Novel Application of Microalgae in Animal Nutrition and Human Health

A Dissertation
Presented to the Faculty of the Graduate School
of Cornell University
in Partial Fulfillment of the Requirements for the Degree of Master of Science

by
Tao Sun
August 2018

ABSTRACT

World population is estimated to reach 8.6 billion by 2030. The increasing population caused huge pressure on food supply which relies on staple crop production such as corn and soybean. However, microalgae as a novel generation of feedstock have drawn great attention due to their various advantages and applications. They can not only be supplemented in animal diets as protein ingredients but also be cultured to produce health beneficial phytochemicals such as astaxanthin and DHA for human consumption. Therefore, we conducted three studies in broiler chicks to: (1) measure the nutrient retention and digestibility of 10% *Nannochloropsis oceanica* as a protein ingredient; (2) investigate the bioavailability of *Haematococcus pluvialis* for producing astaxanthin enriched meat products and its effect against heat stress, and (3) explore the effect of *Aurantiochytrium* on growth performance, health status and meat production and quality. Our finding demonstrated that (1) nutrient retention and digestibility of 10% *Nannochloropsis oceanica* were largely unaffected except retention and digestibility of amino acid were decreased; (2) dietary *Haematococcus pluvialis* supplementation could enrich astaxanthin in meat products and change endogenous antioxidant defense, and (3) feeding up to 2% of *Aurantiochytrium* had no effect on growth performance, health status, and meat quality and production. In summary, dietary microalgae supplementation could be beneficial to animal nutrition and human health by providing potential protein feed ingredient, improving meat production and quality, and producing phytochemical-rich foods.

BIOGRAPHIC SKETCH

Tao Sun was born in Anhui, China on April 26, 1992, where he spent his first eighteen years. After finishing high school, Tao went to China Agricultural University (CAU) and majored in Animal Science. Then he participated "2+2" exchange program from CAU and spent two years in Indiana State after transferring to Animal Science Department of Purdue University. During his stay in Purdue, Tao started his initial research experience in Dr. Applegate's lab as an undergraduate researcher in the area of poultry husbandry and nutrition. Immediately following his graduation, he entered Cornell University to pursue his master's degree in Animal Nutrition under the supervision of Dr. Xingen Lei. His master research projects focus on investigating multiple types of microalgae biomass and their effects on broiler nutrition and human health until August 2018.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Lei for his intellectual guidance through my graduate program. I have developed a strong understanding of experimental research, critical thinking of science and a good habit for research. I have gained so much during my masters' study because of the Dr. Lei's challenge and encouragement all the time. I would also like to thank Dr. Shubing Qian of Genetics and Dr. Dan Brown of Animal Science for serving on my graduate committee and their valuable advice and help.

I would also like to thank Karl and Carol Roneker and Marko Vatamaniuk for their support in every animal study and lab maintenance. I am grateful to Dr. Ling Tao for her patience and guidance that helped me to be familiar with the lab and start my first experiment. I thank Joanne Parson for her cheerful help in and out of the lab. I am also grateful to Andrew Magnuson, Samar Tolba, and Guanchen Liu for their help on animal husbandry and lab analysis as an efficient and great chicken research group. I would also like to thank my other colleagues and friends in the lab: Dr. Matthew Barcus, Dr. Zeping Zhao, Madeleine Keefe, Mengmeng Zhang, and Weiwei Liu.

Finally, I would like to thank my parent Changchun Sun and Huiping Tao, and my close extended family for their greatest support, advice and understanding.

TABIE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AA, amino acid

AST, astaxanthin

BWG, body weight gain

CF, crude fat

CP, crude protein

DHA, docosahexaenoic acid

DM, dry matter

FI, feed intake

G: F ratio, gain to feed ratio

GPx, glutathione peroxidase

GR, glutathione reductase

GSH, glutathione

GSSG, glutathione disulfide

GST, glutathione s transferase

MDA, malondialdehyde

SOD, superoxide dismutase

TPA, texture profile analysis

CHAPTER ONE

Introduction

1.1 Nutritional Application of Microalgae

The world population reached 7.6 billion in 2017 and this number is estimated to reach 8.6 billion by 2030.[1] Total food production has increased every year to meet the massive demand for this rapidly increasing global population. In 2017, the U.S. poultry industry raised 9 billion broiler chickens and more than 40 billion pounds of chicken products were served on a ready-to-cook basis.[2] Total poultry production has increased from 1,500 million pounds of meat in 1,950 to 40,000 million pounds in 2016.[2] An estimated 30 million and 14 million metric tons of corn and soybean meal, respectively, will be used as broiler and breeder feed in 2018.[2] This use directly competes against the need of corn and soybean as a staple for human consumption. Thus, it is necessary to find alternative sources of feed for maintaining sustainable animal production.

Recently, microalgae have gained a great deal of interest for their potential as the third generation of feedstock for biofuel production and the high contents of protein and other nutrients in the defatted biomass as a new source of animal feed. Microalgae are aquatic photoautotrophic single cellular organisms that have potential to convert carbon dioxide to biofuels, foods, and feeds.[3-4] In addition to high levels of protein and amino acids, some microalgae species contain beneficial components including n-3 fatty acids and bioactive compounds.[5] During the past years, our laboratory has evaluated nutrient composition and feeding values of three types of defatted microalgae including *Straurospira sp*., *Desmodesmus sp*., and *Nannochloropsis oceanica* in the diets for weanling pigs, broiler chicks, and laying

hens.[6-8] The defatted microalgae *Nannochloropsis oceanica* were also effective in enriching n-3 fatty acids in the liver and muscle (breast and thigh) of broiler chicks[9] and eggs.[10]

1.2 Nutrient Retention and Digestibility of Microalgae

Few studies in the past have determined nutrient digestibility and retention of microalgae in broiler chicks. The digestibility of crude protein in Chlorella, Spriulina and Coelastrum were reported to be 89.3, 89.2 and 88.6% respectively, when being used as the sole source of protein (10% of the diet) in rats.[11] Meanwhile, the digestibility of crude protein in *Sprirulina maxima* was found to be between 75.5 to 76.7% at 15% level in the rat diet.[12] Although these digestibility values derived from feeding microalgae as the sole source of dietary protein were encouraging, their nutritional relevance was problematic. This was because feeding microalgae over 20% caused adverse growth performance in chicks,[13] let alone feeding them microalgae as the only protein source.[14] Likewise, adverse performance was also observed from feeding chicks with high levels of Diatom microalgae due to relative deficiency in methionine and cysteine.[6] Therefore, the nutrient digestibility or retention of microalgae may be estimated by feeding broiler chicks microalgae to replace both corn and soybean meal in corn-soy basal diet by direct method (total collection of excreta) or indirect method (using indigestible marker such as chromic oxide).

1.3 Health Benefits from Microalgal Phytochemicals

As one of the oldest living organism on earth, over 50,000 species of microalgae live in ocean or lakes. There are various applications of microalgae including supplemented as a protein feed ingredient or selected and genetically modified to produce health benefiting phytochemicals such as astaxanthin and DHA.

Astaxanthin is a xanthophyll carotenoid which is widely used in nutraceutical, cosmetics, food and feed industry. It can be found in aquatic animals including salmon, trout, red seabream, shrimp and lobster. Microalgae *Haematococcus pluvialis* is the richest source of natural astaxanthin.[15] Astaxanthin has a strong antioxidant activity which is several-fold higher than vitamin E and even been called as "super vitamin E".[16-17] Because of that, astaxanthin supplementation is believed to protect the skin and retinal receptor of the eyes against UV-light photo-oxidation.[15] The anti-inflammatory effect from astaxanthin could also be beneficial to heart health associated with the development of chronic heart disease by modifying plasma LDL and HDL cholesterol.[18] Additionally, several studies have demonstrated an anti-cancer effect of astaxanthin in colon,[19] mammary,[20] and urinary bladder cancers.[21] Thus, it has been suggested that daily ingestion of astaxanthin might be a practical and beneficial strategy to human health.[15]

DHA (Docosahexaenoic acid) is an essential omega-3 polyunsaturated fatty acid (PUFA) [22] and plays an important role in maintenance of neural functions and is abundant in the human brain and retina.[23] Supplemental DHA has been associated with a reduced risk of cardiovascular disease[24], non-alcoholic fatty liver disease[25], hypertriacyglycerolemia[26] and Alzheimer disease.[27] Since human have very limited abilities of DHA synthesis, they require dietary DHA supplementation such as DHA rich foods including fatty fish and algae.[28] In consideration of multiple health benefits, World Health Organization recommends 1-2% Omega-3 fatty acid including DHA of energy per day to prevent chronic metabolic disease.[29]

1.4 Research Objectives

Our research objectives included three aspects. The first objective was to determine the retention and digestibility of nutrients including dry matter, crude protein, crude fat, energy, amino acid and mineral from the microalgae *Nannochloropsis oceanica,* and whether 10% microalgae supplement to replace corn and soybean would affect growth performance in broiler chicken. The second objective was to investigate the bioavailability of microalgal astaxanthin from *Haematococcus pluvialis* to broiler chicks under normal or heat stress conditions and its effects on the antioxidative stress, growth performance and meat quality. The third objective was to investigate whether feeding DHA-rich microalgae *Aurantiochytrium* to broiler chicks had any effect on growth performance, health status, meat quality and production.

CHAPTER TWO

Effect of Microalgae on Nutrient Digestibility and Retention in Broiler Chicks

2.1 Abstract

The purpose of this experiment was to evaluate the potential of defatted microalgae (*Nannochloropsis oceanica*, 45% CP and 3.8% ether extraction, CF) from biofuel production as a potential crude protein source for growing broilers. This was evaluated through the addition of 10% of the defatted microalgae into a corn-soybean meal basal diet (BD) and measuring its impact on nutrient retention and digestibility in broiler chicks. Day-old hatchling Cornish Giant cockerels were divided into two groups (5 cages/group, 4-5 chicks/cage) and fed either a control or microalgae diet for 6 weeks. Starting week 3, chicks were fed diets containing 0.2% chromic oxide as an indigestible marker. Total excreta of individual cages was collected daily for 3 consecutive days during week 6. At the end of week 6, chicks were euthanized to collect ileal digesta from 1 chick/cage. Concentration of DM, CP, CF, AA, and chromic oxide in digesta, excreta, and diets were assayed. Apparent nutrient retention was calculated based on total excreta collection and chromic oxide as an indigestible marker. The latter was also used to estimate apparent ileal digestibility of nutrients. Data was analyzed by the Student t-test. Chicks fed the two diets had similar ADFI and G: F ratio, although those who were fed the microalgae diet had a 5% heavier BW ($P < 0.05$) than chicks fed the control. Feeding the microalgae diet decreased ($P < 0.05$) apparent retention of DM by 8.7% when measured via the direct method while apparent retention of DM was enhanced by 1.3% when measured through the indirect method compared with the control. Feeding the microalgae diet elevated (4.17% and 8.11%, $P < 0.05$) apparent retention of CF determined by both methods, respectively. Supplemental defatted

microalgae decreased ($P < 0.05$) the apparent retention and digestibility of CP by 13.2% and 13.3% respectively. Dietary supplementation of microalgae decreased ($P < 0.05$) apparent ileal digestibilities of 8 essential AA and 6 nonessential AA, ranging from 32% for isoleucine to 7% for glutamic acid (list all of the AAs). Microalgal supplementation decreased ($P < 0.05$) apparent retention of 6 essential AA and 5 nonessential AA, ranging from 16% for threonine to 0.6% for leucine. In conclusion, supplementing 10% defatted microalgae in broiler diets did not show consistent effect on apparent retention or ileal digestibility of DM, CF, CP or energy determined by the two methods, but the microalgae diet decreased apparent retention or ileal digestibility of a number of AA.

2.2 Introduction

The United Nations expect the world population to increase from 7.6 billion in 2017 to 8.6 billion by 2030[1]. With this explosive increase of the human population total food production has to increase to meet this massive demand. Within the U.S. alone the poultry industry raised 9 billion broiler chicks and produced more than 40 billion pounds of chicken products in 2017 with this number increasing everyday[2]. Approximately 30 million and 14 million metric tons of corn and soybean meal were used to feed these broilers in 2018[2]. However, limited resources such as arable land and water needed to grow these crops are necessary for both animals and humans. Ultimately this leads to a desire to find an alternative crude protein and energy source for broiler diets to increase sustainability in animal production.

Microalgae biomass has gained a great deal of attention as a third generation feedstock for biofuel production, as an animal feed ingredient, and as a good source to produce phytochemicals for human health. One species of microalgae, *Nannochloropsis oceanica,* is an

excellent candidate to biofuel production as it is raised in salt water so no arable land and fresh water are needed to compete with other staple crops[30]. The by-product after oil extraction of this microalgae is a natural food source which could be implemented to partially replace corn and soybean oil in swine and poultry diets[7]. A previous study our lab conducted utilizing dietary supplementation of up to 23% defatted *Nannochloropsis oceanica* in laying hens demonstrated that the addition of 10% of this algae did not affect hen performance[7].

Therefore the objective of this experiment was to determine if supplementing defatted microalgae *Nannochloropsis oceanica* as a replacement for the macro ingredients corn and soybean in broiler diets would affect the retention and digestibility for dry matter, crude protein, crude fat, energy, amino acids and minerals.

2.3 Materials and Methods

2.3.1 Animals, Diets, and Management

Day-old hatchling Cornish broiler chicks were obtained from Moyer's Chicks (Quakertown, PA). On day 0, birds were divided into control and algae treatments (5 cages/treatment, 4-5 chicks/cage) randomly and housed in thermostatically-controlled cages. Defatted microalgae (*Nannochloropsis oceanica*) in the form of powder were generated from biofuel production (Cellana, Kailua-Kona, HI) and the nutrient composition of microalgae was shown in **Table 2.1**. Chicks from control group were fed corn and soybean meal basal diet (BD), while algae diet was formulated as BD with microalgae inclusion as protein and energy feed ingredient to replace 10% of corn and soybean meal in total. Starter (week0-3) and Grower (week4-6) diets were formulated to meet broiler nutrient requirements from NRC (1994) as shown in **Table 2.2**. Water and feed were accessible to chicks for 24 hours and 22h light: 2h dark light schedule was

provided for whole period. Experimental protocol was approved by the Institution of Animal Care and Use Committee of Cornell University.

2.3.2 Growth Performance and Collection of Excreta and Digesta

Body weight was recorded weekly. Feed troughs were weighed and refilled with fresh diet daily to obtain feed intake. Gain: Feed ratio was calculated as body weight gain divided by average daily feed intake. Over the period of last 3 days in week 6, total excreta from each pen were weighed and sampled from various spots daily after removing any feathers or debris. At the end of week 6, all animals were fasted for 8 hours before euthanization via CO_2 and ileal digesta between Meckel's diverticulum and ceca were collected. All samples were weighed and freeze dried in -20^{o}C by Virtis freeze dryer (Model: 20 SRC-X, Gardiner, NY). Resulting dried samples were then weighed and ground to a fine powder and stored in -20^{o}C until analysis.

2.3.3 Proximate Nutrient Analysis

Dry matter, crude protein, crude fat, amino and mineral profile from excreta, ileal digesta and diets were measured based on methods described in AOAC [31]. Dry matter of experimental diets was determined by measuring the weight loss during drying the sample in 100^{o}C oven for 24 hours. Excreta and digesta dry matter were calculated after sample freeze dried. N analyzer (2300 KjeltecTM Analyzer, FOSS) has been used to determine crude protein (nitrogen times 6.25) and crude fat was determined by Soxhlet ether extraction method using petroleum ether. Chromic oxide in diet, excreta and ileal digesta were assayed using the method from Fenton [32]. The amino acid profile for diet and ileal digesta were determined by using the Shimadzu HPLC system after acid hydrolysis[33]. Sulfur amino acids were measured through performic acid oxidation followed by acid hydrolysis[34]. Mineral profile was determined by inductively coupled

plasma (ICP) trace analyzer emission spectrometry (Model: Thermo Scientific ICAP 6000 trace element analyzer). Energy was determined by bomb calorimeters. All above analyses were done in duplicate.

2.3.4 Nutrient Retention and Digestibility

Nutrient retention was measured and calculated by two methods including 0.2% chromic oxide as indigestible marker and total excreta collection to minimize variation. In addition, nutrient digestibility was determined by chromic oxide method. Corresponding equations were presented as following:

$$Retention(\%) = \left(1 - \frac{Cr_{diet} \times Nutrient_{excreta}}{Cr_{excreta} \times Nutrient_{diet}}\right) \times 100$$

or

$$Retention(\%) = \left(1 - \frac{Total\ collection_{excreta} \times Nutrient_{excreta}}{Total\ collection_{diet} \times Nutrient_{diet}}\right) \times 100$$

or

$$Digestibility(\%) = \left(1 - \frac{Cr_{diet} \times Nutrient_{ileal}}{Cr_{ileal} \times Nutrient_{diet}}\right) \times 100$$

Cr_{diet}, $Cr_{excreta}$ and Cr_{ileal} stand for Chromic Oxide concentration in diet, excreta and ileal digesta, while $Nutrient_{diet}$, $Nutrient_{excreta}$ and $Nutrient_{ileal}$ stand for each nutrient concentration in diet, excreta and ileal digesta, respectively.

2.3.5 Statistical Analyses

Data was analyzed with RStudio (RStudio, Version 1.1.447. Boston, MA). The overall main effect of algae diet was determined by Student t-test and the significant level for difference was $P<0.05$.

Table 2.1. Nutrient analysis of the defatted biomass of *Nannochloropsis oceanica.*

Nutrient (%, "as is")		Amino acid (% "as is")	
DM[1]	95.6	Pro	2.38
CP	45.1	Glu	4.13
CF	3.80	Leu	3.51
ADF	3.10	Asp	3.53
NDF	17.0	Lys	2.37
Ca	0.42	Val	2.33
P	0.69	Arg	2.23
Na	3.27	Gly	2.36
K	1.22	Phe	2.05
Mg	0.51	Thr	1.97
Fe, mg/kg	1970	Ile	1.83
Cu, mg/kg	8.0	Ser	1.63
Mn, mg/kg	166	Tyr	1.52
Zn, mg/kg	33.0	His	0.77
Mo, mg/kg	1.50	Met	0.67
Se, mg/kg	0.18	Trp	0.55
		Cys	0.42

*Abbreviations: ADF, acid detergent fiber; DM, dry mass; CP, crude protein; CF, crude fat; DM, dry mass; MUFA, monounsaturated fatty acid; ADF, acid detergent fiber; NDF, neutral detergent fiber; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Table 2.2. Composition (g/kg) of starter and grower diets for broiler chicks

	Starter		Grower	
Ingredients, g/kg	Control	Algae	Control	Algae
Corn	488.7	510.1	512.7	500.1
Soybean Meal	425.0	332.8	400	314.8
Algae	0	100	0	100
Limestone	13	13	13	13
Dical. Phos	19.5	19.5	19.5	19.5
Corn oil	40	15	40	40
Sodium Chloride	4	0	4	0
Vit. /Min. Mix*	4	4	4	4
DL-methionine	3.5	3.5	3.5	3.5
Lysine	0.5	1.2	0.5	1.2
L-Threonine	0.8	0.8	0.8	0.8
Arginine	1.07	1.07	0	1.07
Chromic oxide	0	0	2	2

Nutritive Value				
ME, kcal/kg	3025	2940	3045	3073
Crude protein, %	23.8	24.1	22.8	23.2
Methionine, %	0.71	0.72	0.70	0.71
Cysteine, %	0.41	0.39	0.39	0.37
Lysine, %	1.45	1.47	1.38	1.41
Phosphorus, %	0.75	0.77	0.74	0.76
Calcium, %	1.14	1.15	1.13	1.14

*Vitamin and mineral mixture provided the following nutrients (per kg of diet): 0.37 mg retinyl palmitate, 4.0 mg cholecalciferol, 50 mg dl-alpha-tocopherol, 0.94 mg menadione, 5.5 mg D-bio tin, 3250 mg choline chloride, 0.84 mg folic acid, 53 mg niacin, 15 mg Ca-D-pantothenate, 5.5 mg riboflavin, 2.68 mg thiamine hydrochloride, 6.4 mg pyridoxine hydrochloride, 0.015 mg vita min B12, 12 mg copper, 0.54 mg iodine, 8.0 mg iron, 91 mg manganese, 0.2 mg selenium, 60 m g zinc.

2.4 Results

2.4.1 Growth Performance

During week 0-3, no differences were found in body weight gain, feed intake and G: F ratio between control and algae treatment (**Table 2.3**). However, birds fed with the microalgae diet had 5.0% and 3.3% higher ($P < 0.05$) body weight gain during week 4-6 and 0-6 compared with the control, respectively. Meanwhile, feed intake and G: F ratio remained unaffected by algae diets over the control during week 4-6 and 0-6.

2.4.2 Retention or Digestibility of DM, CP, CF and Energy

Two methods including chromic oxide and total collection were used to calculate retention or digestibility of DM, CP, CF and energy from control and algae diet (**Table 2.4**). Bird fed algae diet had 8.7% ($P<0.05$) lower retention of DM by total collection method than the control, while there was 1.3% increase of DM retention ($P < 0.05$) by chromic oxide method. Meanwhile, retention of CP and energy from microalgae group were decreased ($P<0.05$) using total collection method by 13.2% and 8.0% over the control, whereas there were no differences from using chromic oxide method. However, retention of CF from algae diet calculated by total

collection and chromic oxide method was 4.2% and 8.1% higher ($P < 0.05$) than the control. In addition, digestibility of DM, CP, CF and energy were unaffected by algae treatment except CP digestibility was decreased ($P < 0.05$) by 13% over the control.

2.4.3 Retention and Digestibility of Amino Acids and Mineral

Chicks supplemented algae treatment exhibited consistent lower amino acid retention and digestibility than the control (**Table 2.5**). There were at least 6 essential amino acids and 5 nonessential amino acids decreased ($P<0.05$) by algae diet ranging from 0.6% to 16%. Meanwhile, feeding algae diet lowered ($P<0.05$) 13 amino acid digestibility (9 essential amino acids, 5 nonessential amino acids) ranging from 50.3% for glycine to 4.3% for arginine. Retention and digestibility of potassium, calcium, sodium, phosphorus and sulfur were shown in **Figure 2.1**. Feeding microalgae treatment resulted in 16% higher ($P < 0.05$) and 17% lower ($P < 0.05$) retention of potassium and sodium, respectively. Meanwhile, there was no different in retention of calcium, phosphorus and sulfur. In addition, mineral digestibility remained largely unchanged except digestibility of sodium was reversely increased ($P < 0.05$) compared with the control.

Table 2.3. Effect of microalgae on growth performance of broiler chicks

Treatment	Week	Control	Algae	SEM	P value
Body weight gain	0-3	35.3	35.1	0.616	0.874
g/chick/d	4-6	86.7a	91.0b	1.10	0.030
	0-6	61.0a	63.0b	0.535	0.031
Feed intake	0-3	46.6	46.6	1.07	0.991
g/chick/d	4-6	120	125	2.49	0.204
	0-6	83.3	85.8	0.69	0.243
Gain: feed ratio	0-3	0.757	0.753	0.0003	0.728
	4-6	0.722	0.726	0.008	0.749
	0-6	0.732	0.734	0.007	0.860

Data are expressed as mean (n=5). Main effects were analyzed by two sample unpaired t test.

Table 2.4. Effect of microalgae on retention and digestibility of broiler chicks

Treatment	Control	Algae	SEM	P value
Retention				
Total collection method				
Dry matter, %	72.4	66.1	1.22	<0.01
Crude protein, %	66.1a	57.4b	2.15	0.015
Crude fat, %	82.7a	86.3b	1.17	0.038
Energy, %	77.5a	71.8b	1.01	<0.01
Chromic Oxide method				
Dry matter, %	94.4a	95.7b	0.17	<0.01
Crude protein, %	62.2	64.7	1.43	0.246
Crude fat, %	83.8a	90.6b	0.91	<0.01
Energy, %	73.8	74.9	0.42	0.073
Digestibility				
Dry matter, %	93.1	91.3	0.73	0.205
Crude protein, %	77.7a	67.4b	2.17	0.031
Crude fat, %	81.5	81.4	2.37	0.978
Energy, %	66.4	63.2	3.48	0.558

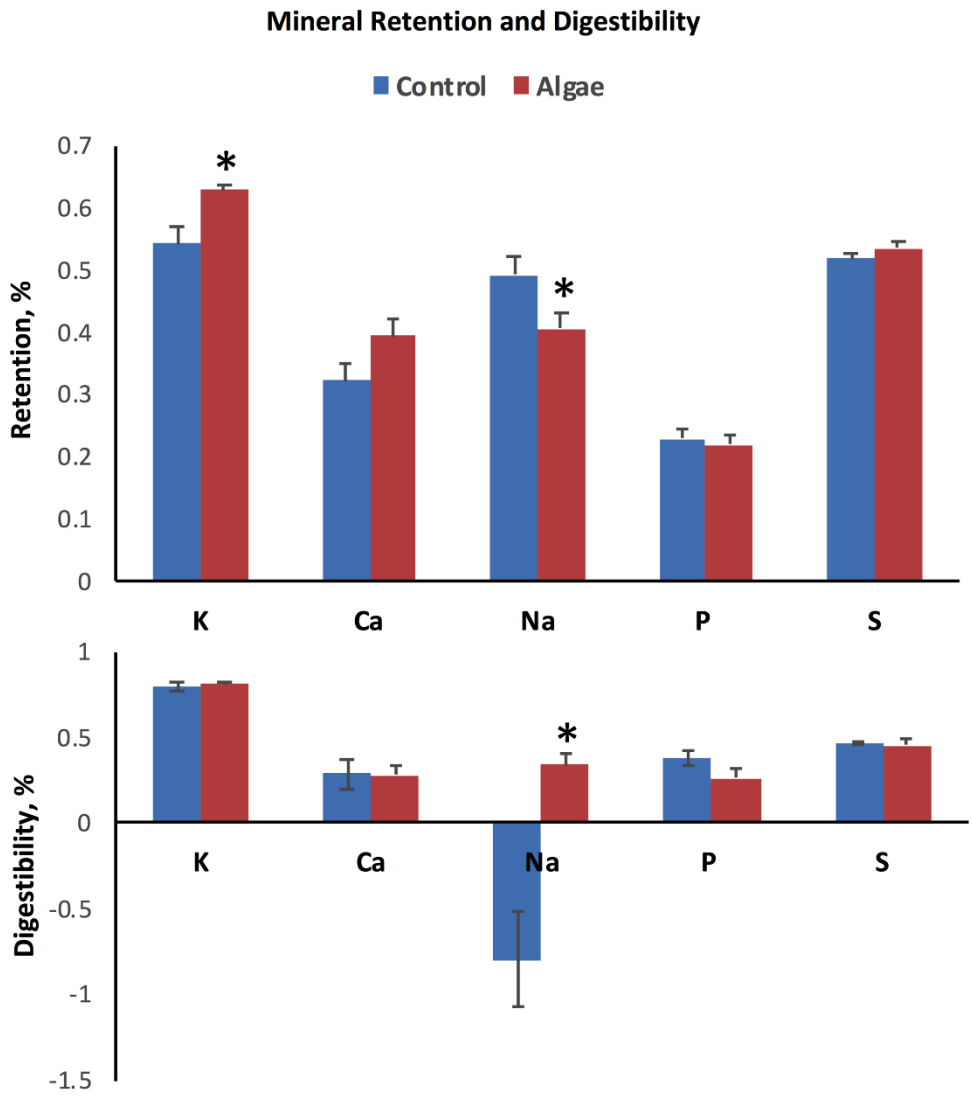
Data are expressed as mean (n=5). Main effects were analyzed by two sample unpaired t test.

Table 2.5. Effect of microalgae on retention and digestibility of broiler chicks

Treatment	Control	Algae	SEM	P value
Retention, %				
Isoleucine	89.3a	81.9b	0.557	<0.001
Leucine	98.7a	98.1b	0.098	0.032
Lysine	91.8a	89.8b	0.474	0.031
Phenylalanine	93.6a	84.7b	0.791	0.004
Threonine	90.4a	75.9b	0.523	<0.001
Valine	85.0a	78.5b	0.685	<0.001
Alanine	94.1a	93.2b	0.168	0.009
Asparagine	96.7a	90.3b	0.224	<0.001
Glutamine	97.3a	95.3b	0.150	<0.001
Serine	96.1a	92.1b	0.200	<0.001
Tyrosine	97.2a	95.4b	0.130	<0.001
Digestibility, %				
Arginine	86.0	82.3	1.54	0.167
Histidine	85.6a	68.8b	3.56	0.014
Isoleucine	79.0a	54.2b	4.87	0.0144
Leucine	95.6a	88.1b	1.61	0.015
Lysine	88.9a	79.2b	2.49	0.043
Phenylalanine	87.0a	64.0b	1.34	<0.001
Threonine	92.3a	74.4b	1.82	0.001
Valine	93.1	83.3	3.03	0.065
Alanine	87.0	77.7	2.62	0.052
Asparagine	94.1a	77.9b	1.25	<0.001
Glutamine	95.1a	88.3b	0.975	0.002
Glycine	82.0a	62.1b	3.19	0.005
Serine	93.1a	79.6b	1.40	0.001
Tyrosine	95.6	91.4	1.25	0.063

Data are expressed as mean (n=5). Main effects were analyzed by two sample unpaired t test.

Figure 2.1. Effect of microalgae on retention and digestibility of mineral



Retention and digestibility of potassium, calcium, sodium, phosphorus and sulfur in broiler chicks fed 0 or 10% algae diet for 6 weeks. Values are means ± SEs, n = 5. *Different from control, $P < 0.05$.

2.5 Discussion

The present study suggested that 10% microalgae inclusion exerted no effect on growth performance including BWG, FI and G: F ratio to replace equal amounts of corn and soybean meal in the basal diet. Meanwhile, 5% increase of BWG was shown in chicks fed microalgae diet over the control. It is probably due to the high CP% (45%) from defatted microalgae *Nannochloropsis oceanica*, which is almost comparable to SBM (CP%: 47.7%) and higher than corn (CP%: 7.3%). Another possibility could be that this microalgae was enriched with bioactive compounds, such as vitamin, polyunsaturated fatty acids and numerous polyphenolic compounds, which could improve growth performance and animal health[35, 36]. From previous research, Gatrell et al. used the same defatted *Nannochloropsis oceani*ca (CP: 38.2%, CF: 3.60%) and conducted a broiler feeding trail with 0, 2, 4, 6, 8 and 16% microalgae inclusion.[37] From that, 16% microalgae decreased BWG and G: F ratio in broiler but 8% microalgae inclusion didn't affect growth performance. Two studies presented similar results and the discrepancy of tolerance levels of microalgae could be attributed to the CP (45% vs. 38%) and related amino acid (AA) concentrations variation. In consideration of growth performance, it is possible to replace corn and soybean meal to defatted microalgae *Nannochloropsis oceanica* with high CP% (45%) as protein source feed ingredients by up to 10%.

Another finding was that retention of CF with 10% microalgae inclusion was increased in both chromic oxide and total collection methods. This could be due to either higher crude fat absorption through gastrointestinal track, or less crude fat excretion, or both. The possible reason would be defatted microalgae has better lipid profile which is easier to absorb or harder to excrete through intestine than that from corn and soybean meal. Interestingly, feeding defatted *Nannochloropsis oceanica* microalgae can enrich n-3 fatty acids including DHA and EPA and

improve n-6: n-3 ratio into broiler breast and thigh tissues,[9] which could contribute to better human health by lowing the risk of metabolic diseases such as cardiovascular and Alzheimer's disease[24, 27]. Therefore, microalgae inclusion in broilers is demonstrated to not only improve crude fat utilization but also produce more valuable and healthier chicken products than feeding corn and soybean meal.

Though crude protein concentration of defatted algae biomass was reported to be comparable to soybean meal (45% vs. 47%), retention and digestibility of CP and AA in algae were significantly lower than those in corn and soybean meal. However, it is not surprising that algae biomass has lower CP retention and digestibility since there are other non-protein nitrogen such as nucleic acid, amines, glucosamides and nitrogen from cell wall material which representing that amino acid percentage would be less as the similar crude protein percentage as corn and soybean meal[38]. Meanwhile the microalgal cell wall has high concentration of polysaccharide cellulose which is difficult to digest in the broiler GI tract and may contribute to lower nutrient retention and digestibility of CP or AA[39]. However, lower CP retention and digestibility could be potentially attenuated by supplementing proteases or additional essential amino acids[6, 39].

Higher potassium retention rate demonstrated that less potassium was excreted from broilers offered the microalgae diet with respect to the control. This is beneficial to the environment as too much potassium from broiler excreta may cause water pollution[40] which has become a concern in agriculture and a sustainable development and rational utilization of mineral should be considered. Furthermore, the decrease of sodium retention by algae treatment could be due to the high sodium chloride concentration (3.3%) from these microalgae as it was grown in sea water. It has been shown that high concentration of salt significantly increases animal water

intake[41], which in turn increases pollution with enhanced excretion. Therefore it should be considered that a process to remove salt from the microalgae is utilized prior to feeding this to animals.

Total collection and chromic oxide are two common methods to estimate retention or digestibility of feed ingredients. Total collection method calculates the total nutrient weight by directly collect the total weight of feed intake and feces produced, while chromic oxide method utilizes indigestible marker such chromic oxide to estimate real nutrient disappearance during digestion process. Though those are two different methodologies, in theory the values from two methods should be consistent to each other. However, we detected some inconsistence of CP, DM and energy retention by two methods. One hypothesis is that since algae biomass has high level of salts which induced higher water intake than birds in control. Due to this increased water intake, excreta from algae group seems waterier and feces scattered around. Even though we tried our best to take sample evenly from different spots, it could still bring in variation on determining retention and digestibility by two methods.

Overall, our present research suggested that 10% defatted microalgae (*Nannochloropsis oceania*) could be used as protein and energy feed ingredient to replace parts of corn and soybean meal in broiler diets without causing any growth performance loss. Besides, higher retention of CF would be beneficial to produce more valuable chicken products. It remains to be further studied why the retention of CP and AA from microalgae treatment was lower than corn and soybean meal containing control and how to compensate for this nutrient loss.

CHARPTER THREE

Dose-Dependent Enrichments and Improved Redox Status in Tissues of Broiler Chicks under Heat Stress by Dietary Supplemental Microalgal Astaxanthin

3.1 Abstract

Astaxanthin (AST) is a well-known carotenoid with a high antioxidant capacity. This study was designed to evaluate the nutritional and metabolic effects of microalgal AST added to the diets of broiler chicks under heat stress. A total of 240 Cornish male chicks (1 day old) were divided into six cages per treatment (eight chicks per cage) and fed a corn-soybean meal diet supplemented with AST from Haematococcus pluvialis at 0, 10, 20, 40, and 80 mg/kg for 6 weeks. Heat stress was employed during weeks 4−6. The supplementation led to dose-dependent enrichments ($P < 0.05$) of AST and total carotenoids in the plasma, the liver, and the breast and thigh muscles. There were similar enhancements ($P < 0.05$) of oxygen-radical-absorbance capacities, but there were decreases or mixed responses ($P < 0.05$) of glutathione concentrations and glutathione peroxidase activities in the tissues. In conclusion, supplemental dietary microalgal AST was bioavailable to the chicks and enriched in their tissues independent of heat stress, leading to coordinated changes in their endogenous antioxidant defense and meat quality.

3.2 Introduction

Research on microalgal biomass as animal feed has been previously conducted.[6-7, 9] However, there have been only a few studies on microalgal phytochemicals in animal nutrition.[42-43] Astaxanthin (AST) is a xanthophyll carotenoid abundantly distributed in microalgae.[44-46] Because of its antioxidant, anti-inflammatory, UV-light protective, and coloring properties, AST

has been used in the food, feed, nutraceutical, and cosmetics industries.[15, 47-49] The compound is often formulated into diets for salmon, trout, shrimp, and lobster.[15] Rahman et al.[50] reported that formulated AST at 50 mg/kg in the diet showed little impact on the growth performance or feed-use efficiency of juvenile rainbow trout, but it did increase their antioxidant status, the contents of AST and total carotenoids, and the color in the fish muscles. Additionally, the plasma activities of catalase (CAT) and superoxide dismutase (SOD) were decreased by increasing AST supplementation.

Supplemental AST from yeast (*Phaffia rhodozyma*) at 2.3 and 4.6 mg per kilogram of diet for broiler chicks produced slight improvements in body-weight gain and the feed-use conversion ratio,[51] but it had no effect on tissue thiobarbituric acid reactive substances or meat color. Perenlei et al.[52] reported that supplemental AST in broiler diets at 10 and 20 mg/kg improved the meat's texture, water-retaining ability, and sensory qualities and prevented meat-protein oxidation during postmortem storage, but it exerted no effect on growth performance. In addition, supplemental AST protected chick embryos from glucocorticoid-induced cataract formation.[53-54] Although these reported effects of AST in the diets of broilers were apparently inconsistent or conflicting, there has been little research on the role of AST in broilers exposed to high temperatures or under heat stress.

Heat stress is a practical problem to broiler chicks in the summer in the southern states of the United States, where a major portion of U.S. chicks are produced.[55] Heat stress results in over $128 million in losses for the poultry industry in the United States.[56] Physiologically, heat stress is characterized as a thermoregulatory imbalance between the net energy flowing from the body to the surrounding area and the heat generation of the animal. It decreases growth performance

and animal product quality.[57-59] Heat stress induces the generation of reactive oxygen species (ROS) which impair cellular structure and function.[60-62] With its known antioxidant activities, AST might be used to protect animals from heat-stress-mediated oxidative insults.[49, 63] Therefore, the objective of this experiment was to investigate the bioavailability of microalgal to broiler chicks under normal or heat-stress conditions and its effects on the antioxidative status, growth performance, and meat quality of these animals.

3.3 Materials and Methods

3.3.1 Animals, Diets, and Management

A total of 240 Cornish male broiler chicks (1 day old) were purchased from Moyer's Chicks (Quakertown, PA), housed in an environmentally controlled room with cages, and randomly separated into five treatments (each treatment had six replicates of eight chicks per replicate). The chicks were fed a corn−soybean meal basal diet supplemented with AST from *Haematococcus pluvialis* (Heliae, Gilbert, AZ) at 0, 10, 20, 40, and 80 mg/kg for 6 weeks. Supplemental AST for 10 and 40 mg/kg AST diets was provided by defatted H. pluvialis, and for 20 and 80 mg/kg AST diets, it was provided by full-fatted H. pluvialis. The two forms of microalgal biomass were used for the accuracy and convenience of supplementing the intended amounts of AST into the diets, for minimizing the impacts of the supplementations on the compositions of the diets, and for fulfilling our interest in exploring the potential of defatted microalgal biomass. The light schedule was 2:22h dark−light cycles for the whole period. The animals were given free access to feed and water. All experimental diets were formulated according to the nutrient requirements for broiler by the National Research Council.[64] The nutrient compositions of the diets with the supplemental AST concentrations are shown in **Supplemental Table 1**. The temperature schedule was set according to the industrial guide[65]

(week 1: 34 °C, week 2: 31 °C, and week 3:27 °C) during weeks 1 to 3. Starting from week 4, heat stress was applied to broilers by raising the room temperature 10°F above the recommended temperature (week 4: 32.5 °C, week 5: 30 °C, and week 6: 28.3 °C). Our animal protocol was approved by the Institutional Animal Care and Use Committee of Cornell University.

3.3.2 Blood Collection, Tissue Examination, and Biochemical Assays

Growth performance was recorded weekly. At the end of weeks 3 and 6, the chicks were euthanized by carbon dioxide following cervical dislocation after an 8 h fast. Blood was centrifuged at 3000g for 15 min (Beckman GS-6R centrifuge, Brea, CA) and kept on ice before analysis. The liver, breast muscle, and legs were removed and weighed, and a portion of each was frozen on dry ice and stored at -20°C until analysis. Plasma alanine aminotransferase (ALT) activities were determined by an Infinity ALT liquid-stable reagent kit (Thermo Electron Corporation, Waltham, MA). Plasma alkaline phosphatase (AKP) activities were determined using the method of Bowers and McComb.[66] Plasma tartrate-resistant acid phosphatase (TRAP) was determined with the method of Lau et al.[67] Plasma inorganic phosphorus (PIP) was analyzed following the method of Gomori.[68] All samples were tested in duplicate. A glucose-assay kit was purchased from Sigma-Aldrich (St. Louis, MO). Kits for total cholesterol (TC), triglyceride (TG), and non-esterified fatty acid (NEFA) were purchased from Wako Chemicals USA (Richmond, VA). The uric acid and ALT reagents were obtained from Thermo Scientific, Inc. (Waltham, MA).

3.3.3 Analyses of Astaxanthin and Total Carotenoid Content

Total AST in diet, plasma, and tissues were extracted using the method of Lopez et al.[69] with modifications. Samples of tissues (0.5−3 g) and plasma (500 μL) were incubated in acetone and

ethyl acetate for 10 min on ice. Water was then added, and the solutions were centrifuged at 3000g for 15 min at 4 °C. The upper layers were collected and dried under N_2 gas. The residues were dissolved in chloroform (HPLC grade) for the HPLC-UV analyses. The AST concentrations in the samples were measured on the basis of the methods of Sowell et al.,[70] Breithaupt et al.,[71] and Rohrle et al.[72] with modifications. Briefly, the AST extraction was eluted isocratically with methanol and acetonitrile (50:50) containing 0.1% triethylamine (TEA) at a flow rate of 1 mL/ min on an Agilent Eclipse plus C18 reverse-phase column (5 μm, 4.6×250 mm) using a Shimadzu HPLC system with an LC-10ADmicroplunger pump and an SPD-10 AV vp UV detector. The column temperature was set to 30 °C. The mobile phase was sonicated for 15 min before use. All the chemicals were HPLC grade, and the solutions were freshly prepared. The detected peaks were identified by comparison with the retention times of a standard AST. To validate the results, the sample extracts were spiked with the standard AST to determine its appearance on the chromatogram in relation to the sample peak being identified. Pure AST and β-carotene were purchased from Sigma-Aldrich (St. Louis, MO). The AST and β-carotene concentrations were calculated by the areas under the curves (AUCs) of the samples against the AUCs of the spiked AST and β-carotene standards in the HPLC-chromatogram results. The extraction efficiencies (recoveries) of AST and β-carotene were 92 and 90%, respectively.

3.3.4 Oxygen-Radical-Absorbance-Capacity (ORAC) Assay

The oxygen-radical-absorbance capacities (ORACs) of the plasma, the livers, and the thigh and breast muscles were measured using the method of Ou et al.[73] with modifications. Briefly, the tissues were homogenized, extracted by hexane twice, and then centrifuged at 12000g for 5 min. The supernatants were then combined and dried under N_2 gas. The lipophilic fragments were redissolved in a 7% methylated cyclodextrin, 50% acetone, and 50% water solution. Extraction

residues were extracted in a 7% acetic acid and 80% methanol solution, sonicated at 37 °C for 5 min, and incubated at room temperature for 15 min, with frequent shaking. The hydrophilic fragments were dissolved in 75 mM phosphate buffer (pH 7.4). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the antioxidant-activity standard, and all results were expressed as micromolar trolox equivalence. The samples and standards were detected under 350 nm excitation and 450 nm emission (SpectraMax M2, Molecular Devices, Sunnyvale, CA).

3.3.5 Determination of MDA, GSH, GSSG, and Antioxidant Enzymes

Malondialdehyde (MDA) levels were determined by the method of McDonald and Hultin,[74] using a 2-thiobarbituric acid assay with 1,1,3,3-tetraethoxypropane as the standard. The glutathione (GSH) and glutathione disulfide (GSSG) contents and the glutathione peroxidase (GPX), glutathione reductase (GR), glutathione S-transferase (GST), and superoxide dismutase (SOD) activities in the livers, breasts, and thighs were determined using the methods of Anderson,[75] Flohe and Gunzler,[76] Massey and William,[77] Guthenberg and Mannervik,[78] and McCord and Fridovich,[79] respectively.

3.3.6 Meat pH, Water-Holding-Capacity, and Texture-Profile Analyses

Texture-profile analysis was used to measure the compression force by compressing the meat samples with a texturometer (TA.XTplus, Stable Micro Systems, Hamilton, MA). Frozen breast and thigh muscles were thawed and cut into 2 in. diameter cubes; cooked in an oven at 175 °C for 30 min; and then subjected to analyses of their chewiness, springiness, and hardiness using the method from Huidobro et al.[80] The meat pH was determined using the iodoacetate method[81] with modification. Approximately 250 mg of meat was homogenized for 30 s in 2.5 mL of a

solution composed of 5 mM sodium iodoacetate and 150 mM potassium chloride at pH 7. The mixture pH was measured using an Accumet pH probe (AB150, Fisher Scientific, Waltham, MA).

The water-holding capacity (WHC) was determined using a centrifugal method.[82-83] Approximately 1 g of meat was added to a centrifuge tube that contained three pieces of Whatman filter paper that were folded into a thimble. The tube and its contents were subjected to 7710g of force for 30 min. The meat samples were separated from the filter paper and weighed to determine the WHC.

3.3.7 Determination of the Fatty Acid Profile

Total lipid was extracted from plasma and tissues according to the method of Folch et al.[84] and Fristche et al.[85] Then, the lipids were methylated using anhydrous methanol in 4% sulfuric acid at 90 °C for 60 min, and tridecanoic acid was used as an internal standard. Each fatty acid was identified by comparing its retention time and peak area against individual fatty acid methyl ester standards. The methyl esters of the fatty acids were analyzed by gas chromatography−mass spectrometry (model HP 5890 A with an HP 5970 series mass-selective ion-monitoring detector, Hewlett-Packard, Palo Alto, CA).[86-87]

3.3.8 Statistical Analyses

Data were collected using the cage as the experimental unit and analyzed by one-way ANOVA. Mean comparisons were conducted with Duncan's multiple-range method. Data were presented as means ± SEM, and the significance level for differences was $P < 0.05$. Correlations between variables were analyzed by Pearson's correlation, and linear or polynomial regression was

performed using the Proc General Linear Models procedures of SAS (version 9.2, SAS Institute, Cary, NC).

3.4 Results

3.4.1 Total Carotenoid and Astaxanthin Concentrations

There were dose-dependent elevations (linear or polynomial, $R2 > 0.9$, $P < 0.05$) of total carotenoids and AST in the plasma, livers, and breast and thigh muscles with increasing dietary AST supplementation (**Table 3.1**). The highest concentrations of AST reached 17 μg/mL in the plasma and 5.8, 2.2, and 2.1 mg/kg in the livers, breasts, and thighs at week 6, respectively. The highest concentrations of total carotenoids were 218 μg/mL in the plasma and 53, 12, and 7.0 mg/kg in the livers, breasts, and thighs at week 6, respectively.

3.4.2 Growth Performance and Plasma Health

Body-weight gain, feed intake, and gain−feed ratio were not influenced by AST supplementation during the starter period (weeks 1−3, **Table 3.2**). However, during the grower period (weeks 4−6), the 20 and 80 mg/kg AST treatments decreased ($P < 0.05$) the gain−feed ratios by 14 and 18% ($P < 0.05$), respectively, without affecting body-weight gain or feed intake. Likewise, the four doses of supplemental AST led to similar decreases (7−11%, $P < 0.05$) in the gain−feed ratios compared with the control grain−feed ratio during the entire period (weeks 1−6). The health indicators in the plasma biochemical assays were largely unaffected at weeks 3 and 6 by AST inclusion (**Supplemental Table 2**).

3.4.3 Antioxidant Status

There were dose-dependent elevations (linear or polynomial, R2 > 0.9, P < 0.05) of ORAC in the liver, breast, and thigh with increasing dietary AST supplementation at weeks 3 and 6 (**Table 3.3**). However, the MDA concentrations in these tissues, except for the thigh muscle at week 6, remained similar across the dietary treatments. Additionally, GSH and GSSG in the liver were affected by the AST supplementation at week 3 but not at week 6. Chicks fed with 40 or 80 mg/kg AST had a 28 or 45% decrease (P < 0.05) in hepatic GSH compared with the level of the control at week 3. Breast GSH at both time points and thigh GSH at week 6 were decreased (P < 0.05) by the highest dose of supplemental GST, compared with those of the controls.

3.4.4 Antioxidant Enzyme Activities

The four doses of supplemental AST enhanced hepatic GR activities by 1.4−2.2-fold over the controls at week 3 (**Table 3.4**). These supplementations caused dose-dependent increases (P<0.05) in GPX and GST activities in the liver and GPX activities in the thigh muscle at the same time point. The thigh GPX activities at week 6 showed linear decreases (P < 0.05) in response to AST supplementation, whereas 80 and 10 mg/kg AST diets decreased (P < 0.05) GPX activities in the liver and breast, respectively, compared with the activities in the controls.

3.4.5 Meat Quality

The meat-color scores of the breast and thigh muscles at weeks 3 and 6 were elevated (P < 0.05) as dietary AST concentration increased, but they were not simply linear (**Table 3.5**). The 20, 40, and 80 mg/kg AST diets elevated (P < 0.05) the breast pH compared with that of the control at week 3. The 80 mg/kg AST diet decreased (P < 0.05) the water-holding capacity (WHC) of the breast muscle by 17% compared with that of the control at both time points. The chewiness and hardness of the thigh muscle were affected (P <0.05) by AST supplementation, but the changes

were inconsistent at the two time points. The dressing percentage of the carcass and the meat−bone ratio in the thigh muscle at week 6 were not affected by the supplemental AST (**Supplemental Table 3**).

3.4.6 Fatty Acid Concentration Profile

Saturated fatty acid (SFA), monounsaturated fatty acid (MUFA), and polyunsaturated fatty acid (PUFA) concentrations in the thigh and breast muscles were largely unaffected by supplemental AST at both time points (**Table 3.6**). Compared with the control, the 40 and 80 mg/kg AST diets decreased ($P < 0.05$) hepatic SFA, MUFA, and PUFA concentrations by 23 to 46% at weeks 3 and 6. More details on the effects of supplemental AST on individual fatty acid profiles are summarized in **Supplemental Tables 4 and 5**.

Table 3.1. Effect of dietary microalgal astaxanthin on total carotenoid and astaxanthin concentr ations in diets, plasma, and tissues*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Astaxanthin							
Plasma, µg/mL							
Week 3	0^{c}	2.24^{d}	4.92^{b}	4.52^{c}	11.6^{a}	0.44	<0.01
Week 6	0^{d}	4.08^{c}	2.92^{d}	5.17^{b}	16.5^{a}	0.56	<0.01
Liver, mg/kg							
Week 3	0^{c}	2.49^{b}	2.50^{b}	2.45^{b}	3.07^{a}	0.15	<0.01
Week 6	0^{d}	3.77^{c}	4.10^{c}	4.87^{b}	5.81^{a}	0.26	<0.01
Breast, mg/kg							
Week 3	0^{d}	0.40^{c}	0.51^{b}	0.54^{b}	0.68^{a}	0.06	<0.01
Week 6	0^{c}	1.51^{b}	1.89^{ab}	2.05^{ab}	2.16^{a}	0.34	<0.01
Thigh, mg/kg							
Week 3	0^{c}	0.90^{b}	0.96^{ab}	0.98^{ab}	1.06^{a}	0.08	<0.01
Week 6	0^{c}	1.79^{b}	1.81^{b}	1.93^{ab}	2.08^{a}	0.12	<0.01
Total carotenoid							
Diet, mg/kg							
Starter	10.9^{e}	37.5^{d}	114^{b}	105^{c}	310^{a}	5.36	<0.01
Grower	9.50^{e}	22.3^{d}	125^{b}	98.8^{c}	301^{a}	8.39	<0.01
Plasma, µg/mL							
Week 3	0.32^{d}	12.2^{c}	35.8^{c}	122^{b}	175^{a}	33.6	<0.01
Week 6	0.52^{e}	4.69^{d}	37.2^{c}	131^{b}	218^{a}	7.71	<0.01
Liver, mg/kg weight							
Week 3	6.07^{e}	9.63^{d}	17.7^{b}	14.5^{c}	40.3^{a}	0.38	<0.01
Week 6	2.80^{e}	8.37^{d}	26.9^{b}	10.1^{c}	53.4^{a}	0.30	<0.01
Breast, mg/kg weight							
Week 3	1.17^{d}	1.79^{c}	3.75^{b}	2.00^{c}	4.46^{a}	0.20	<0.01
Week 6	0.96^{e}	2.32^{d}	2.83^{c}	3.21^{b}	12.0^{a}	0.24	<0.01
Thigh, mg/kg weight							
Week 3	0.73^{e}	1.58^{d}	6.63^{b}	4.12^{c}	10.4^{a}	0.16	<0.01
Week 6	1.34^{d}	3.55^{c}	5.23^{b}	3.64^{c}	7.04^{a}	0.28	<0.01

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. ^{a,b,c,d,e}Means in the same row without a common letter differ ($P < 0.05$).

Table 3.2. Effects of dietary microalgal astaxanthin on growth performance of broiler chicks*

		Astaxanthin, mg/kg						
	Week	0	10	20	40	80	SEM	p value
Body weight gain	0-3	40.8	41.6	41.1	39.0	44.4	4.61	0.39
(g/chick/d)	4-6	58.4	54.1	55.4	55.7	48.7	11.1	0.13
	0-6	49.6	47.9	48.2	47.4	46.6	6.24	0.94
Feed intake	0-3	60.4	65.1	65.8	65.8	66.8	6.48	0.48
(g/chick/d)	4-6	110	111	115	111	103	8.90	0.13
	0-6	85.6	92.2	86.8	88.4	85.0	6.75	0.45
Gain: feed ratio	0-3	0.72	0.68	0.69	0.65	0.69	0.040	0.06
	4-6	0.55^{a}	0.51^{ab}	0.48^{b}	0.50^{ab}	0.45^{b}	0.045	0.02
	0-6	0.64^{a}	0.59^{b}	0.58^{b}	0.57^{b}	0.57^{b}	0.032	0.02

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. ^{a,b}Means in the same row without a common letter differ ($P < 0.05$).

Table 3.3. Effects of dietary microalgal astaxanthin on malondialdehyde (MDA) concentrations and oxygen radical absorbance capacity (ORAC) in broiler tissues*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
MDA**, μmol/g protein							
Liver							
Week 3	0.110	0.116	0.151	0.130	0.118	0.027	0.16
Week 6	0.095	0.071	0.075	0.073	0.085	0.022	0.41
Breast							
Week 3	0.257	0.262	0.203	0.197	0.194	0.042	0.10
Week 6	0.193	0.186	0.170	0.193	0.127	0.057	0.36
Thigh							
Week 3	0.150	0.140	0.136	0.121	0.138	0.057	0.95
Week 6	0.419^{a}	0.296^{ab}	0.326^{ab}	0.225^{b}	0.237^{b}	0.116	0.13
ORAC**, μmol/g weight							
Liver							
Week 3	0.728^{c}	1.14^{b}	1.15^{b}	1.16^{b}	1.35^{a}	0.032	<0.01
Week 6	0.948^{d}	0.960^{d}	1.11^{c}	1.17^{b}	1.37^{a}	0.024	<0.01
Breast							
Week 3	0.357^{c}	0.648^{b}	0.869^{a}	0.880^{a}	0.886^{a}	0.023	<0.01
Week 6	0.200^{d}	0.884^{c}	0.928^{b}	1.17^{a}	1.17^{a}	0.031	<0.01
Thigh							
Week 3	0.605^{d}	0.910^{c}	0.949^{b}	0.963^{b}	1.08^{a}	0.028	<0.01
Week 6	0.497^{c}	0.690^{b}	0.712^{b}	1.03^{a}	1.06^{a}	0.025	<0.01
GSH**, μM/g weight							
Liver							
Week 3	2.29^{a}	1.95^{ab}	1.88^{ab}	1.71^{bc}	1.27^{c}	0.58	<0.01
Week 6	2.62	2.39	1.98	2.31	1.98	0.65	0.64
Breast							
Week 3	1.72^{ab}	2.15^{a}	1.94^{ab}	1.97^{ab}	0.98^{b}	0.47	<0.01
Week 6	7.00^{a}	7.48^{a}	5.48^{ab}	3.82^{b}	4.94^{ab}	1.23	<0.01
Thigh							
Week 3	4.01	6.37	4.06	3.66	3.51	1.51	0.32
Week 6	7.50^{ab}	10.2^{a}	10.2^{a}	5.92^{ab}	4.81^{b}	2.44	<0.01
GSSG**, μM/g weight							
Liver							
Week 3	0.28^{a}	0.32^{a}	0.26^{a}	0.19^{ab}	0.06^{b}	0.17	<0.01
Week 6	0.27	0.23	0.30	0.39	0.31	0.10	0.47
Breast							
Week 3	0.09	0.11	0.07	0.09	0.09	0.04	0.86
Week 6	0.35	0.35	0.48	0.54	0.33	0.20	0.55
Thigh							
Week 3	0.24	0.34	0.21	0.29	0.16	0.12	0.39
Week 6	0.27	0.34	0.42	0.31	0.29	0.14	0.21

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. ^{a,b,c,d}Means in the same row without a common letter differ ($P < 0.05$).

*MDA, malondialdehyde; ORAC, oxygen radical absorbance capacity; GSH: glutathione; GSSG: glut athione disulfide.

Table 3.4. Effect of dietary microalgal astaxanthin on redox enzyme activities of broil er tissues*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Week 3							
Liver							
GPX**	51.4[b]	74.8[ab]	71.3[ab]	105[a]	63.8[b]	9.70	0.010
GR**	8.39[b]	20.4[a]	20.6[a]	26.4[a]	23.0[a]	2.09	<0.01
GST**	204[b]	270[ab]	321[ab]	398[a]	282[ab]	42.4	0.045
SOD**	0.379	0.430	0.38	0.495	0.476	0.07	0.773
Breast							
GPX	6.24	6.35	5.63	5.68	5.46	0.33	0.392
GR	6.20	5.76	5.67	5.84	5.49	0.61	0.959
GST	6.02	6.29	5.67	6.79	6.43	0.57	0.704
SOD	0.157	0.161	0.15	0.134	0.157	0.01	0.501
Thigh							
GPX	9.58[ab]	12.0[ab]	8.76[b]	10.1[a]	15.0[a]	1.43	0.055
GR	10.8	10.6	9.85	9.82	9.80	1.18	0.973
GST	12.2	13.1	11.2	12.9	13.0	1.30	0.899
SOD	0.217	0.189	0.211	0.22	0.211	0.03	0.982
Week 6							
Liver							
GPX	157[ab]	184[a]	146[ab]	159[ab]	129[b]	15.4	0.227
GR	27.8	35.7	33.5	31.9	30.1	4.96	0.833
GST	202[b]	266[ab]	277[ab]	376[a]	313[ab]	34.7	0.073
SOD	0.587	0.756	0.862	0.733	0.517	0.20	0.794
Breast							
GPX	7.63[a]	5.47[b]	7.78[a]	7.40[a]	6.49[ab]	0.32	<0.01
GR	7.98	6.35	9.72	8.63	7.34	1.26	0.380
GST	7.77	7.10	8.36	7.87	7.30	0.51	0.488
SOD	0.169	0.160	0.180	0.179	0.168	0.02	0.862
Thigh							
GPX	24.3[a]	21.5[ab]	15.9[bc]	16.5[bc]	11.7[c]	2.09	<0.01
GR	17.2	16.3	14.4	16.2	14.9	2.31	0.915
GST	11.4	11.3	9.75	13.5	12.0	1.24	0.394
SOD	0.232	0.197	0.214	0.239	0.163	0.04	0.791

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's mult iple-range test. [a,b,c]Means in the same row without a common letter differ (P < 0.05). **GP X: glutathione peroxidase; GR: glutathione reductase; GST: glutathione transferase; SOD: superoxide dismutase. Units of GPX, GR, GST and SOD: U/mg protein/min.

Table 3.5. Effect of dietary microalgal astaxanthin on meat quality of broiler chicks*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Week 3							
Breast							
Color	1.11^{c}	1.11^{c}	2.00^{b}	1.22^{c}	4.22^{a}	0.77	<0.01
pH	5.50^{b}	5.82^{ab}	5.90^{a}	5.98^{a}	5.90^{a}	0.21	<0.01
WHC**	46.2^{ab}	43.0^{ab}	41.4^{b}	49.0^{a}	38.6^{b}	4.27	<0.01
Chewiness	1197	1308	1378	1274	1437	577	0.62
Springiness	0.79	0.81	0.83	0.87	0.87	0.97	0.77
Hardness	2662	2688	2603	2452	2823	1043	0.73
Thigh							
Color	1.00^{c}	1.00^{c}	1.56^{b}	1.00^{c}	3.11^{a}	0.58	<0.01
pH	6.26	6.41	6.16	6.17	6.16	0.18	0.46
WHC	53.6	44.3	49.6	46.3	43.8	5.23	0.39
Chewiness	1204^{ab}	808^{b}	1731^{a}	1565^{a}	871^{b}	622	<0.01
Springiness	0.90	0.86	0.91	0.94	0.87	0.09	0.68
Hardness	2320^{ab}	1653^{b}	2872^{a}	2418^{ab}	1589^{b}	820	<0.01
Week 6							
Breast							
Color	1.00^{c}	1.11^{c}	2.22^{b}	1.44^{c}	4.11^{a}	0.69	<0.01
pH	5.91	5.90	5.85	6.08	5.79	0.18	0.426
WHC	51.5^{a}	51.3^{a}	45.2^{ab}	46.5^{ab}	42.7^{b}	2.72	<0.01
Chewiness	1541	1857	1909	1759	1765	549	0.67
Springiness	0.79	0.79	0.80	0.81	0.79	0.07	0.59
Hardness	3112	3597	3747	3094	3623	989	0.74
Thigh							
Color	1.00^{d}	1.67^{c}	2.67^{b}	2.00^{c}	4.44^{a}	0.53	<0.01
pH	6.05	5.81	6.07	6.02	6.02	0.17	0.19
WHC	45.1	39.2	44.9	53.2	48.0	6.77	0.43
Chewiness	800^{ab}	742^{b}	620^{b}	849^{ab}	1189^{a}	416	<0.01
Springiness	0.80	0.79	0.81	0.80	0.83	0.08	0.49
Hardness	1662^{ab}	1595^{ab}	1302^{b}	1709^{ab}	2278^{a}	776	<0.01

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. ^{a,b,c}Means in the same row without a common letter differ ($P < 0.05$).
*WHC: water holding capacity.

Table 3.6. Effect of dietary microalgal astaxanthin on fatty acids concentrations of tissues of broiler chicks*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Fatty Acid, mg/g of tissue							
Week 3							
Breast							
SFA**	2.10^{abc}	2.16^{ab}	1.90^{bc}	1.52^{c}	2.65^{a}	0.22	<0.01
MUFA**	3.21^{a}	2.87^{ab}	2.44^{ab}	1.53^{b}	3.14^{a}	0.57	<0.01
PUFA**	1.68^{b}	1.66^{b}	1.49^{b}	1.55^{b}	2.19^{a}	0.20	<0.01
n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
Thigh							
SFA	3.58	3.94	3.15	3.07	4.55	0.87	0.32
MUFA	4.81	5.08	3.78	3.66	6.01	1.24	0.41
PUFA	3.46	3.36	2.99	2.89	3.52	0.88	0.29
n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
Liver							
SFA	7.46^{a}	7.37^{a}	7.37^{a}	5.73^{b}	5.71^{b}	1.13	<0.01
MUFA	3.79^{a}	3.30^{a}	3.27^{a}	2.92^{ab}	2.06^{b}	0.88	<0.01
PUFA	5.69^{ab}	6.62^{a}	6.48^{ab}	4.55^{b}	4.67^{ab}	0.75	<0.01
n-3	0.00	0.358	0.442	0.189	0.167	0.209	0.43
Week 6							
Breast							
SFA	2.18	2.63	2.60	2.49	2.52	0.42	0.85
MUFA	2.47	2.90	3.03	3.11	2.89	0.54	0.39
PUFA	1.87	2.11	2.01	1.84	2.05	0.39	0.41
n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
Thigh							
SFA	2.89	2.63	3.16	3.50	3.04	0.46	0.29
MUFA	3.27	3.20	3.80	4.16	3.62	0.77	0.35
PUFA	2.44^{ab}	2.15^{b}	2.92^{ab}	3.31^{a}	2.79^{ab}	0.46	<0.01
n-3	0.076	0.000	0.055	0.069	0.076	0.045	0.51
Liver							
SFA	13.72^{a}	12.17^{ab}	10.0^{abc}	8.82^{bc}	7.32^{c}	3.36	<0.01
MUFA	10.29^{a}	9.28 ^{ab}	6.52^{abc}	5.12^{bc}	4.10^{c}	3.51	<0.01
PUFA	8.58^{a}	7.63^{ab}	6.07^{ab}	6.30^{ab}	4.77^{b}	2.60	<0.01
n-3	0.336	0.147	0.138	0.194	0.692	0.620	0.62

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. ^{a,b,c}Means in the same row without a common letter differ ($P < 0.05$).
**SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

3.5 Discussion

The plasma, liver, breast and thigh concentrations of AST and total carotenoids showed dose-dependent elevations with increasing AST supplementation for the chicks under normal- and high-temperature conditions. The liver was the organ where AST accumulated the most,

followed by the breast and thigh muscles. Similar AST and carotenoid depositions in edible meat and liver were achieved by feeding AST-containing yeast to broiler chicks.[88-90] As a xanthophyll carotenoid, AST contains a long carbon chain with conjugated double bonds and keto (C=O) moieties on each ionone ring, which makes it as a potent antioxidant.[91] Therefore, the AST and total carotenoid accumulation led to dose-dependent increases of ORAC in the livers, breasts, and thighs of the chicks. The ORAC assay has been widely used as a method of choice to assess antioxidant capacity.[92]

Antioxidant status in the body is controlled by the production of reactive oxygen species and the supply of endogenous and exogenous antioxidants. Endogenous antioxidants include GSH and enzymes such as GPX, GR, and SOD. Both functions of GPX and GR affect intracellular GSH levels.[93] Our results indicate that the highest dose of AST supplementation actually decreased GSH concentrations in the tissues compared with the control, whereas the other two lower doses of supplementation produced mixed effects. Additionally, the AST supplementations, with the exception of the highest dose, enhanced GPX, GR, and GST activities in the liver and the breast and thigh muscles of chicks at week 3 at the normal or recommended temperature. However, it seemed that only the GPX activities were affected in the three tissues of the chicks under the high temperature at week 6. This implies that multiple antioxidant enzymes might coordinate and operate under specific conditions. It also suggests that GSH and related antioxidant enzymes might respond to the elevated AST in the tissues for the homeostasis of antioxidant balance in broiler bodies. It remains unclear why the AST supplementations led to dose-dependent decreases of GPX activities in the thigh muscles of chicks at week 6.

The highest dose of AST supplementation enhanced the color of the breast and thigh muscles at both weeks 3 and 6. Akiba et al.[88] also reported that the redness (a* value) of broiler meats was intensified by supplementing 15 and 30 mg/ kg AST (from yeast). Similar color changes were produced in the blood, muscle, and skin by feeding broilers 22.5 mg/kg AST.[94] Because meat color is a critical indicator for the choice of consumers,[95] the H. pluvialis biomass used in the present study can be a potent food colorant to color broiler meat and skin for different customers' preferences.

The water-holding capacity (WHC) represents the capacity of muscle samples to retain fluid during handling and processing.[96] In addition, water deprivation reduces the nutrient value of meat because of the loss of the leachate and results in tougher and flavorless meat.[97] Our data indicate that the WHC of the breast muscle was decreased by 80 mg/kg AST compared with that of the control at both time points. Jeong et al.[51] discovered that there was no difference in WHC as a result of feeding 2.3 and 4.6 mg/kg AST (from P. rhodozyma yeast) for 4 weeks. Perenlei et al.[52] found that supplementing 10 and 20 mg/kg AST (from yeast) for 4 weeks failed to change WHC. These findings were similar to the effects of lower doses of AST supplementation on the WHC of muscles in our present study. Heat stress has proved to negatively affect WHC. Breast drip loss, another method of quantifying WHC,[96] was adversely affected by acute heat stress in broilers of 35 days old.[59] Lu et al.[98] reported that chronic heat stress aggravated drip loss in Arbor Acres broiler chicken muscle. Although our data showed a negative effect from the highest dose of AST supplementation on the WHC of the breast muscle of the chicks, the associations with chewiness and hardness were inconsistent.

Double-bond-containing fatty acids such as MUFA and PUFA are susceptible to oxidation and peroxidation. Because antioxidants such as vitamin E are suggested to protect PUFA in broiler meat products from oxidation,[99] supplemental AST was expected to protect unsaturated fatty acids in the tissues against oxidation, leading to enrichment. However, the AST supplementation, even the highest concentration, did not enhance MUFA or PUFA concentrations in any of the assayed tissues. In contrast, the AST supplementation caused strong dose-dependent decreases in the hepatic concentrations of SFA, MUFA, and PUFA in the chicks at both weeks 3 and 6. Because excessive SFA is considered detrimental to health due to its linear structure, packing ability in cell membranes, and signaling properties,[100] the potential of AST to decrease hepatic SFA may improve animal health. However, the resultant decreases in hepatic MUFA or PUFA by the supplemental AST may not be desirable.

Dietary supplementation of AST did not affect the growth performance of chicks during the starter period. This was similar to the lack of such effects due to supplemental AST from H. pluvialis at 179 mg/kg[89] or supplemental AST from P. rhodozyma yeast at 22.5 mg/kg for 5 weeks.[94] Heat stress exerts deleterious effect on growth performance, carcass yield and survival rates of meat type chickens.[101] Supplementation of the diet with antioxidants has been shown to be an effective method of protecting against heat stress. Although there is little research on role of AST as an antioxidant in protecting poultry against heat stress, supplemental vitamin E (250 vs 125 mg/kg) resulted in improved feed intake, body-weight gain, and feed efficiency in Japanese quail exposed to heat stress.[102] Because of the space and resource limitation, our present study did not include chicks raised under normal temperature to compare with the high-temperature effects on their growth performance or other measures. However, our data indicate that supplemental AST at various doses did not affect the growth performance of chicks under

the high-temperature conditions during weeks 4−6. In contrast, the supplementation decreased the feed-use efficiency. The absorption and deposition of microalgal AST and the enriched AST in the tissues of chicks might have created a metabolic burden, consuming extra energy to reduce the overall feed efficiency of the chicks under heat stress.

In conclusion, supplemental microalgal AST enriched this phytochemical in the tissues of broiler chicks, changed the pigment of the chicken products, elevated the total antioxidant capacities, and down-regulated the intrinsic antioxidant systems in the tissues. It remains to be further studied why supplemental microalgal AST did not show obvious benefits in the growth performance or meat quality of the chicks exposed to high temperatures. Overall, 20−40 mg/kg AST seemed to be the appropriate inclusion rate for the best potential of this phytochemical in broiler feeding.

CHARPTER FOUR

Effect of DHA Rich Microalgae on Growth Performance and Meat Quality of Broiler Chicks

4.1 Abstract

Docosahexaenoic acid (DHA) is an n-3 polyunsaturated fatty acid. This study aimed to investigate the effects of feeding a DHA-rich microalgal biomass on growth performance, health status, and meat characteristics in broilers. A total of 190 (day-old) Cornish male chicks were housed in an environmental control room (6 cages/treatment, 8 chicks/cage), and fed a corn-soybean meal basal diet supplemented with the microalgal biomass (*Aurantiochytrium*, Heliae, Gilbert, AZ) at 0, 1, 2, and 4% (0, 1.7, 3.4 and 6.8 g DHA/kg diet) for 6 weeks. Growth performance were measured weekly. Blood samples were collected at weeks 3 and 6 (2 chicks/cage). Liver, breast, thigh and adipose tissue were sampled (2 chicks/cage) for biochemical and meat quality analysis. Data were analyzed by one-way ANOVA and linear regression analysis. Growth performance was not affected by 1 or 2% microalgae compared with the control, but 4% microalgae decreased ($P < 0.05$) body weight gain (19%) and gain to feed ratio (19%) during weeks 4-6. The microalgae treatments resulted in dose-dependent decreases ($P < 0.05$, $R^2 = 0.21$- 0.54) in plasma alanine amino transferase activity and glucose, cholesterol and non-esterified fatty acid concentrations, but had little effect on plasma activity of alkaline phosphatase or concentrations of inorganic phosphorus, uric acid and triglyceride at weeks 3 and 6. The carcass dressing percentage was not affected by the microalgae supplementation, but breast muscle weight was 21% lower ($P < 0.05$) in chicks fed 4% microalgae than the control, which was associated with the decrease of mammalian target of rapamycin and ribosomal s6 protein and increase of muscle RING finger protein 1 and muscle atrophy F box protein. The

microalgae supplementation caused linear increases in lipid peroxidation ($P < 0.01$, $R^2 = 0.62$-0.90) and hardness and chewiness ($P < 0.01$, $R^2 = 0.34$-0.44) of breast and thigh muscles, although springiness, pH, water holding capacity, and lipid profiles of both muscles in the microalgae-fed chicks remained similar to the control. In conclusion, feeding chicks with 3.4 g DHA/kg diet (up to 2% microalgae) had positive or no adverse effects on growth performance, health status, and meat characteristics.

4.2 Introduction

Docosahexaenoic acid (DHA) is an essential omega-3 polyunsaturated fatty acid (PUFA) which is abundant in neural tissue such as brain and retina in human [22]. It not only plays an important role in growth and development of the brain in infants but is also required for maintenance of ordinary brain function in adults [23]. DHA has also been shown to exhibit anti-inflammatory and hypolipidemic properties, and therefore has the potential to lower the risk of cardiovascular disease [24], ameliorate non-alcoholic fatty liver disease [25], reduce hypertriacyglycerolemia [26] and alleviate Alzheimer disease[27]. Because of these multiple health benefits, the World Health Organization (WHO) recommends intake of DHA and other omega-3 fatty acids as 1-2% of energy/day to reduce the risk of chronic metabolic disease [29].

Dietary DHA primarily originates from marine food such as fatty fish or algae[28]. While some animal species are capable of converting DHA from alpha-linolenic acid (ALA), human has very limited abilities to synthesize DHA on their own. [103] Therefore, dietary supplementation through fish consumption is necessary for human to maintain adequate amounts of DHA in their systems. However, concerns regarding oceanic contamination[28],, as well as issues with affordability and consumer preferences have led researchers to search for alternative availabilities of DHA, such

as incorporation of DHA and other FA into livestock through dietary supplementation. The most promising meat source for DHA supplementation is poultry. In 2015, 46.3 billion pounds of poultry was slaughtered, surpassing that for commercial beef (23.7 billion pounds) and pork (24.5 billion pounds)[104] . In addition, shorter growth period, higher feed efficiency and low cost make poultry the more favorable option to beef and pork, making it the most popular animal protein source worldwide.

Given the above benefits, scientists started to focus on producing n-3 fatty acid enriched meat products. During the past 30 years, multiple other fatty acid sources have been incorporated into broiler chick diet including linseed oil[105], fish meal[106], fish oil[107], flaxseed oil[108], and microalgae[109-110]. Most of these studies were capable of enriching n-3 PUFA including DHA by either direct deposition or in vivo synthesis. However, previous research on microalgae *Aurantiochytrium* in broiler chicks remained limited. The objective of the study was to investigate whether feeding DHA-rich microalgae has any effect on growth performance, health status, meat characteristics and production of broiler chicks. Results would aid in determining the best inclusion of DHA-rich microalgae in broiler diet.

4.3 Materials and Methods

4.3.1 Animal, Diets and Management

A total of 190 Cornish Broiler chicks were ordered from local hatchery (Moyer's Hatchery, PA) and housed in an environmental control room for 6 weeks. Broiler chicks were weighed and randomly allocated to 4 treatments (6 cages/treatment, 8 chicks/cage) to maintain consistent body weight on day 0. DHA-rich microalgae biomass (*Aurantiochytrium*, Heliae, Gilbert, AZ) was supplemented as 1, 2, 4% (0, 1.7, 3.4 and 6.8 g DHA/kg diet) in corn-soybean meal basal

diet. All experimental diets (**Table 4.1**) were iso-caloric and proteinic mixed based on the broiler nutrient requirement from NRC (1994). Feed and water were provided ad libitum and light schedule was regulated at 22:2 h light: dark cycles during the study. Temperature were maintained at 34, 31, 27, 24, 21, 19 °C from week 1 to 6, respectively, as suggested by COBB broiler management guide [65]. Body weight and feed intake were recorded once per week. All protocols were approved by the Institutional Animal Care and Use Committee of Cornell University.

4.3.2 Blood Collection, Tissue Examination, and Biochemical Analysis

At the end of week 3 and 6, 2 chicks from each cage were fasted for 8 hours, the euthanized by carbon dioxide asphyxiation for sampling. Blood was drawn from heart by heparinized needle, and spun in a tabletop centrifuge for 15 minutes at 3000g. The plasma supernatant was collected and stored in -20^{o}C fridge. Activities of plasma alanine aminotransferase (ALT) was determined by Infinity ALT liquid stable reagent kit (Thermo Electron Crop.). Plasma alkaline phosphatase (AKP) and Tartrate-resistant acid phosphatase (TRAP) was analyzed using the method of Bowers et al. [66] and Lau et al. [67], respectively. The method of Gomori, G [68] was used to measure plasma inorganic phosphorus (PIP). Glucose assay kit was purchased from Sigma Aldrich (St. Louis, Missouri, USA) and kit for determining uric acid was obtained from Thermo Scientific, Inc. (Waltham, MA, USA).

4.3.3 Determination of Carcass Yield, Meat Quality and Malondialdehyde (MDA) Levels

Texture profile analysis (TPA) was performed by measuring the compression force of meat samples by the texturometer (TA.XT*plus*, Stable Micro Systems, Hamilton, MA). Frozen breast and thigh muscle were thawed and cut into 2-inch diameter cubes, cooked in oven at 175^{o}C for

30 min, then subjected to analysis of chewiness, springiness, and hardiness using the method from Huidobro et al[80]. The meat pH was determined using iodacetate method [81] with modification. Approximately 250 mg of meat was homogenized for 30 seconds in 2.5 mL of solution composed of 5 mM sodium iodoacetate and 150 mM potassium chloride at pH 7. The pH of this mixture was measured using Accumet pH probe (AB150, Fisher Scientific, Waltham, MA). The water holding capacity (WHC) was determined using a centrifugal method.[82-83] Approximately 1g of meat was added to a centrifuge tube that contained three pieces of Whatman filter paper which were folded into a thimble. The tube and its contents were subjected to 7,710 g force for 30 min. The meat samples were separated from the filter paper and weighed to determine the WHC. Malondialdehyde (MDA) was measured by the method modified from Jo et al., reacting with 2-thiobarbituric acid and using 1,1,3,3-tetraethoxypropane as standard.[111]

4.3.4 Real Time PCR

Total mRNA was isolated and purified using TRIzol Reagent (Life Technologies, Carlsbad, CA) from liver or breast tissue (20 mg). Total RNA was analyzed both qualitatively and quantitatively using SpectraDrop Micro-Volume Microplate (SpectraMax® Plus 384 Absorbance Plate Reader, Molecular Devices). Spectrophotometric analysis showed purity of RNA as ~2.0 (260/280). The integrity of RNA was found to be good in agarose gel electrophoresis with intact 28S and 18S r RNA bands. The reverse transcription was performed using cDNA synthesis kit (Applied Biosystems, Grand Island, NY) according to kit guidelines.

The subsequent quantification was carried out by real-time polymerase chain reaction (qPCR) (7900 HT; Applied Biosystems) in a 10 μL reaction mixture containing 5 μL of Power SYBR green master mix (Invitrogen, Warrington, UK); 0.5 μL of each forward and reverse primer;

1 µL of cDNA template and 3 µL nuclease free water. All samples were run in duplicate reactions. The qPCR condition was as follows: 95^{o}C for 10 min, followed by 40 cycles of 95^{o}C for 15s, annealing 60^{o}C for 20 s, and then 72^{o}C for 20 s. At the end of each run, melt curve analysis was performed to ascertain single sharp peak. The primer sequences used for all the assayed genes are shown in **Supplemental Table 6**. The relative quantification of gene expression for each sample was adjusted with the control gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), using the 2-delta delta Ct (ΔΔCt) equation [112] and normalized to the control birds (as ‘‘1’’).

4.3.5 Immunoblotting Analysis

Protein from 50 mg liver or breast tissue was extracted by homogenizing with protein lysis buffer. The homogenates were then centrifuged for 15 mins at 14,000 × g at 4°C. The protein contents of the resulting supernatants were determined by bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL). Liver or breast homogenates (75 µg protein) were dissolved in SDS reducing sample buffer and boiled for 5 mins before loading onto 12% SDS-PAGE reducing mini-gels (Bio-Rad Laboratories Inc., Hercules, CA). Proteins in the gels were transferred onto nitrocellulose membranes using a mini-trans blot cell (Bio-Rad Laboratories Inc.) at 100 V for 60 min. Membranes were blocked in 5% milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature on a rocking platform. After three 5-mins washes with TBST, membranes were incubated with the appropriate primary antibodies overnight at 4°C with constant gentle agitation. Information on the primary antibodies used for western blot analysis is included in **Supplemental Table 7**. Membranes were washed 3 times in TBST before incubating for 1 h at room temperature with the horseradish peroxidase-conjugated secondary antibody diluted 1:2000 in 3% milk TBST. After 3 washes in TBST and 5

rinses in distilled water, membranes were incubated for 5 min at room temperature (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL) before exposing to a file (Kodak BioMax XAR Film; Carestream, Rochester, NY). The relative density of the protein bands was quantified by using ImageJ software (NIH) and normalized to GAPDH as the loading control [113].

Table 4.1. Composition (g/kg) of experimental diets for broiler chicks

	Starter				Grower			
Ingredients, g/kg	0%	1%	2%	4%	0%	1%	2%	4%
Corn	501	526	510	477	582	607	591	558
Soybean Meal	406	399	400	401	330	324	325	325
Omega-3 Biomass	0	10.0	20.0	40.0	0	10.0	20.0	40.0
Limestone	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Dical. Phos	18.1	18.1	18.1	18.1	13.6	13.6	13.6	13.6
Corn oil	30.0	15.0	15.0	15.0	30.0	15.0	15.0	15.0
Salt	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Vit. /Min. Mix*	7.50	7.50	7.50	7.50	7.50	7.50	7.50	7.50
DL-methionine	3.00	2.80	2.60	2.20	1.80	1.60	1.40	1.20
Lysine	0	0	0	0	0.60	0.60	0.60	0.60
L-Threonine	0.80	0.80	0.80	0.80	1.00	1.00	1.00	1.00
Celite	15.1	0.99	7.41	20.26	14.29	0.20	6.62	19.47
Nutritive Value								
ME, kcal/kg	2935	2935	2935	2935	3025	3025	3025	3025
Crude protein, %	23.0	23.0	23.0	23.0	20.0	20.0	20.0	20.0
Methionine, %	0.65	0.65	0.65	0.66	0.50	0.50	0.50	0.50
Cysteine, %	0.40	0.40	0.40	0.39	0.35	0.36	0.35	0.35
Lysine, %	1.35	1.35	1.35	1.35	1.19	1.20	1.19	1.19
Phosphorus, %	0.79	0.80	0.80	0.81	0.66	0.67	0.68	0.69
Calcium, %	1.01	1.01	1.01	1.01	0.90	0.90	0.90	0.91

*Vitamin and mineral mixture provided the following nutrients per kilogram of diets: vitamin A, 2550 IU; vitamin E, 15 IU; vitamin D, 300 IU; menadione, 0.75 mg; riboflavin, 5.4 mg; pantothenate, 15 mg; niacin, 52.5 mg; vitamin B1 2, 0.015 mg; biotin, 0.23 mg; folic acid, 0.83 mg; thiamine, 2.7 mg; pyridoxine, 5.3 mg; Choline, 1950 mg; $CuSO_4 \cdot 5H_2O$, 48 mg; KI, 0.69 mg; $MnSO_4 \cdot H_2O$, 277 mg; Na_2SeO_3, 0.50 mg; ZnO, 75 mg; $FeSO_4 \cdot 7H_2O$, 603 mg.

4.4 Results

4.4.1 Growth Performance

Growth performance including body weight gain (BWG), feed intake (FI) and gain: feed ratio (G: F ratio) was summarized during starter (week 0-3), grower (week 4-6) and overall (week 0-6) period in **Table 4.2**. Birds fed microalgae (1% and 2%) had similar BWG, FI and G: F ratio with the control for all periods, whereas 4% microalgae significantly decreased ($P<0.05$) BWG (18.7-12.7%) and G: F (19.4-14.7%) during both the grower and overall period. BWG and G: F ratio decreased linearly ($P<0.01$, R2=0.32-0.60) with increasing microalgae concentration during week 4-6 and week 0-6, however FI was not affected by increasing microalgae concentration.

4.4.2 Plasma Health Parameters

Chicks supplemented with microalgae exhibited a linear decrease ($P<0.01$, $R^2=0.34$) ALT activity at week 6, but AKP activity and PIP concentration were minimally affected during both week 3 and 6 as shown in **Table 4.3**. Glucose concentration decreased dose-dependently ($P<0.05$, $R^2=0.22-0.54$) with increasing microalgae concentration, and 4% microalgae caused 38.4% ($P<0.05$) and 9.93% ($P<0.05$) lower concentration of glucose at week 3 and 6, respectively. In addition, uric acid was largely unaffected at both time points except 2% microalgae diet increased by 1.1-fold over the control at week 3. For the plasma lipid profile, chicks fed with 1% and 4% microalgae diet exhibited significant decreases ($P<0.05$) of TC concentration at week 3 and 6. In addition, the concentration of NEFA from 2% and 4% microalgae treatments were 42.4% and 51% lower ($P<0.05$) than the control, while plasma TG remained consistent between all treatments.

4.4.3 Meat Production and Quality

Effect of DHA-rich microalgae on carcass traits and meat quality was shown in **Table 4.4**. Although BWG decreased as microalgae increased, there was no significant difference in carcass weight and dressing percentage at week 6. However, breast muscle weight decreased linearly ($P<0.05$, $R^2=0.29$), with the 4% microalgal diet resulting in 21% lower ($P<0.05$) breast muscle weight than the control. Similarly, muscle yield, measured as the percentage of breast muscle weight in overall carcass, decreased ($P<0.01$, $R^2=0.40$) with increasing microalgal concentration. In contrast, while thigh muscle weight was not affected by microalgal concentration, muscle yield decreased linearly ($P<0.05$, $R^2=0.18$) as microalgae inclusion increased. However, weight and percentage of other organs were largely not influenced except heart and liver. Both heart weight percentage and liver weight had a linear increasing trend ($P<0.05$, $R=0.22$ or 0.18) when microalgae concentration rose. In summary, breast muscle, thigh weight and thigh weight percentage had decreased, while carcass and organ weight and percentage remain generally unchanged at week 6.

As for meat quality, WHC, pH and springiness of breast and thigh muscle tissue were largely unaffected. However, chewiness and hardness of breast and thigh muscle were dose dependently increased ($P<0.05$, $R=0.24$-0.442) in the higher microalgal concentration diet. More specifically, birds fed a 4% microalgae treatment exhibited over 70% higher ($P<0.01$) of chewiness and hardness in both breast and thigh than the control. In addition, while there was no significant difference of MDA concentration at all tissues during week 3 (**Table 4.5**), MDA did show an increase ($P<0.05$, $R=0.62$-0.91) with increasing microalgae concentration in breast, thigh, liver and adipose tissue at week 6.

4.4.4 Meat Protein Gene Regulation

Breast and liver mRNA expression after week 3 or 6 are shown in **Figure 4.1 and 4.2**. 4% microalgae inclusion decreased (P<0.05) mRNA levels of *AMPK*, *mTOR*, *S6K1*, *4E-BP1*, and *MAFbx* in breast at week 3, while 2% microalgal concentration increased mRNA Levels of *MURF1* (P<0.05) and *MAFbx* (P>0.05). However, dietary microalgae inclusion had no effects on gene expression in breast at week 6 of above genes except 4% microalgae increased (P<0.05) *4E-BP1* mRNA level. Meanwhile, chicks fed 4% microalgae resulted in lower (P<0.05) mRNA levels of *mTOR*, *S6K1*, *4E-BP1* and *MURF1* at week 3 and *mTOR* and *4E-BP1* at week 6 in the liver.

Highest microalgae inclusion (4%) decreased (P<0.05) phosphor-S6 to S6 ratio by 40% in the breast at week 3, while mTOR protein amount and P70 to PP70 ratio were not affected by microalgae inclusion at week 3 or 6 (**Figure 4.3, 4.4**). Meanwhile, mTOR protein was 47% lower (P<0.05) compared with the control in the liver at week 3, but there was no different of mTOR protein, phosphor P70 to P70, and phosphor-S6 to S6 between microalgae treatments and control at week 6.

Table 4.2. Effect of different concentrations of microalgae on growth performance in broiler chicks

Treatment	Week	Control	Trt1	Trt2	Trt3	SEM	P value		R^2
Microalgae (%)		0	1	2	4		ANOVA	Linear	
Body weight gain	0-3	41.3	40.0	41.4	42.1	1.02	0.531	0.345	0.041
g/chick/d	4-6	97.0[a]	94.7[a]	91.4[a]	78.9[b]	2.40	<0.01	<0.01	0.600
	0-6	71.6[a]	69.7[a]	68.7[a]	62.5[b]	1.55	<0.01	<0.01	0.474
Feed intake	0-3	53.5[ab]	52.1[b]	55.8[ab]	57.4[a]	1.48	0.091	0.028	0.202
g/chick/d	4-6	164	180	166	165	5.59	0.192	0.606	0.012
	0-6	109	116	111	111	3.04	0.419	0.955	<0.01
Gain: feed ratio	0-3	0.773	0.767	0.772	0.736	0.018	0.423	0.028	0.210
	4-6	0.594[a]	0.534[ab]	0.551[a]	0.479[b]	0.022	0.014	<0.01	0.342
	0-6	0.660[a]	0.606[ab]	0.619[ab]	0.563[b]	0.020	0.021	<0.01	0.317

Data analysis using one-way ANOVA and Duncan's multiple comparison.
[a, b, c, d] Means in the same row without a common letter differ (P < 0.05)

Table 4.3. Effect of different concentrations of microalgae on plasma indicator of broiler chicks

Treatment	Control	Trt1	Trt2	Trt3	SEM	P value		R^2
Microalgae (%)	0	1	2	4		ANOVA	Linear	
ALT, U/L								
Week 3	2.40	2.77	2.98	2.61	0.198	0.409	0.653	0.015
Week 6	1.29[a]	1.02[ab]	0.947[ab]	0.422[b]	0.194	0.060	<0.01	0.335
AKP, U/mL								
Week 3	298	305	208	252	45.3	0.573	0.406	0.041
Week 6	157	161	131	172	15.9	0.492	0.741	0.006
PIP, mg/dL								
Week 3	78.2	85.4	91.4	91.3	11.3	0.854	0.428	0.035
Week 6	66.2[a]	60.8[ab]	57.6[b]	63.3[ab]	2.43	0.114	0.501	0.022
Glucose, g/L								
Week 3	2.79[a]	2.28[b]	1.94[bc]	1.72[c]	0.144	0.0002	<0.01	0.536
Week 6	3.02[a]	2.93[ab]	2.89[ab]	2.72[b]	0.091	0.200	0.028	0.219
Uric acid, mmol/L								
Week 3	0.125[b]	0.189[ab]	0.262[a]	0.218[ab]	0.027	0.072	0.095	0.175
Week 6	0.195	0.218	0.281	0.205	0.034	0.453	0.714	0.009
TC, mg/dL								
Week 3	57.2[a]	45.4[b]	53.4[ab]	43.0[b]	3.07	0.012	0.026	0.205
Week 6	72.7[a]	60.4[b]	63.7[ab]	61.9[b]	3.09	0.069	0.138	0.102
TG, mg/dL								
Week 3	33.8	29.0	28.7	30.0	4.45	0.839	0.635	0.010
Week 6	24.4	25.6	29.3	23.4	9.55	0.124	0.841	0.002
NEFA, µmol/L								
Week 3	391	476	703	395	78.0	0.047	0.851	0.002
Week 6	604[a]	464[ab]	348[b]	296[b]	72.7	0.034	<0.01	0.300

Data analysis using one-way ANOVA and Duncan's multiple comparison.

[a,b,c,d] Means in the same row without a common letter differ ($P < 0.05$)

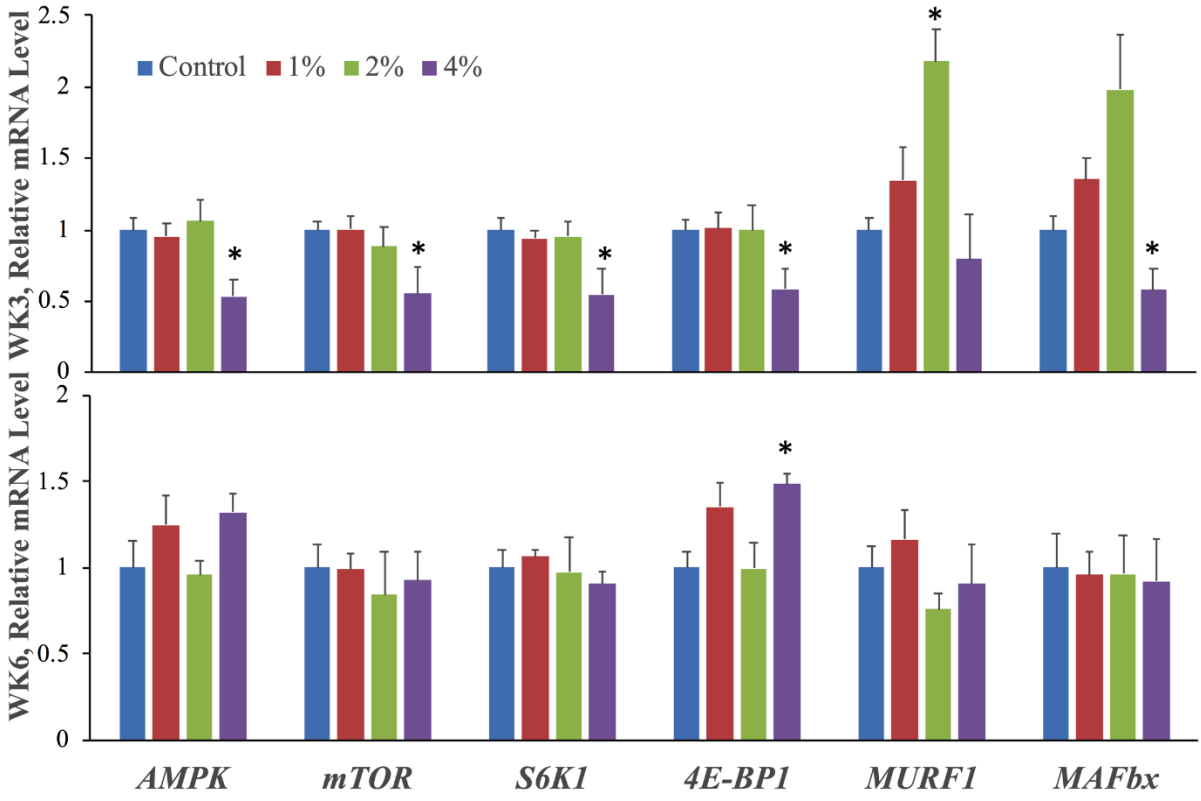
ALT: Alanine aminotransferase; AKP: Alkaline phosphatase; PIP: plasma inorganic phosphorus; TC: total choleste rol; TG: triglycerides; NEFA: non-esterified fatty acid

Table 4.4. Effect of different concentrations of microalgae on Carcass traits of broiler chicks at week 6

Treatment	Control	Trt1	Trt2	Trt3	SEM	P value		R^2
Microalgae (%)	0	1	2	4		ANOVA	Linear	
Breast muscle								
Weight, g	656[a]	581[ab]	622[a]	520[b]	28.5	0.029	0.011	0.285
Muscle yield, %	27.4[a]	25.8[ab]	26.1[a]	24.1[b]	0.56	0.01	<0.01	0.400
pH	6.31[ab]	6.11[ab]	6.00[b]	6.53[a]	0.144	0.0775	0.233	0.064
Water holding capacity (%)	40.3	39.3	41.1	39.2	1.44	0.783	0.731	0.005
Chewiness	1373[c]	2168[b]	2291[ab]	2993[a]	250	0.0091	<0.01	0.442
Springiness	0.765[ab]	0.756[b]	0.758[b]	0.797[a]	0.001	0.0351	0.021	0.237
Hardness	3656[b]	6368[a]	6335[a]	7189[a]	563	0.0029	<0.01	0.362
Thigh muscle								
Weight (with bone), g	583	564	588	574	21.5	0.871	0.927	<0.01
Muscle yield, %	24.3	25.0	24.7	25.8	0.49	0.201	0.046	0.177
pH	6.30	6.39	6.32	6.43	0.131	0.901	0.558	0.017
Water holding capacity (%)	39.1	37.9	39.9	39.2	0.876	0.472	0.651	<0.01
Chewiness	1964[b]	3008[ab]	3163[a]	3855[a]	361	0.0202	<0.01	0.369
Springiness	0.880	0.994	0.875	0.870	0.006	0.4673	0.162	0.100
Hardness	3607[b]	5426[a]	5292[a]	6129[a]	455	0.0118	<0.01	0.340
Other organs								
Heart weight, g	14.8	15.6	16.4	16.2	0.827	0.577	0.256	0.061
Heart percentage, %	0.620[b]	0.690[ab]	0.692[ab]	0.729[a]	0.029	0.098	0.023	0.224
Liver weight, g	64.4[ab]	59.3[b]	67.2[ab]	71.7[a]	3.23	0.106	0.050	0.179
Liver percentage, %	2.67	2.75	2.96	2.95	0.203	0.711	0.292	0.053
Intestine weight, g	114	126	118	117	8.01	0.767	0.989	<0.01
Intestine percentage, %	4.74	5.60	4.96	5.23	0.259	0.144	0.541	0.018
Carcass weight, g	2397	2248	2379	2229	86.9	0.431	0.299	0.051
Dressing percentage, %	76.5	75.6	76.1	74.6	0.58	0.181	0.514	0.022

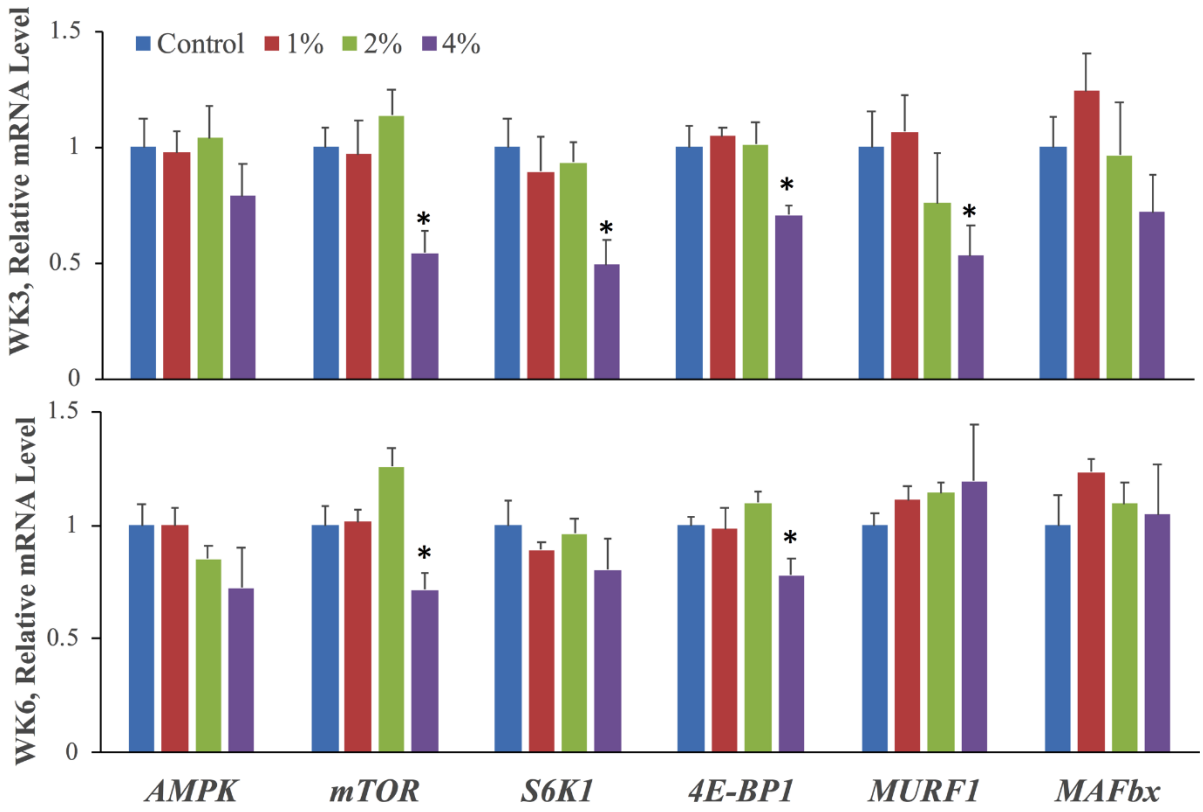
Data are expressed as mean (n=6).
Data analysis: one-way ANOVA, Duncan's multiple comparison and linear regression.

Figure 4.1. Relative mRNA levels of protein metabolism in the breast of broiler chicks at weeks 3 (top) and 6 (bottom)



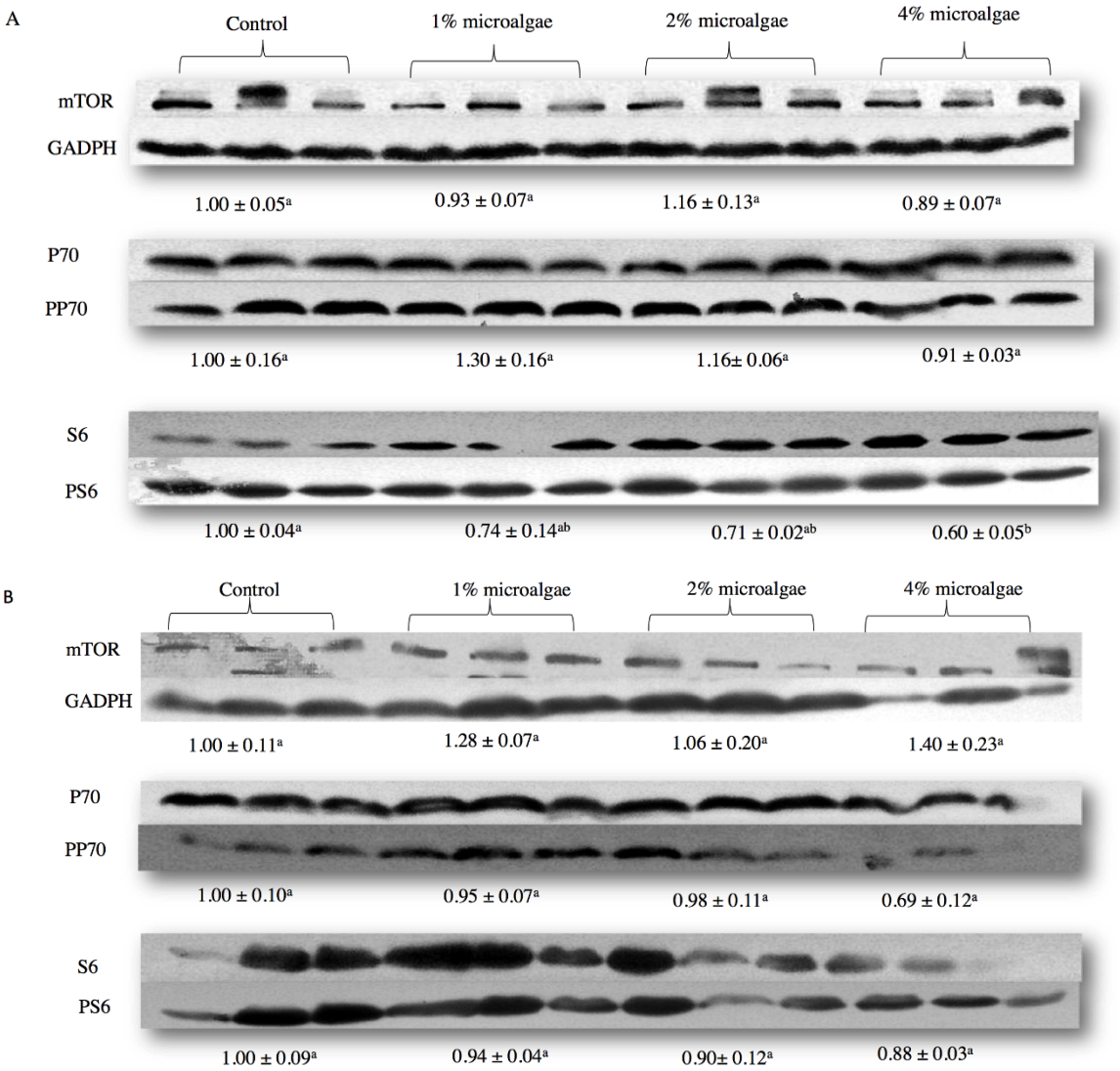
Relative mRNA levels of protein synthesis and degradation in the breast during week 3 (top) and week 6 (bottom) of broiler chicks fed 0, 1, 2, and 4% DHA microalgae diets for 6 weeks. Values are means ± SEs, n = 6. Asterisk symbol indicates significance at $P < 0.05$. AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; S6K1: ribosomal protein S6 kinase beta-1; 4EBP1: eukaryotic translation initiation factor 4E-binding protein 1; MURF1: muscle RING finger 1. MAFbx: muscle atropy F-box.

Figure 4.2. Relative mRNA levels of protein metabolism in the liver of broiler chicks at weeks 3 (top) and 6 (bottom)



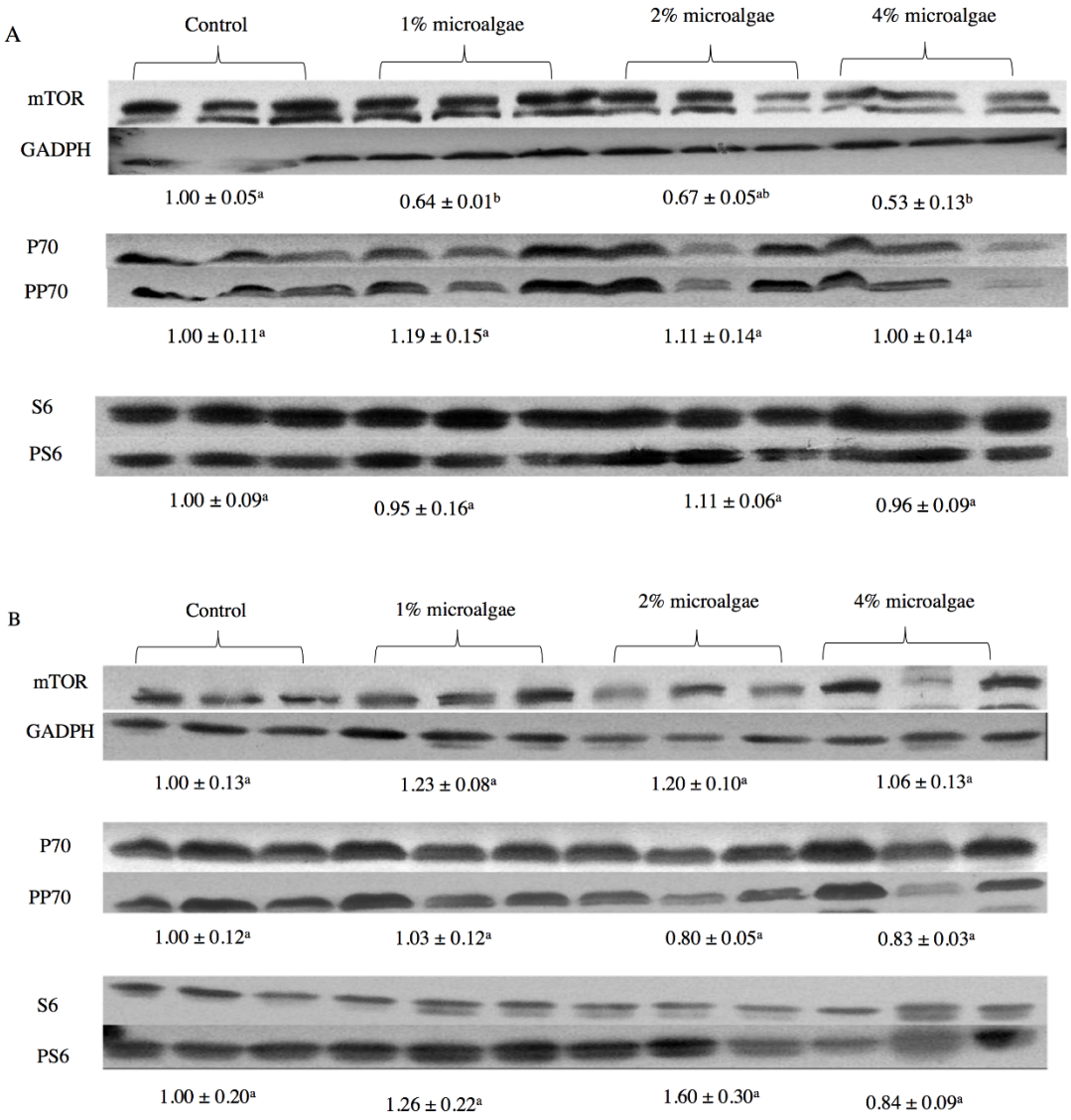
Relative mRNA levels of protein synthesis and degradation in the liver during week 3 (top) and week 6 (bottom) of broiler chicks fed 0, 1, 2, and 4% DHA microalgae diets for 6 weeks. Values are means ± SEs, n = 6. Asterisk symbol indicates significance at $P < 0.05$. AMPK: AMP-activated protein kinase; mTOR: mammalian target of rapamycin; S6K1: ribosomal protein S6 kinase beta-1; 4EBP1: eukaryotic translation initiation factor 4E-binding protein 1; MURF1: muscle RING finger 1. MAFbx: muscle atropy F-box.

Figure 4.3. Relative protein concentration in the breast of broiler chicks tissue at weeks 3 (A) and week 6 (B)



Relative protein concentration of breast mTOR, P70, PP70, S6 and PS6 in broiler fed 0, 1, 2, and 4% DHA microalgae diets at week 3 (A) and week 6 (B). Values below the protein band were relative densities and are expressed as means ± SEs, n = 3. Means without a common letter differ, $P < 0.05$. mTOR: mammalian target of rapamycin; GADPH: Glyceraldehyde 3-phosphate dehydrogenase; P70: ribosomal protein S6 kinase; PP70: phosphorylated ribosomal protein S6 kinase; S6: ribosomal protein S6; PS6: phosphorylated ribosomal protein S6.

Figure 4.4. Relative protein concentration in the liver of broiler chicks tissue at weeks 3 (A) and week 6 (B)



Relative protein concentration of liver mTOR, P70, PP70, S6 and PS6 in broiler fed 0, 1, 2, and 4% DHA microalgae diets at week 3 (A) and week 6 (B). Values below the protein band were relative densities and are expressed as means ± SEs, n = 3. Means without a common letter differ, $P < 0.05$. mTOR: mammalian target of rapamycin; GADPH: Glyceraldehyde 3-phosphate dehydrogenase; P70: ribosomal protein S6 kinase; PP70: phosphorylated ribosomal protein S6 kinase; S6: ribosomal protein S6; PS6: phosphorylated ribosomal protein S6.

4.5 Discussion

Feeding Microalgae *Aurantiochytrium*, containing up to 12% DHA concentration by weight, dose-dependently deposited DHA in all tissues such as breast, thigh and liver in broiler (data will be published in another paper). Surprisingly, growth performance was impaired by microalgae inclusion and chicks fed 4% microalgae diet had significantly lower BWG and G: F ratio compared with the control. Since FI remained unchanged among treatments, it was possible that the highest dose of DHA treatment decreased G: F ratio which contributed to BWG decrease. Although DHA rich ingredients such as fish oil or other microalgae species have been previously incorporated into chicken products, results have indicated that feeding red fish meal or red fish oil (rich with omega-3 fatty acids) to broilers resulted in lower body weight, feed consumption and poorer feed conversion [106]. Nevertheless, there were some studies suggested that dietary DHA enrichment could improve growth performance. Broiler chicks fed with 7.4% DHA Gold, a product extracted from *Schizochytrium* microalgae, demonstrated the improvement of body weight gain, feed intake and feed conversion ratio [109]. A study by Long et al. which supplemented other DHA rich microalgae (*Schizochytrium limacinum*) by up to 2% showed a higher average body weight as well as better feed conversion ratio [110]. Besides, there were studies that didn't exert any effect on growth performance by feeding 0.2% microalgae *Schizochytrium JB5* [114] or up to 8% microalgae *Nannochloropsis Oceanica* with detectable DHA deposition increase in broiler tissues.[9] The discrepant effects of DHA on growth performance may be attributed to varying concentrations of DHA, difference between feed ingredient, or feeding period and methods. It also remains unknown whether DHA or other nutrients from microalgae causes impaired growth performance. Therefore, more studies are required to investigate the individual effect of DHA or other microalgal composition in broiler chicks. This

sudy conclude that feeding up to 2% microalgae *Schizochytrium* supplementation could enrich DHA in broiler tissues without affecting growth performance.

Carcass results suggested that the breast and thigh weight or yield were adversely affected by microalgae treatments, while weight of organs such as heart, liver and intestine were increased or not affected. However, breast muscle weight loss does show a correlation with gene level expression. Our results demonstrated that mRNA levels of protein anabolic gene including *mTOR* and *S6K1* were downregulated by feeding 4% microalgae, while mRNA levels of protein catabolic gene such as *MURF1* and *MAFbx* were upregulated in breast muscle by feeding 2% microalgae at week 3. At week 6, mRNA levels of most genes remained unchanged among treatments which may be due to the decreased growth rate of broiler chicks. The relative protein amounts of mTOR and ribosomal protein S6 decreased which was in accordance with the trend of mRNA expression data. Little has been studied regarding the effects of DHA in broiler chicks model, as most of the experiments were conducted in mouse or human trail. However, a recent series of studies have reported that DHA is a potentially useful agent in stimulating muscle growth. Smith et al. found that n-3 fatty acid had muscle protein properties in health young and middle age adults.[115] Dietary DHA-rich supplement promoted muscle growth in pigs at fed state by upregulating mTOR and downregulate 4E-BP1.[116] Kamolrat et al. found that DHA can enhance protein synthesis by increasing p70s6k phosphorylation but had no effect on protein breakdown in C2C12 myotube.[117] The muscle retardation from 4% DHA-rich microalgae treatment could be inferred as the negative effect of such high dietary DHA concentration supplementation (6.8 g DHA/kg diet) or the difference in animal species and fed state. However, more studies are still needed to investigate whether DHA or other nutrients in microalgae interfere with protein metabolism in broiler by dietary microalgae supplementation.

Even though breast and thigh weight or yield decreased with increasing dietary concentration of microalgae, the weight or percentage of liver and heart were found to increase. It has been reported that increased liver weight is associated with elevated immunity which may improve the general health of broiler chicks.[110] In addition, plasma activity of ALT was dose dependently decreased by treatments. Alanine aminotransferase (ALT) is being used as a critical plasma indicator in diagnosis and assessment of liver disease. It will increase after acute liver damage such as non-alcoholic fatty liver disease.[118] However, dose dependent decline of ALT may indicate that dietary DHA could reduce the incident of liver disease in broiler and improve overall liver health. In addition, plasma glucose concentration was significantly decreased at week 3 and there was a decreasing trend at week 6 after 8 hours fasting. So far there was no clear evidence to prove plasma glucose can be downregulated due to n-3 PUFA. But it has been reported that the DHA/EPA supplement in animal and human can increase insulin sensitivity.[119] It is possible the increased sensitivity of insulin increased the adipose uptake of glucose which decreased plasma glucose concentration. In summary, DHA-rich microalgae may improve broiler chick liver health and insulin sensitivity in broiler chicks.

Our results showed no effect on water holding capacity and pH of breast and thigh muscle by microalgae treatments. This is in accordance with previous studies such as that by Long et al. who reported that pH and water holding capacity were not affected after feeding DHA rich microalgae for 6 weeks as well.[110] However, chewiness and hardness increased with increasing microalgae concentration. The similar finding has been reported by Betti et al. which found that shear force before cooking from n-3 fatty acid enriched meat was increased by feeding flaxssed oil.[120] Therefore, it is safe to say that DHA-rich microalgae appears to have an impact on meat texture profile after cooking. On the other hand, with the deposition of DHA in breast and thigh

muscle (data were discussed in another paper), the MDA concentration was increased dose dependently as microalgae levels increased. MDA is the secondary products from lipid peroxidation and a practical indicator for determination of food lipid peroxidation which is responsible for off flavor of meat products. [121] Higher MDA concentration in tissues means the more lipid have been oxidized and shorter shelf life it could be. DHA contains 6 double carbon bonds which is prone to be oxidized and cause off flavor. Over enrichment of DHA might affect shelf life of breast and thigh muscle products. Anjum et al. also demonstrated that n-3 PUFA enrichment by extruded flaxseed meal elevate MDA concentration. [122]

In conclusion, feeding up to 2% DHA-rich microalgae largely had no effect on growth performance, meat quality and products, and broiler health. However, 4% microalgae could result in a decrease body weight by upregulating protein catabolic genes and downregulating protein anabolic genes. It is not yet clear whether high concentration of DHA or other nutrients from microalgae contributed this effect on broiler chicks. Future research will focus to elicit the causes of protein metabolism change in broiler chicks.

APPENDIX

Supplemental Table 1. Composition (g/kg) of experimental diets for broiler chicks

	Starter Astaxanthin, mg/kg					Grower Astaxanthin, mg/kg				
	0	10	20	40	80	0	10	20	40	80
Corn	518	515	517	507	512	602	599	601	592	600
Soybean Meal	403	400	403	389	402	327	324	327	314	326
Defatted algae	0	5	0	20	0	0	5	0	20	0
Full fat algae	0	0	0.8	0	3.2	0	0	0.8	0	3.2
Limestone	12.9	12.9	12.9	12.9	12.9	8.6	8.6	8.6	8.6	8.6
Dical. Phos	18.4	18.4	18.4	18.4	18.4	18.2	18.2	18.2	18.2	18.2
Corn oil	20	20	20	20	20	20	20	20	20	20
Salt	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Vit. /Min. Mix*	4	4	4	4	4	4	4	4	4	4
DL-methionine	3.5	3.5	3.5	3.5	3.5	2.5	2.5	2.5	2.5	2.5
L-Lysine HCL	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Threonine	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Celite	14.9	16	15.1	18.9	15.3	11.9	13	12.1	15.8	12.3
Nutritive Value										
ME, kcal/kg	2900	2900	2900	2900	2900	3000	3000	3000	3000	3000
Crude protein, %	23	23	23	23	23	20	20	20	20	20
Methionine, %	0.70	0.70	0.70	0.70	0.70	0.57	0.57	0.57	0.57	0.57
Cysteine, %	0.40	0.39	0.39	0.39	0.40	0.36	0.35	0.36	0.35	0.36
Lysine, %	1.39	1.39	1.39	1.40	1.39	1.18	1.19	1.18	1.19	1.19
Phosphorus, %	0.76	0.76	0.76	0.77	0.76	0.69	0.69	0.69	0.70	0.69
Calcium, %	1.00	1.00	1.00	1.00	1.00	0.90	0.90	0.90	0.90	0.90

*Vitamin and mineral mixture provided the following amount (per kilogram of diets): vitamin A, 4,550 IU; vitamin E, 7.5 IU; vitamin D_3, 450 IU; vitamin K, 0.752 mg; riboflavin, 3.75 mg; pantothenic acid, 3 mg; niacin, 15.2 mg; vitamin B_{12}, 0.006 mg; biotin, 0.152 mg; folic acid, 0.376 mg; thiamine, 1.07 mg; pyridoxine, 3.78 mg; choline, 1575mg; Cu, 12mg; I, 0.053mg; Mn, 30.2mg; Se, 0.09mg; Zn, 53.0mg; Fe, 67.8mg.

Supplemental Table 2. Effect of dietary microalgal astaxanthin on plasma indicators of broiler chicks[*]

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
ALT**, U/L							
Week 3	0.759[c]	0.83[c]	1.11[bc]	1.31[ab]	1.64[a]	0.31	<0.01
Week 6	2.02	2.56	2.75	3.08	3.08	1.54	0.78
AKP**, U/L							
Week 3	101	101	99.6	97.0	85.9	30.2	0.93
Week 6	46.4[a]	46.9[a]	39.8[a]	34.9[a]	21.3[b]	10.6	<0.01
TRAP**, U/L							
Week 3	0.227	0.259	0.335	0.300	0.301	0.076	0.28
Week 6	0.446	0.489	0.435	0.452	0.424	0.098	0.83
Cholesterol, mg/dL							
Week 3	64.5[b]	67.7[b]	65.0[b]	71.6[b]	83.6[a]	8.07	<0.01
Week 6	60.1	58.2	66.4	62.3	69.7	8.15	0.15
Triglycerides, mg/dL							
Week 3	36.9[a]	28.3[ab]	33.6[a]	21.5[b]	26.9[ab]	7.55	0.034
Week 6	17.1	18.6	16.2	19.8	20.5	4.65	0.53
NEFA**, µmol/L							
Week 3	672[a]	495[b]	263[c]	375[bc]	463[b]	102	<0.01
Week 6	760	817	700	787	754	303	0.98
PIP**, mg/dL							
Week 3	1.24	1.38	1.32	1.48	1.52	0.197	0.13
Week 6	1.02	1.07	0.989	1.06	1.08	0.146	0.80
Glucose, mmol/L							
Week 3	16.2	16.8	18.4	18.2	16.4	2.17	0.28
Week 6	9.52	9.78	8.72	9.01	9.28	0.75	0.28
Uric acid, µmol/L							
Week 3	181	218	280	178	224	104	0.47
Week 6	177	216	227	183	231	79.5	0.71

[*]Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. [a,b,c]Means in the same row without a common letter differ ($P < 0.05$). [**]ALT: alanine aminotransferase; AKP: alkaline phosphatase; TRAP: tartrate-resistant acid phosphatase; NEFA: non-esterified fatty acid; PIP: plasma inorganic phosphorus.

Supplemental Table 3. Effect of dietary microalgal astaxanthin on dressing percentage of broiler chicks[*]

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Dressing percentage	88.4	83.8	83.0	84.1	82.3	1.27	0.31
Meat to bone ratio, thigh	80.3	82.5	82.3	82.7	81.0	2.89	0.56

[*]Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test.

Supplemental Table 4. Effect of dietary microalgal astaxanthin on fatty acid composition of broiler chicks*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Fatty Acid, %							
Week 3							
Breast							
SFA[**]	30.1	33.9	32.6	33.1	33.3	1.29	0.29
MUFA[**]	45.9[a]	42.9[a]	41.9[ab]	33.2[b]	39.4[a]	3.41	<0.01
PUFA[**]	24.0[b]	24.9[b]	25.5[b]	33.7[a]	27.5[ab]	2.87	<0.01
Thigh							
SFA	30.2	31.8	31.7	31.9	32.3	1.31	0.87
MUFA	40.6	41.0	38.1	38.0	42.7	3.53	0.56
PUFA	29.3	27.1	30.2	30.1	25.0	2.82	0.41
Liver							
SFA	44.0[b]	42.6[b]	43.0[b]	43.4[b]	45.9[a]	0.61	<0.01
MUFA	22.4	19.1	19.1	22.1	16.6	2.68	0.61
PUFA	33.6[b]	38.3[a]	37.9[a]	34.5[ab]	37.5[a]	2.59	<0.01
Week 6							
Breast							
SFA	33.5	34.4	34.1	33.5	33.8	0.95	0.17
MUFA	37.9	38.0	39.6	41.8	38.7	2.92	0.45
PUFA	28.6	27.6	26.3	24.8	27.5	2.57	0.63
Thigh							
SFA	33.6	33.0	32.0	31.9	32.2	0.80	0.23
MUFA	38.0	40.1	38.5	37.9	38.3	3.29	0.46
PUFA	28.4	26.9	29.5	30.2	29.5	2.97	0.79
Liver							
SFA	42.1	43.6	44.4	41.9	45.2	6.09	0.74
MUFA	31.6	25.3	28.8	31.9	25.3	6.85	0.81
PUFA	26.3	31.1	26.8	26.2	29.5	8.03	0.92

[*]Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. [a,b]Means in the same row without a common letter differ ($P < 0.05$). [**]SFA, saturated fatty acid; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids.

Supplemental Table 5. Effect of dietary microalgal astaxanthin on fatty acid concentration of broiler chicks*

	Astaxanthin, mg/kg						
	0	10	20	40	80	SEM	p value
Fatty Acid, mg/g of tissue							
Week 3							
Breast							
C16	1.65	1.22	1.45	1.11	1.94	0.43	0.21
C16:1	0.46	0.19	0.28	0.17	0.41	0.18	0.44
C18	0.45	0.45	0.44	0.41	0.71	0.12	0.19
C18:1	2.74	1.77	2.16	1.36	2.74	0.85	0.56
C18:2	1.51	1.28	1.41	1.33	1.85	0.324	0.74
C20:4	0.166[ab]	0.169[ab]	0.081[b]	0.220[ab]	0.343[a]	0.099	<0.01
C22:6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
n-6	1.68	1.45	1.49	1.55	2.19	0.356	0.33
n-3/n-6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
Thigh							
C16	2.66	3.00	2.34	2.34	3.51	0.730	0.63
C16:1	0.746	0.874	0.579	0.546	0.966	0.252	0.17
C18	0.917	0.943	0.738	0.809	1.03	0.178	0.25
C18:1	4.06	4.21	3.20	3.11	5.05	1.13	0.39
C18:2	2.95	2.86	2.54	2.51	3.02	0.654	0.46
C18:3	0.052	0.065	0.056	0.062	0.108	0.062	0.89
C20:4	0.468	0.439	0.400	0.317	0.394	0.0734	0.44
C22:6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
n-3	0.0517	0.0654	0.0558	0.0620	0.1077	0.0615	0.64
n-6	3.42	3.29	2.94	2.83	3.41	0.693	0.41
n-3/n-6	0.0134	0.0159	0.0158	0.0185	0.0315	0.0166	0.37
Liver							
C16	3.80	3.54	3.44	2.79	3.75	0.879	0.56
C16:1	0.190	0.265	0.175	0.131	0.351	0.241	0.27
C18	3.66	3.84	3.94	2.95	3.59	0.709	0.52
C18:1	3.60	3.04	3.10	2.79	3.80	1.16	0.41
C18:2	3.49	3.57	3.50	2.54	3.90	0.79	0.33
C18:3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
C20:4	2.20	2.53	2.54	1.83	2.02	0.473	0.71
C22:6	0.00	0.358	0.442	0.189	0.167	0.208	0.38
n-3	0.00	0.358	0.442	0.189	0.167	0.208	0.46
n-6	5.69	6.09	6.04	4.36	5.93	1.12	0.52
n-3/n-6	0.00	0.08	0.73	0.44	0.23	0.045	0.27
Week 6							
Breast							
C16	1.65	1.22	1.45	1.11	1.94	0.43	0.41
C16:1	0.31	0.37	0.39	0.38	0.40	0.10	0.39
C18	0.57	0.63	0.62	0.59	0.60	0.10	0.28
C18:1	2.17	2.53	2.64	2.73	2.49	0.45	0.24
C18:2	1.60	1.81	1.73	1.66	1.73	0.370	0.56
C20:4	0.271	0.272	0.278	0.165	0.316	0.088	0.30

C22:6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
n-3	0.00	0.00	0.00	0.00	0.00	0.00	N/A
n-6	1.87	2.08	2.01	1.82	2.05	0.372	0.41
n-3/n-6	0.00	0.00	0.00	0.00	0.00	0.00	N/A
Thigh							
C16	2.06	1.98	2.29	2.62	2.68	0.464	0.30
C16:1	0.444	0.516	0.562	0.562	0.626	0.151	0.21
C18	0.835	0.651	0.875	0.885	0.887	0.136	0.41
C18:1	2.83	2.69	3.25	3.60	3.71	0.718	0.52
C18:2	2.07	1.86	2.45	2.86	2.80	0.535	0.63
C18:3	0.00	0.00	0.055	0.069	0.076	0.045	0.29
C20:4	0.373^{ab}	0.290^{b}	0.418^{a}	0.385^{ab}	0.403^{ab}	0.062	<0.01
C22:6	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N/A
n-3	0.00	0.00	0.0554	0.0686	0.076	0.045	0.41
n-6	2.45^{ab}	2.15^{b}	2.86^{ab}	3.24^{a}	3.20^{ab}	0.562	<0.01
n-3/n-6	0.00	0.00	0.0178	0.0189	0.0212	0.012	0.19
Liver							
C16	7.80^{a}	4.65^{bc}	5.18^{abc}	6.70^{ab}	3.79^{c}	2.22	<0.01
C16:1	1.03^{a}	0.569^{ab}	0.600^{ab}	0.740^{ab}	0.371^{b}	0.379	<0.01
C18	5.92^{a}	4.17^{bc}	4.86^{abc}	5.47^{ab}	3.52^{c}	1.33	<0.01
C18:1	9.27^{a}	4.55^{b}	5.92^{ab}	8.54^{a}	3.73^{b}	3.18	<0.01
C18:2	5.89^{a}	4.02^{ab}	3.75^{ab}	5.28^{ab}	2.63^{b}	2.14	<0.01
C18:3	0.118	0.091	0.00	0.082	0.492	0.544	0.21
C20:4	2.36^{a}	2.13^{ab}	2.18^{ab}	2.16^{ab}	1.45^{b}	0.567	<0.01
C22:6	0.218	0.0562	0.138	0.112	0.199	0.178	0.31
n-3	0.354	0.147	0.138	0.194	0.692	0.620	0.27
n-6	8.25^{a}	6.15^{ab}	5.93^{ab}	7.43^{a}	4.08^{b}	2.38	<0.01
n-3/n-6	0.039	0.019	0.021	0.022	0.368	0.290	0.45

*Data are expressed as mean (n = 6) and analyzed by one-way ANOVA and Duncan's multiple-range test. [a,b,c]Means in the same row without a common letter differ ($P < 0.05$).

Supplemental Table 6. List of primers used for q-PCR analysis

Gene Name	Forward (5'---3') Reverse (5'---3')
mTOR	GGAATGAACCGTGATGACCG AGCATTTGACTGAGAGGGCT
S6K1	CAATTTGCCTCCCTACCTCA AAGGAGGTTCCACCTTTCGT
AMPK	CCAGTGTTTTCAGCTCCCAC GAGGTCCAGGATAGCGACAA
MURF1	GCCAAGCAGCTCATTAAAACG CATGTTCTCATAGCCTTGCTCAAT
MAFbx1	AGGCCGCAGTGTGTTGTTCT GTGTGAATGGCTGGTTGCAT

Supplemental Table 7. List of primary and secondary antibodies for western blot analysis

Antigen	Species	Working dilution	Buffer	Source
mTOR	Rabbit	1:1000	3% milk	Cell Signaling Technology
S6	Rabbit	1:1000	3% milk	Cell Signaling Technology
P-S6	Rabbit	1:2000	3% milk	Cell Signaling Technology
P70	Rabbit	1:1000	3% milk	Cell Signaling Technology
P-P70	Rabbit	1:1000	3% milk	Cell Signaling Technology
GADPH	Rabbit	1:200	3% milk	Cell Signaling Technology

CITATION

1. Nation, U. *World Population Prospects: The 2017Revision*; 2017.
2. Council, N. C. Statistics. https://www.nationalchickencouncil.org/about-the-industry/statistics/.
3. Chisti, Y., Biodiesel from microalgae. *Biotechnol Adv* **2007,** *25* (3), 294-306.
4. Brune, D. E.; Lundquist, T. J.; Benemann, J. R., Microalgal Biomass for Greenhouse Gas Reductions: Potential for replacement of fossil fuels and animal feeds. *J Environ Eng* **2009,** *135* (11), 1136-1144.
5. Spolaore, P.; Joannis-Cassan, C.; Duran, E.; Isambert, A., Commercial applications of microalgae. *J Biosci Bioeng* **2006,** *101* (2), 87-96.
6. Austic, R. E.; Mustafa, A.; Jung, B.; Gatrell, S.; Lei, X. G., Potential and limitation of a new defatted diatom microalgal biomass in replacing soybean meal and corn in diets for broiler chickens. *J Agric Food Chem* **2013,** *61* (30), 7341-8.
7. Ekmay, R.; Gatrell, S.; Lum, K.; Kim, J.; Lei, X. G., Nutritional and metabolic impacts of a defatted green marine microalgal (Desmodesmus sp.) biomass in diets for weanling pigs and broiler chickens. *J Agric Food Chem* **2014,** *62* (40), 9783-91.
8. Leng, X.; Hsu, K. N.; Austic, R. E.; Lei, X. G., Effect of dietary defatted diatom biomass on egg production and quality of laying hens. *J Anim Sci Biotechnol* **2014,** *5* (1), 3.
9. Gatrell, S. K.; Kim, J.; Derksen, T. J.; O'Neil, E. V.; Lei, X. G., Creating omega-3 fatty-acid-enriched chicken using defatted green microalgal biomass. *J Agric Food Chem* **2015,** *63* (42), 9315-22.
10. Kim, J.; Barcus, M.; Magnuson, A.; Tao, L.; Lei, X. G., Supplemental defatted microalgae affects egg and tissue fatty acid composition differently in laying hens fed diets containing corn and flaxseed oil. *J Appl Poultry Res* **2016,** *25* (4), 528-538.
11. Saleh, A. M.; Hussein, L. A.; Abdalla, F. E.; Elfouly, M. M.; Shaheen, A. B., The nutritional quality of drum-dried algae produced in open-door mass-culture. *Z Ernahrungswiss* **1985,** *24* (4), 256-263.
12. Clement, G.; Giddey, C.; Menzi, R., Amino acid composition and nutritive valve of the alga Spirulina maxima. *J Sci Food Agric* **1967,** *18* (11), 497-501.
13. Combs, G. F., Algae (Chlorella) as a source of nutrients for the chick. *Science* **1952,** *116* (3017), 453-4.
14. Grau, C. R.; Klein, N. W., Sewage-grown algae as a feedstuff for chicks. *Poult Sci* **1957,** *36* (5), 1046-1051.
15. Guerin, M.; Huntley, M. E.; Olaizola, M., Haematococcus astaxanthin: applications for human health and nutrition. *Trends Biotechnol* **2003,** *21* (5), 210-6.
16. Shimidzu, N.; Goto, M.; Miki, W., Carotenoids as singlet oxygen quenchers in marine organisms. *Fisheries Sci* **1996,** *62* (1), 134-137.
17. Lorenz, R. T.; Cysewski, G. R., Commercial potential for Haematococcus microalgae as a natural source of astaxanthin. *Trends Biotechnol* **2000,** *18* (4), 160-7.
18. Tracy, R. P., Inflammation markers and coronary heart disease. *Curr Opin Lipidol* **1999,** *10* (5), 435-41.
19. Tanaka, T.; Kawamori, T.; Ohnishi, M.; Makita, H.; Mori, H.; Satoh, K.; Hara, A., Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the postinitiation phase. *Carcinogenesis* **1995,** *16* (12), 2957-63.

20. Chew, B. P.; Park, J. S.; Wong, M. W.; Wong, T. S., A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. *Anticancer Res* **1999,** *19* (3A), 1849-53.
21. Tanaka, T.; Makita, H.; Ohnishi, M.; Mori, H.; Satoh, K.; Hara, A., Chemoprevention of rat oral carcinogenesis by naturally-occurring xanthophylls, astaxanthin and canthaxarathin. *Cancer Res* **1995,** *55* (18), 4059-4064.
22. Arterburn, L. M.; Hall, E. B.; Oken, H., Distribution, interconversion, and dose response of n-3 fatty acids in humans. *Am J Clin Nutr* **2006,** *83* (6 Suppl), 1467S-1476S.
23. Horrocks, L. A.; Yeo, Y. K., Health benefits of docosahexaenoic acid (DHA). *Pharmacol Res* **1999,** *40* (3), 211-25.
24. Mozaffarian, D.; Lemaitre, R. N.; King, I. B.; Song, X.; Huang, H.; Sacks, F. M.; Rimm, E. B.; Wang, M.; Siscovick, D. S., Plasma phospholipid long-chain omega-3 fatty acids and total and cause-specific mortality in older adults: a cohort study. *Ann Intern Med* **2013,** *158* (7), 515-25.
25. Scorletti, E.; Bhatia, L.; McCormick, K. G.; Clough, G. F.; Nash, K.; Hodson, L.; Moyses, H. E.; Calder, P. C.; Byrne, C. D.; Study, W., Effects of purified eicosapentaenoic and docosahexaenoic acids in nonalcoholic fatty liver disease: results from the Welcome* study. *Hepatology* **2014,** *60* (4), 1211-21.
26. Kris-Etherton, P. M.; Harris, W. S.; Appel, L. J.; Association, A. H. A. N. C. A. H., Omega-3 fatty acids and cardiovascular disease: new recommendations from the American Heart Association. *Arterioscler Thromb Vasc Biol* **2003,** *23* (2), 151-2.
27. Zarate, R.; El Jaber-Vazdekis, N.; Tejera, N.; Perez, J. A.; Rodriguez, C., Significance of long chain polyunsaturated fatty acids in human health. *Clin Transl Med* **2017,** *6* (1), 25.
28. Arterburn, L. M.; Oken, H. A.; Hoffman, J. P.; Bailey-Hall, E.; Chung, G.; Rom, D.; Hamersley, J.; McCarthy, D., Bioequivalence of docosahexaenoic acid from different algal oils in capsules and in a DHA-fortified food. *Lipids* **2007,** *42* (11), 1011-24.
29. WHO *Joint WHO/FAO expert consultation on diet, nutrition and the prevention of chronic diseases*; 2003.
30. Li, D. W.; Cen, S. Y.; Liu, Y. H.; Balamurugan, S.; Zheng, X. Y.; Alimujiang, A.; Yang, W. D.; Liu, J. S.; Li, H. Y., A type 2 diacylglycerol acyltransferase accelerates the triacylglycerol biosynthesis in heterokont oleaginous microalga Nannochloropsis oceanica. *J Biotechnol* **2016,** *229*, 65-71.
31. AOAC International., Official methods of analysis of AOAC International. AOAC International: Arlington, Va., 1995; pp v. (loose-leaf).
32. Fenton, T. W.; Fenton, M., Improved procedure for the determination of chromic oxide in feed and feces. *Can J Anim Sci* **1979,** *59* (3), 631-634.
33. Landry, J.; Delhaye, S.; Jones, D. G., Determination of tryptophan in feedstuffs - comparison of 2 methods of hydrolysis prior to hplc analysis. *J Sci Food Agr* **1992,** *58* (3), 439-441.
34. Elkin, R. G.; Griffith, J. E., Amino acid analysis of feedstuff hydrolysates by cation exchange high performance liquid chromatography. *J Assoc Off Anal Chem* **1985,** *68* (5), 1028-32.
35. Yuan, J. P.; Peng, J.; Yin, K.; Wang, J. H., Potential health-promoting effects of astaxanthin: a high-value carotenoid mostly from microalgae. *Mol Nutr Food Res* **2011,** *55* (1), 150-65.

36. Raposo, M. F.; de Morais, R. M.; Bernardo de Morais, A. M., Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Mar Drugs* **2013,** *11* (1), 233-52.
37. Gatrell, S. K.; Derksen, T. J.; O'Neil, E. V.; Lei, X. G., A new type of defatted green microalgae exerts dose-dependent nutritional, metabolic, and environmental impacts in broiler chicks. *J Appl Poultry Res* **2017,** *26* (3), 358-366.
38. Becker, E. W., Micro-algae as a source of protein. *Biotechnol Adv* **2007,** *25* (2), 207-10.
39. Gerken, H. G.; Donohoe, B.; Knoshaug, E. P., Enzymatic cell wall degradation of Chlorella vulgaris and other microalgae for biofuels production. *Planta* **2013,** *237* (1), 239-53.
40. Hatten, N. R.; Borazjani, H.; Diehl, S.; Prewitt, L., Effects of composting on removal of nitrogen, phosphorus, and potassium from sawdust amended with chicken litter. *Compost Sci Util* **2009,** *17* (3), 166-172.
41. Marks, H. L.; Washburn, K. W., The relationship of altered water/feed intake ratios on growth and abdominal fat in commercial broilers. *Poult Sci* **1983,** *62* (2), 263-72.
42. Norambuena, F.; Hermon, K.; Skrzypczyk, V.; Emery, J. A.; Sharon, Y.; Beard, A.; Turchini, G. M., Algae in fish feed: performances and fatty acid metabolism in juvenile Atlantic Salmon. *Plos One* **2015,** *10* (4), e0124042.
43. de Jesus Raposo, M. F.; de Morais, R. M.; de Morais, A. M., Health applications of bioactive compounds from marine microalgae. *Life Sci* **2013,** *93* (15), 479-86.
44. David-Palma, M.; Libkind, D.; Sampaio, J. P., Global distribution, diversity hot spots and niche transitions of an astaxanthin-producing eukaryotic microbe. *Mol Ecol* **2014,** *23* (4), 921-32.
45. Goodwin, T. W.; Srisukh, S., Some observations on astaxanthin distribution in marine crustacea. *Biochem J* **1949,** *45* (3), 268-70.
46. Huangfu, J.; Liu, J.; Sun, Z.; Wang, M.; Jiang, Y.; Chen, Z. Y.; Chen, F., Antiaging effects of astaxanthin-rich alga Haematococcus pluvialis on fruit flies under oxidative stress. *J Agric Food Chem* **2013,** *61* (32), 7800-4.
47. Lawlor, S. M.; Obrien, N. M., Astaxanthin - antioxidant effects in chicken-embryo fibroblasts. *Nutr Res* **1995,** *15* (11), 1695-1704.
48. Yang, Y.; Fuentes, F.; Shu, L.; Wang, C.; Pung, D.; Li, W.; Zhang, C.; Guo, Y.; Kong, A. N., Epigenetic CpG methylation of the promoter and reactivation of the expression of GSTP1 by astaxanthin in human prostate LNCaP cells. *AAPS J* **2017,** *19* (2), 421-430.
49. Pashkow, F. J.; Watumull, D. G.; Campbell, C. L., Astaxanthin: a novel potential treatment for oxidative stress and inflammation in cardiovascular disease. *Am J Cardiol* **2008,** *101* (10A), 58D-68D.
50. Rahman, M. M.; Khosravi, S.; Chang, K. H.; Lee, S. M., Effects of dietary inclusion of astaxanthin on growth, muscle pigmentation and antioxidant capacity of juvenile rainbow trout (Oncorhynchus mykiss). *Prev Nutr Food Sci* **2016,** *21* (3), 281-288.
51. Jeong, J. S.; Kim, I. H., Effect of astaxanthin produced by Phaffia rhodozyma on growth performance, meat quality, and fecal noxious gas emission in broilers. *Poult Sci* **2014,** *93* (12), 3138-44.
52. Perenlei, G.; Tojo, H.; Okada, T.; Kubota, M.; Kadowaki, M.; Fujimura, S., Effect of dietary astaxanthin rich yeast, Phaffia rhodozyma, on meat quality of broiler chickens. *Anim Sci J* **2014,** *85* (10), 895-903.

53. Ishikawa, S.; Hashizume, K.; Nishigori, H.; Tezuka, Y.; Sanbe, A.; Kurosaka, D., Effect of astaxanthin on cataract formation induced by glucocorticoids in the chick embryo. *Curr Eye Res* **2015,** *40* (5), 535-40.
54. Masters, P. M.; Bada, J. L.; Zigler, J. S., Jr., Aspartic acid racemisation in the human lens during ageing and in cataract formation. *Nature* **1977,** *268* (5615), 71-3.
55. Quinteiro-Filho, W. M.; Ribeiro, A.; Ferraz-de-Paula, V.; Pinheiro, M. L.; Sakai, M.; Sa, L. R.; Ferreira, A. J.; Palermo-Neto, J., Heat stress impairs performance parameters, induces intestinal injury, and decreases macrophage activity in broiler chickens. *Poult Sci* **2010,** *89* (9), 1905-14.
56. St-Pierre, N. R.; Cobanov, B.; Schnitkey, G., Economic losses from heat stress by US livestock industries. *J Dairy Sci* **2003,** *86*, E52-E77.
57. Geraert, P. A.; Padilha, J. C.; Guillaumin, S., Metabolic and endocrine changes induced by chronic heat exposure in broiler chickens: growth performance, body composition and energy retention. *Br J Nutr* **1996,** *75* (2), 195-204.
58. Bartlett, J. R.; Smith, M. O., Effects of different levels of zinc on the performance and immunocompetence of broilers under heat stress. *Poult Sci* **2003,** *82* (10), 1580-8.
59. Sandercock, D. A.; Hunter, R. R.; Nute, G. R.; Mitchell, M. A.; Hocking, P. M., Acute heat stress-induced alterations in blood acid-base status and skeletal muscle membrane integrity in broiler chickens at two ages: implications for meat quality. *Poult Sci* **2001,** *80* (4), 418-25.
60. Lin, H.; Decuypere, E.; Buyse, J., Acute heat stress induces oxidative stress in broiler chickens. *Comp Biochem Physiol A Mol Integr Physiol* **2006,** *144* (1), 11-7.
61. Azad, M. A.; Kikusato, M.; Maekawa, T.; Shirakawa, H.; Toyomizu, M., Metabolic characteristics and oxidative damage to skeletal muscle in broiler chickens exposed to chronic heat stress. *Comp Biochem Physiol A Mol Integr Physiol* **2010,** *155* (3), 401-6.
62. Altan, O.; Pabuccuoglu, A.; Altan, A.; Konyalioglu, S.; Bayraktar, H., Effect of heat stress on oxidative stress, lipid peroxidation and some stress parameters in broilers. *Br Poult Sci* **2003,** *44* (4), 545-50.
63. Kuroki, T.; Ikeda, S.; Okada, T.; Maoka, T.; Kitamura, A.; Sugimoto, M.; Kume, S., Astaxanthin ameliorates heat stress-induced impairment of blastocyst development in vitro:--astaxanthin colocalization with and action on mitochondria. *J Assist Reprod Genet* **2013,** *30* (5), 623-31.
64. Council, N. R., *Nutrient Requirements of Poultry*. The National Academies Press: Washington, DC, 1994; p 176.
65. COBB Broiler Management Guide. http://www.cobb-vantress.com/docs/default-source/management-guides/broiler-management-guide.pdf.
66. Bowers, G. N., Jr.; McComb, R. B., Measurement of total alkaline phosphatase activity in human serum. *Clin Chem* **1975,** *21* (13), 1988-95.
67. Lau, K. H.; Onishi, T.; Wergedal, J. E.; Singer, F. R.; Baylink, D. J., Characterization and assay of tartrate-resistant acid phosphatase activity in serum: potential use to assess bone resorption. *Clin Chem* **1987,** *33* (4), 458-62.
68. Gomori, G., A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. *J Lab Clin Med* **1942,** *27*, 955-960.
69. Lopez, A. B.; Van Eck, J.; Conlin, B. J.; Paolillo, D. J.; O'Neill, J.; Li, L., Effect of the cauliflower Or transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. *J Exp Bot* **2008,** *59* (2), 213-23.

70. Sowell, A. L.; Huff, D. L.; Yeager, P. R.; Caudill, S. P.; Gunter, E. W., Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. *Clin Chem* **1994,** *40* (3), 411-6.
71. Breithaupt, D. E., Identification and quantification of astaxanthin esters in shrimp (Pandalus borealis) and in a microalga (Haematococcus pluvialis) by liquid chromatography-mass spectrometry using negative ion atmospheric pressure chemical ionization. *J Agric Food Chem* **2004,** *52* (12), 3870-5.
72. Rohrle, F. T.; Moloney, A. P.; Osorio, M. T.; Luciano, G.; Priolo, A.; Caplan, P.; Monahan, F. J., Carotenoid, colour and reflectance measurements in bovine adipose tissue to discriminate between beef from different feeding systems. *Meat Sci* **2011,** *88* (3), 347-53.
73. Ou, B.; Huang, D.; Hampsch-Woodill, M.; Flanagan, J. A.; Deemer, E. K., Analysis of antioxidant activities of common vegetables employing oxygen radical absorbance capacity (ORAC) and ferric reducing antioxidant power (FRAP) assays: a comparative study. *J Agric Food Chem* **2002,** *50* (11), 3122-8.
74. Mcdonald, R. E.; Hultin, H. O., Some characteristics of the enzymatic lipid-peroxidation system in the microsomal fraction of flounder skeletal-muscle. *J Food Sci* **1987,** *52* (1), 15-&.
75. Anderson, M. E., Determination of glutathione and glutathione disulfide in biological samples. *Methods Enzymol* **1985,** *113*, 548-55.
76. Flohe, L.; Gunzler, W. A., Assays of glutathione peroxidase. *Methods Enzymol* **1984,** *105*, 114-21.
77. Massey, V.; Williams, C. H., Jr., On the reaction mechanism of yeast glutathione reductase. *J Biol Chem* **1965,** *240* (11), 4470-80.
78. Guthenberg, C.; Mannervik, B., Glutathione S-transferase (transferase pi) from human placenta is identical or closely related to glutathione S-transferase (transferase rho) from erythrocytes. *Biochim Biophys Acta* **1981,** *661* (2), 255-60.
79. McCord, J. M.; Fridovich, I., Superoxide dismutase. An enzymic function for erythrocuprein (hemocuprein). *J Biol Chem* **1969,** *244* (22), 6049-55.
80. de Huidobro, F. R.; Miguel, E.; Blazquez, B.; Onega, E., A comparison between two methods (Warner-Bratzler and texture profile analysis) for testing either raw meat or cooked meat. *Meat Sci* **2005,** *69* (3), 527-36.
81. Jeacocke, R. E., Continuous measurements of the pH of beef muscle in intact beef carcases. *J. Fd Technol.* **1977,** *12*, 375-386.
82. Karmas, E.; Turk, K., Water binding of cooked fish in combination with various proteins. *J Food Sci* **1976,** *41* (4), 977-979.
83. Miller, W. O.; Saffle, R. L.; Zirkle, S. B., Factors which influence water-holding capacity of various types of meat. *Food Technol-Chicago* **1968,** *22* (9), 1139-&.
84. Folch, J.; Lees, M.; Sloane Stanley, G. H., A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **1957,** *226* (1), 497-509.
85. Fritsche, K. L.; Johnston, P. V., Effect of dietary alpha-linolenic acid on growth, metastasis, fatty acid profile and prostaglandin production of two murine mammary adenocarcinomas. *J Nutr* **1990,** *120* (12), 1601-9.
86. Awad, J. A.; Morrow, J. D.; Hill, K. E.; Roberts, L. J.; Burk, R. F., Detection and localization of lipid-peroxidation in selenium-deficient and vitamin-E-deficient rats using f-2-isoprostanes. *J Nutr* **1994,** *124* (6), 810-816.

87. Morrow, J. D.; Roberts, L. J., Mass-spectrometry of prostanoids - f-2-isoprostanes produced by noncyclooxygenase free radical-catalyzed mechanism. *Method Enzymol* **1994,** *233*, 163-174.
88. Akiba, Y.; Sato, K.; Takahashi, K.; Matsushita, K.; Komiyama, H.; Tsunekawa, H.; Nagao, H., Meat color modification in broiler chickens by feeding yeast Phaffia rhodozyma containing high concentrations of astaxanthin. *J Appl Poultry Res* **2001,** *10* (2), 154-161.
89. Waldenstedt, L.; Inborr, J.; Hansson, I.; Elwinger, K., Effects of astaxanthin-rich algal meal (Haematococcus pluvalis) on growth performance, caecal campylobacter and clostridial counts and tissue astaxanthin concentration of broiler chickens. *Anim Feed Sci Tech* **2003,** *108* (1-4), 119-132.
90. Takahashi, K.; Watanabe, M.; Takimoto, T.; Akiba, Y., Uptake and distribution of astaxanthin in several tissues and plasma lipoproteins in male broiler chickens fed a yeast (Phaffia rhodozyma) with a high concentration of astaxanthin. *Br Poult Sci* **2004,** *45* (1), 133-8.
91. Murillo, A. G.; DiMarco, D. M.; Fernandez, M. L., The potential of non-provitamin A aarotenoids for the prevention and treatment of non-alcoholic fatty liver disease. *Biology (Basel)* **2016,** *5* (4).
92. Wu, C.; Duckett, S. K.; Neel, J. P.; Fontenot, J. P.; Clapham, W. M., Influence of finishing systems on hydrophilic and lipophilic oxygen radical absorbance capacity (ORAC) in beef. *Meat Sci* **2008,** *80* (3), 662-7.
93. Mahmoud, K. Z.; Edens, F. W., Influence of selenium sources on age-related and mild heat stress-related changes of blood and liver glutathione redox cycle in broiler chickens (Gallus domesticus). *Comp Biochem Physiol B Biochem Mol Biol* **2003,** *136* (4), 921-34.
94. An, G. H.; Song, J. Y.; Chang, K. S.; Lee, B. D.; Chae, H. S.; Jang, B. C., Pigmentation and delayed oxidation of broiler chickens by the red carotenoid, astaxanthin, from chemical synthesis and the yeast, Xanthophyllomyces dendrorhous. *Asian Austral J Anim* **2004,** *17* (9), 1309-1314.
95. Barbut, S., Acceptance of fresh chicken meat presented under three light sources. *Poult Sci* **2001,** *80* (1), 101-4.
96. Kauffman, R. G.; Eikelenboom, G.; van der Wal, P. G.; Engel, B.; Zaar, M., A comparison of methods to estimate water-holding capacity in post-rigor porcine muscle. *Meat Sci* **1986,** *18* (4), 307-22.
97. Hamm, H., The effect of water on the quality of meat and meat products: Problems and research needs. In *Properties of water in Foods*, Simatos, D.; Multon, J. L., Eds. Martinus Nijhoff 1985; pp 591-602.
98. Lu, Q.; Wen, J.; Zhang, H., Effect of chronic heat exposure on fat deposition and meat quality in two genetic types of chicken. *Poult Sci* **2007,** *86* (6), 1059-64.
99. Barroeta, A. C., Nutritive value of poultry meat: relationship between vitamin E and PUFA. *World Poultry Sci J* **2007,** *63* (2), 277-284.
100. Vannice, G.; Rasmussen, H., Position of the academy of nutrition and dietetics: dietary fatty acids for healthy adults. *J Acad Nutr Diet* **2014,** *114* (1), 136-53.
101. Mujahid, A.; Yoshiki, Y.; Akiba, Y.; Toyomizu, M., Superoxide radical production in chicken skeletal muscle induced by acute heat stress. *Poult Sci* **2005,** *84* (2), 307-14.
102. Sahin, K.; Kucuk, O., Effects of vitamin E and selenium on performance, digestibility of nutrients, and carcass characteristics of Japanese quails reared under heat stress (34 degrees C). *J Anim Physiol Anim Nutr (Berl)* **2001,** *85* (11-12), 342-8.

103. Burdge, G. C.; Calder, P. C., Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. *Reprod Nutr Dev* **2005,** *45* (5), 581-597.
104. USDA *Overview of the United States Slaughter Industry*; 2016.
105. Lopez-Ferrer, S.; Baucells, M. D.; Barroeta, A. C.; Galobart, J.; Grashorn, M. A., n-3 enrichment of chicken meat. 2. Use of precursors of long-chain polyunsaturated fatty acids: linseed oil. *Poult Sci* **2001,** *80* (6), 753-61.
106. Hulan, H. W.; Ackman, R. G.; Ratnayake, W. M. N.; Proudfoot, F. G., Omega-3 fatty-acid levels and performance of broiler-chickens fed redfish meal or redfish Oil. *Can J Anim Sci* **1988,** *68* (2), 533-547.
107. Lopez-Ferrer, S.; Baucells, M. D.; Barroeta, A. C.; Grashorn, M. A., n-3 enrichment of chicken meat. 1. Use of very long-chain fatty acids in chicken diets and their influence on meat quality: fish oil. *Poult Sci* **2001,** *80* (6), 741-52.
108. Haug, A.; Eich-Greatorex, S.; Bernhoft, A.; Wold, J. P.; Hetland, H.; Christophersen, O. A.; Sogn, T., Effect of dietary selenium and omega-3 fatty acids on muscle composition and quality in broilers. *Lipids Health Dis* **2007,** *6*, 29.
109. Ribeiro, T.; Lordelo, M. M.; Costa, P.; Alves, S. P.; Benevides, W. S.; Bessa, R. J.; Lemos, J. P.; Pinto, R. M.; Ferreira, L. M.; Fontes, C. M.; Prates, J. A., Effect of reduced dietary protein and supplementation with a docosahexaenoic acid product on broiler performance and meat quality. *Br Poult Sci* **2014,** *55* (6), 752-65.
110. Long, S. F.; Kang, S.; Wang, Q. Q.; Xu, Y. T.; Pan, L.; Hu, J. X.; Li, M.; Piao, X. S., Dietary supplementation with DHA-rich microalgae improves performance, serum composition, carcass trait, antioxidant status, and fatty acid profile of broilers. *Poult Sci* **2018,** *97* (6), 1881-1890.
111. Jo, C.; Ahn, D. U., Fluorometric analysis of 2-thiobarbituric acid reactive substances in turkey. *Poult Sci* **1998,** *77* (3), 475-80.
112. Livak, K. J.; Schmittgen, T. D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* **2001,** *25* (4), 402-8.
113. Yan, X.; Pepper, M. P.; Vatamaniuk, M. Z.; Roneker, C. A.; Li, L.; Lei, X. G., Dietary selenium deficiency partially rescues type 2 diabetes-like phenotypes of glutathione peroxidase-1-overexpressing male mice. *J Nutr* **2012,** *142* (11), 1975-82.
114. Yan, L.; Kim, I. H., Effects of dietary omega-3 fatty acid-enriched microalgae supplementation on growth performance, blood profiles, meat quality, and fatty acid composition of meat in broilers. *J Appl Anim Res* **2013,** *41* (4), 392-397.
115. Smith, G. I.; Atherton, P.; Reeds, D. N.; Mohammed, B. S.; Rankin, D.; Rennie, M. J.; Mittendorfer, B., Omega-3 polyunsaturated fatty acids augment the muscle protein anabolic response to hyperinsulinaemia-hyperaminoacidaemia in healthy young and middle-aged men and women. *Clin Sci (Lond)* **2011,** *121* (6), 267-78.
116. Wei, H. K.; Zhou, Y.; Jiang, S.; Tao, Y. X.; Sun, H.; Peng, J.; Jiang, S., Feeding a DHA-enriched diet increases skeletal muscle protein synthesis in growing pigs: association with increased skeletal muscle insulin action and local mRNA expression of insulin-like growth factor 1. *Br J Nutr* **2013,** *110* (4), 671-80.
117. Kamolrat, T.; Gray, S. R., The effect of eicosapentaenoic and docosahexaenoic acid on protein synthesis and breakdown in murine C2C12 myotubes. *Biochem Biophys Res Commun* **2013,** *432* (4), 593-8.
118. Kim, W. R.; Flamm, S. L.; Di Bisceglie, A. M.; Bodenheimer, H. C.; Public policy committee of the american association for the study of liver, d., serum activity of alanine

aminotransferase (ALT) as an indicator of health and disease. *Hepatology* **2008,** *47* (4), 1363-70.
119. Nobili, V.; Bedogni, G.; Alisi, A.; Pietrobattista, A.; Rise, P.; Galli, C.; Agostoni, C., Docosahexaenoic acid supplementation decreases liver fat content in children with non-alcoholic fatty liver disease: double-blind randomised controlled clinical trial. *Arch Dis Child* **2011,** *96* (4), 350-3.
120. Betti, M.; Schneider, B. L.; Wismer, W. V.; Carney, V. L.; Zuidhof, M. J.; Renema, R. A., Omega-3-enriched broiler meat: 2. Functional properties, oxidative stability, and consumer acceptance. *Poult Sci* **2009,** *88* (5), 1085-95.
121. Guillen-Sans, R.; Guzman-Chozas, M., The thiobarbituric acid (TBA) reaction in foods: a review. *Crit Rev Food Sci Nutr* **1998,** *38* (4), 315-30.
122. Anjum, F. M.; Haider, M. F.; Khan, M. I.; Sohaib, M.; Arshad, M. S., Impact of extruded flaxseed meal supplemented diet on growth performance, oxidative stability and quality of broiler meat and meat products. *Lipids Health Dis* **2013,** *12*, 13.