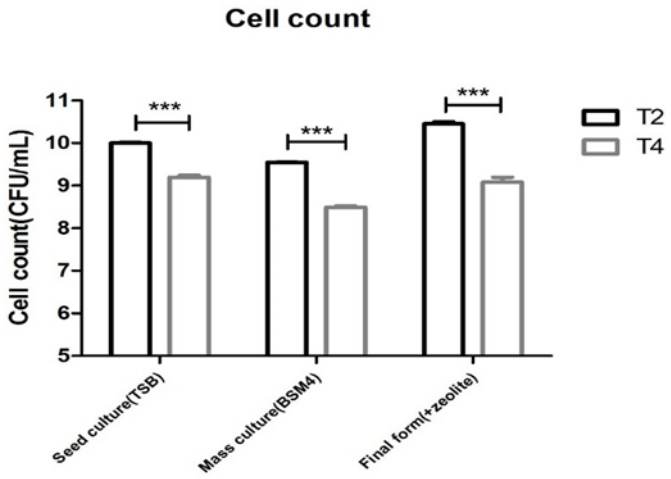

Figure 35. Viable cell count of *Bacillus licheniformis* T2 (T2) and *Bacillus subtilis* T4 (T4) in seed culture, mass culture, and final form.

② Target gene identification

The target genes to identify using T2 were fatty acid desaturase genes (***des***, gene encoded enzyme converting OA to LA; ***fad6***, gene encoded enzyme converting DGLA to AA and ETA to EPA). The gene ***d6d*** was not amplified designed primer. To detect these 2 genes, the primers were designed by primer BLAST based on *Bacillus licheniformis* ATCC 9789 reference genome (Figure 36). By amplifying PCR with primers, T2 was confirmed that 2 fatty acid desaturase genes were encoded although the gene ***d6d*** was not detected (Figure 37). Then, their expression level when treated PUFA were investigated by qRT-PCR, which are upregulated when treating especially ALA (Figure 38). In conclusion, T2 encoded 2 fatty acid desaturase genes which were all activated. Thus, T2 was selected to use as *Bacillus* in laying hen feed experiment.

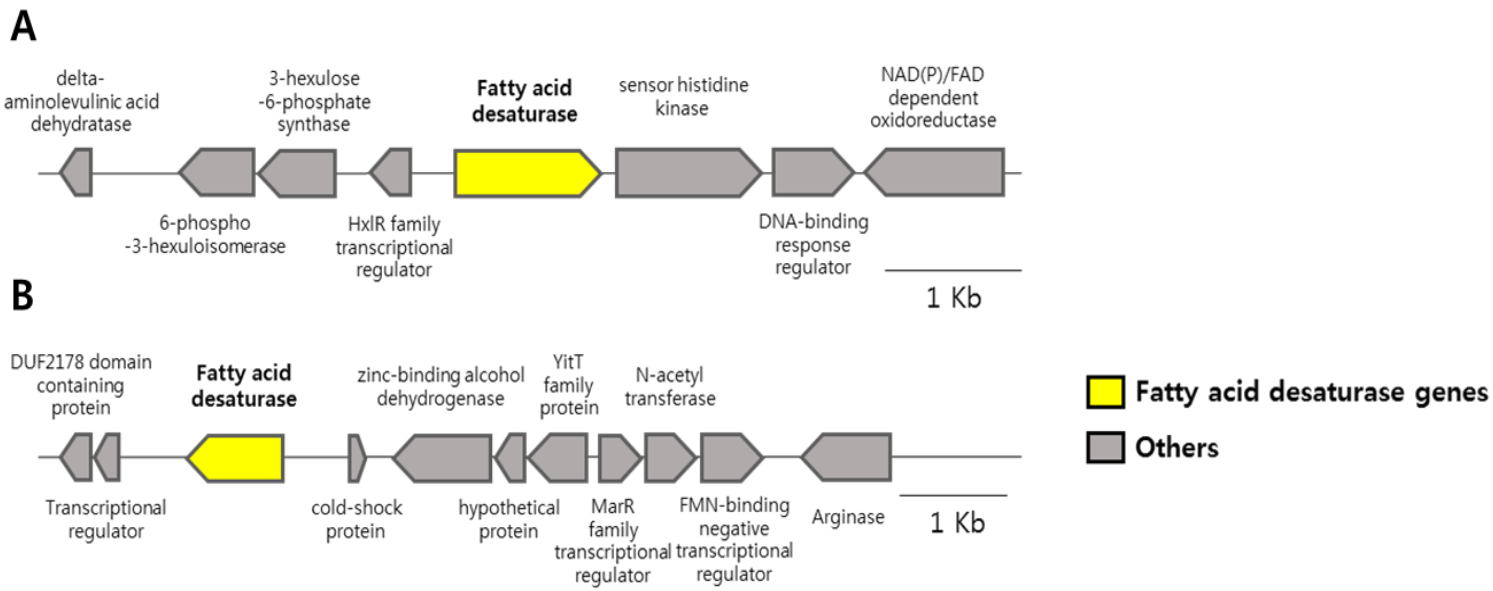

Figure 36. Flanking region of fatty acid desaturase genes.

(A) Delta-12 desaturase (*des*) (B) Delta-5 desaturase (fad6).

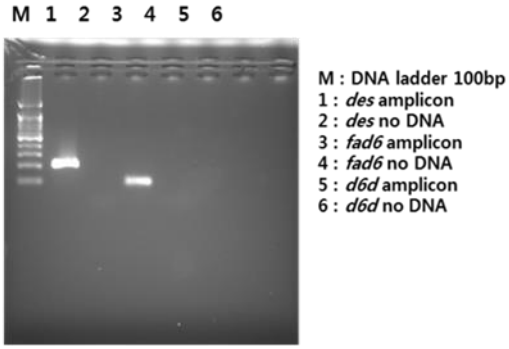

Figure 37. Gel loading pics for *des*, *fad6* in T2

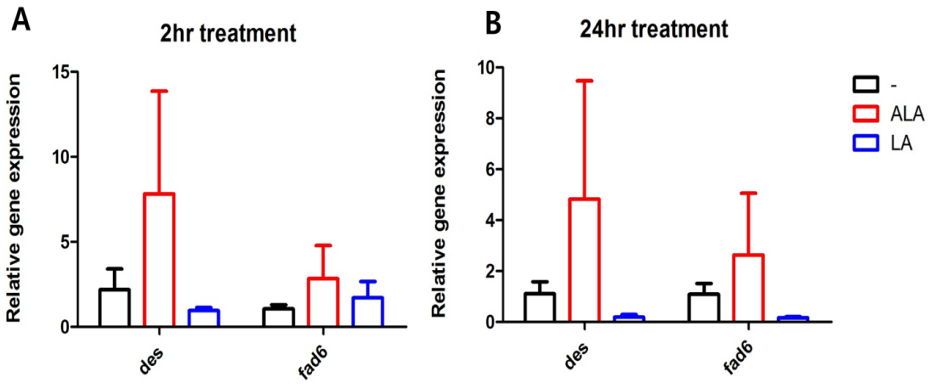

Figure 38. qRT results of *des*, *fad6* genes in *Bacillus licheniformis* T2 when treated ALA, LA 2uM.

(A) 2hr (B) 24hr.

(3) Storage property of probiotics

To confirm probiotic effect during feed experiment, the number of viable cell should be more than minimum useful count. Therefore, viable cell of probiotics stored at refrigerator (4ºC) were monitored once a week during experimental period. Also, as feed for laying hen was manually manufactured once a week and stored at cold room (5-8 ºC), changes of viable cell during cold room storage also monitored by CFU counting. Under refrigerator condition, T2 kept its original viable cell number (8.36×10^8 CFU/g lyophilized powder) throughout the overall experimental period while the number of Lp was decreased gradually as the period went on and remained about 3.69×10^7 CFU/g lyophilized powder (Figure 39A). Likewise, during the cold room storage mixed in feed, T2 maintained its original number whereas Lp was decreased by 1.14 % of cell viable number compared to pre-cold room state (Figure 39B). However, both 2 probiotics were included in feed at least 2×10^7 CFU/kg feed that is more than minimum useful count.

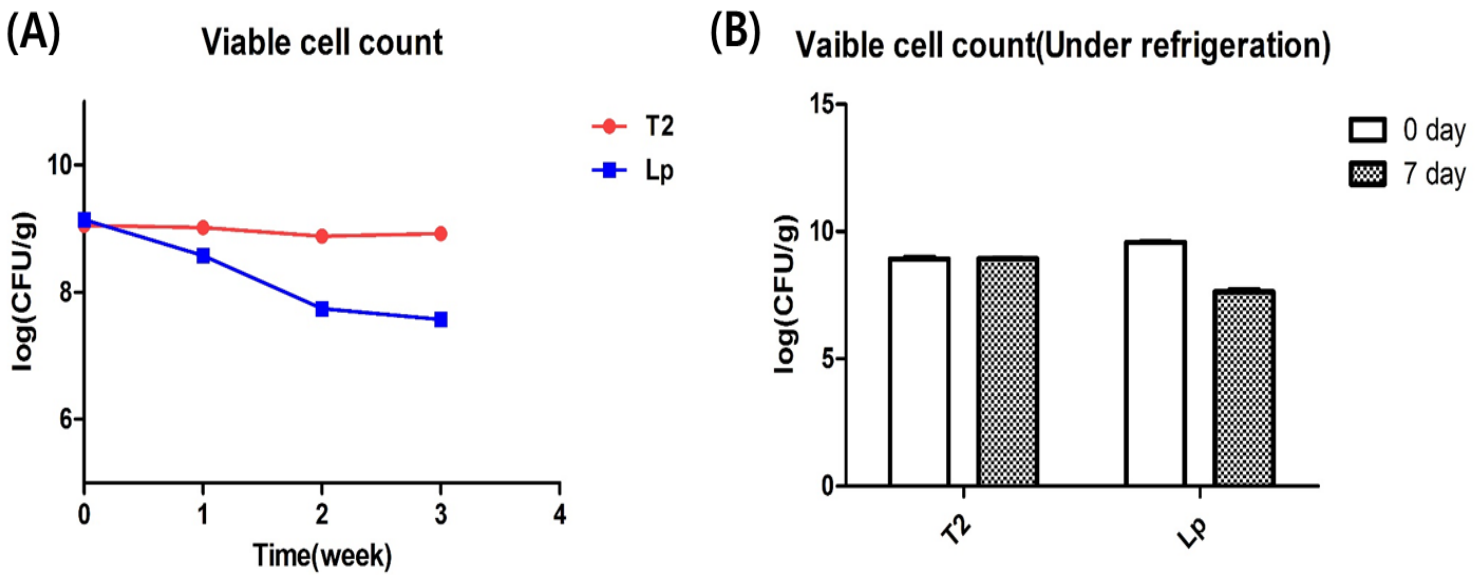

Figure 39. (A) Viable cell count of 2 probiotics during storage at 4 ℃ refrigerator and (B) cold room.

Each group of viable cells was counted weekly and under refrigerating 0 and 7 days to monitor storage property. All groups were 3 replicates.

2) Changes in fatty acid profile by feeding flaxseed and probiotics

(1) The ratio of omega-6 to omega-3 in egg

As each fatty acid has distinct biological effect, fatty acid profile were monitored and compared among group to investigate effects of flaxseed and probiotics. First, the ratio of omega-6 to omega-3 were measured by once a week from the week 0 to 4 (Figure 40). The ratio was decreased except 5C group since week 1, which means supplementing flaxseed mainly affect the changes of the ratio. There was no significant difference in the ratio among flaxseed-fed group (5F, 5FL, 5FB, 5FBL). The average values of the ratio on week 4 were 21.76 (5C), 6.57 (5F), 6.47 (5FL), 6.30 (5FB), 7.07 (5FBL) respectively.

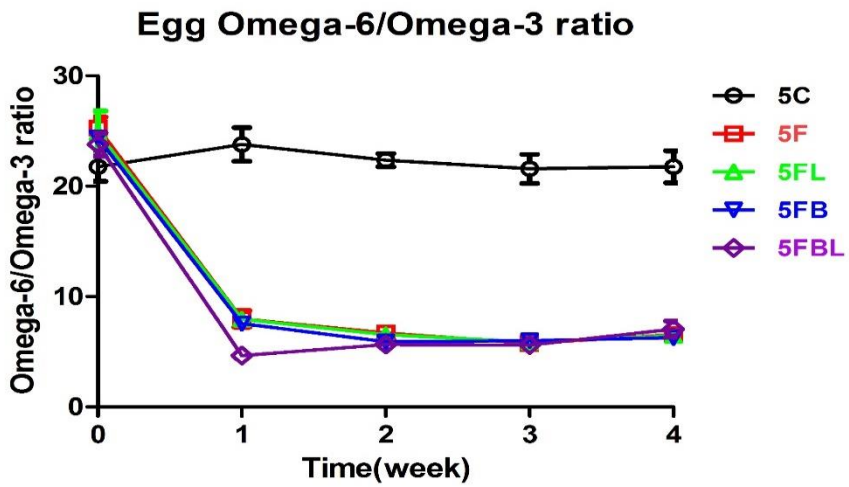

Figure 40. Changes in egg omega-6 to omega-3 fatty acid ratios during the study 2.

The ratios were calculated by data of gas chromatography standardized with FAME 37 mix.

(2) Investigation of egg fatty acid profile

More detailed fatty acid profiles were also investigated to identify significantly changed fatty acid using PLS-DA plot and VIP plot. PLS-DA plot was used to confirm distribution among groups based on their overall fatty acid profile. Through this, the groups mainly clustered by flaxseed supplementation similar to the change of the omega-6 to omega-3 ratio (Figure 41A). Interestingly, the fatty acid profiles of 5FL group (pink circle) were clustered distinctly apart from other flaxseed-fed groups. To determine which fatty acid contributed to separate fatty acid profiles, VIP plot was used and identified 15 fatty acids which mostly influenced (Figure 41B). Omega-3 fatty acids (EPA, DHA, ETA, ALA) were ranked in top 5, which means flaxseed supplementation was main factor to modulate fatty acid profile consistent with results of PLS-DA plot. Also, omega-6 fatty acids (GLA, AA) were ranked 6^{th} and 7^{th} respectively abundant in fatty acid profile of 5C group.

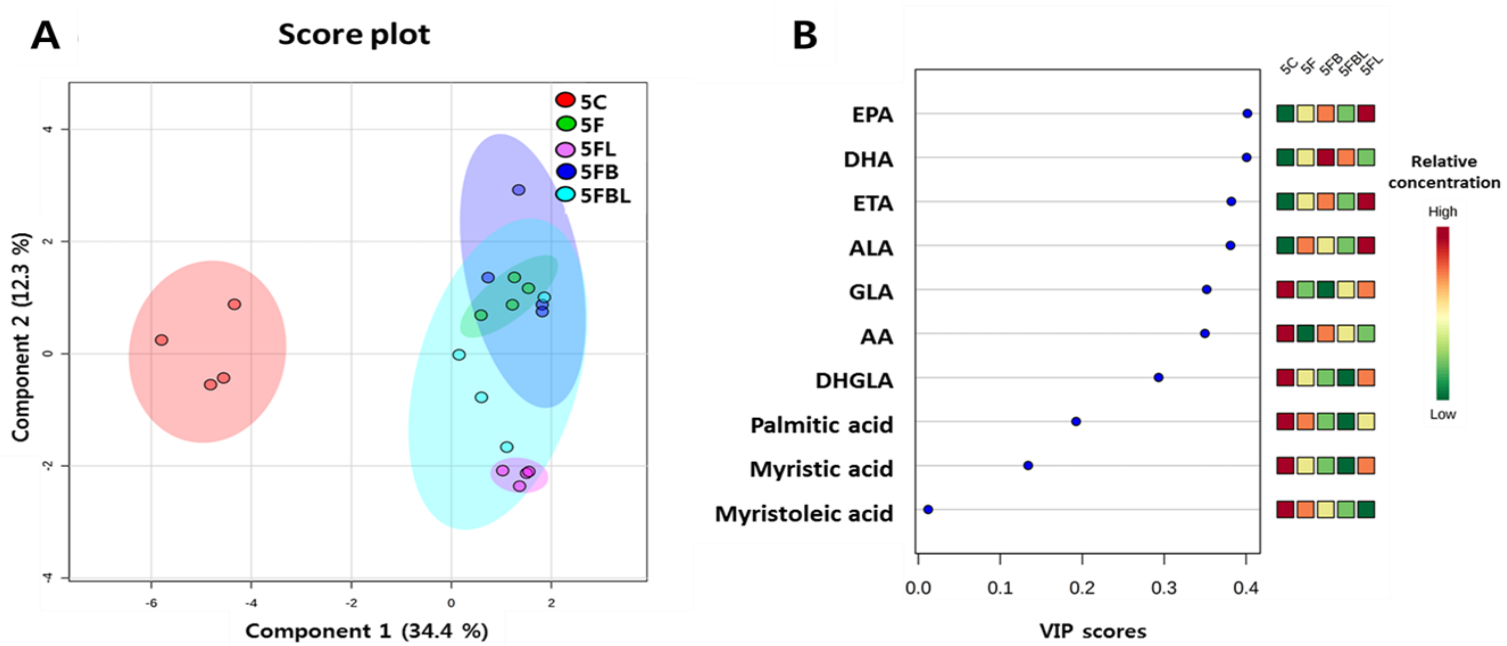

Figure 41. Multi-variant analysis of fatty acid profile in week 4 egg samples.

(A) Partial least squares discriminant analysis (PLS-DA) plot of fatty acids.
(B) Variable importance in projection (VIP) plot of 10 fatty acids (VIP scores Top 10) that were differentially regulated among groups.

Next, to investigate probiotic effect on fatty acid profile, the relative concentration of each fatty acids were compared by one-way ANOVA and post hoc Tukey's HSD test followed by generating bar plots which was significantly differed among group (Figure 42). In addition to enriching omega-3 fatty acid (Figure 42F-I) and reducing omega-6 fatty acid contents (Figure 42C-E), there were several changes in saturated fatty acid and monounsaturated fatty acid. Palmitic acid was significantly decreased in T2-fed group (5FB, 5FBL) compared to 5C group (Figure 42A). Also, myristoleic acid which is monounsaturated fatty acid were significantly decreased only in 5FL group (Figure 42B). The detailed fatty acid profile of egg samples were listed in Table 17.

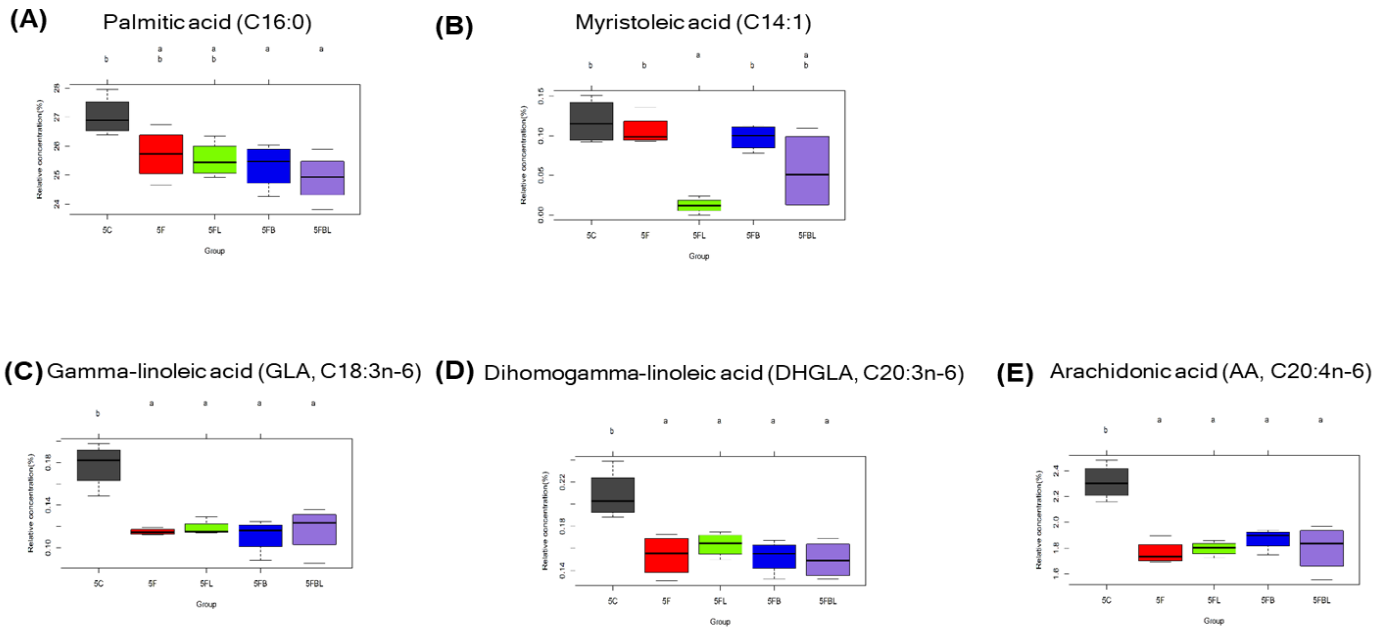

Figure 42. Bar plots of relative concentrations of fatty acids in week 4 egg samples.

(A) Saturated fatty acid (Palmitic acid).
(B) Monounsaturated fatty acid (Myristoleic acid).
(C-E) Omega-6 fatty acids (GLA, DHGLA, AA).

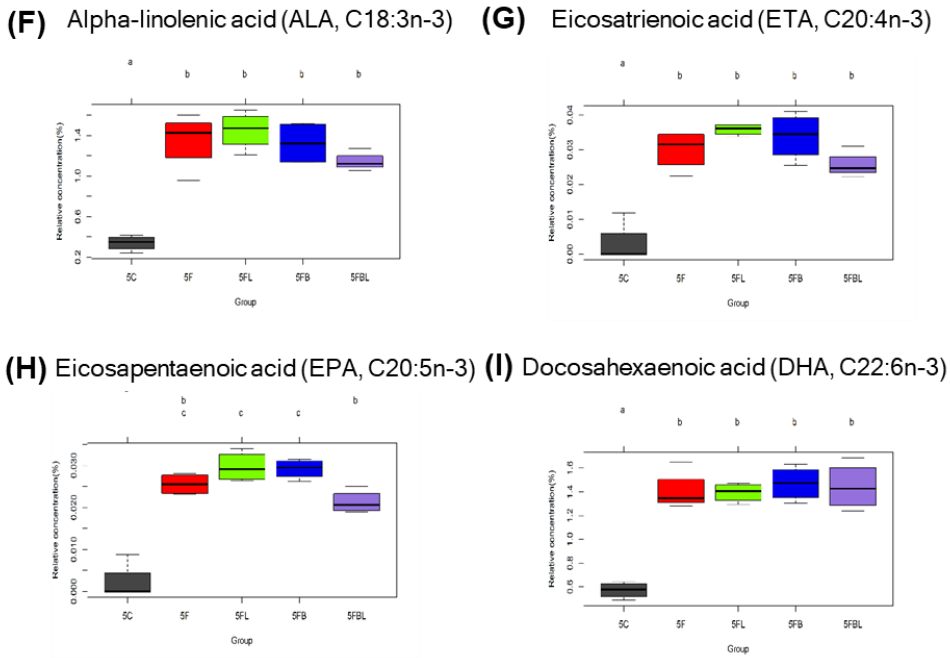

Figure 48. continued.

(F-I) Omega-3 fatty acids (ALA, ETA, EPA, DHA).

Table 17. Relative fatty acid concentration among groups on week 4 egg samples of study 2[1].

Egg fatty acid (%)	5C	5F	5FL	5FB	5FBL	*p-value*
Myristic acid (C14:0)	0.51±0.06^{a}	0.44±0.04^{a}	0.45±0.02^{a}	0.43±0.03^{a}	0.41±0.03^{a}	0.0510
Myristoleic acid (C14:1)	0.12±0.03^{b}	0.11±0.02^{b}	0.01±0.01^{a}	0.10±0.02^{b}	0.06±0.05^{ab}	<0.001***
Pentadecanoic acid (C15:0)	0.09±0.02^{a}	0.08±0.01^{a}	0.00±0.00^{a}	0.09±0.01^{a}	0.06±0.03^{a}	<0.001***
Palmitic acid (C16:0, PA)	27.04±0.68^{b}	25.72±0.88^{ab}	25.54±0.62^{ab}	25.31±0.78^{a}	24.89±0.86^{a}	0.015*
Palmitoleic acid (C16:1)	3.55±0.52^{b}	2.49±1.64^{b}	3.35±0.43^{a}	3.24±0.34^{b}	3.04±0.33^{b}	0.453
Margaric acid (C17:0)	0.22±0.01^{a}	0.20±0.01^{a}	0.21±0.02^{a}	0.23±0.02^{a}	0.23±0.03^{a}	0.328
Margaroleic acid (C17:1)	0.06±0.01^{a}	0.03±0.03^{a}	0.00±0.00^{a}	0.09±0.10^{a}	0.00±0.00^{a}	0.065
Stearic acid (C18:0, SA)	7.38±0.71^{a}	7.04±0.28^{a}	7.34±0.87^{a}	7.19±0.57^{a}	7.71±0.37^{a}	0.616
Oleic acid (C18:1n9c, OA)	40.39±1.62^{a}	42.76±1.02^{a}	41.29±1.03^{a}	42.37±1.53^{a}	42.49±2.66^{a}	0.284
Linoleic acid (C18:2n-6c, LA)	16.8±1.46^{a}	16.09±0.99^{a}	16.59±1.34^{a}	15.72±1.73^{a}	16.2±2.56^{a}	0.905
Arachidic acid (C20:0)	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.838
Gamma-linolenic acid (C18:3n6, GLA)	0.18±0.02^{b}	0.11±0.00^{a}	0.12±0.01^{a}	0.11±0.02^{a}	0.12±0.02^{a}	<0.001***
Gondoic acid (C20:1)	0.05±0.00^{a}	0.05±0.00^{a}	0.05±0.01^{a}	0.08±0.07^{a}	0.04±0.01^{a}	0.503
α-linolenic acid (C18:3n-3, ALA)	0.34±0.08^{a}	1.35±0.28^{b}	1.45±0.18^{b}	1.33±0.21^{b}	1.14±0.09^{b}	<0.001***
Eicosadienoic acid (C20:2)	0.15±0.01^{a}	0.13±0.01^{a}	0.15±0.01^{a}	0.14±0.01^{a}	0.14±0.02^{a}	0.217
Dihomogamma-linolenic acid (C20:3n-6, DHGLA)	0.21±0.02^{b}	0.15±0.02^{a}	0.16±0.01^{a}	0.15±0.01^{a}	0.15±0.02^{a}	0.001**
Erucic acid (C22:1)	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.01^{a}	0.00±0.00^{a}	0.438

Eiosatrienoic acid(C20:3n-3, ETA)	0.00±0.01[a]	0.03±0.01[b]	0.04±0.00[b]	0.03±0.01[b]	0.03±0.00[b]	<0.001***
Arachidonic acid (C20:4)	2.31±0.14[b]	1.76±0.09[a]	1.80±0.06[a]	1.87±0.09[a]	1.80±0.18[a]	<0.001***
Docosadienoic acid (C22:2n6)	0.01±0.02[a]	0.01±0.01[a]	0.01±0.00[a]	0.01±0.01[a]	0.01±0.01[a]	0.936
Eicosapentaenoic acid(C20:5n-3, EPA)	0.00±0.00[a]	0.03±0.00[bc]	0.03±0.00[c]	0.03±0.00[c]	0.02±0.00[b]	<0.001***
Docosahexaenoic acid(C22:6n-3, DHA)	0.57±0.07[a]	1.41±0.17[b]	1.39±0.08[b]	1.47±0.15[b]	1.44±0.20[b]	<0.001***

[1] Each value represents the mean±SD of five replicate eggs.

[a-b] Means in a row analyzed using one-way ANOVA and post hoc TUKEY's test (*p-value<0.05 **p-value<0.01 ***p-value<0.001).

(3) The changes in egg c9, t11-CLA content

As target genes of Lp were CLA-related genes, c9, t11-CLA content in egg samples were measured to determine effect of Lp administration. Even though there was no significant difference among group during experimental period, 5FL group tend to contain highest amount of c9,t11-CLA (0.344 mg/g egg yolk) in egg samples among group especially 1.57 fold increase compared to the content of 5C group (0.219 mg/g egg yolk) (Figure 43).

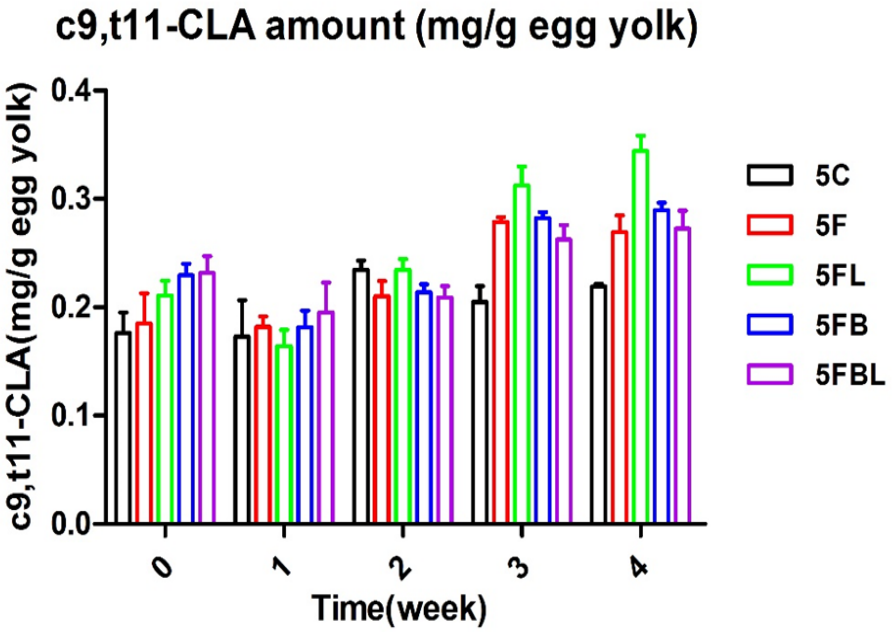

Figure 43. Changes of c9, t11-CLA amount in eggs during the study 2.

The c9, t11-CLA amounts of egg samples were calculated by data of gas chromatography standardized with pure c9, t11-CLA.

3) Effects on inflammatory indices

(1) Serum pro-inflammatory cytokine level

Serum levels of pro-inflammatory cytokine were monitored to evaluate the effect of flaxseed and probiotics on inflammatory processes. No significant differences were observed in levels of TNF-alpha, IL-1beta, or IL-6 in serum samples, although levels of TNF-alpha (Figure 44A) and IL1beta (Figure 44B) tended to decrease in 5FL and 5FB groups.

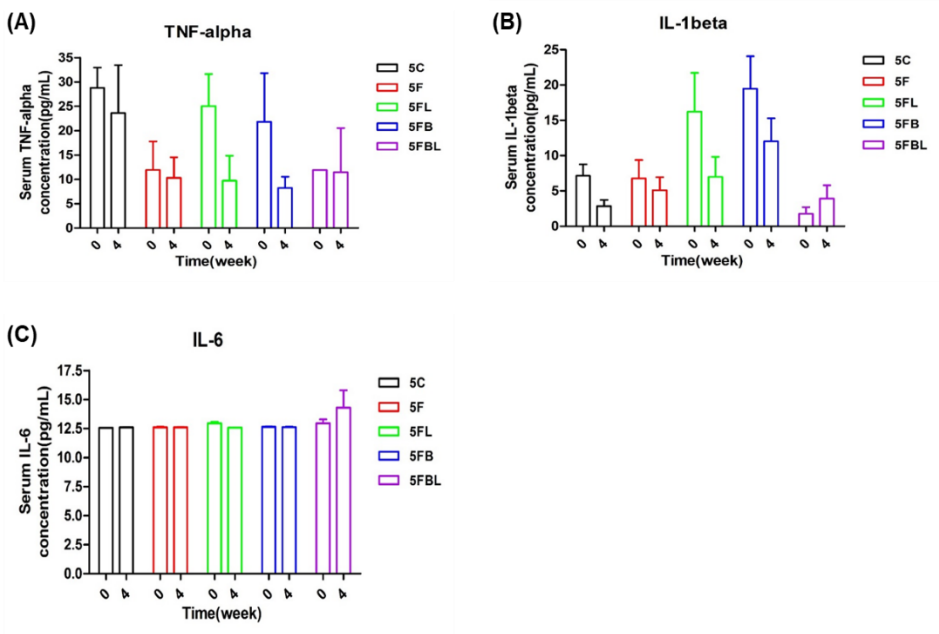

Figure 44. Serum pro-inflammatory cytokine levels in experimental hens at weeks 0 and 4.

(A) TNF-α (B) IL-1β (C) IL-6.

(2) Inflammatory-related gene expression in liver

Liver mRNA samples collected from each group were extracted and used to investigate expression levels of inflammatory-related genes. The genes encoded pro-inflammatory cytokine (***tnf-alpha***, ***il-1beta***, ***il-6***) and the gene encoded lipid mediator synthesis (***cox-2***) were checked. Although there was no significant difference among groups in pro-inflammatory cytokine-encoded genes, there were tendency to decrease expression levels of ***il-1beta*** (Figure 45B), ***il-6*** (Figure 45C) probiotics-fed groups (5FL, 5FB, 5FBL) especially 5FL group. Moreover, 5FL group were significantly decreased expression of ***cox2*** compared to 5F group (Figure 45D).

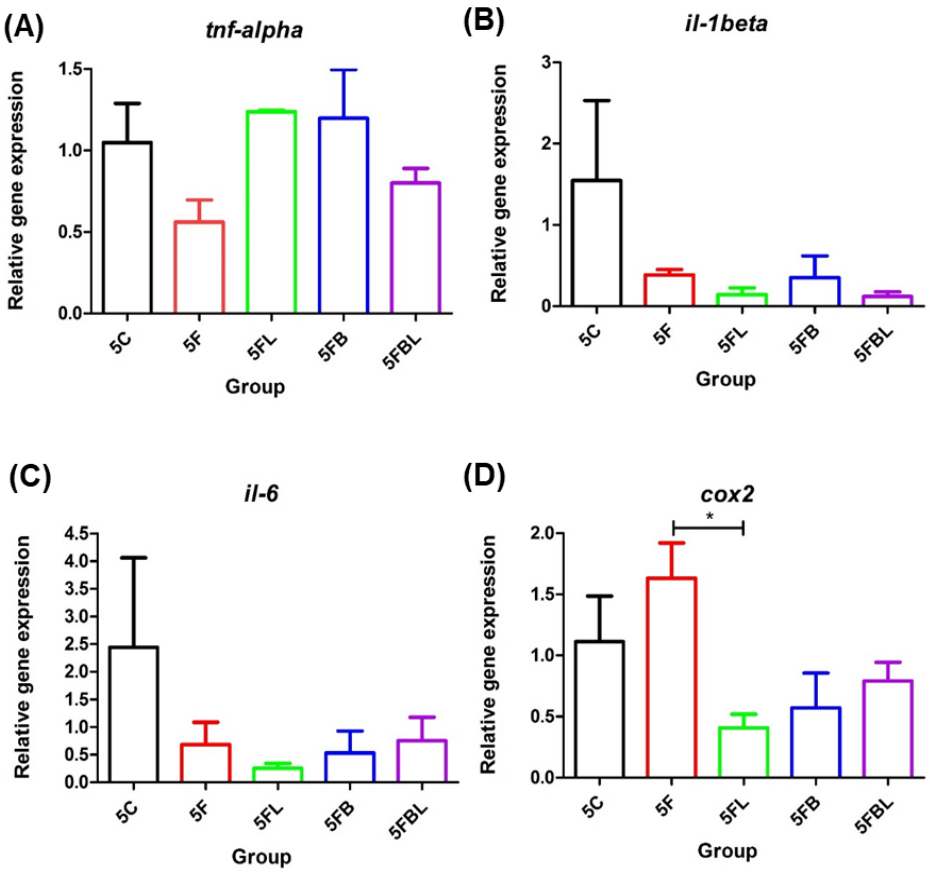

Figure 45. Analysis of gene expression of pro-inflammatory cytokines and lipid mediator synthesis in experimental hens at week 4.

(A) The transcription expression of pro-inflammatory cytokine *tnf-α*, (B) *il-1β*, and (C) *il-6*, and (D) lipid mediator synthesis related gene *cox2* relative to β-actin was quantified by qRT-PCR.

4) Effects on stress indices

To investigate the effect of flaxseed and probiotics on stress state of laying hen under normal condition, three stress indices, serum corticosterone level, liver HSP70 gene expression level and H:L ratio, were examined (Figure 46). Even though there was no significant difference on serum corticosterone level (Figure 46A) and H:L ratio (Figure 46C), ***hsp70*** (Figure 46B)showed significant decrease in probiotics-fed groups (5FL, 5FB, 5FBL) compared to 5C group. Especially, the expression of 5FL group was mostly decreased.

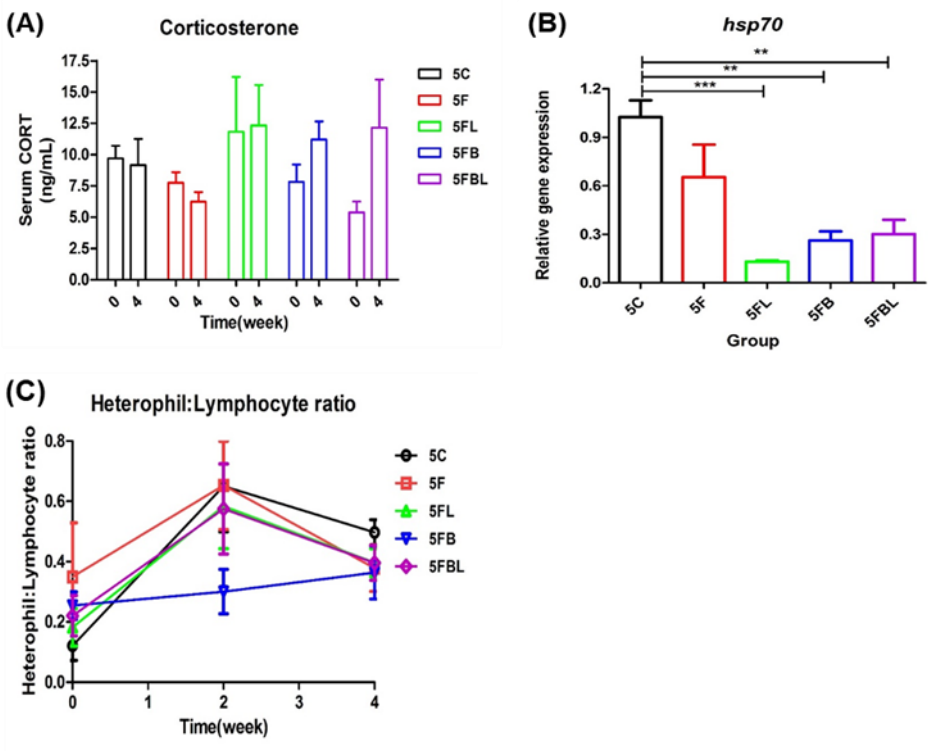

Figure 46. Effects of dietary flaxseed and probiotics on stress indices in experimental hens.

(A) Serum corticosterone level at weeks 0 and 4.
(B) HSP70 gene expression of liver samples at week 4.
(C) Changes in H:L ratios at weeks 0,2 and 4.

5) Effects on gut microbiota

To investigate effects of flaxseed and probiotics on gut microbiota, microbial community and its predicted metagenome were analyzed with cecum samples collected from each group. At the 97% similarity level, all of the OTUs observed were classified into 18 phyla and 300 genera.

(1) Microbial community analysis

In this study, the microbial diversity were determined within samples (alpha diversity) and between samples (beta diversity). The observed OTUs, which was used as a parameter of alpha diversity, were as follows: 1) 5C: 370.30 (±24.49); 2) 5F: 371.46 (±24.49); 3) 5FL: 330.48 (±20.54); 4) 5FB: 343.60 (±22.77); 5) 5FBL: 339.98 (±25.26) respectively. There was significant difference among groups. The 5FL group was significantly decreased compared to the 5C group (Figure 47A). The beta diversity were visualized by PCoA plot based on unweighted and weighted UniFrac distances of the 16S rRNA sequences. It revealed that the microbial communities assessed by unweighted UniFrac distances were separated by treatment (Figure 47B).

To determine which bacterial taxa contributed to separate microbial communities, relative abundance of taxa among groups were compared at the phylum and genus level. At the phylum level, all groups shared the following 15

phyla: *Euryarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Elusimicrobia*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *TM7*, *Tenericutes*, and *Verrucomicrobia*. Also, there were 2 phyla (*Euryarchaeota*, *Tenericutes*) that significantly differed among groups. 5FB and 5FBL group were significantly more abundant level of *Euryarchaeota*, *Tenericutes* compared to 5C group respectively (Table 18).

At the genus level, all groups shared 110 genera, and there were 4 genera that significantly differed among groups. *Epulopiscium*, *Aquincola* were significantly abundant in 5F group compared to 5C group. *Parabacteroides* was significantly abundant in 5FL group. Also, *Methanobrevibacter* was significantly abundant in 5FB group (Table 18).

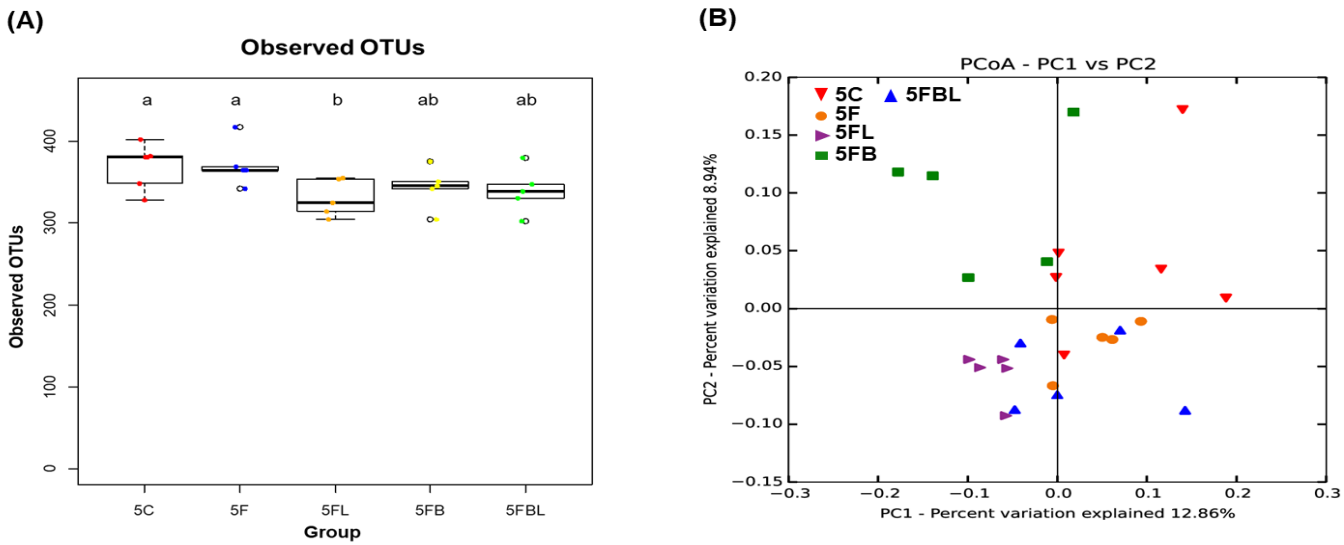

Figure 47. Differences in the gut microbial diversity among groups.

(A) Alpha diversity (observed OTUs).

(B) Beta diversity (PCoA plot).

The alpha diversity index were calculated from 3,000 sequence reads with 10 iterations. PCoA plot were designed based on unweighted UniFrac distances. One-way ANOVA with Tukey's post hoc test was used, and different superscript letters indicate significant difference (*p-value* < 0.05).

Table 18. Differences in the gut microbiota among groups at the phylum and genus level.

Taxon	Relative abundance (%)					*p-value*
	5C	5F	5FL	5FB	5FBL	
Phylum						
Euryarchaeota	1.27±1.01^{a}	0.75±0.65^{a}	1.76±1.09^{ab}	3.27±1.02^{b}	2.12±1.27^{ab}	<0.001***
Tenericutes	1.88±0.32^{a}	2.49±0.43^{ab}	2.10±0.80^{ab}	3.44±0.65^{ab}	3.50±1.64^{b}	0.017**
Genus						
Methanobrevibacter	1.17±1.04^{a}	0.66±0.56^{a}	1.64±1.02^{ab}	3.23±1.01^{b}	2.04±1.28^{ab}	0.007**
Parabacteroides	1.04±0.33^{a}	1.66±0.74^{ab}	2.29±0.50^{b}	1.52±0.46^{ab}	1.56±0.27^{ab}	0.008**
Paenibacillus	0.03±0.03^{a}	0.02±0.02^{a}	0.00±0.00^{a}	0.01±0.00^{a}	0.00±0.00^{a}	0.042*
Enterococcus	0.74±0.34^{a}	0.70±0.40^{a}	0.18±0.09^{a}	0.57±0.33^{a}	0.32±0.32^{a}	0.035*
Streptococcus	0.00±0.01^{a}	0.02±0.02^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.044*
Coprococcus	0.19±0.06^{a}	0.19±0.05^{a}	0.11±0.03^{a}	0.11±0.07^{a}	0.16±0.06^{a}	0.045*
Epulopiscium	0.00±0.00^{a}	0.01±0.01^{b}	0.00±0.00^{ab}	0.00±0.01^{ab}	0.00±0.00^{ab}	0.035*
Aquincola	0.00±0.00^{a}	0.01±0.01^{b}	0.01±0.00^{ab}	0.01±0.01^{ab}	0.00±0.00^{a}	0.013*

The data were expressed as the mean values ± standard deviation (SD).
The *p-value*s were determined using Welch's *t*-test (* *p-value*< 0.05; ** *p-value* < 0.01; *** *p-value* < 0.001).

(2) Predicted functions of the gut microbiota of laying hen fed flaxseed and probiotics

To compare the functions of the gut microbiota among groups, the KEGG pathways were predicted using PICRUSt. Then, their relative abundance were compared among groups with one-way ANOVA and post hoc TUKEY's HSD test to determine significantly differed. 'Phosphotransferase system (PTS)' was predicted at significantly lower levels in 5FL group (*p-value* = 0.027) (Figure 48A). 'Energy metabolism' was significantly differed, enriching in bacillus-fed group (5FB, 5FBL) compared to 5C group (*p-value* = 0.019) (Figure 48B).

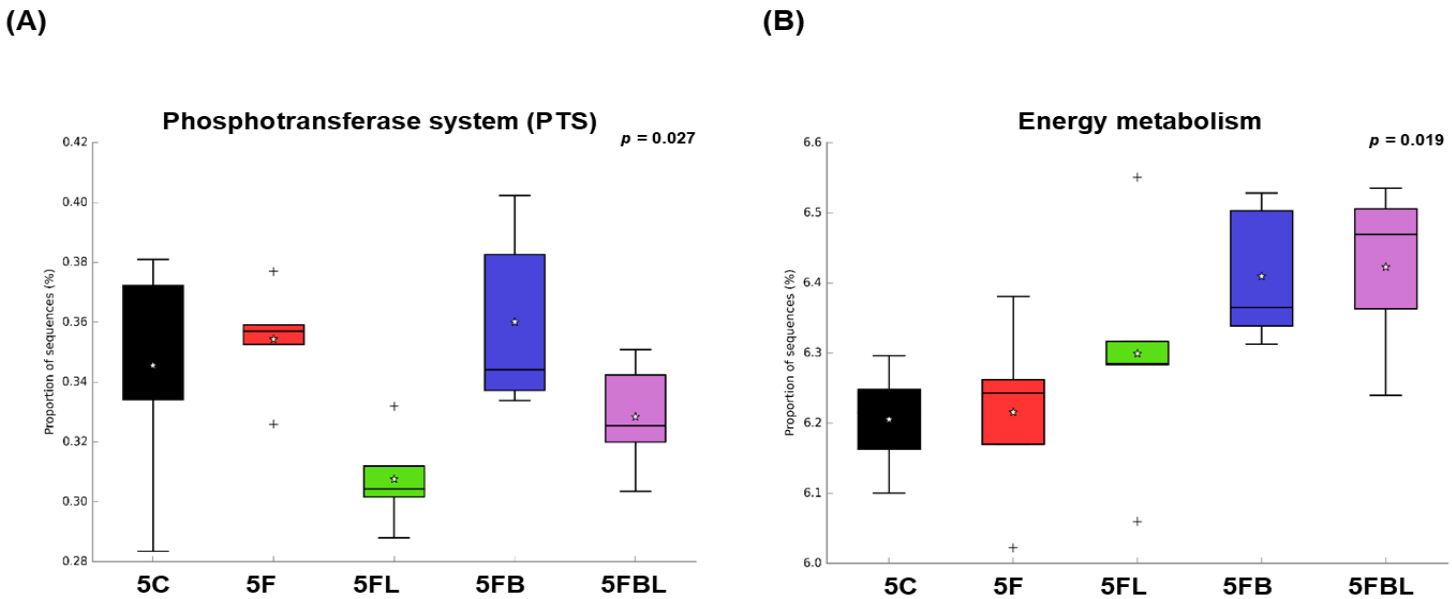

Figure 48. The different functions of the laying hen cecum microbiota when fed flaxseed and probiotics.

(A) Phosphotransferase system (PTS) (B) Energy metabolism.

The microbial functions were predicted using PICRUSt at the third level of the KEGG pathway and were expressed as relative abundances. The difference between the levels of the predicted functions were tested using a two-sided Welch's *t*-test, and *p-value* < 0.05 was considered significant.

6) Laying performance and egg quality when fed flaxseed and probiotics

The effects of flaxseed and probiotics on laying performance and egg quality were evaluated. Laying performance was evaluated by daily feed intake, average egg weight, feed conversion ratio and hen-day egg production as same as study 1.

Overall, average egg weight, feed conversion ratio and hen-day egg production were significantly differed by 4 week feed experiment (*p-values* <0.001, <0.001, <0.001, respectively). Especially, 5FL group was measured the most improved feed conversion ratio compared to the others whereas some adverse effects were measured by bacillus-fed group (5FB, 5FBL) in average egg weight, feed conversion ratio and hen-day egg production.

In case of effect of flaxseed supplementation, there was a tendency to improvement in overall traits compared to the 5C group although there was no significance in values of week 1, 2, 3, 4 and 1 to 4.

Meanwhile, in case of effects of probiotics administration, there were several significantly differed among groups in laying performance traits. On week 1, average egg weight and feed conversion ratio were significantly higher in 5FL group compared to 5FB group (*p-values* = 0.019, 0.019, respectively). On week 2, average egg weight was significantly higher in 5F group than 5FBL group (*p-value* = 0.048). On week 3, average egg weight, feed conversion ratio and hen-

day egg production were all significantly higher in 5F group than bacillus-fed groups (5FB, 5FBL) (*p-values* <0.001, <0.001, <0.001, respectively). On week 4, average egg weight, feed conversion ratio and hen-day egg production were all significantly worse in bacillus-fed groups (5FB, 5FBL) compared to others (5C, 5F, 5FL) (*p-values* <0.001, <0.001, <0.001, respectively) (Table 19).

Meanwhile, the indices of egg quality such as albumen height, Haugh unit and eggshell thickness showed no predominant improvement by flaxseed and probiotics supplementation for 4 weeks among group (Table 20).

Table 19. Effects of flaxseed and probiotics in diet on performance of laying hens during 52-56 weeks of age[1].

Items	Group					*p-value*
	5C	5F	5FL	5FB	5FBL	
Week 1						
Daily feed intake(g/head)	119.73±0.02[a]	116.38±2.67[a]	116.93±2.07[a]	116.61±1.79[a]	112.61±3.89[a]	0.415
Average egg weight(g)	57.64±2.39[ab]	59.94±1.49[ab]	62.19±1.46[a]	51.26±1.78[b]	54.61±3.68[ab]	0.019*
Feed conversion ratio (g feed/g egg mass)	2.10±0.09[ab]	1.95±0.05[ab]	1.89±0.04[a]	2.29±0.09[b]	2.11±0.13[ab]	0.019*
Hen-day egg production, %	98.10±4.04[a]	100.00±2.52[a]	102.86±2.86[a]	85.71±3.39[a]	91.43±7.08[a]	0.055
Week 2						
Daily feed intake(g/head)	119.71±0.00[a]	119.10±0.55[a]	118.91±0.49[a]	118.99±0.61[a]	117.28±2.21[a]	0.603
Average egg weight(g)	57.33±1.01[ab]	59.39±1.22[a]	57.09±1.12[ab]	55.92±1.04[ab]	54.36±1.22[b]	0.048*
Feed conversion ratio (g feed/g egg mass)	2.09±0.04[a]	2.01±0.04[a]	2.09±0.04[a]	2.13±0.04[a]	2.16±0.05[a]	0.153
Hen-day egg production, %	94.29±1.74[ab]	97.14±1.98[a]	92.38±1.74[ab]	92.38±1.74[ab]	89.52±1.98[b]	0.079
Week 3						
Daily feed intake(g/head)	119.71±0.00[a]	117.58±2.02[a]	118.61±0.85[a]	118.72±0.88[a]	119.26±0.37[a]	0.695
Average egg weight(g)	56.99±1.26[ab]	59.92±1.20[a]	60.95±0.74[a]	53.12±0.81[b]	54.04±1.37[b]	<0.001***
Feed conversion ratio (g feed/g egg mass)	2.11±0.05[ab]	1.97±0.04[a]	1.95±0.02[a]	2.24±0.03[b]	2.22±0.06[b]	<0.001***

Hen-day egg production, %	93.33±2.06[ab]	98.10±1.90[a]	98.10±1.23[a]	87.62±1.74[b]	88.57±2.40[b]	<0.001***
Week 4						
Daily feed intake(g/head)	117.43±2.29[a]	119.07±0.59[a]	119.12±0.42[a]	118.91±0.69[a]	115.68±3.40[a]	0.653
Average egg weight(g)	60.02±1.88[a]	59.10±0.79[a]	61.39±1.14[a]	52.42±1.85[b]	51.92±1.03[b]	<0.001***
Feed conversion ratio (g feed/g egg mass)	1.97±0.07[a]	2.02±0.03[ab]	1.94±0.04[a]	2.29±0.08[c]	2.23±0.04[bc]	<0.001***
Hen-day egg production, %	96.19±3.21[a]	97.14±1.35[a]	99.05±1.74[a]	85.71±3.06[b]	84.76±1.90[b]	<0.001***
Week 1 to 4						
Daily feed intake(g/head)	119.15±0.57[a]	118.03±0.81[a]	118.40±0.56[a]	118.31±0.55[a]	116.20±1.41[a]	0.170
Average egg weight(g)	58.00±0.84[a]	59.59±0.57[a]	60.40±0.66[a]	53.18±0.76[b]	53.73±1.02[b]	<0.001***
Feed conversion ratio (g feed/g egg mass)	2.07±0.03[ace]	1.99±0.02[ae]	1.97±0.02[e]	2.24±0.03[bd]	2.18±0.04[cd]	<0.001***
Hen-day egg production, %	95.48±1.42[a]	98.10±0.96[a]	98.10±1.18[a]	87.86±1.33[b]	88.57±1.94[b]	<0.001***

[1] Each value represents the mean±SD of three replicate cages (five birds per cage).

[a-e] Means in a row without a common superscript letter differ as analyzed by one-way ANOVA and the TUKEY test (*p-value<0.05, **p-value<0.01, ***p-value<0.001).

Table 20. Effects of flaxseed and probiotics in diet on egg quality of laying hens during 52-56 weeks of age[1].

Items	Time, week	Group					*p-value*
		5C	5F	5FL	5FB	5FBL	
Albumen height, mm	**0**	8.25±2.18	9.23±2.20	8.93±1.64	8.57±2.35	9.32±0.97	0.926
	1	10.4±0.94	10.80±1.26	10.78±1.63	8.98±0.53	9.10±1.28	0.105
	2	7.95±1.35	9.73±1.45	9.68±1.27	10.40±0.41	9.38±1.46	0.132
	3	8.57±0.35	10.53±1.44	9.25±1.20	10.68±1.08	10.00±0.73	0.056
	4	8.45±0.53	8.68±1.19	9.28±1.72	9.40±1.98	9.23±0.26	0.804
Haugh unit	**0**	89.77±12.89	94.95±10.76	93.53±7.43	91.73±13.11	96.63±4.49	0.891
	1	101.03±3.92	102.24±5.32	102.08±7.25	94.45±2.71	94.65±6.21	0.114
	2	88.20±8.67	97.28±5.89	96.90±6.57	100.58±1.94	95.55±7.01	0.141
	3	92.43±1.02	101.06±6.36	94.94±5.96	101.42±4.35	98.63±2.92	0.056
	4	91.12±3.01	93.34±6.36	94.73±8.09	95.13±9.73	95.37±0.98	0.877
Egg shell thickness, mm	**0**	0.39±0.01	0.41±0.01	0.41±0.02	0.40±0.01	0.41±0.01	0.420
	1	0.40±0.04	0.40±0.03	0.40±0.02	0.39±0.02	0.40±0.02	0.999
	2	0.38±0.02	0.40±0.01	0.41±0.02	0.40±0.01	0.39±0.03	0.232
	3	0.39±0.02	0.40±0.03	0.41±0.03	0.39±0.02	0.38±0.02	0.477
	4	0.39±0.01	0.41±0.03	0.40±0.01	0.40±0.02	0.38±0.02	0.404

[1]Each value represents the mean±SD of ten replicates with fifty hen per replicate.

4. Discussion

In the study 2, the aim was to evaluate flaxseed and probiotic effects on the laying hen physiology and its gut microbiota. As probiotics encoded enzymes that lack or insufficient in host, their administration can enrich the microbiome so that affecting host physiology. In case of *Lactobacillus*, it has been reported that several species including *Lactobacillus plantarum* can synthesis CLA via LA transformation (Khosravi et al., 2015). *Bacillus licheniformis* was reported to encode various enzymes such as fatty acid desaturase and keratinase (Diomande et al., 2015;Abdel-Fattah et al., 2018). Thus, their administration can induce several changes such as CLA enrichment and protein utilization in host. Also, some synergistic effects can be expected when using with flaxseed or under overcrowding condition.

To evaluate their effects, the probiotics used for laying hen feed experiment were screened by production yield and target gene identification, which resulted in selecting Lp and T2. Then, the 52-week-old laying hen were divided into five groups according to their treatment. They were fed experimental diet which was mixed commercial basal diet, flaxseed (Lintex 170) and probiotics (Lp, T2) for 4 weeks. During the experimental period, egg, blood, liver and cecal samples were collected from each group.

Analyzed with these samples, it was demonstrated that the fatty acid profile was modulated by supplementing flaxseed and probiotics. Flaxseed enriched the contents of omega-3 fatty acids in the eggs whereas reduced that of omega-6 fatty acids. The administration of probiotics altered the fatty acid profile, which reduced palmitic acid, a kind of saturated fatty acid. There were similar results that probiotics altered fatty acid profile in pig feed experiment (Ross *et al.*, 2012). Likewise, the kefir grains altered fatty acid profile of milk during fermentation (Vieira *et al.*, 2015). Interestingly, Lp lowered the content of myristoleic acid, which modulated fatty acid profile in a way of reducing MUFA. In other laying hen study, it was reported that probiotic supplementation decrease myristoleic acid similar to the result of this study (Luo *et al.*, 2010). Thus, even though more studies are needed to clarify the probiotic effect on livestock fatty acid profile, the probiotic encoded enzyme regarding fatty acid biotransformation may affect host fatty acid profile. The increment of c9, t11-CLA content in egg samples was monitored by supplementing Lp which encodes genes for CLA synthesis. Also, there was possibility to modulate the expression of host gene regarding fatty acid biotransformation as reported in a previous study (Park *et al.*, 2013).

As various fatty acid have distinct biological effects, its alteration may affect host physiology including inflammatory state. Actually, serum pro-inflammatory cytokines levels and their gene expression in liver samples were monitored. Although there were several reports that probiotics were downregulated pro-inflammatory cytokines among human (Howarth and Wang, 2013) and various

animal models so that resulting in improvement in disease (Matsumoto *et al.*, 2005;Donato *et al.*, 2010), there was no significant difference on the serum pro-inflammatory cytokine level by probiotic administration in this study. It was assumed that serum indicated comprehensive state of host while probiotics affect mainly in the gut. The previous studies reporting downregulated levels of pro-inflammatory cytokine in intestinal cells serve as a basis for this thought (Wang *et al.*, 2016). Also, there were a few studies reporting upregulation of pro-inflammatory cytokines by probiotics (Laskowska *et al.*, 2019). In case of stress indices, there was significantly difference in *hsp70* gene expression while no differences were observed in the other two indices, which could be consistent with the thought that the indices measured by serum samples didn't represent the host state. Meanwhile, as liver was experienced external changes continuously, its gene expression may be more appropriate to determine the state of host. Actually, various researches were used liver samples to monitor host physiological state (Esposito *et al.*, 2009;Zhong *et al.*, 2012). In particular, the gut-liver axis has been suggested recently (Endo *et al.*, 2013), which indicates the importance of monitoring liver when evaluating alteration of gut microbiota. Similar to the effect on inflammatory state, probiotic effect on host stress state were controversial as of now. For example, some studies showed improvement in stress index such as cortisol (Sohail *et al.*, 2010), ratio of neutrophil to lymphocyte (Noori *et al.*, 2016) while there were some studies showing opposite result or no effect of probiotics (Shim *et al.*, 2005). In this study, the group fed Lp showed the lowest gene

expression of pro-inflammatory cytokines and stress-regarding gene (HSP70) among groups. It may be associated with Lp supplementation as the group fed Lp was increased c9, t11-CLA content and c9, t11-CLA was widely studied its anti-inflammatory effect by acting as a PPAR-gamma activator (Bassaganya-Riera et al., 2002). However, there was limitation of collecting liver and cecum samples during experimental period. Thus, further researches are needed to elucidate the probiotic effects on host inflammatory and stress state.

As probiotics were mainly activated in the gut and the gut microbiota is involved in host physiology variously, the effects of flaxseed and probiotics (LP and T2) on laying hen gut microbiota were evaluated. Also, flaxseed was reported to affect gut microbiota (Lee *et al.*, 2016b). In the model of chicken, cecum was mostly used because it contains most diverse and large amount of microbial communities (Zhu *et al.*, 2002). Thus, the cecum samples were collected on the end of the feed experiment. As a result, some significant effects of flaxseed and probiotics were monitored. Alpha diversity was decreased when fed Lp similar to other study with mouse that there were similar result that mixture of 2 lactobacilli decreased the alpha diversity index (Jeung *et al.*, 2018). The alteration of the gut microbiota with probiotic administration were observed in this study similar to other studies. For example, *Lactobacillus* GG modulate gut microbiome reducing *Enterobacteriaceae* and increasing *Clostridiales* (Bajaj *et al.*, 2014). The study using *Bacillus licheniformis* also reported alteration of gut microbiota (Xu *et al.*, 2018). As microbial metagenome prediction using PICRUSt was based upon 16S

rRNA database, the alteration of taxa induced changes in the predicted metagenome (Langille *et al.*, 2013). In this study, 'phosphotransferase (PTS) system' was reduced its abundance by Lp administration. Although there was few study analyzing the effect of probiotics on phosphotransferase system of gut microbiota, it can be assumed that its reduction may increase host nutrient efficiency since *Firmicutes* including *Lactobacillus* species were used phosphotransferase system to transport carbohydrate source from outside (Looft *et al.*, 2014). In other words, Lp administration may have positive effects on host digestion efficiency by inhibiting gut microbiota taking nutrients from host. Actually, it was reported that *Lactobacillus* supplementation increase host nutrition digestibility (Yu *et al.*, 2008).

Monitoring laying performance and egg quality are important as well as investigating livestock health state by collecting their body samples. Also, it can be easily monitored because it is well visible. Therefore, they were also monitored to observe effects of flaxseed and probiotics. Contrary to the study 1 using 33-week-old laying hen, flaxseed didn't show significant improvement on all laying performance traits in study 2 using 52-week-old laying hen. It may suggest that the effect of nutraceutical such as flaxseed varies according to the growth stage of the livestock since livestock altered their physiology and the gut microbiota to the time order (Han *et al.*, 2018a). Meanwhile, each probiotics showed distinct effects on laying performance. Lp induced improvement on laying performance while T2 had adverse effects on them. Associated with results of predicted metagenome,

Lp-induced phosphotransferase system reduction may improve host nutrient digestibility, which resulted in laying performance improvement. On the other hand, T2 increased the activation of energy metabolism, which may be depriving nutrients from host so that laying performance got worse.

In the study 2, the physiological effects of flaxseed and probiotics were assessed (Table 21). Although more studies are required, these findings will broaden the understanding the importance of nutraceutical selection suitable to livestock and its growth stage. Also, the experimental design of this study will be applied to evaluate novel nutraceutical as feed additives.

Table 21. Summary of study 2.

Contents	Results
Probiotics characterization	Selected LP177 as probiotics (High production yield, CLA-related enzyme expression) Selected BLT2 as probiotics (High production yield, Fatty acid desaturase expression)
***In vivo* laying hen feed study (flaxseed + probiotics)**	Effects of flaxseed - Egg omega-6/omega-3 ratio ↓ - Inflammatory indices ↓ (serum cytokine, Liver mRNA) - Laying performance ↑ (average egg weight, feed conversion ratio) Effects of Lp - Egg CLA content ↑ - Inflammatory indices ↓ (serum cytokine, Liver mRNAs esp. COX2) - Stress indices ↓ (Liver HSP70 mRNA) - Laying performance ↑↑ (average egg weight, feed conversion ratio) Effects of BLT2 - Inflammatory indices ↓ (serum cytokine, Liver mRNA) - Stress indices ↓ (serum corticosterone, Liver mRNA) - Laying performance ↓ (average egg weight, feed conversion ratio, hen-day egg production)

Study 3. Physiological effects of flaxseed and probiotics in laying hen with overcrowding stress condition

1. Introduction

Excessive or chronic stress induces damage to host, which is impairing their physiology and lowering the performance in both human and livestock (Ceballos *et al.*, 2018). As livestock animals were raised in harsh environment, they are threatened by various stress factors. For example, livestock suffered from high-density farming, heat, cold, long transportation and unsanitary housing (Olfati *et al.*, 2018). In particular, overcrowding stress caused by high-density farming and unsanitary housing are representatives of factors that can act chronically throughout the overall raising period. Moreover, overcrowding stress is an inevitable part of the livestock industry focusing on efficient production whereas unsanitary housing can be improved by continuous cleanliness management.

Actually, previous studies were reported about the adverse effects of stress on livestock. Heat stress were reported to reduce reproduction in cow (Sirotkin and Kacaniova, 2010), alter body composition parameters in pig (Boddicker *et al.*, 2014) and reduce egg production in laying hens (Mashaly *et al.*, 2004). Regard to overcrowding stress, some researchers also studied. Zhang *et al.* reported that high

stocking densities could decrease growth performance of broilers (Zhang *et al.*, 2013). Also, some studies were suggested that overcrowding stress provoked physiological changes such as disruption of body component by stress-induced oxidative stress (Rashidi *et al.*, 2019), induction of pro-inflammatory cytokines (Kang *et al.*, 2011) and increment of mortality (Rashidi *et al.*, 2019).

Meanwhile, there were some trials to alleviate livestock stress state by feed additives. Wang *et al.* reported that the levels of oxidative stress and inefficient feed efficiency were improved by supplementing anti-oxidant nutraceutical to broilers under high ambient temperature condition. Likewise, polyphenol such as resveratrol were fed to reduce various stress effects on livestock animal (Liu *et al.*, 2014;Zhang *et al.*, 2017)

Also, probiotics fed to laying hen could improve laying performance and reduce the effects of overcrowding stress (Ferrante *et al.*, 2009;Cesari *et al.*, 2014). Moreover, it can be assumed that nutraceutical with anti-inflammatory properties such as flaxseed may be applied to alleviate stress state because of the crosstalk between stress and inflammation.

The aim of the study 3 was to investigate the physiological effects of flaxseed and probiotics on laying hen under overcrowded state (Table 22). First, the physiological effects of overcrowding stress were investigated in study 3-1. To accomplish this goal, 52-week-old laying hens were divided into 2 groups (**Control, Overcrowded**) by stocking density (**Control**: 5 head/cage,

Overcrowded: 8 head/cage) Then, they were monitored with the changes of egg PUFA profile, serum pro-inflammatory cytokine levels and serum corticosterone levels during 4-week-experiment. Also, their liver and ceca samples were collected at the end of the experiment to analyze their physiological state. Liver samples were used to detect gene expression of inflammatory cytokines, lipid mediator synthesis and HSP70 gene expression. Ceca samples were used to analyze cecum microbial community and its predicted metagenome. Then, its effect on laying performance and egg quality were also investigated.

In study 3-2, flaxseed and probiotics were evaluated their possibility to alleviate adverse effects of overcrowding stress with 52-week-old laying hens. Lintex 170 as flaxseed supplements and *Lactobacillus plantarum* 177 (Lp), *Bacillus licheniformis* T2 (T2) screened in study 2 were used as probiotics. The traits that monitored to investigate physiological effects were same as study 3-1. The laying performance and egg quality were also checked.

Table 22. Summarization of study 3.

Study	Category	Item
<Study 3-1> *In vivo* validation of overcrowding stress on laying hen <Study 3-2> *In vivo* validation of flaxseed and probiotics as feed additives when overcrowding condition on laying hen	Experimental design	Animal (laying hen)
		Stress condition
		Sampling (Egg, blood, liver, cecum)
		Feed information
	Effects on PUFAs	Multi-variant analysis
		Relative concentrations of fatty acid profile
	Inflammatory and stress indices	Inflammatory indices
		Stress indices
	Gut microbiota	Composition of OTUs (Richness, Diversity, Relative abundance)
		Metagenome prediction
	Laying performance	Performance
		Egg quality

2. Materials and Methods

1) Animals and treatment

The experiment using laying hens (Study 3-1: n = 39, study 3-2: n=135; 52-week-old; *Lohmann Brown-Lite*) was conducted at Seoul National University animal farm (PyeongChang, Republic of Korea). The protocol for this experiment was reviewed and approved by the Institutional Animal Care and Use Committee of Seoul National University (IACUC No. SNU-180219-1).

In study 3-1, the hens were divided randomly into two groups as follows: 1) control group (**Control**, five hens per cage); 2) overcrowded group (**Overcrowded**, eight hens per cage) and three cages per group respectively. Both groups were fed commercial basal diet daily with 120 g per head.

In study 3-2, the groups consisted of one normal state group (**5C**) and five overcrowded state groups (**8C, 8F, 8FL, 8FB, 8FBL**). Overcrowded state groups were further subdivided according to their diet as follows: 1) commercial basal diet group (**8C**); 2) basal diet with 1.8% (w/w) of Lintex 170 **(8F)**; 3) basal diet with 1.8% (w/w) of Lintex 170 and 0.1% (w/w) Lp **(8FL)**; 4) basal diet with 1.8% (w/w) of Lintex 170 and 0.1% (w/w) T2 (8**FB**); 5) basal diet with 1.8% (w/w) of Lintex 170, 0.05% (w/w) Lp, and 0.05% (w/w) T2 (8**FBL**) (Table 23). Commercial basal diet used in study 3 was same commercial product used in study 1 and its formulation was described in study 1. Feed was offered 120 g per bird

daily and fresh water was offered *ad libitum* during the 4-week experimental period.

Table 23. Feed information of experimental groups used in study 3-2.

Group	Feed
5C	**Commercial basal diet (C)**
8C	**Commercial basal diet (C)**
8F	**C + 1.8% (w/w) Lintex 170**
8FL	**C + 1.8% (w/w) Lintex 170 + 0.1% (w/w) LP177**
8FB	**C + 1.8% (w/w) Lintex 170 + 0.1% (w/w) BLT2**
8FBL	**C + 1.8% (w/w) Lintex 170 + 0.05% (w/w) LP177 + 0.05% (w/w) BLT2**

2) Samples and data collection

All samples used in study 3 such as egg, blood, liver and ceca samples were collected and analyzed as described in study 2. The traits to check physiological effects on laying hen were also same as study 2.

3) Gut microbiome analysis

The overall step of DNA extraction, amplification of 16S rRNA gene, sequencing, microbial community analysis and prediction of the functions of the microbial communities were described in study 2. The 16S rRNA gene sequences determined in this study were deposited in the NCBI SRA database with accession number SRP197263 for the study 3 experiments. To compare effect of overcrowding stress on predicted gut metagenome between 2 groups in study 3-1, principal component analysis (PCA) was performed using STAMP v2.1.3 (Parks *et al.* 2014).

3. Results

1) Effects of overcrowding stress

To investigate physiological effects of overcrowding stress, fatty acid profile, inflammatory indices, stress indices, gut microbiota, laying performance and egg quality were monitored as same as study 2.

(1) Changes in fatty acid profile under overcrowding stress

The fatty acid profile between two groups were estimated to investigate effects of overcrowding stress. The ratio of omega-6 to omega-3 between two groups was no significant difference during experimental period (Figure 49). The average values of the ratio on week 4 were 21.76 (Control), 23.83 (Overcrowded) respectively.

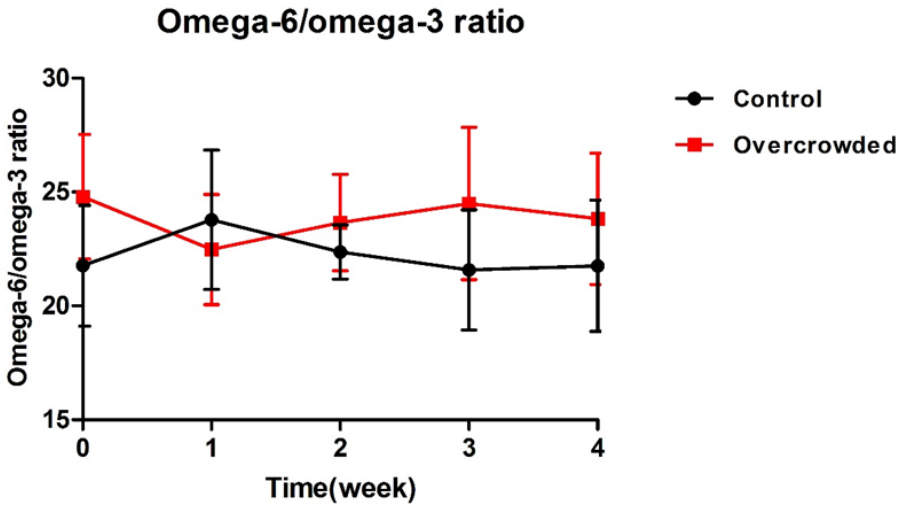

Figure 49. Changes in egg omega-6 to omega-3 fatty acid ratios during the study 3-1.

The ratios were calculated by data of gas chromatography standardized with FAME 37 mix. Each group was 5 replicates.

More detailed fatty acid profiles were also investigated to identify significantly changed fatty acid using PLS-DA plot and VIP plot. Using PLS-DA plot, the distribution of overall fatty acid profile between two groups was confirmed, which was clustered by stress condition (Figure 50A). To determine which fatty acid contributed to separate fatty acid profiles, VIP plot was used and identified the mostly influenced fatty acids (Figure 50B).

Next, the relative concentration of each fatty acids were compared between two groups followed by generating bar plots which was significantly differed among group (Figure 51). Margaroleic acid, a kind of MUFA with 17 carbons, and gamma-linolenic acid, a kind of omega-6 fatty acid with 3 double bonds, were showed significantly higher in the Control group than the Overcrowded group. The detailed fatty acid profile were listed in Table 24.

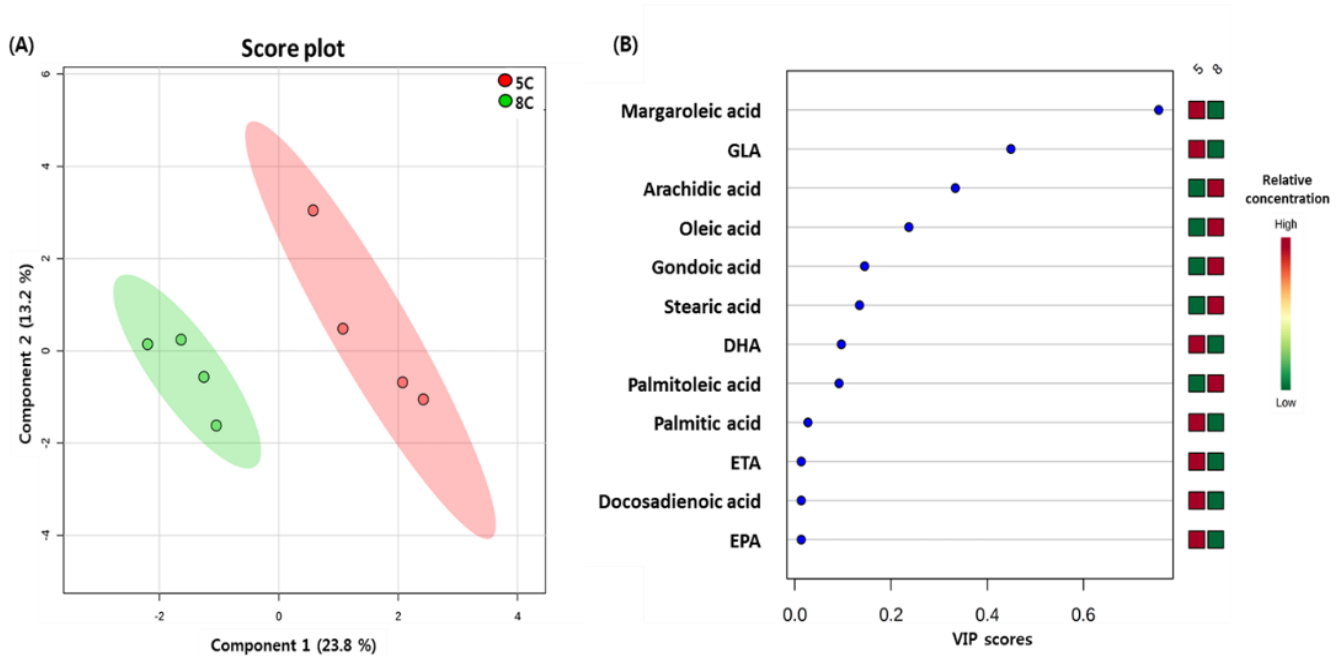

Figure 50. Multi-variant analysis of fatty acid profile in week 4 egg samples with/without overcrowding stress.

(A) Partial least squares discriminant analysis (PLS-DA) plot of fatty acids (B) Variable importance in projection (VIP) plot of 10 fatty acids (VIP scores Top 10) that were differentially regulated between groups.

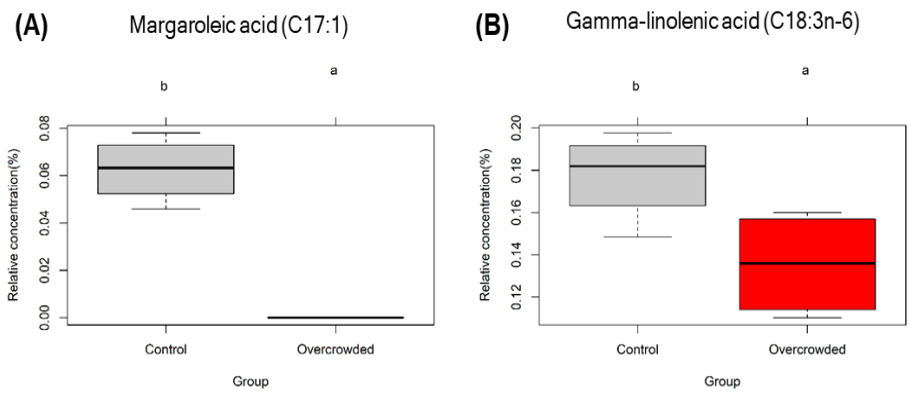

Figure 51. Bar plots of relative concentrations of fatty acids in week 4 egg samples.

(A) Monounsaturated fatty acid (Margaroleic acid).
(B) Omega-6 fatty acid (GLA).

Table 24. Relative fatty acid concentration between groups on week 4 egg samples of study 3-1[1].

Egg fatty acid (%)	Control	Overcrowded	*p-value*
Myristic acid (C14:0)	0.51±0.06[a]	0.55±0.10[a]	0.518
Myristoleic acid (C14:1)	0.12±0.03[a]	0.14±0.03[a]	0.371
Pentadecanoic acid (C15:0)	0.09±0.02[a]	0.08±0.01[a]	0.560
Palmitic acid (C16:0, PA)	27.04±0.68[a]	20.37±12.88[a]	0.341
Palmitoleic acid (C16:1)	3.55±0.52[a]	4.06±0.66[a]	0.275
Margaric acid (C17:0)	0.22±0.01[a]	0.20±0.04[a]	0.542
Margaroleic acid (C17:1)	0.06±0.01[b]	0.00±0.00[a]	<0.001***
Stearic acid (C18:0, SA)	7.38±0.71[a]	8.40±1.38[a]	0.235
Oleic acid (C18:1n9c, OA)	40.39±1.62[a]	46.22±6.94[a]	0.153
Linoleic acid (C18:2n-6c, LA)	16.8±1.46[a]	16.31±3.6[a]	0.806
Arachidic acid (C20:0)	0.02±0.00[a]	0.02±0.00[a]	0.093
Gamma-linolenic acid (C18:3n6, GLA)	0.18±0.02[b]	0.14±0.03[a]	0.043*
Gondoic acid (C20:1)	0.05±0.00[a]	0.10±0.07[a]	0.226
α-linolenic acid (C18:3n-3, ALA)	0.34±0.08[a]	0.30±0.07[a]	0.416
Eicosadienoic acid (C20:2)	0.15±0.01[a]	0.16±0.04[a]	0.910
Dihomogamma-linolenic acid (C20:3n-6, DHGLA)	0.21±0.02[a]	0.21±0.04[a]	0.904
Eiosatrienoic acid(C20:3n-3, ETA)	0.00±0.01[a]	0.00±0.00[a]	0.356
Arachidonic acid (C20:4)	2.31±0.14[a]	2.26±0.35[a]	0.792
Docosadienoic acid (C22:2n6)	0.01±0.02[a]	0.00±0.00[a]	0.356
Eicosapentaenoic acid(C20:5n-3, EPA)	0.00±0.00[a]	0.00±0.00[a]	0.356
Docosahexaenoic acid(C22:6n-3, DHA)	0.57±0.07[a]	0.51±0.08[a]	0.270

[1] Each value represents the mean±SD of five replicate eggs.

[a-b] Means in a row without a common superscript letter differ as analyzed using unpaired t test (*p-value<0.05 **p-value<0.01 ***p-value<0.001).

The content of c9, t11-CLA in egg samples were also monitored once a week to determine whether overcrowding stress affected its content (Figure 52). As a result, there was no significant difference between the amount of c9, t11-CLA of two groups during overall experimental period (Control: 0.202 ± 0.042 mg/g egg yolk, Overcrowded: 0.237 ± 0.042 mg/g egg yolk).

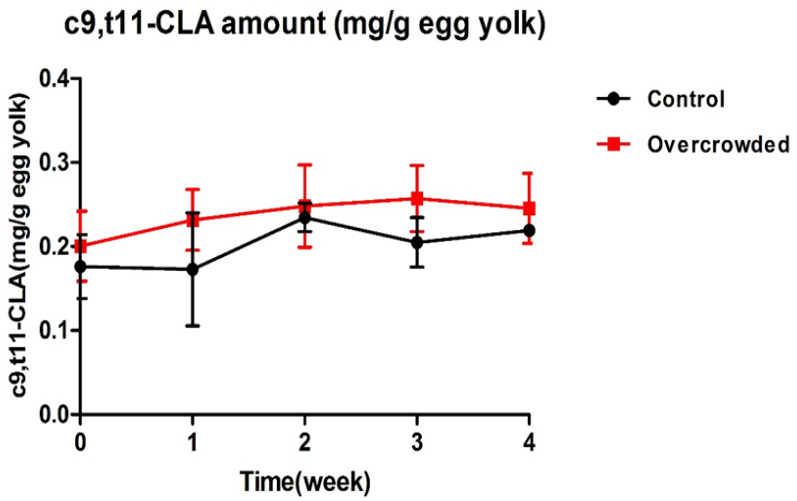

Figure 52. Changes of c9, t11-CLA amount in eggs during study 3-1.

The c9, t11-CLA amounts of egg samples were calculated by data of gas chromatography standardized with FAME 37 mix. Each group was 5 replicates.

(2) Effects on inflammatory indices

① Serum pro-inflammatory cytokine levels

Serum level of pro-inflammatory cytokine were monitored to evaluate the effect of overcrowding stress on inflammatory processes (Figure 53). Although there were no significant differences in levels of TNF-alpha, IL-1beta, or IL-6 in serum samples, the Overcrowded groups had tendency to increase the levels of TNF-alpha (Figure 53A) and IL-1beta (Figure 53B).

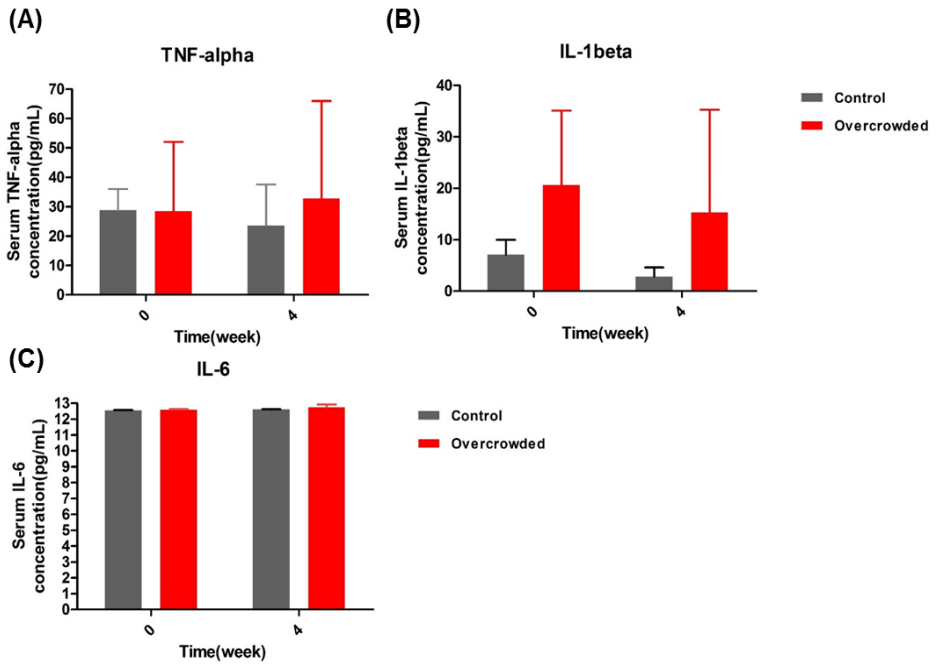

Figure 53. Serum pro-inflammatory cytokine level in experimental hens on week 0 and 4.

(A) TNF-α (B) IL-1β (C) IL-6.

② Inflammatory-related gene expression in liver

Liver mRNA samples collected from each group were extracted and used to investigate expression levels of inflammatory-related genes (***tnf-alpha***, ***il-1beta***, ***il-6***, ***cox-2***) which were checked in study 2 (Figure 54). Although there was no significant difference among groups in pro-inflammatory cytokine-encoded genes, there were tendency to increase expression levels of ***tnf-alpha*** (Figure 54A), ***il-1beta*** (Figure 54B), ***il-6*** (Figure 54C) and ***cox-2*** (Figure 54D) in the Overcrowded group.

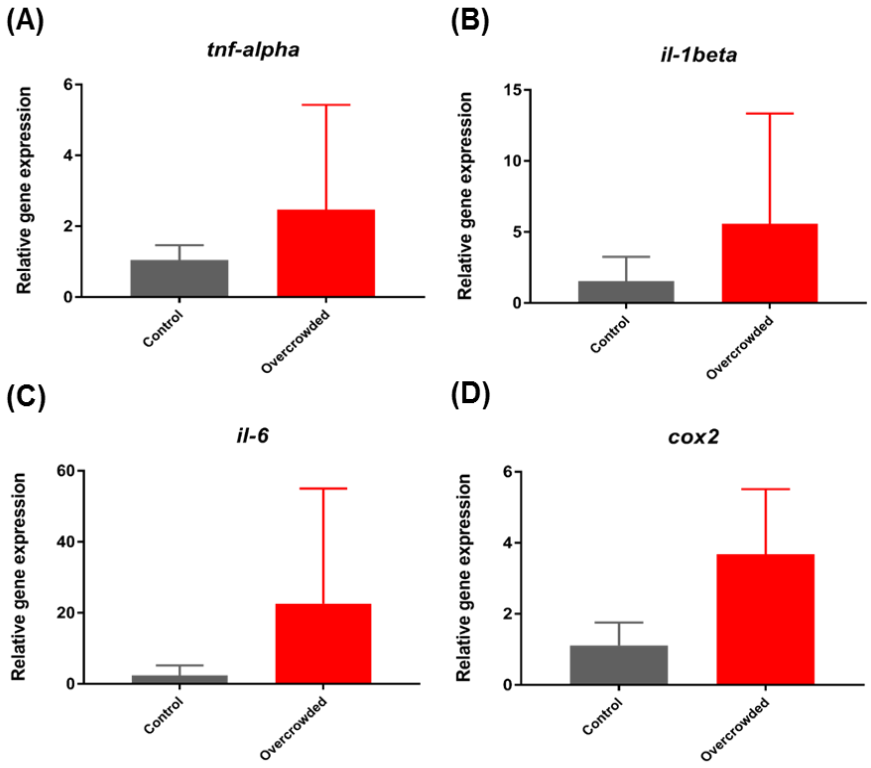

Figure 54. Analysis of gene expression of pro-inflammatory cytokines and lipid mediator synthesis in experimental hens liver sample at week 4.

(A) The transcription expression of pro-inflammatory cytokine *tnf-α*, (B) *il-1β*, and (C) *il- 6*, and (D) lipid mediator synthesis related gene *cox2* relative to β-actin was quantified by qRT-PCR.

(3) Effects on stress indices

To investigate the effect of overcrowding stress on the stress state of laying hen, three stress indices (serum corticosterone level, liver HSP70 gene expression level and H:L ratio) were examined (Figure 55). Even though there was no significant difference in all indices, ***hsp70*** (Figure 55B) tended to increase under overcrowding stress condition contrary to serum corticosterone level (Figure 55A) and H:L ratio (Figure 55C).

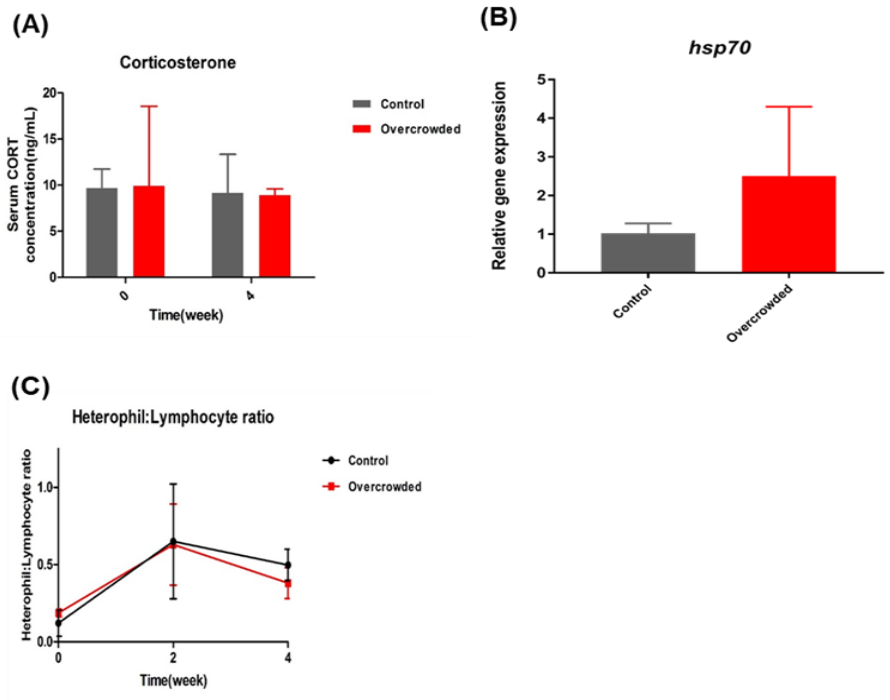

Figure 55. Effects of overcrowding stress on stress indices in experimental hens.

(A) Serum corticosterone level at weeks 0 and 4.
(B) HSP70 gene expression of liver samples at week 4.
(C) Changes in H:L ratios at weeks 0,2 and 4.

(4) Effects on gut microbiota

① Microbial community analysis

First, the microbial diversity were determined within samples (alpha diversity) and between samples (beta diversity). The observed OTUs, which was used as a parameter of alpha diversity, were as follows: 1) Control: 370.30 (±24.49); 2) Overcrowded: 346.18 (±16.11) respectively. There was no significant difference between groups in spite of tendency to decrease in the Overcrowded group (Figure 56A). The beta diversity were visualized by PCoA plot based on unweighted and weighted UniFrac distances of the 16S rRNA sequences. It revealed that the microbial communities assessed by weighted UniFrac distances were distinctly separated by overcrowding stress condition (Figure 56B)

To identify distinct differed bacteria between two groups, relative abundance of taxa among groups were compared at the phylum and genus level. 18 phyla and 212 genera were detected. At the phylum level, all groups shared the following 15 phyla: *Euryarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Elusimicrobia*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*, *Spirochaetes*, *Synergistetes*, *TM7*, *Tenericutes*, and *Verrucomicrobia*. Also, there were 1 phylum (*Elusimicrobia*) that significantly differed between groups (Table 25).

At the genus level, all groups shared 140 genera, and there were 8 genera that significantly differed between groups. *Mucilaginibacter*, *Lactobacillus*, *Enterobacter* and *Pseudomonas* were significantly abundant in the Control group compared to the Overcrowded group whereas *Propionibacterium*, *Bacteroides*, *Parabacteroides*, and *[Clostridium]* were significantly abundant in the Overcrowded group compared to the Control group (Table 25).

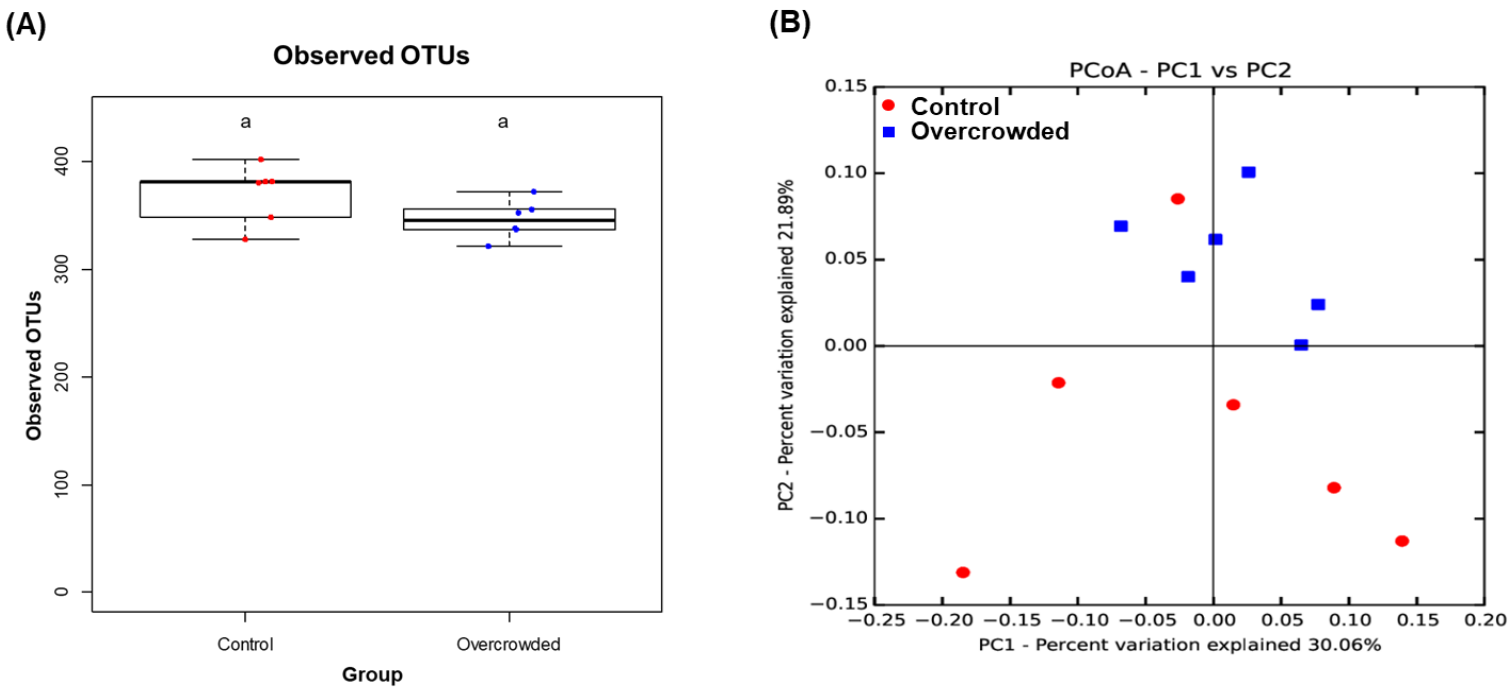

Figure 56. Diversity of gut microbiota of laying hen fed flaxseed and probiotics.

(A) Alpha diversity index (Observed OTUs).
(B) Principal coordinate analysis (PCoA) plot based on weighted UniFrac distances.

Table 25. Relative abundances of phyla and genera at laying hen with overcrowding stress condition.

Taxon	Relative abundance (%)		*p-value*
	Control	Overcrowded	
Phylum			
Elusimicrobia	2.84 ± 1.38^{b}	1.25 ± 0.94^{a}	0.041*
Genus			
Propionibacterium	0.00 ± 0.00^{a}	0.01 ± 0.01^{b}	0.026*
Bacteroides	9.10 ± 2.39^{a}	15.25 ± 3.91^{b}	0.008**
Parabacteroides	1.04 ± 0.33^{a}	2.33 ± 0.93^{b}	0.009**
Mucilaginibacter	0.03 ± 0.02^{b}	0.00 ± 0.00^{a}	0.009**
Lactobacillus	2.94 ± 1.56^{b}	1.27 ± 0.31^{a}	0.027*
[Clostridium]	0.01 ± 0.02^{a}	0.08 ± 0.06^{b}	0.043*
Enterobacter	0.01 ± 0.01^{b}	0.00 ± 0.00^{a}	0.039*
Pseudomonas	0.21 ± 0.16^{b}	0.05 ± 0.05^{a}	0.035*

Data shown as the mean ± standard deviation (SD).
One-way ANOVA with Tukey's post hoc test was used. Within a row, different superscript letters indicate significant difference (**p-value* < 0.05; ***p-value<0.01*).

② Predicted functions of the gut microbiota of laying hen under overcrowding stress condition

To determine the effects of overcrowding stress on the functions of the gut microbiota, the KEGG pathways were predicted using PICRUSt followed by one-way ANOVA and post hoc TUKEY's HSD test using the relative abundances of groups. Then, several KEGG pathways were significantly differentially identified in the microbiota of the two groups. 'Insulin signaling pathway', 'Biosynthesis of unsaturated fatty acids', 'African trypanosomiasis', 'Staphylococcus aureus infection', 'Fluorobenzoate degradation', 'Pyruvate metabolism', 'beta-Alanine metabolism', 'Taurine and hypotaurine metabolism', 'Pantothenate and CoA biosynthesis', 'Tryptophan metabolism', 'Metabolism of Terpenoids and Polyketides', 'Chagas disease (American trypanosomiasis)' and 'Bacterial invasion of epithelial cells' were predicted at significantly higher levels in the microbiota of the Control group than those in that of the Overcrowded group. Meanwhile, 'Adipocytokine signaling pathway', 'Phenylpropanoid biosynthesis', 'Cyanoamino acid metabolism' and 'Bacterial toxins' were predicted at significantly higher levels in the Overcrowded group (Figure 57).

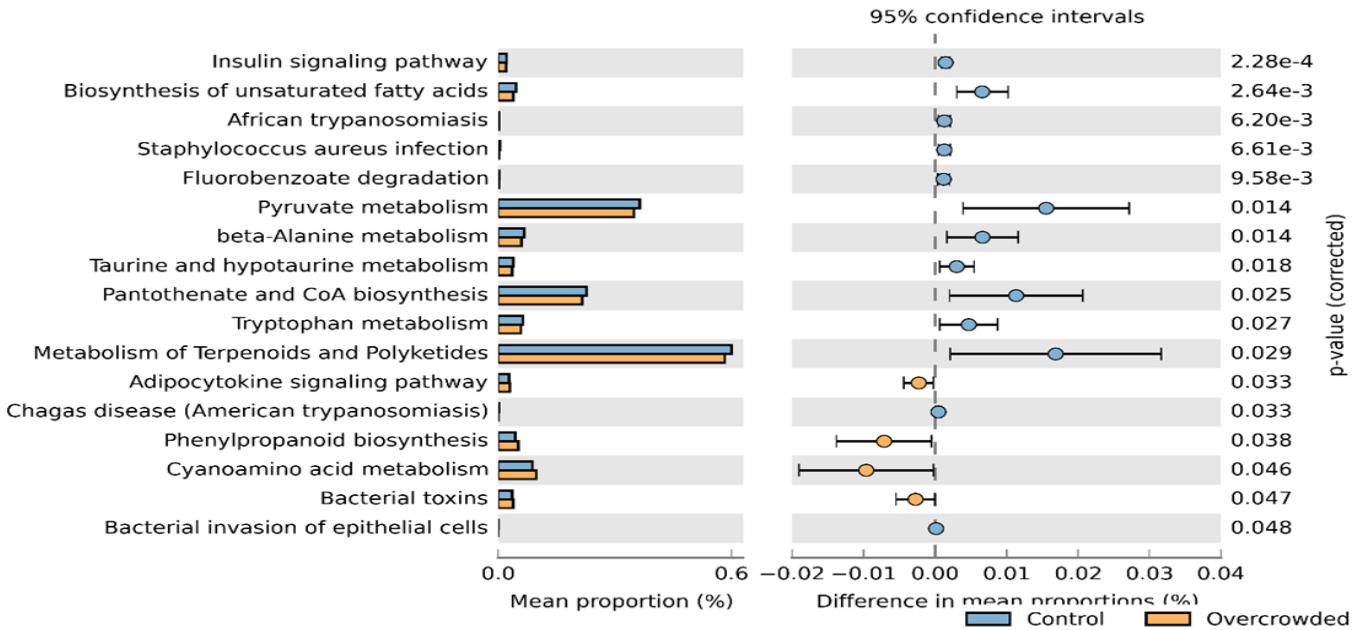

Figure 57. Changes in laying hen gut microbiota function when overcrowding stress condition.

The microbial functions were predicted using PICRUSt at the third level of the KEGG pathway and were expressed as relative abundances. The difference between the levels of the predicted functions were tested using a two-sided Welch's *t*-test, and $P < 0.05$ was considered significant.

(5) Laying performance and egg quality under overcrowding stress condition

The effects of overcrowding stress on laying performance and egg quality were evaluated as same traits used in study 1 and 2.

Overall, average egg weight, feed conversion ratio and hen-day egg production were significantly differed by 4 week feed experiment (*p-values* <0.001, 0.007, <0.001, respectively), which were confirmed adverse effects of overcrowding stress. While there was no significant difference on all the traits do evaluate laying performance on week 1, 2 and 3, the values of average egg weight (Control: 60.02±1.88; Overcrowded: 54.17±1.47) and feed conversion ratio (Control: 1.97±0.07; Overcrowded: 2.17±0.06) were significantly differed on week 4. As a result, the overcrowding stress reduced average egg weight and hen-day egg production, and increased the feed conversion ratio, which all showed adverse effects of overcrowding stress (Table 26).

Meanwhile, the indices of egg quality such as albumen height, Haugh unit and eggshell thickness showed no predominant improvement by overcrowding stress except the Haugh unit values of week 1 (Control: 101.03±3.92, Overcrowded: 93.79±2.98; *p-value* = 0.026) between groups (Table 27).

Table 26. Effects of overcrowding stress on performance of laying hens during 52-56 weeks of age[1].

Items	Group		*p-value*
	Control	Overcrowded	
Week 1			
Daily feed intake (g day^{-1} hen^{-1})	119.73±0.02[a]	115.80±2.59[a]	0.268
Average egg weight (g/egg)	57.64±2.39[a]	52.81±2.39[a]	0.178
Feed conversion ratio (g feed/g egg mass)	2.10±0.09[a]	2.22±0.11[a]	0.418
Hen-day egg production (%)	98.10±4.04[a]	88.69±4.24[a]	0.134
Week 2			
Daily feed intake (g day^{-1} hen^{-1})	119.71±0.00[a]	117.56±1.16[a]	0.205
Average egg weight (g/egg)	57.33±1.01[a]	52.75±2.51[a]	0.129
Feed conversion ratio (g feed/g egg mass)	2.09±0.04[a]	2.26±0.11[a]	0.195
Hen-day egg production (%)	94.29±1.74[a]	86.90±4.21[a]	0.144
Week 3			
Daily feed intake (g day^{-1} hen^{-1})	119.71±0.00[a]	118.50±1.08[a]	0.378
Average egg weight (g/egg)	56.99±1.26[a]	53.55±1.47[a]	0.102
Feed conversion ratio (g feed/g egg mass)	2.11±0.05[a]	2.22±0.07[a]	0.181
Hen-day egg production (%)	93.33±2.06[a]	87.50±2.41[a]	0.091
Week 4			
Daily feed intake (g day^{-1} hen^{-1})	117.43±2.29[a]	116.88±2.17[a]	0.871
Average egg weight (g/egg)	60.02±1.88[b]	54.17±1.47[a]	0.031*
Feed conversion ratio (g feed/g egg mass)	1.97±0.07[a]	2.17±0.06[b]	0.042*
Hen-day egg production (%)	96.19±3.21[a]	88.10±2.48[a]	0.070
Week 1 to 4			
Daily feed intake (g day^{-1} hen^{-1})	119.15±0.57[a]	117.18±0.85[a]	0.070
Average egg weight (g/egg)	58.00±0.84[b]	53.32±0.96[a]	<0.001***
Feed conversion ratio (g feed/g egg mass)	2.07±0.03[a]	2.22±0.04[b]	0.007**
Hen-day egg production (%)	95.48±1.42[b]	87.80±1.63[a]	<0.001***

[1] Each value represents the mean±SD of three replicate cages (five hens per control cage and eight hens per overcrowded cage).

[a-b] Means in a row without a common superscript letter differ as analyzed using unpaired t test (*p-$value$<0.05, ** p-$value$<0.01, ***p-$value$<0.001).

Table 27. Effects of overcrowding stress on egg quality of laying hens during 52-56 weeks of age[1].

Items	Time, week	Group		*p-value*
		Control	Overcrowded	
Albumen height, mm	**0**	8.25±2.18	8.82±2.00	0.711
	1	10.40±0.94	9.07±0.67	0.061
	2	7.95±1.35	8.30±1.04	0.696
	3	8.57±0.35	9.30±1.47	0.375
	4	8.45±0.53	9.90±1.15	0.062
Haugh unit	**0**	89.77±12.89	93.21±9.23	0.679
	1	101.03±3.92[b]	93.79±2.98[a]	0.026*
	2	88.20±8.67	92.10±4.89	0.464
	3	92.43±1.02	96.02±6.59	0.323
	4	91.12±3.01	97.84±5.59	0.079
Egg shell thickness, mm	**0**	0.39±0.01	0.39±0.02	0.626
	1	0.40±0.04	0.37±0.02	0.336
	2	0.38±0.02	0.38±0.02	0.598
	3	0.39±0.02	0.40±0.01	0.779
	4	0.39±0.01	0.38±0.02	0.409

[1]Each value represents the mean±SD of ten replicates with fifty hen per replicate.

[a,b] Values within the same row with different superscript letters significantly differ ($p<0.05$).

2) Effects of flaxseed and probiotics under overcrowding stress condition

(1) Changes in fatty acid profile by feeding flaxseed and probiotics under overcrowding stress condition

The fatty acid profile among groups were estimated to investigate effects of overcrowding stress. The ratio of omega-6 to omega-3 were decreased except basal diet-fed group (5C, 8C) since week 1, which is similar to that of study 2 (Figure 58). The average values of the ratio on week 4 were 21.76 (5C), 23.83 (8C), 6.11 (8F), 7.45 (8FL), 6.93 (8FB), 6.63 (8FBL) respectively.

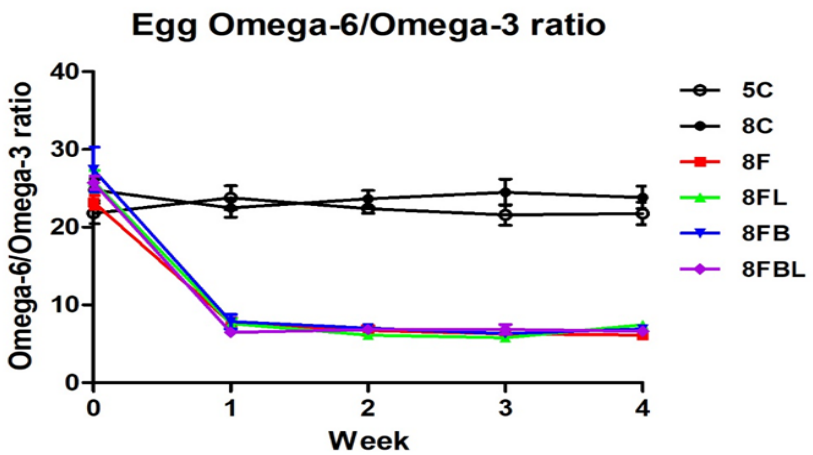

Figure 58. Changes in the ratio of omega-6 to omega-3 fatty acid in eggs during study 3-2.

The ratios were calculated by data of gas chromatography standardized with FAME 37 mix. Each group was 5 replicates.

More detailed fatty acid profiles were also investigated using PLS-DA plot and VIP plot as same as study 2 and 3-1. Using PLS-DA plot, the distribution of overall fatty acid profile among groups was confirmed, which was mainly clustered by flaxseed supplementation. Besides, the 8FL group was distinctly clustered from flaxseed-fed group (8F, 8FL, 8FB, 8FBL), which is similar result with study 2. Also, the 5C group and 8C group were clustered by stress condition consistent results with study 3-1. (Figure 59A). To determine which fatty acid contributed to separate fatty acid profiles, VIP plot was used and identified the mostly influenced fatty acids (Figure 59B). Similar to the VIP plot of fatty acid profile described in study 2, omega-3 fatty acids (ETA, ALA, EPA, and DHA) were the most influential factors to cluster among groups. Also, omega-6 fatty acids (AA, GLA) were ranked 6^{th} and 7^{th} respectively similar to that of study 2 (AA: 7^{th}, GLA: 6^{th}).

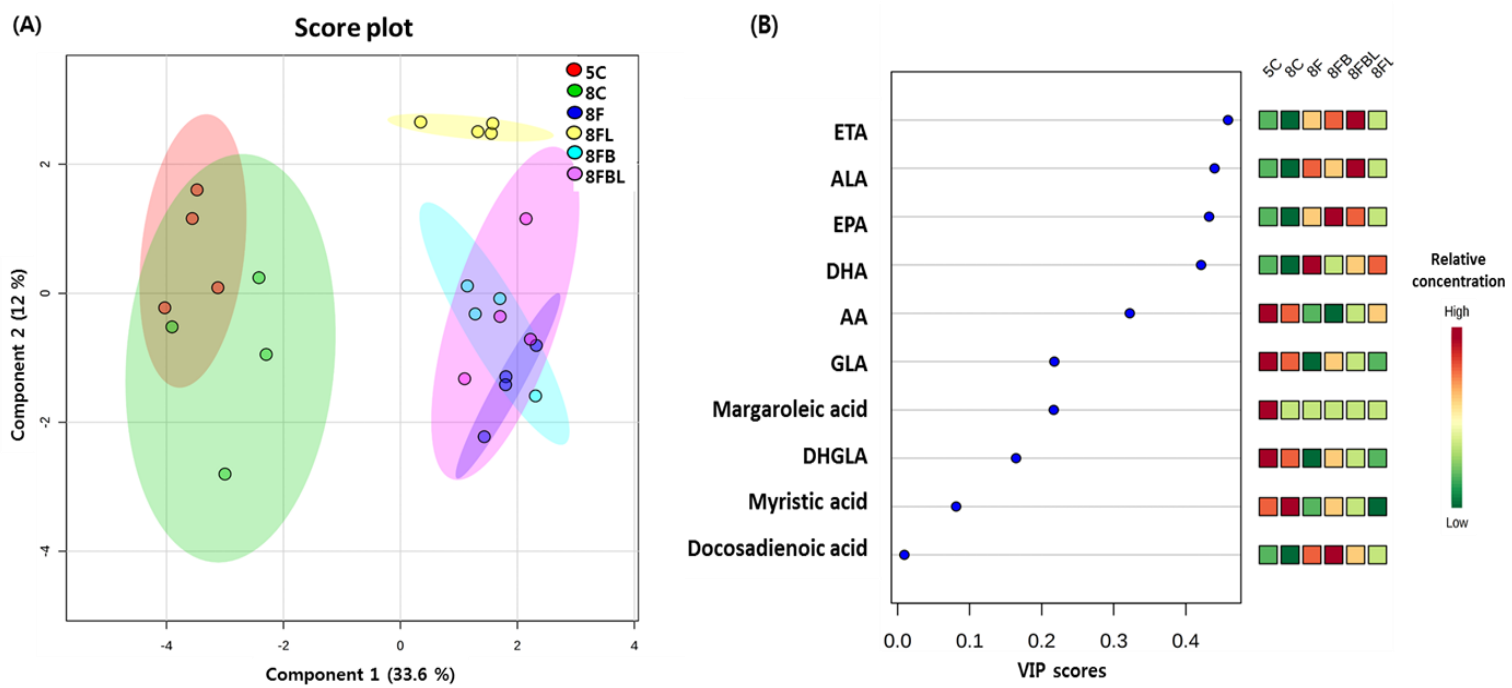

Figure 59. Multi-variant analysis of fatty acid profile in week 4 egg samples.

(A) Partial least squares discriminant analysis (PLS-DA) plot of fatty acids.
(B) Variable importance in projection (VIP) plot of 10 fatty acids (VIP scores Top 10) that were differentially regulated among groups.

The relative concentration of each fatty acids in each groups were also investigated and bar plots of significantly differed fatty acids were generated as same as study 2 and study 3-1 (Figure 60). Similar to study 2, the enrichment of omega-3 fatty acids (ALA, ETA, EPA, DHA) (Figure 60I-L) and the reduction of omega-6 fatty acids (GLA, DHGLA, AA) (Figure 60F-H) were confirmed. Interestingly, 8FB group was significantly higher concentration of ETA among groups. There were also several changes in saturated fatty acid and monounsaturated fatty acid content in egg samples. 2 saturated fatty acids (myristic acid and pentadecanoic acid) were significantly differed among groups especially being reduced in 8FL group (Figure 60A-B). Moreover, 3 monounsaturated fatty acids (myristoleic acid, palmitoleic acid, margaroleic acid) were significantly differed among groups. In case of myristoleic acid, 8FL group was significantly reduced its content compared to 5C, 8C, 8F groups (Figure 60C). Palmitoleic acid was significantly decreased only in 5FL group (Figure 60D). Margaroleic acid were only detected in non-stressed group (5C) (Figure 60E). The detailed fatty acid profile of egg samples were listed in Table 28.

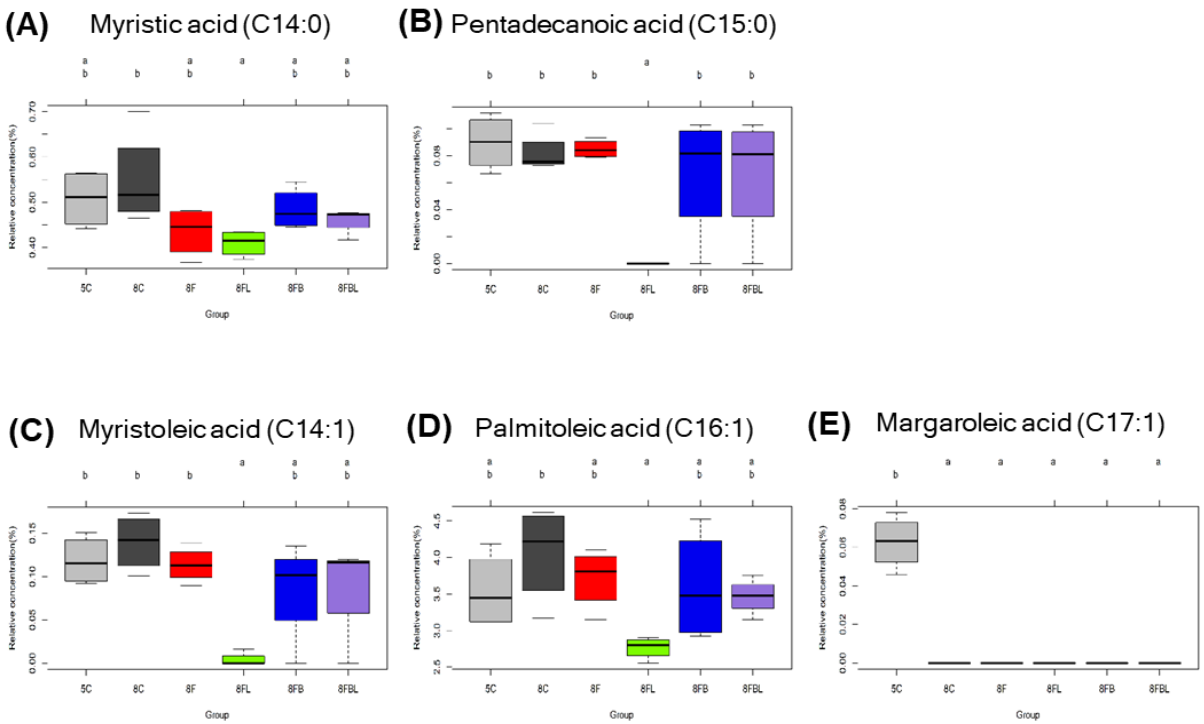

Figure 60. Bar plots of relative concentrations of fatty acids in week 4 serum samples.

(A-B) Saturated fatty acids (Myristic acid, Pentadecanoic acid).
(C-E) Monounsaturated fatty acids (Myristoleic acid, Palmitoleic acid, Margaroleic acid).

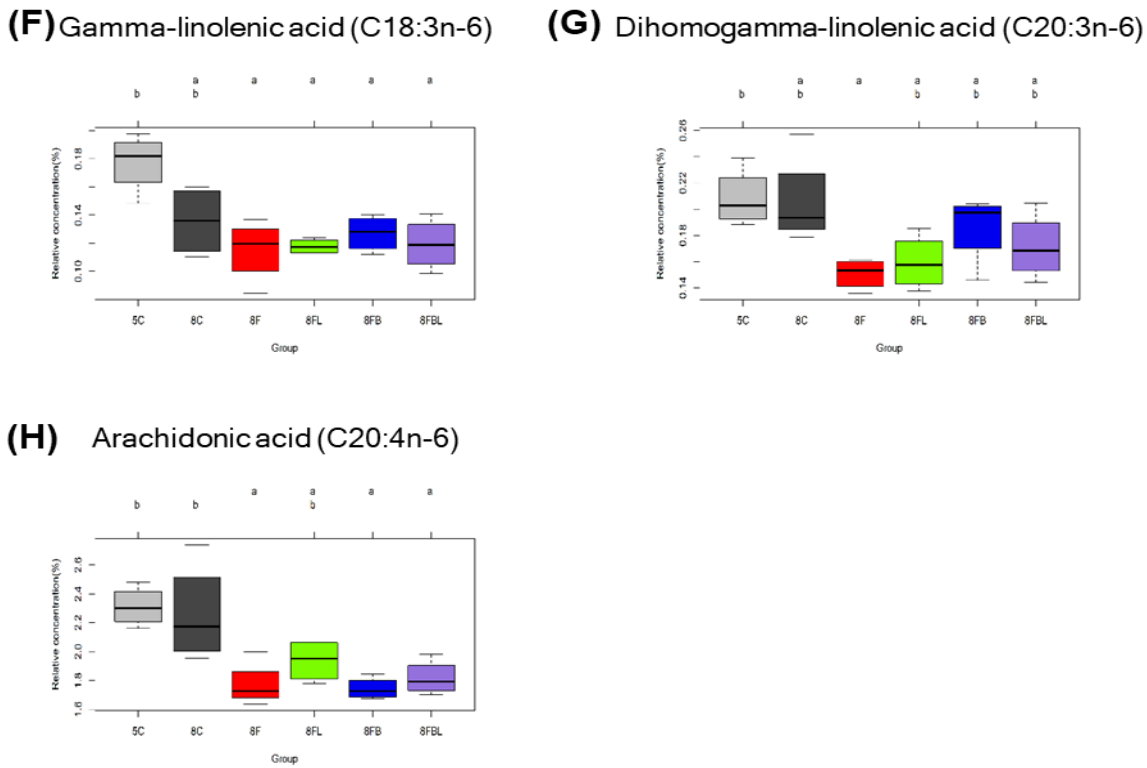

Figure 66. Continued.

(F-H) Omeag-6 fatty acids (GLA, DHGLA, AA).

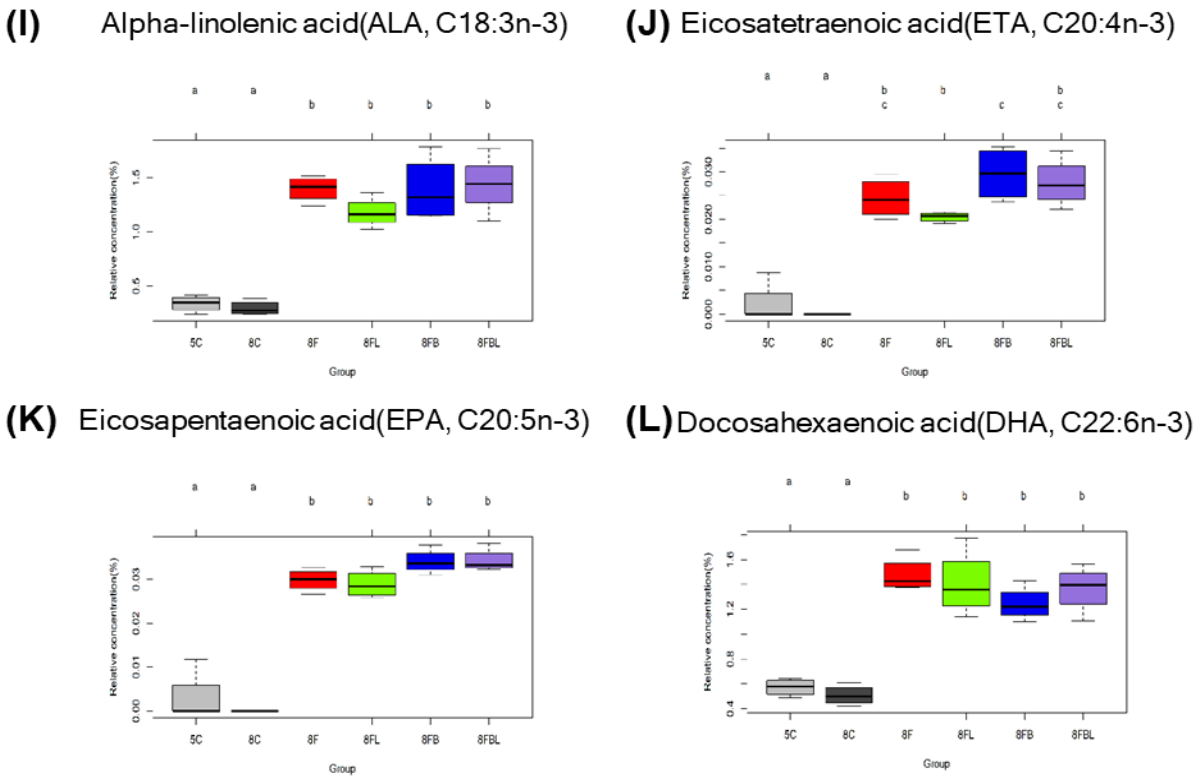

Figure 66. Continued.

(I-L) Omeag-3 fatty acids (ALA, ETA, EPA, DHA).

Table 28. Relative fatty acid concentration among groups on week 4 egg samples of study 3-2[1].

Egg fatty acid (%)	5C	8C	8F	8FL	8FB	8FBL	*p-value*
Myristic acid (C14:0)	0.51±0.06^{ab}	0.55±0.10^{b}	0.43±0.05^{ab}	0.41±0.03^{a}	0.48±0.05^{ab}	0.46±0.03^{ab}	0.048*
Myristoleic acid (C14:1)	0.12±0.03^{b}	0.14±0.03^{b}	0.11±0.02^{b}	0.00±0.01^{a}	0.08±0.06^{ab}	0.09±0.06^{ab}	0.002**
Pentadecanoic acid (C15:0)	0.09±0.02^{b}	0.08±0.01^{b}	0.09±0.01^{b}	0.00±0.00^{a}	0.07±0.05^{b}	0.07±0.05^{b}	0.004**
Palmitic acid (C16:0, PA)	27.04±0.68^{a}	20.37±12.88^{a}	25.5±0.33^{a}	25.18±0.87^{a}	26.32±1.72^{a}	25.74±0.47^{a}	0.567
Palmitoleic acid (C16:1)	3.55±0.52^{ab}	4.06±0.66^{b}	3.72±0.41^{ab}	2.77±0.15^{a}	3.60±0.76^{ab}	3.47±0.25^{ab}	0.049*
Margaric acid (C17:0)	0.22±0.01^{a}	0.20±0.04^{a}	0.20±0.01^{a}	0.24±0.02^{a}	0.22±0.03^{a}	0.22±0.02^{a}	0.311
Margaroleic acid (C17:1)	0.06±0.01^{b}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	<0.001***
Stearic acid (C18:0, SA)	7.38±0.71^{a}	8.40±1.38^{a}	7.27±0.27^{a}	7.50±0.39^{a}	7.75±0.88^{a}	7.15±0.38^{a}	0.272
Oleic acid (C18:1n9c, OA)	40.39±1.62^{a}	46.22±6.94^{a}	41.84±0.75^{a}	41.83±1.18^{a}	40.21±1.43^{a}	41.02±1.90^{a}	0.124
Linoleic acid (C18:2n-6c, LA)	16.80±1.46^{a}	16.31±3.60^{a}	15.65±0.52^{a}	17.00±0.76^{a}	16.25±1.17^{a}	16.57±1.70^{a}	0.924
Arachidic acid (C20:0)	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.00^{a}	0.02±0.01^{a}	0.02±0.00^{a}	0.865
Gamma-linolenic acid (C18:3n6, GLA)	0.18±0.02^{b}	0.14±0.03^{ab}	0.12±0.02^{a}	0.12±0.01^{a}	0.13±0.01^{a}	0.12±0.02^{a}	0.002**
Gondoic acid (C20:1)	0.05±0.00^{a}	0.10±0.07^{a}	0.05±0.00^{a}	0.03±0.02^{a}	0.06±0.01^{a}	0.05±0.00^{a}	0.100
α-linolenic acid (C18:3n-3, ALA)	0.34±0.08^{a}	0.30±0.07^{a}	1.40±0.12^{b}	1.18±0.14^{b}	1.39±0.30^{b}	1.44±0.27^{b}	<0.001***
Eicosadienoic acid (C20:2)	0.15±0.01^{a}	0.16±0.04^{a}	0.13±0.00^{a}	0.16±0.01^{a}	0.16±0.01^{a}	0.16±0.02^{a}	0.501
Dihomogamma-linolenic acid (C20:3n-6, DHGLA)	0.21±0.02^{b}	0.21±0.04^{ab}	0.15±0.01^{a}	0.16±0.02^{ab}	0.19±0.03^{ab}	0.17±0.03^{ab}	0.018

Eiosatrienoic acid(C20:3n-3, ETA)	0.00±0.01[a]	0.00±0.00[a]	0.03±0.00[b]	0.03±0.00[b]	0.03±0.00[b]	0.03±0.00[b]	<0.001***
Arachidonic acid (C20:4)	2.31±0.14[b]	2.26±0.35[b]	1.77±0.16[a]	1.94±0.15[ab]	1.74±0.08[a]	1.82±0.12[a]	<0.001***
Docosadienoic acid (C22:2n6)	0.01±0.02[a]	0.00±0.00[a]	0.01±0.00[a]	0.01±0.01[a]	0.01±0.00[a]	0.01±0.01[a]	0.237
Eicosapentaenoic acid(C20:5n-3, EPA)	0.00±0.00[a]	0.00±0.00[a]	0.02±0.00[bc]	0.02±0.00[b]	0.03±0.01[c]	0.03±0.01[bc]	<0.001***
Docosahexaenoic acid(C22:6n-3, DHA)	0.57±0.07[a]	0.51±0.08[a]	1.48±0.14[b]	1.41±0.27[b]	1.24±0.14[b]	1.37±0.19[b]	<0.001***

[1] Each value represents the mean±SD of five replicate eggs.

[a-b] Means in a row analyzed using one-way ANOVA and post hoc TUKEY's test (*p-value<0.05 **p-value<0.01 ***p-value<0.001).

Next, c9, t11-CLA content in the egg samples were monitored to determine whether Lp-induced CLA enriching effect was maintained under overcrowding stress condition. Even though there was no significant difference among group during experimental period, 5FL group tend to contain highest amount of c9,t11-CLA in egg samples among group on week 4 (0.335 mg/g egg yolk) especially 1.53 fold increase compared to the average content of 5C group (0.219 mg/g egg yolk) which is similar to the result of study 2 (The c9, t11-CLA content of eggs from 5FL was 1.57 fold increase compared to that of 5C) that (Figure 61).

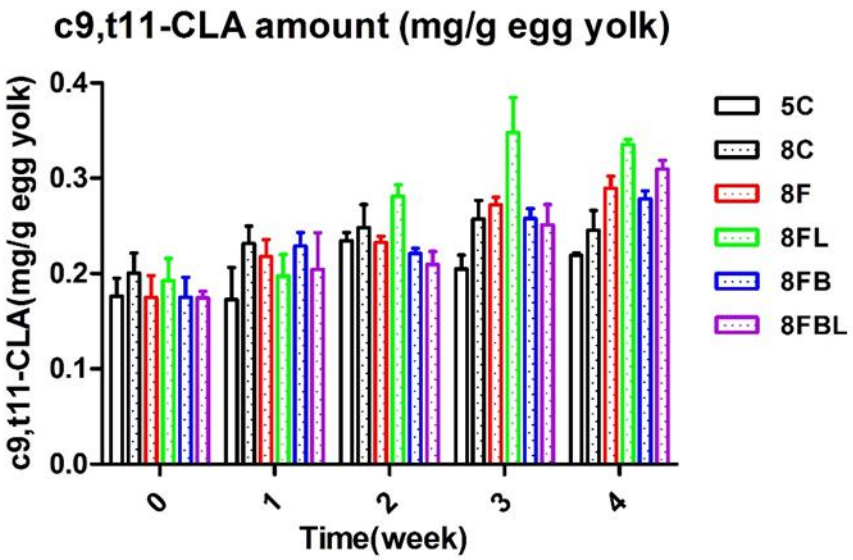

Figure 61. Changes of c9, t11-CLA amount in eggs during the study 3.

The c9, t11-CLA amounts of egg samples were calculated by data of gas chromatography standardized with FAME 37 mix. Each group was 5 replicates.

(2) Effects on inflammatory indices

① Serum pro-inflammatory cytokine levels

Serum levels of pro-inflammatory cytokine were monitored as inflammatory index evaluating the effect of flaxseed and probiotics on inflammatory state of overcrowded laying hen (Figure 62). No significant differences were observed in levels of TNF-alpha, IL-1beta, or IL-6 in serum samples, although the level of TNF-alpha on week 4 tend to decrease compared to the level of week 0 in 8FB group (Figure 62A). Likewise, the levels of IL-1beta on week 4 tend to decrease in 8FL and 8FB groups (Figure 62B).

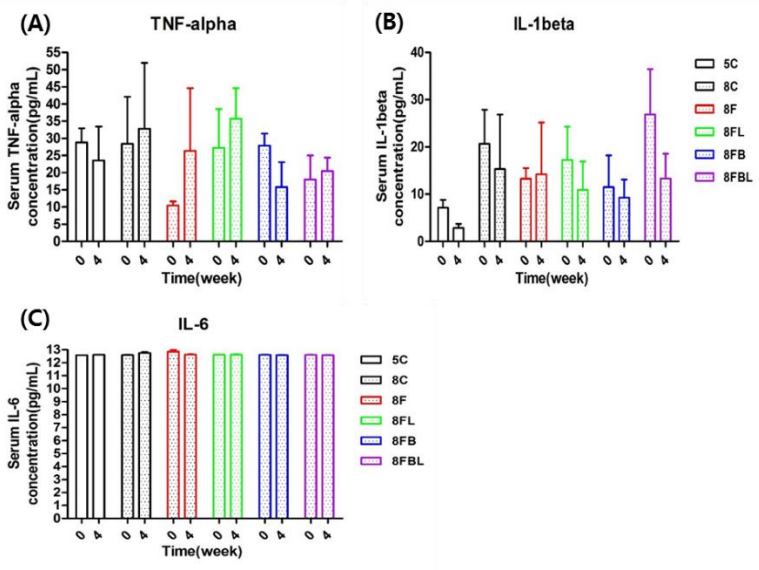

Figure 62. Serum pro-inflammatory cytokine levels in experimental hens at weeks 0 and 4.

(A) TNF-α (B) IL-1β (C) IL-6.

② Inflammatory-related gene expression in liver

As same as study 2 and study 3-1, gene expression levels of inflammatory-related genes (***tnf-alpha***, ***il-1beta***, ***il-6***, and ***cox-2***) were investigated using liver mRNA samples (Figure 63). There was no significant difference among groups in pro-inflammatory cytokine-encoded genes. However, 8F, 8FL, 8FB, 8FBL groups had tendencies to decrease levels of gene expression which were upregulated by overcrowding stress. Especially, the decrease in the groups fed probiotics (8FL, 8FB, 8FBL) were much larger than 8F group, which were observed in all 3 genes (***tnf-alpha***, ***il-1beta***, ***il-6***) (Figure 63A-C). Meanwhile, 8FL and 8FB groups were significantly decreased expression of ***cox2*** compared to 8C group (Figure 63D). Therefore, probiotics had effects to alleviate and improve host inflammatory state when under overcrowding stress condition.

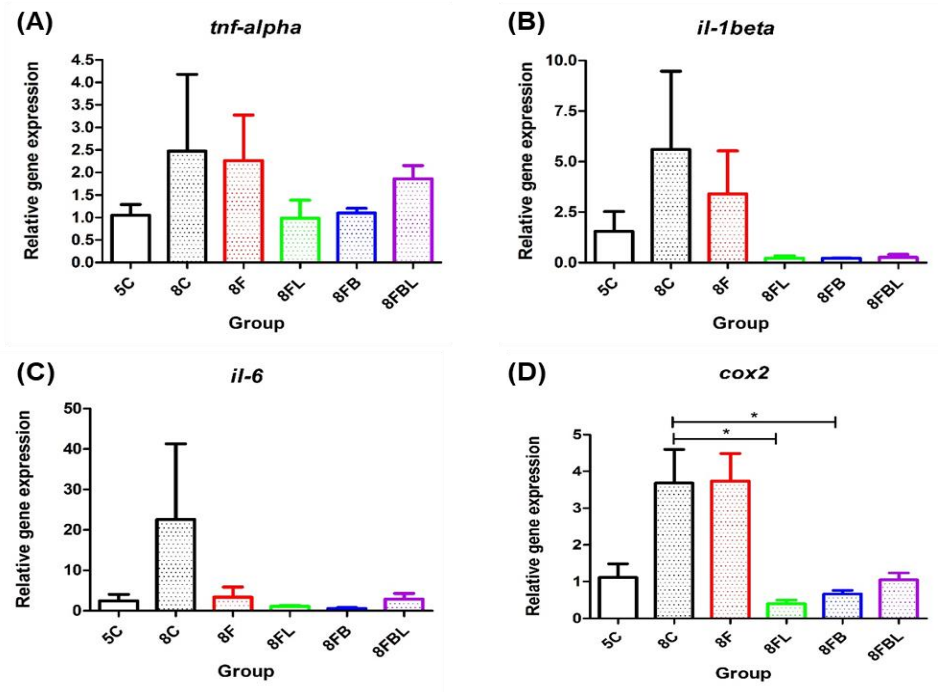

Figure 63. Analysis of gene expression of pro-inflammatory cytokines and lipid mediator synthesis in experimental hens at week 4.

(A) The transcription expression of pro-inflammatory cytokine *tnf-α*, (B) *il-1β*, (C) *il- 6*, and (D) lipid mediator synthesis related gene *cox2* relative to β-actin was quantified by qRT-PCR.

(3) Effects on stress indices

As same as study 2 and study 3-1, the effect of flaxseed and probiotics on the stress state of overcrowded laying hen, three stress indices (serum corticosterone level, liver HSP70 gene expression level and H:L ratio) were examined (Figure 64). Even though there was no significant difference in serum corticosterone level (Figure 64A) and H:L ratio (Figure 64C), the gene ***hsp70*** (Figure 64B) were significantly reduced its expression in probiotics-fed group (8FL, 8FB, 8FBL) compared to that of the 8C group. Among 3 probiotic-fed group, the 8FL group were mostly reduced its expression.

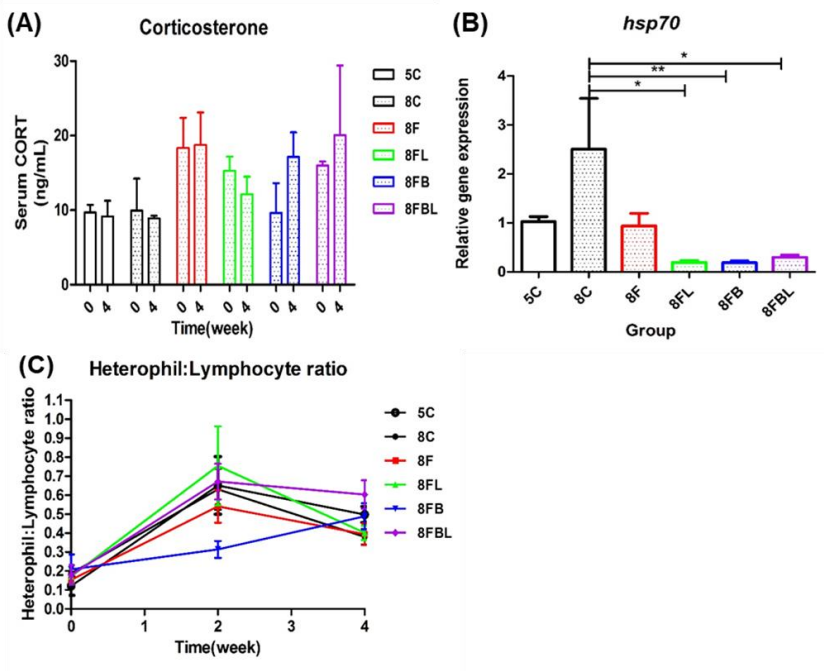

Figure 64. Effects of dietary flaxseed and probiotics on stress indices in experimental hens.

(A) Serum corticosterone level at weeks 0 and 4.
(B) HSP70 gene expression of liver samples at week 4.
(C) Changes in H:L ratios at weeks 0,2 and 4.

(4) Effects on gut microbiota

① Microbial community analysis

The observed OTUs, the alpha diversity index were as follows: 1) 5C: 370.30 (±24.49); 2) 8C: 346.18 (±16.11); 3) 8F: 372.56 (±16.11); 4) 8FL: 392.36 (±29.15); 5) 8FB: 381.62 (±24.69); 6) 8FBL: 352.46 (±38.14) respectively. There was significant difference among groups especially the index of 8C and 8FBL groups were significantly lower than that of 8FL group (Figure 65A). The PCoA plot based on unweighted UniFrac distances of the 16S rRNA sequences showed that the microbial communities were distinctly separated by each treatment (Figure 65B). The probiotic administration mainly influenced so that dividing groups into 2 parts (5C, 8C, 8F vs 8FL, 8FB, 8FBL). In addition, the 5C group was apart from 8C and 8F group, which meant that overcrowding stress influenced the clustering among groups. Among the probiotics-fed groups (8FL, 8FB and 8FBL) were divided by their treatment so that 8FBL were placed between the 8FL and 8FB group.

To identify distinct bacteria among groups, relative abundance of taxa among groups were compared at the phylum and genus level. 24 phyla and 334 genera were detected. At the phylum level, all groups shared the following 15 phyla: *Euryarchaeota*, *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Elusimicrobia*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Proteobacteria*,

Spirochaetes, *Synergistetes*, *TM7*, *Tenericutes*, and *Verrucomicrobia*. These were same as the result of study 3-1. Also, there were 2 phyla (*Elusimicrobia*, *Actinobacteria*) that significantly differed among groups (Table 29). *Elusimicrobia* was significantly abundant in the 5C group compared to the 8FL group. *Actinobacteria* was significantly abundant in the probiotics-fed group (8FL, 8FB, 8FBL) compared to probiotics-unfed group (5C, 8C, 8F).

At the genus level, all groups shared 98 genera, and there were 29 genera that significantly differed among groups. *Chryseobacterium*, *Mucilaginibacter*, *Paenibacillus*, *Agrobacterium*, *Enterobacter*, *Erwinia* and *Pseudomonas* were significantly abundant in the 5C group. *Zea* was significantly abundant in the 8F group compared to 8C and 8FBL group. *Corynebacterium*, *Glycomyces*, *Janibacter*, *Bacteroides*, *Oceanobacillus*, *Staphylococcus* were abundant in probiotics-fed group (8FL, 8FB, 8FBL) compared to probiotics-unfed group (5C, 8C, 8F). There were several genus that differed by specific probiotic administration. *Brachybacterium*, *Aeriscardovia*, *Lysinibacillus*, *Jeotgalicoccus*, *Aerococcus*, *Facklamia*, *Turicibacter*, *SMB53*, *Faecalibacterium* and *Gallicola* were significantly differed by Lp administration. *Brevibacterium*, *Butyricimonas*, *Bacillus*, *Phascolarctobacterium* and *Alcaligenes* were bacteria that significantly differed by T2 administration (Table 29).

Control group compared to the Overcrowded group whereas *Propionibacterium*, *Bacteroides*, *Parabacteroides*, and *[Clostridium]* were significantly abundant in the Overcrowded group compared to the Control group (Table 29).

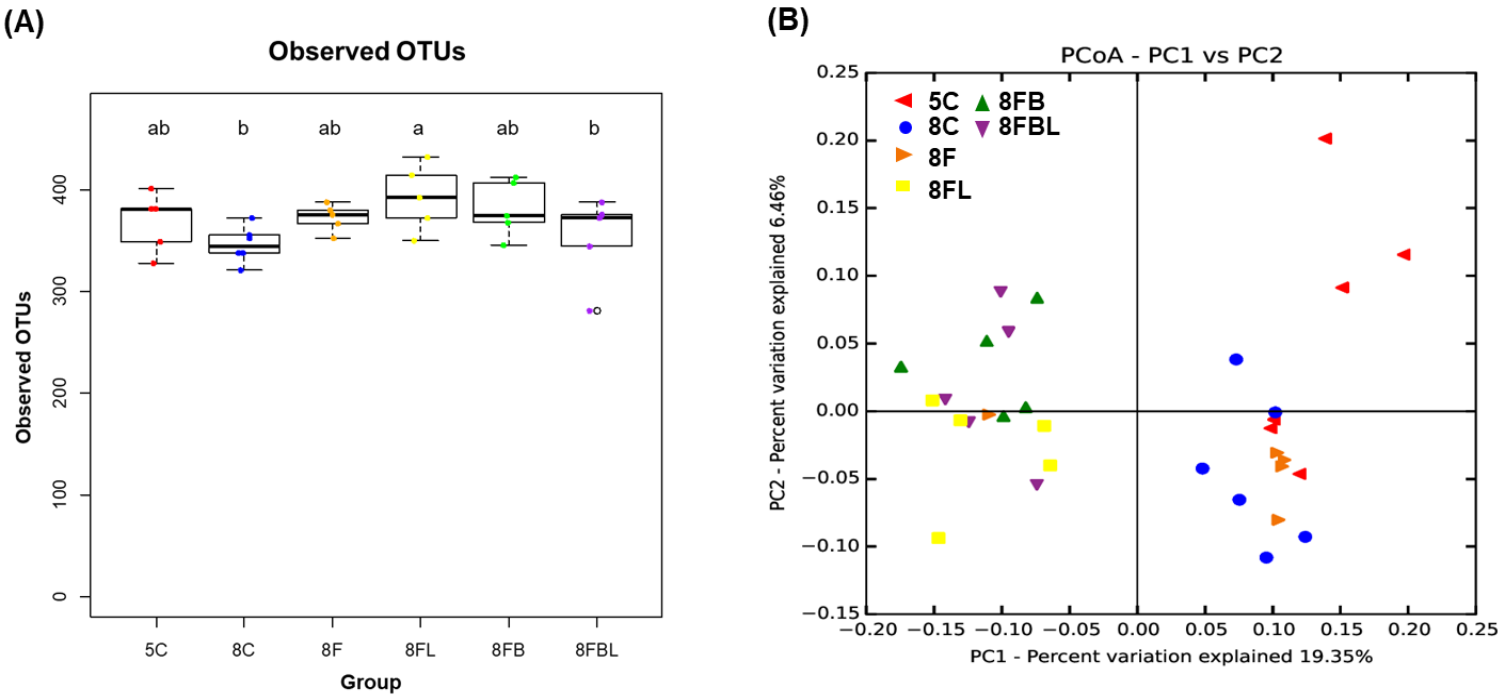

Figure 65. Diversity of gut microbiota of overcrowded laying hen fed flaxseed and probiotics.

(A) Alpha diversity index (Observed OTUs).
(B) Principal coordinate analysis (PCoA) plot based on unweighted UniFrac distanaces.

Table 29. Relative abundances of phyla and genera at overcrowded laying hen fed flaxseed and probiotics.

Taxon	Relative abundance (%)						*p-value*
	5C	8C	8F	8FL	8FB	8FBL	
Phylum							
Actinobacteria	0.24±0.13^{a}	0.16±0.08^{a}	0.40±0.43^{a}	1.21±0.48^{b}	1.11±0.34^{b}	1.08±0.20^{b}	<0.001***
Cyanobacteria	5.57±3.26^{a}	5.03±2.30^{a}	5.82±4.12^{a}	2.56±2.26^{a}	1.20±1.00^{a}	2.29±1.31^{a}	0.031*
Elusimicrobia	2.84±1.38^{b}	1.25±0.94^{ab}	2.57±1.82^{ab}	0.63±0.23^{a}	1.15±1.21^{ab}	0.83±0.60^{ab}	0.015*
Genus							
Brevibacterium	0.00±0.00^{a}	0.00±0.00^{a}	0.03±0.05^{ab}	0.14±0.07^{bc}	0.21±0.11^{c}	0.16±0.07^{c}	<0.001***
Corynebacterium	0.01±0.01^{a}	0.01±0.01^{a}	0.07±0.15^{a}	0.55±0.32^{b}	0.44±0.21^{b}	0.44±0.19^{b}	<0.001***
Brachybacterium	0.01±0.02^{a}	0.00±0.00a	0.05±0.09^{ab}	0.19±0.11^{c}	0.15±0.06^{bc}	0.16±0.02^{bc}	<0.001***
Glycomyces	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.01±0.01^{b}	<0.001***
Janibacter	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.01^{a}	0.00±0.00^{a}	0.01±0.01^{a}	0.06±0.02^{b}	<0.001***
Mycetocola	0.03±0.03^{a}	0.01±0.01^{a}	0.01±0.01^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.044*
Yaniella	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.01^{a}	0.02±0.02^{a}	0.01±0.01^{a}	0.00±0.00^{a}	0.031*
Aeriscardovia	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{ab}	0.05±0.06^{b}	0.00±0.01^{ab}	0.00±0.00^{ab}	0.026*
Bacteroides	9.10±2.39^{a}	15.25±3.91^{ab}	9.18±2.47^{ab}	12.87±4.95^{ab}	10.33±2.43^{ab}	17.69±8.47^{b}	0.023*
Parabacteroides	1.04±0.33^{a}	2.33±0.93^{b}	1.71±0.44^{ab}	2.56±1.06^{b}	1.44±0.56^{ab}	1.98±0.68^{ab}	0.015*
Butyricimonas	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.01^{a}	0.02±0.02^{ab}	0.05±0.04^{b}	0.04±0.03^{b}	<0.001***
Spirosoma	0.05±0.06^{a}	0.02±0.03^{a}	0.02±0.01^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.030*
Chryseobacterium	0.12±0.11^{b}	0.04±0.05^{ab}	0.02±0.02^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.006**
Mucilaginibacter	0.03±0.02^{b}	0.00±0.00^{a}	0.01±0.02^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	<0.001***
Pedobacter	0.17±0.16^{a}	0.07±0.12^{a}	0.11±0.09^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.039*
Elusimicrobium	0.97±0.66^{a}	0.56±0.46^{a}	0.61±0.30^{a}	0.24±0.21^{a}	0.24±0.24^{a}	0.27±0.21^{a}	0.030*
Bacillus	0.00±0.01^{ab}	0.00±0.00^{a}	0.01±0.02^{ab}	0.03±0.03^{ab}	0.05±0.03^{b}	0.04±0.03^{ab}	0.010*
Oceanobacillus	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{ab}	0.00±0.00^{ab}	0.00±0.00^{ab}	0.00±0.00^{b}	0.032*
Paenibacillus	0.03±0.03^{b}	0.01±0.01^{ab}	0.01±0.01^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.014*
Lysinibacillus	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.01±0.01^{b}	0.01±0.01^{ab}	0.01±0.01^{ab}	0.002**
Sporosarcina	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.02±0.03^{a}	0.02±0.01^{a}	0.00±0.00^{a}	0.015*
Jeotgalicoccus	0.00±0.00^{a}	0.00±0.00^{a}	0.04±0.07^{ab}	0.18±0.10^{c}	0.12±0.04^{bc}	0.08±0.02^{ac}	<0.001***
Staphylococcus	0.00±0.01^{a}	0.01±0.02^{a}	0.02±0.05^{a}	0.27±0.13^{b}	0.23±0.08^{b}	0.25±0.21^{b}	<0.001***
Aerococcus	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{ab}	0.02±0.02^{b}	0.01±0.01^{ab}	0.00±0.00^{a}	0.003**
Facklamia	0.00±0.00^{a}	0.00±0.01^{a}	0.02±0.05^{ab}	0.10±0.09^{b}	0.08±0.03^{ab}	0.07±0.03^{ab}	0.002**
Turicibacter	0.04±0.04^{a}	0.13±0.13^{ab}	0.12±0.04^{ab}	0.27±0.13^{b}	0.19±0.09^{ab}	0.09±0.07^{ab}	0.012*

SMB53	0.24±0.08[a]	0.45±0.24[ab]	0.41±0.21[ab]	0.76±0.39[b]	0.68±0.20[ab]	0.46±0.35[ab]	0.033*
Faecalibacterium	0.18±0.10[a]	0.22±0.11[a]	0.33±0.11[ab]	1.18±1.26[b]	0.41±0.13[ab]	0.54±0.19[ab]	0.040*
Phascolarctobacterium	0.01±0.01[a]	0.02±0.01[ab]	0.01±0.01[a]	0.03±0.02[ab]	0.05±0.02[b]	0.03±0.02[ab]	0.019*
Gallicola	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.06±0.07[b]	0.00±0.01[ab]	0.02±0.01[ab]	0.016*
[Eubacterium]	0.16±0.16[a]	0.20±0.11[a]	0.15±0.06[a]	0.14±0.06[a]	0.32±0.24[a]	0.43±0.24[a]	0.039*
Bosea	0.00±0.00[a]	0.00±0.00[a]	0.00±0.01[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.038*
Agrobacterium	0.23±0.21[b]	0.11±0.13[ab]	0.14±0.10[ab]	0.01±0.01[a]	0.01±0.01[a]	0.00±0.00[a]	0.016*
Zea	0.01±0.01[ab]	0.00±0.00[a]	0.03±0.03[b]	0.01±0.01[ab]	0.00±0.01[ab]	0.00±0.00[a]	0.026*
Alcaligenes	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.01±0.01[ab]	0.02±0.02[b]	0.00±0.00[a]	0.002**
Enterobacter	0.01±0.01[b]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.003**
Erwinia	0.22±0.18[b]	0.09±0.05[ab]	0.08±0.05[ab]	0.01±0.01[a]	0.00±0.00[a]	0.00±0.01[a]	0.002**
Pseudomonas	0.21±0.16[b]	0.05±0.05[a]	0.03±0.02[a]	0.05±0.04[a]	0.03±0.03[a]	0.02±0.02[a]	0.001**
Pseudofulvimonas	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.01[a]	0.00±0.00[a]	0.00±0.00[a]	0.032*
Akkermansia	2.04±2.65[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.028*

The data were expressed as the mean values ± standard deviation (SD). The *p-value*s were determined using Welch's *t*-test (* *p-value*< 0.05; ** *p-value* < 0.01; *** *p-value* < 0.001).

② Changes in predicted functions of the gut microbiota of overcrowded laying hen when fed flaxseed and probiotics

The KEGG pathways predicted using PICRUSt were compared their relative abundance among groups with one-way ANOVA and post hoc TUKEY's HSD test to determine significantly differed. 'Amino acid metabolism', 'D-Glutamine and D-glutamate metabolism', 'Glycine, serine and threonine metabolism' and 'Lysine biosynthesis' were predicted at significantly higher levels in 8FB group (*p-value* = 0.044, 0.030, 0.034, 5.00e-3) (Figure 66).

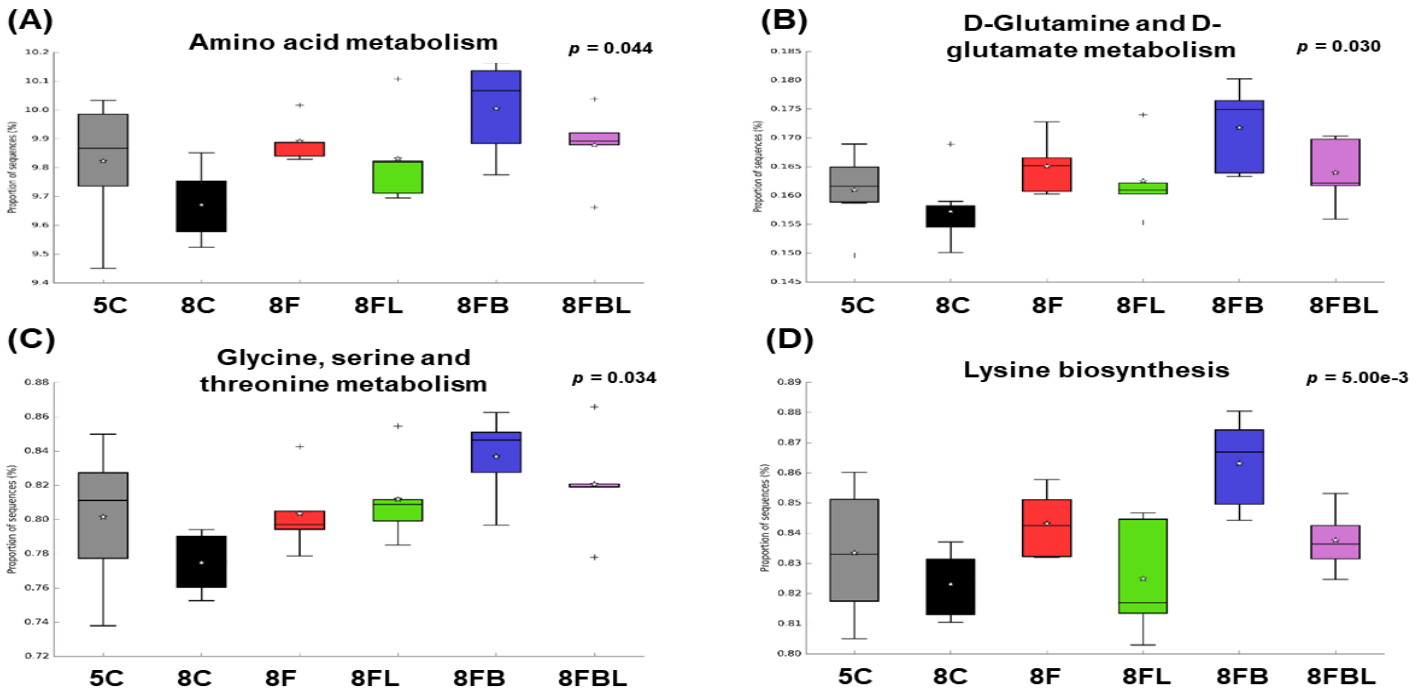

Figure 66. Changes in overcrowded laying hen gut microbiota function when fed flaxseed and probiotics.

The microbial functions were predicted using PICRUSt at the third level of the KEGG pathway and were expressed as relative abundances. The difference between the levels of the predicted functions were tested using a two-sided Welch's *t*-test, and *p-value* < 0.05 was considered significant.

(5) Laying performance and egg quality of overcrowded laying hen when fed flaxseed and probiotics

The laying performance and egg quality of each group were monitored to determine alleviating or improving effect of flaxseed and probiotics on overcrowded laying hen. The traits to be observed were the same as study 1, 2 and 3-1.

Overall, daily feed intake, average egg weight and hen-day egg production were significantly differed by 4 week feed experiment (*p-values* = 0.026, 0.011, 0.015, respectively). Daily feed intake was reduced in 8C and 8FL group among groups. Average egg weight of 8C group was significantly lighter than that of 5C group. Hen-day egg production was significantly increased in 8FB group compared to other overcrowded group (8C, 8F, 8FL and 8FBL) while the value of 8C group was significantly decreased compared to 5C group and 8FB group. On week 4, there were also several significant difference on laying performance among groups. Average egg weight of 8F and 8FL group were significantly lighter than that of 5C group whereas that of 8FB and 8FBL were not significantly differed (*p-value* = 0.017). Also, feed conversion ratio of 8F group was significantly increased compared to that of 5C group contrary to others (Table 30).

Meanwhile, the indices of egg quality such as albumen height, Haugh unit and eggshell thickness showed no significant difference among groups on week 0, 1, 2, 3 and 4, which were similar to the results of study 2 and 3-1 (Table 31).

Table 30. Effects of flaxseed and probiotics in diet on performance of overcrowded laying hens during 52-56 weeks of age[1].

Items	Group						*p-value*
	Normal	Overcrowded					
	5C	8C	8F	8FL	8FB	8FBL	
Week 1							
Daily feed intake(g/head)	119.73±0.02[a]	115.80±2.59[a]	118.19±0.88[a]	114.43±0.67[a]	118.79±0.64[a]	115.85±0.87[a]	0.066
Average egg weight(g)	57.64±2.39[a]	52.81±2.39[a]	56.98±2.00[a]	55.00±2.56[a]	57.06±4.22[a]	55.40±2.10[a]	0.820
Feed conversion ratio	2.10±0.09[a]	2.22±0.11[a]	2.09±0.08[a]	2.11±0.10[a]	2.15±0.16[a]	2.11±0.07[a]	0.951
Hen-day egg production, %	98.10±4.04[a]	88.69±4.24[a]	97.02±3.37[a]	94.05±4.62[a]	97.02±7.31[a]	94.05±3.50[a]	0.748
Week 2							
Daily feed intake(g/head)	119.71±0.00[a]	117.56±1.16[a]	119.51±0.12[a]	117.87±1.24[a]	119.54±0.15[a]	118.33±0.97[a]	0.294
Average egg weight(g)	57.33±1.01[a]	52.75±2.51[a]	56.41±1.82[a]	55.82±0.69[a]	56.83±2.54[a]	54.57±2.24[a]	0.580
Feed conversion ratio	2.09±0.04[a]	2.26±0.11[a]	2.13±0.07[a]	2.11±0.03[a]	2.13±0.09[a]	2.19±0.09[a]	0.656
Hen-day egg production, %	94.29±1.74[a]	86.90±4.21[a]	95.24±3.21a	94.05±1.24[a]	94.64±4.33[a]	91.67±3.64[a]	0.483
Week 3							
Daily feed intake(g/head)	119.71±0.00[a]	118.50±1.08[a]	118.94±0.26[a]	118.18±0.71[a]	119.37±0.13[a]	119.04±0.68[a]	0.544
Average egg weight(g)	56.99±1.26[a]	53.55±1.47[a]	55.23±1.46[a]	54.37±0.54[a]	55.65±1.24[a]	55.42±1.15[a]	0.484
Feed conversion ratio	2.11±0.05[a]	2.22±0.07[a]	2.16±0.06[a]	2.17±0.02[a]	2.15±0.05[a]	2.15±0.05[a]	0.717

Hen-day egg production, %	93.33±2.06[a]	87.50±2.41[a]	92.86±1.97[a]	91.67±0.91[a]	92.86±1.97[a]	92.86±1.97[a]	0.290
Week 4							
Daily feed intake(g/head)	117.43±2.29[a]	116.88±2.17[a]	118.92±0.73a	116.30±1.42[a]	119.19±0.31[a]	122.18±3.34[a]	0.385
Average egg weight(g)	60.02±1.88[a]	54.17±1.47[ab]	53.89±1.14[b]	53.30±1.32[b]	56.74±1.19[ab]	56.01±1.24[ab]	0.017*
Feed conversion ratio	1.97±0.07[a]	2.17±0.06[ab]	2.21±0.05[b]	2.19±0.06[ab]	2.11±0.04[ab]	2.19±0.05[ab]	0.026*
Hen-day egg production, %	96.19±3.21[a]	88.10±2.48[a]	89.88±1.79[a]	89.29±2.00[a]	92.86±1.97[a]	93.71±2.25[a]	0.147
Week 1 to 4							
Daily feed intake(g/head)	119.15±0.57[a]	117.18±0.85[a]	118.89±0.29[a]	116.69±0.64[a]	119.22±0.18[a]	118.85±1.03[a]	0.026*
Average egg weight(g)	58.00±0.84[a]	53.32±0.96[b]	55.63±0.81[ab]	54.62±0.73[ab]	56.57±1.23[ab]	55.35±0.83[ab]	0.011*
Feed conversion ratio	2.07±0.03[a]	2.22±0.04[b]	2.15±0.03[ab]	2.15±0.03[ab]	2.13±0.05[ab]	2.16±0.03[ab]	0.113
Hen-day egg production, %	95.48±1.42[a]	87.80±1.63[b]	93.75±1.36[ab]	92.26±1.30[ab]	94.35±2.13[ac]	93.07±1.40[ab]	0.015*

[1] Each value represents the mean±SD of three replicate cages (five birds per cage).

[a-c] Means in a row without a common superscript letter differ as analyzed by one-way ANOVA and the TUKEY test (*p-$value$<0.05, **p-$value$<0.01, ***p-$value$<0.001).

Table 31. Effects of flaxseed and probiotics in diet on egg quality of overcrowded laying hens during 52-56 weeks of age compared to normal state[1].

Items	Time, week	Group						*p-value*
		Normal	Overcrowded					
		5C	8C	8F	8FL	8FB	8FBL	
Albumen height, mm	**0**	8.25±2.18	8.82±2.00	9.00±0.85	7.68±2.47	9.05±1.02	8.13±1.31	0.717
	1	10.40±0.94	9.07±0.67	9.38±1.79	9.57±1.20	9.98±0.56	10.88±0.25	0.109
	2	7.95±1.35	8.30±1.04	9.70±0.96	9.57±1.38	9.15±0.53	10.58±2.33	0.166
	3	8.57±0.35	9.30±1.47	9.48±1.07	9.05±0.71	9.32±1.08	8.95±2.04	0.839
	4	8.45±0.53	9.90±1.15	9.65±0.97	9.00±0.57	9.43±1.37	8.95±1.18	0.270
Haugh unit	**0**	89.77±12.89	93.21±9.23	94.98±3.29	86.25±16.21	94.71±4.75	90.58±6.58	0.675
	1	101.03±3.92	93.79±2.98	95.91±7.30	98.05±5.61	98.84±2.45	102.67±0.50	0.061
	2	88.20±8.67	92.10±4.89	97.82±3.43	96.70±6.57	94.46±1.99	99.94±11.09	0.231
	3	92.43±1.02	96.02±6.59	96.80±3.54	94.68±3.28	95.48±5.03	92.59±10.63	0.741
	4	91.12±3.01	97.84±5.59	96.29±4.28	94.60±2.20	95.34±6.86	93.24±5.69	0.358
Egg shell thickness, mm	**0**	0.39±0.01	0.39±0.02	0.42±0.02	0.40±0.02	0.40±0.04	0.39±0.02	0.878
	1	0.40±0.04	0.37±0.02	0.39±0.01	0.40±0.03	0.39±0.02	0.38±0.01	0.682
	2	0.38±0.02	0.38±0.02	0.39±0.02	0.39±0.01	0.39±0.02	0.39±0.01	0.590
	3	0.39±0.02	0.40±0.01	0.40±0.01	0.40±0.02	0.39±0.02	0.40±0.03	0.955
	4	0.39±0.01	0.38±0.02	0.40±0.01	0.38±0.03	0.39±0.02	0.39±0.02	0.960

[1]Each value represents the mean±SD of ten replicates with fifty hen per replicate.

4. Discussion

In the study 3, the aim was to evaluate flaxseed and probiotic effects on the overcrowded laying hen physiology and its gut microbiota. First, the effect of overcrowding stress on 52-week-old laying hen were investigated in study 3-1. After adverse effects of overcrowding stress condition, the supplementation of flaxseed and probiotics were conducted to observe alleviating or improving effects on laying hen under stress condition with the same age in study 3-2.

To determine adverse effect of overcrowding stress, 52-week-old laying hen were randomly divided into two groups according to stress condition with same basal diet and housing farm, which can exclude dietary and other environmental effects. The experiments were progressed for 4 weeks and biological samples were collected with measuring laying performance and egg quality to investigate its physiological effect.

Interestingly, overcrowding stress was demonstrated to alter fatty acid profile of egg. Although there were few study to observe alteration of fatty acid profile by stress condition, it can be possible when considering the relationship between gut microbiota and stress condition. As various stress can modulate the gut microbiota as reported in other studies (Bello *et al.*, 2018;He *et al.*, 2019;Zhu *et al.*, 2019), its alteration may affect composition of gut microbiota and its abundance of fatty acid-related genes. In this study, myristoleic acid and gamma-

linolenic acid were observed as significantly decreased fatty acids under stress condition. In particular, gamma-linolenic acid is related to 1-series PGs, particularly PGE_1, which was synthesized by dihomo gaama-linolenic acid and have anti-inflammatory effect (Borgeat *et al.*, 1976). Thus, its reduction in egg may imply overcrowding stress-induced pro-inflammatory response. Conversely, they can be applied as non-invasive stress marker only using livestock product. As there was few reference about the alteration of fatty acid by stress condition, more studies are needed.

In case of inflammatory and stress indices, the levels of liver gene expression were commonly increased by overcrowding stress while indices measured from serum samples were not significantly differed. Taken together with other results such as laying performance, these results can support the idea that liver is more appropriate to judge the effect of nutraceutical. Also, the gut microbiota was altered by overcrowding stress. Especially, the predicted metagenome showed activation of 'bacterial toxins' representing the vulnerable gut microbiota by stress. Overcrowding stress adversely affected the overall index of laying performance in this study. Thus, high density stocking when farming livestock is not good for the health status and productivity of livestock.

In study 3-2, flaxseed and probiotics (Lp, T2) were administrated to overcrowded 52-week-old laying hen to evaluate whether these nutraceuticals can alleviate the overcrowding stress effects. Several positive effects were observed

in various indices. Several alteration of fatty acid profile were also observed by supplementing nutraceuticals, which were interesting discoveries. Margaroleic acid, which was reported its reduction when stress condition in study 3-1, was not rescued by flaxseed and probiotics. There were various researches that stress caused by rapid changes in environment may affect permanent and irreversible effect on gut microbiota even after the stress had disappeared in case of pig (Campbell *et al.*, 2013) and ruminant (Scheffler *et al.*, 2014). Likewise, it can be hypothesized that the fatty acid disappeared in stressed groups is a result of stress-induced imprinting on host. This is likely to be applied as a biological marker for stress history of livestock animals. Meanwhile, ETA, an omega-3 fatty acid, was significantly increased in eggs from bacillus-fed group. As omega-3 fatty acids were reported to have anti-inflammatory effect (Nohe *et al.*, 2003), its enrichment may be related to improvement of laying performance.

The liver gene expression regarding inflammation and stress levels that upregulated by overcrowding stress were overall downregulated especially when using both flaxseed and probiotics. In particular, the administration of *Bacillus* showed the most improvement on stress index. Those results suggested that using both flaxseed and probiotics together could be effective in managing livestock stress state.

However, it seems to be careful about which probiotics are appropriate considering the circumstances. In study 3-2, the inefficiency usage by mixing 2

probiotics resulted in adverse effects on gut microbiota. Microbial diversity is a parameter of gut microbiota health in general (Lozupone *et al.*, 2012), while 8FBL group showed diversity reduction similar to 8C group. In addition, *Lactobacillus* administration didn't show significant improvement contrary to the result of study 2. The alteration of various phyla and genera were observed by each treatment, which result in changes of metagenome. Especially, *Bacillus* activated several KEGG pathways regarding protein and amino acid such as 'Amino acid metabolism', 'D-Glutamine and D-glutamate metabolism', 'Glycine, serine and threonine metabolism', and 'Lysine biosynthesis'. As *Bacillus* including *Bacillus licheniformis* encoded various proteinase such as keratinase (Okoroma *et al.*, 2012), the administration might enhance digestibility of nitrogen sources. Actually, improvement in crude protein digestibility (Giang *et al.*, 2011) or amino acid utilization (Wang *et al.*, 2006) were reported by previous studies. Under stress condition, laying hen are getting out of hair, so the substrates for *Bacillus licheniformis*-origin keratinase may enrich in cage. Also, problematic proteins such as glycinin or β–conglycinin damaging intestinal morphology can be degraded by *Bacillus*–origin enzymes (Sun *et al.*, 2008;Wang *et al.*, 2011). The result of laying performance also supported this hypothesis as 8FB group showed the most improved in some parameters. As of now, further studies to understand their biological meaning as well as repeating experiment with other growth stage or other livestock animals are needed to clarify the effects of flaxseed and probiotics.

In the study 3, the physiological effects of flaxseed and probiotics on overcrowded laying hen were assessed (Table 32). Although more studies are required, these results may provide insights into understanding the physiological state of livestock and its alteration by environment. Also, the distinct fatty acids or microbes differed by stress condition or nutraceutical supplementation can be initial targets to evaluate the health of livestock and to develop the feed additives improving the health.

Table 32. Summary of study 3.

Contents	Results
***In vivo* laying hen feed study (flaxseed + probiotics with overcrowding stress)**	Effects of overcrowding stress condition - Fatty acid profile alteration (SFA, MUFA ↑) - Inflammatory indices ↑ - Stress indices ↑ - Laying performance ↓
	Effect of flaxseed when stress condition - Egg omega-6/omega-3 ratio ↓ - Laying performance ↑ (average egg weight, feed conversion ratio, hen-day egg production)
	Effect of flaxseed and Lp when stress condition - Egg CLA content ↑ - Inflammatory indices ↓ (Liver mRNA) - Stress indices ↓ (Liver HSP70 mRNA) - Laying performance ↑ (average egg weight, feed conversion ratio, hen-day egg production)
	Effect of flaxseed and T2 when stress condition - Inflammatory indices ↓ (Liver mRNA) - Stress indices ↓ (Liver HSP70 mRNA) - Metagenome - Amino acid-related metabolism ↑ - Laying performance ↑↑ (average egg weight, feed conversion ratio, hen-day egg production)

Overall Conclusion

The issue of chronic inflammation issue in livestock animals has been raised due to an unsanitary environment and various stressors such as high density stocking, heat, cold, and transportation. Also, there is growing demand for safe feed additives as alternatives to antibiotics that also improve animal welfare. Nutraceuticals are food ingredients promoting the health state of the host when administered. Various natural compounds originating from animals, plants, and microbes can be classified as nutraceuticals. In general, their effects are mainly anti-inflammatory and anti-oxidant, which can help manage host inflammatory and stress states. In particular, flaxseed and probiotics are representative materials that have potential to accomplish goals of both safe products and animal health-promoting effects. To evaluate physiological effects of nutraceuticals, biological markers as well as livestock performance should be monitored. However, only a limited number of studies have been performed with biological markers in the livestock industry. The aim of this study was to reveal the physiological effects of flaxseed and probiotics on laying hens under normal and overcrowding stress conditions.

In study 1, the physiological effect of flaxseed was investigated in 33-week-old laying hens. Similar to other previous results, the eggs were bio-fortified with omega-3 fatty acids to rebalance the ratio of omega-6 to omega-3. Furthermore, it was observed that the PUFA and lipid mediator profiles were altered toward

enriched omega-3 fatty acids and derivatives of ALA, EPA, and DHA, downregulating levels of pro-inflammatory cytokines and H:L ratio. These changes resulted in improving laying performance. Therefore, flaxseed may be used as a nutraceutical to modulate the host physiology regarding inflammation and stress.

In study 2, a combination of flaxseed and probiotics was evaluated. *Lactobacillus plantarum* WT177 (Lp) and *Bacillus licheniformis* T2 (T2) were screened by their highest production yield and identified target gene (Lp: *cla-er*, *cla-dc*, *cla-dh*, *cla-hy*; T2: *des*, *fad6*) among three lactobacilli and two bacilli respectively. The effects of their administration with flaxseed on fatty acid profile, inflammatory indices, stress indices, gut microbiota and laying performance were investigated. Overall, distinct probiotic effects were observed. Significantly differed fatty acids in eggs by nutraceuticals were observed as follows: 1) flaxseed: enrichment of omega-3 fatty acids and depletion of omega-6 fatty acids; 2) probiotic: depletion of palmitic acid, depletion of myristoleic acid only in 5FL group, enrichment of c9, t11-CLA amount in 5FL group. Also, both inflammatory (*il-1beta*, *il-6* gene expression) and stress indices (*hsp70* gene expression) measured from liver samples were decreased in probiotic-fed groups, especially the 5FL group. Moreover, there were also some changes in gut microbiota such as diversity, taxa, and the predicted metagenome. In particular, the reduction of the phosphotransferase system (PTS) in the 5FL group and the activation of energy metabolism in the 5FB group were

likely linked to their laying performance in the context of host nutrient or energy utilization.

In study 3, the adverse effects of overcrowding stress and its alleviation by nutraceuticals were investigated. The nutraceuticals used were flaxseed and two probiotics, as in study 2. First, the overall effects of overcrowding stress on host physiology were observed with the same traits as used in study 2. Some fatty acids such as margaroleic acid and gamma-linolenic acid were depleted under a stress condition, which can indicate host stress state. The overall gene expression levels in liver regarding inflammatory (*tnf-alpha*, *il-1beta*, *il-6*) and stress indices (*hsp70*) were increased by overcrowding stress. Overcrowding stress also affects gut microbiota in terms of diversity, taxa, and predicted metagenome. In particular, overcrowding stress activated the KEGG pathway such as bacterial toxins, indicating a vulnerable gut microbiota condition. The alleviating effects of flaxseed and probiotics against overcrowding stress were then assessed by measuring the same traits. Overall, some recovery and improvement effects on inflammatory and stress indices were observed. The inflammatory indices (*tnf-alpha*, *il-1beta*, *il-6*, and *cox-2*) were rescued and their expression, which was upregulated by overcrowding stress, was downregulated. Likewise, a stress index (*hsp70*) was rescued and its expression against the overcrowding stress effect was improved. Also there were significant differences in the diversity, taxa, and predicted metagenome of gut microbiota. In particular, amino acid metabolism, D-glutamine and D-glutamate metabolism, glycine, serine and threonine

metabolism, and lysine biosynthesis were activated in the 8FB group, which were likely linked to improvement of laying performance.

In this study, the physiological effects of nutraceuticals on the host were investigated with flaxseed and probiotics (Figure 67A). While the overall physiological traits of the host were likely to be threatened by overcrowding stress, a recovery effect of flaxseed and probiotics in inflammatory-related and stress-related traits was observed under both normal and stress conditions, especially from liver samples. Also, there were some alterations in the host fatty acid profile not only by flaxseed but also by probiotics and by overcrowding stress. This may indicate that fatty acids can be used as biological markers to monitor the host state. Although the idealistic ratio of omega-6 to omega-3 ratio is approximately from 1:1 to 4:1 in human clinical studies, the proper ratio to maintain livestock homeostasis and eustress state should be investigated in various livestock animal models. However, excessive content of omega-6 fatty acid compared to that of omega-3 fatty acid in the feed seems to need to be improved. In the gut microbiota analysis, the treatments affected the diversity, taxa, and predicted metagenome. Also, some traits can be linked to the results of laying performance. The results show that flaxseed and probiotics have distinct effects on laying hen physiology in many aspects under both normal and stress states, modulating apparent laying performance.

As of now, the reliability of biological markers is controversial, and thus novel markers are needed to assess host health more accurately. Distinct traits depending on the treatment such as fatty acids, lipid mediators, and microbial taxa that were not reported in other studies would be an interesting research subject to enlarge our understanding of the mechanism underlying the regulation of immune homeostasis in livestock animals can potentially serve as novel biological markers, although cumulated data from continuous further research are needed.

The final goal of developing biological markers is to improve animal welfare. The animal welfare issue has garnered much attention in recent years, but research on how to improve animal welfare is limited and biased. Most studies have only been focused on the environmental management of livestock housing, but other areas such as feed management should be considered. Since feed is provided daily to livestock, it can greatly affect the physiology of the livestock. This study was evaluated the various physiological effects of feed additives regarding livestock health, thereby showed possibility of managing livestock welfare by nutritional approach. As various distinct effects by nutraceuticals, the experimental flow of this study can be applied to evaluate the physiological effects of other nutraceuticals regarding the host health state and this work may be cited as a foundation study as a ‘proof of concept’ of animal welfare feed additives (Figure 67B). In other words, the physiological traits measured in this study may be assessed under other stress conditions to investigate whether the same tendency appears. Besides, studies should also be conducted on other parameters that can

represent chronic inflammation and stress conditions. For example, since blood circulating LPS is a causative agent of chronic inflammation, the level of this may be used as an marker for confirming the chronic inflammatory state of livestock animals (Lim et al., 2019). In the case of stress, the activation of the HPA axis in livestock has been mainly measured using cortisol or corticosterone levels, but this is a very responsive such as being significantly affected by stress at the time of sampling, so the levels in blood are limited to indicate chronic stress conditions. However, the cortisol or corticosterone levels in hair sample are emerging measure of cumulative activation of HPA axis (Wright et al., 2015). Therefore, if the hairs are removed at the start of the experiment and measure the concentration of cortisol or corticosterone in the freshly grown hair until the end of the experiment, it will be able to measuring the accumulated activation of HPA axis during the experiment. Taken together all these traits, screening a trait that differing in the same way under various conditions, it can be developed as a novel biological marker indicating inflammation and stress state. Meanwhile, it can also be applied as a screening platform to evaluate the possibility of novel nutraceuticals for animal welfare feed additives (Figure 68).

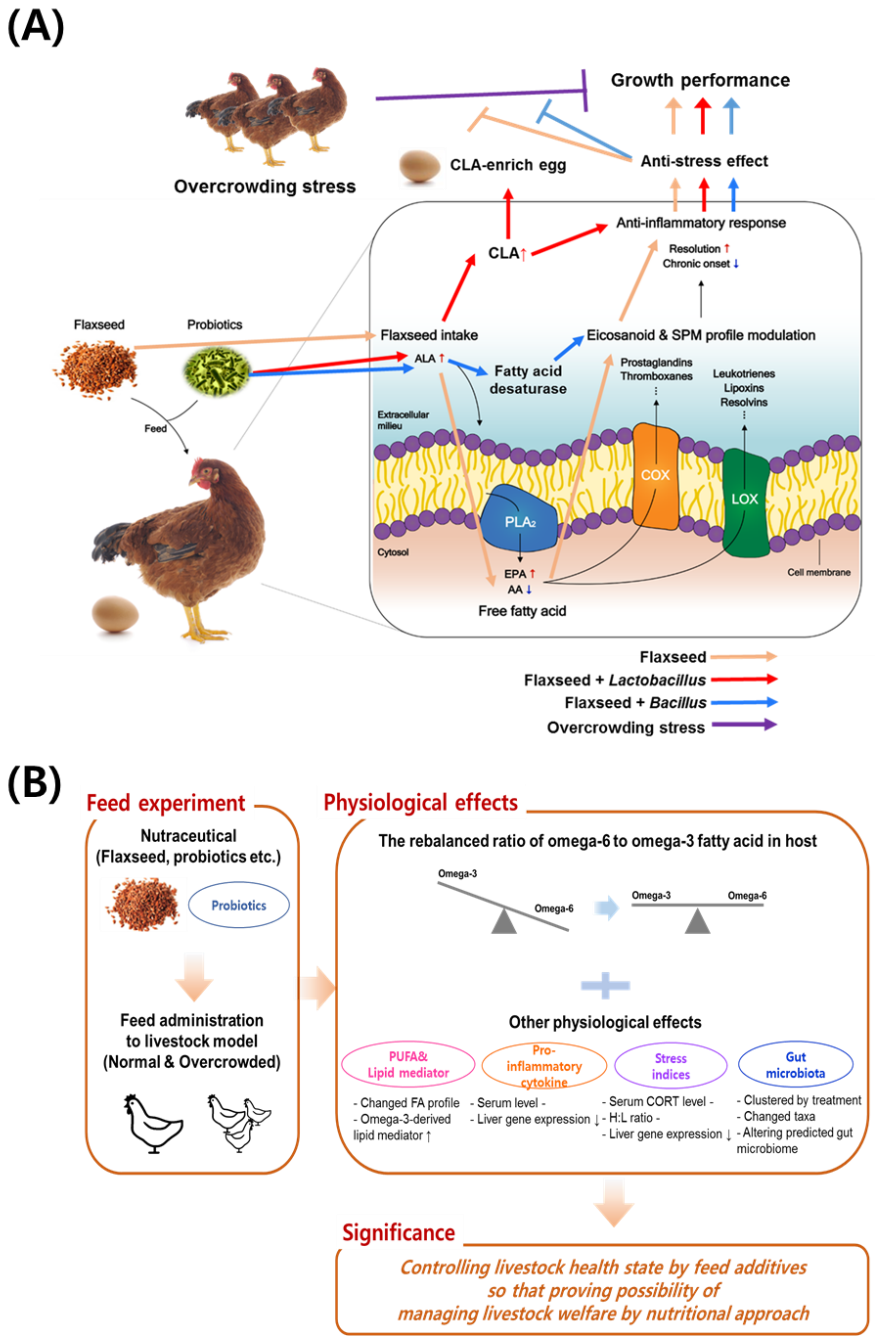

Figure 67. Overall conclusion of the study.

(A) Graphical conclusion of the study. (B) Summary of study and its significance.

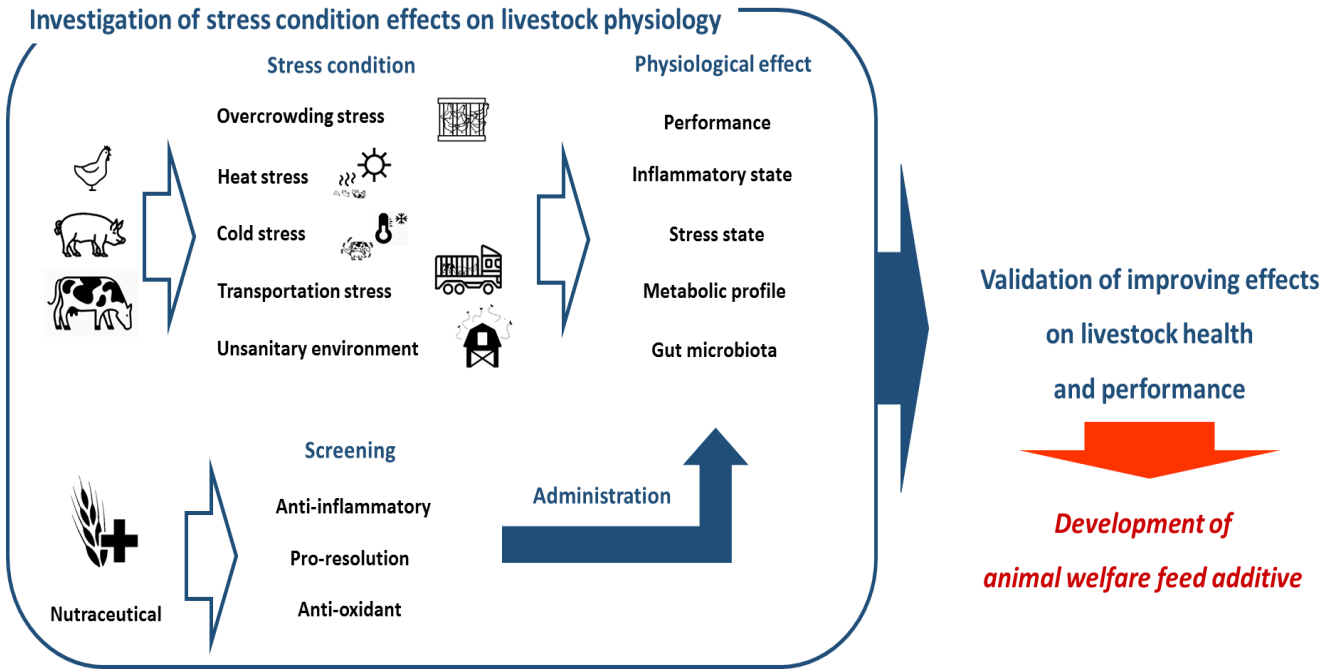

Figure 68. Application example for experimental flow of this study.

The experimental flow of this study including investigation of stress condition effects and screening of nutraceuticals can be applied to develop animal welfare feed additives by validating livestock health and performance.

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Summary in Korean

염증은 외부의 해로운 자극으로부터 숙주를 보호하는 필요한 생물학적 반응이지만, 만섬 염증은 개체의 부상과 세포의 죽음을 야기한다. 현대인들은 불균형한 식단이나 스트레스와 같은 만성염증을 유발하는 것들에 노출되어 있는데, 이는 당뇨병, 비만 그리고 암과 같은 질병의 증가와 밀접한 연관이 있다. 가축의 경우 역시 비슷한 환경이기 때문에, 이는 그들의 능력에 악영향을 줄 수 있다.

한편, 뉴트라수티컬은 질병을 예방하거나 치료하는 식품 성분을 뜻한다. 이는 기본적인 영양적 기능을 넘어 추가적인 치료적 혹은 생리적인 이익을 가져다 주기 때문에, 생리활성 물질을 규명하고 이를 정제한 형태로 제품을 개발하는 연구가 많이 진행되고 있다. 같은 맥락으로, 뉴트라수티컬의 사료첨가제로써 활용 역시 시도되고 있다. 많은 뉴트라수티컬의 경우 항염증 혹은 항산화 기능을 가지고 있기 때문에, 그들은 염증과 산화 스트레스를 감소시키는 데에 효과적일 수 있다. 특히나, 오메가-3 지방산은 염증 과정에 작용하는 대표적인 영양 성분인 다가불포화지방산이기 때문에 가축 건강 상태를 조절할 가능성이 있다. 또한 그 함량이 오메가-6 지방산에 비해 현대인과 가축의 식단 내에서 부족하기 때문에, 이 불균형은 숙주의 염증

항상성을 위협하여 많은 질병을 유발할 수 있다. 이외에도, *Lactobacillus*와 *Bacillus*와 같은 생균제는 항병원균 효과, 발효, 장관막 증진 그리고 면역조절 효과와 같은 기능을 가지고 있기 때문에 동물의 건강 상태를 증진시키기 위한 뉴트라수티컬로써 적용이 가능하다.

뉴트라수티컬의 건강 증진 효과를 평가하기 위해, 염증과 스트레스를 나타내는 생물학적 지표가 조사되어야 한다. 하지만, 많은 연구들은 오직 가축의 능력 변화에 대한 조사에만 집중하고 있었기 때문에, 뉴트라수티컬에 의한 생리학적 변화를 이해하는 데 있어서 불충분하다. 이번 연구에서는, 아마씨와 생균제를 뉴트라수티컬의 소재로써 선정하여 산란계의 염증, 스트레스 그리고 장내 균총을 포함한 다양한 생리적 변화를 조사하였다. 먼저, 아마씨가 산란계의 다가불포화지방산과 지질매개인자의 목록, 염증성 지표 그리고 스트레스 지표에 미치는 영향을 조사하였다. 다음으로, 아마씨와 2 가지 생균제 (*Lactobacillus plantarum*, Lp; *Bacillus licheniformis*, T2)를 함께 사용하여 같은 항목에 대해 조사하였다. 더욱이, 밀식 스트레스가 산란계에 미치는 영향을 확인하여 아마씨와 2 가지 생균제가 스트레스의 부정적 영향을 경감시킬 수 있는지 확인하였다.

첫 번째 연구에서는 알파-리포산의 생리적 효과를 *in vitro* 와 *in vivo* 에서 조사하였다. *In vitro* 연구에서는 지질다당류로 자극한 쥐 대식세포주 RAW264.7 에 알파-리포산을 처리하였다. 종양괴사인자-alpha, 인터루킨-6, 인터루킨-1beta 와 같은 전염증성 사이토카인 유전자의 발현이 줄어들었다. 또한, 고리형 산소화효소-2 와 같은 지질매개인자 생성 효소의 유전자 발현 역시 감소하는 것을 확인하였다. *In vivo* 연구에서는, 알파리포산이 풍부한 물질인 아마씨의 상용화 제품인 Lintex 170 을 33 주령 산란계에 4 주간 급여하였다. 아마씨를 급여함으로써, 달걀과 혈청에서의 오메가-6 와 오메가-3 의 비율이 모두 감소하였고, 이는 오메가-3 유래 지질매개 인자의 증가에 영향을 주었다. 이는 숙주의 염증과 스트레스 상태에 변화를 가져왔다.

두 번째 연구에서는, 아마씨와 생균제의 조합이 52 주령 산란계에 미치는 생리적 영향을 조사하였다. 생균제는 생산 수율과 목적 유전자 발현 유무를 기준으로 각각 3 가지 *Lactobacillus* 균주와 2 가지 *Bacillus* 균주로부터 Lp 와 T2 가 선정되었다. 아마씨와 생균제의 조합을 급여함으로써 생기는 특이적 변화들이 조사되었다. 아마씨의 효과는 오메가-6 와 오메가-3 의 비율의 감소, 간 내 전염증성 사이토카인 유전자의 발현이 감소와 같이 첫 번째 연구의 결과와 유사하였다. 한편, 아마씨와 Lp 조합의 특이적 효과로 계란 내 c9, t11-CLA 함량이

증가하였다. 또한, 염증과 스트레스 지표에 있어 부가적인 개선 효과가 있었고, 이는 산란 성적의 개선으로 이어졌다. 반면에, 아마씨와 T2 조합은 산란 성적에 있어 부정적인 영향을 확인하였다. 사양한 생균제의 종류에 따른 산란 성적의 차이는 장내 균총의 메타게놈 예측 결과와 연관지어 생각할 수 있다. 아마씨와 생균제 급여가 장내 균총에 유의적 변화를 가져왔기 때문에, 그들의 메타게놈 역시 실험군 별로 다르게 예측된 것들이 있었다. 예를 들어, 아마씨와 Lp 를 급여한 실험군은 포스포엔올피루브산 당 인산기 전이체계의 활성화가 감소하는 것을 확인하였고, 이는 숙주의 영양소 이용가능성에 이익을 주어 산란 성적의 증가와 연관 지어질 수 있다. 아마씨와 T2 를 급여한 실험군의 경우, 에너지 대사의 활성화가 예측되었는데, 이는 숙주의 에너지 이용성에 비효율을 초래하고 산란 성적을 감소시킬 수 있다.

세 번째 연구에서는, 아마씨와 생균제 조합이 밀식 상태의 52 주령 산란계에 미치는 영향을 조사하였다. 이를 위해, 동일한 일반 사료를 급여하면서 밀식 스트레스가 산란계의 염증과 스트레스 상태 그리고 장내균총에 미치는 영향을 먼저 검사하였다. 이후, 같은 항목들을 이용하여 아마씨와 생균제가 밀식 상태의 52 주령 산란계에 미치는 영향을 조사하였다. 염증 지표, 스트레스 지표 그리고 산란 성적과

같은 전반적인 생리적 지표들이 밀식스트레스에 의해 악화되는 것을 확인하였고, 또 이것이 아마씨와 생균제에 의해 회복되는 것을 확인하였다. 또한, 이들이 각각의 실험군에 대해 장내 균총과 그 예측된 메타게놈에 미치는 영향에 대해서도 확인하였다. 그룹 중에서, 아마씨와 T2 를 급여한 실험군이 특이적으로 아미노산과 관련된 대사들을 활성화함을 확인하였고, 이는 산란 성적의 증가와 연관이 있을 수 있을 것이다.

이번 연구에서, 뉴트라수티컬 (아마씨와 생균제)과 밀식스트레스가 미치는 생리적 효과에 대해 조사하였다. 첫 번째 연구 결과를 바탕으로 했을 때, 아마씨의 급여는 오메가-6와 오메가-3의 비율의 변화와 지질 매개인자의 목록을 변경하여 산란계의 염증 및 스트레스 지표와 산란 성적에 영향을 준 것을 알 수 있었다. 이는 가축 식단 내 오메가-3 지방산 강화를 통한 균형된 불포화지방산 섭취가 스트레스 상황 등에서 숙주의 생리에 도움을 줄 수 있다고 결론지을 수 있다. 또한, *Lactobacillus*와 *Bacillus*와 같은 생균제는 c9, t11-CLA 생산이나 아미노산 관련 대사의 활성화 등을 통해 아마씨를 급여한 숙주의 항염증과 항스트레스 효과에 있어서 추가적인 효과를 줄 수 있다. 현재까지로는, 만성 염증과 스트레스가 실제적으로 가축의 생리에 영향을 끼치는지 명확히 하기 위해 더 많은 연구가 필요하다. 또한,

특정 사료첨가제가 염증과 스트레스 상태를 조절함으로써 숙주의 건강 유지에 도움을 줄 수 있는지를 증명하기 위한 반복실험이 진행되어야 한다. 그럼에도 불구하고, 이러한 결과들은 혹독한 환경 내에 있는 가축의 복지가 영양적 관리를 통해 개선될 수 있음을 제안하며, 이는 '동물 복지형 사료첨가제'라는 신규한 사료첨가제의 범주에 대한 가능성을 보여준다.

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주요어: 오메가-6와 3 지방산 균형, 뉴트라수티컬, 염증, 스트레스, 동물 복지

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