Food and Nutrition Sciences, 2018, 9, 1160-1173
http://www.scirp.org/journal/fns
ISSN Online: 2157-9458
ISSN Print: 2157-944X

Broiler Tissue Enrichment with Docosahexaenoic Acid (DHA) through Dietary Supplementation with *Aurantiochytrium limacinum* Algae

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How to cite this paper: Moran, C.A., Keegan, J.D., Vienola, K. and Apajalahti, J. (2018) Broiler Tissue Enrichment with Docosahexaenoic Acid (DHA) through Dietary Supplementation with *Aurantiochytrium limacinum* Algae. *Food and Nutrition Sciences*, **9**, 1160-1173.
https://doi.org/10.4236/fns.2018.910084

Received: September 10, 2018
Accepted: October 15, 2018
Published: October 18, 2018

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Abstract

The omega-3 fatty acid (n-3 FA) content of broiler tissues can be increased through dietary supplementation of hens with n-3 FA-rich microalgae. The aim of this study was to evaluate the effect of three different dietary inclusion levels of a docosahexaenoic acid (DHA)-rich microalgae (AURA) on broiler performance and the enrichment of tissues with n-3 FA. The randomized study was conducted using 352 birds, housed in 32 pens with 11 birds per pen. Pens were randomly assigned to one of four treatments, with each treatment replicated 8 times. The treatments included one unsupplemented control (0%) and three wheat-soya based experimental diets supplemented with AURA at a level of 0.5%, 1.5% and 2.5% for the starter, grower and finisher periods. Birds were weighed on days 0, 10, 24, 35 and 41, and feed intake was recorded per pen. On day 41, five birds per treatment were euthanized and individually weighed. Thigh muscle, breast muscle, liver, kidney and skin samples were taken post-mortem, freeze dried and DHA content quantified, following fat extraction and methylation, by GC-FID (AOAC 996.06 method). Performance and tissue data were analyzed by ANOVA with Dunnett's (2-sided) post-hoc test to determine the differences between the mean values for each treatment. Dietary supplementation with AURA had no effect on body weight or feed intake during any period of the study. For thigh muscle, kidney and skin the DHA increased linearly ($P < 0.05$) with increasing level of dietary AURA, whilst there was a quadratic response in uptake of DHA in breast muscle and liver. The study demonstrated the potential of efficiently enriching broiler meat and organs with DHA by feeding AURA.

Keywords

Algae, Broilers, Meat, DHA Enrichment, Omega 3

1. Introduction

Consuming adequate amounts of omega-3 fatty acids (n-3 FA) can reduce the risk of various diseases including cardiovascular disease and depressive disorders [1] [2] [3]. However, Western diets are deficient in n-3 FA and high in omega-6 fatty acids (n-6 FA), with a ratio of 15-20/1 n-6/n-3 compared to a more favorable 1/1 ratio [4]. In recent years, this negative trend to a high n-6:n-3 ratio has being observed on a global basis and no longer a "Western" phenomenon, with reports of an urban Indian population consuming a diet with 38-50:1 n-6:n-3 [5] and concomitantly witnessing a sharp rise in obesity, diabetes and coronary heart disease [6] [7]. This is mainly due to a dietary shift to cereal grains and vegetable oils and a reduction in the consumption of green leafy vegetables, fruits, seeds and nuts [4]. Beyond the simple ratio the n-3 FA have important physiological roles in the human body. Docosahexaenoic (DHA) is one such n-3 FA which is essential for reproduction (production and protection of gametes) and early infant brain and nervous system development [8]. In adults, increased DHA intake has been linked to reduced heart disease, cardiac arrhythmia, atherosclerosis, diabetes and reduced risk of colon, prostate and breast cancers [9], [10]. However, in many countries daily n-3 FA consumption falls below the recommended level of 400 - 1000 mg [11] [12] [13]. To address this deficiency, increasing human consumption of n-3 FA through the enrichment of commonly consumed foods, especially those foods derived from livestock and poultry production, has become the focus of much research [14].

Due to the simplicity of modifying FA composition of animal meat, milk or eggs through the diet, early attempts focused on two strategies, either through supplementation with fish meal or fish oils to provide EPA and DHA directly or alternatively to supplement flaxseed as a source of alpha linoleic acid (ALA n-3) [15]. However, there are drawbacks to both strategies which have impeded progress. Initially fishmeal and fish oil were considered expensive ingredients to use in the diets of agricultural species and more recently there are sustainability questions as to continued use, but more compelling for the consumer are the concerns regarding these ingredients imparting a "fishy" taste to the tissue [16]. Furthermore, the reduction in oxidative stability of both the feed and enriched animal tissue means a reduction in shelf-life with concerns for the distribution chain stakeholders and final consumer [17] [18].

Flaxseed can be a good source of omega-3, however it is required in higher doses which cause issues for formulators due to dilution of other nutrients in the feed and changes in processing quality when pelleting and crumbling, for example. Moreover, the addition of a high percentage of flaxseed in the diet has been

associated with a laxative effect and poor uptake of nutrients in laying hens [19]. In recent years, research has focused on the use of marine heterotrophic microalgae as a source of DHA, such as those found at the base of the pelagic food chain. Rymer and coworkers [20] concluded that the algae *Schizochytrium* (DHA Gold) was as effective as fish oil in enriching broiler meat with omega-3, without changing oxidative stability and without the negative organoleptic properties. The use of microalgal oil has similar limitations as the use of fish oil in terms of oxidative stability and the ability to use in broiler feeds is restricted to a post-pellet application. Our research group has investigated the transfer efficiency of DHA from an unextracted heterotrophically grown *Aurantiochytrium limacinum* microalgal biomass to dairy milk [21] [22], pork meat and fat [23] [24] and laying hen eggs [25] [26]. For this study, the microalgal biomass was produced, following scale up from a pure culture, in a fed-batch production fermenter with a variable glucose feed and a fixed feed of urea and micronutrients. The culture was grown at 30˚C for approximately 72 hours under sterile conditions. After adequate growth the content was harvested by centrifugation and concomitantly spray-dried to achieve approximately 67% crude fat, 16% DHA and 2% dry matter final product. Heterotrophic fermentation in a low salt media has been demonstrated to produce a microalgal biomass which protects the DHA and allows the ingredient to be added to feeds that undergo conditioning and pelleting, an essential part of modern broiler diet production [27].

The aim of the study was to evaluate the efficacy of the dietary inclusion of an unextracted *Aurantiochytrium limacinum* biomass, on the enrichment of select edible broiler chicken tissues, including breast meat, thigh meat, liver, kidney and skin with adhering fat.

2. Materials and Methods

2.1. Animals and Housing

The research protocol and animal care were in accordance with European Union Directive 2010/63/EU covering the protection of animals used for experimental or other scientific purposes. The 41-day trial was conducted in the broiler house of Alimetrics Ltd. in Southern Finland. 352-day-old broiler chicks were housed in 88 open pens (1.125 m^2 each) with wood shavings litter, in an environmentally controlled room. Feed and water were supplied *ad libitum* for the 41-day study. Pens were randomly assigned to one of four dietary treatments, with each treatment replicated 8 times (11 birds per pen). Each chick was marked with permanent color to feathers to identify the treatment but not the individual animal. A veterinarian checked the health of the chicks at the beginning of the trial. The birds were observed twice a day. Chicks with compromised health were excluded from the trial.

The temperature of the barn was raised to 32˚C two days before chicks arrived. Luminosity was adjusted to 20 lux and air humidity was adjusted according to recommendations. Brooder lamps were adjusted to provide extra heating

to the chicks during the first week. The temperature was gradually decreased to 22˚C over the rearing period. Temperature, ventilation and humidity was monitored and recorded throughout the experiment on a daily basis. The dark hours were gradually increased within a week, so that light-dark cycle was 18 hours light and 6 hours dark daily. The standard temperature and lighting program were followed.

2.2. Dietary Treatments

The diet was wheat-soy based feed for broiler chicks as specified in the **Table 1**. The starter formulation was fed during the first 10 days, grower diet for days 10 - 24 and finisher for the rest of the trial (24 - 41 days). The starter diets were prepared as 2 mm pellets and the grower and finisher diets as 4 mm pellets. The feeds were manufactured at the Nature Resources Institute, Finland. Diets were formulated to meet or exceed the nutrient requirements according to the Nutrient Requirements of Poultry (1994) [28]. Birds were assigned to 1 of 4 isonitrogenous and isoenergetic diets: Control diet or treatment diets containing 0.5%, 1.5% or 2.5% heterotrophically grown, unextracted *Aurantiochytrium limacinium* (AURA; CCAP 4087/2) biomass, provided by Alltech Inc. (All-G-Rich®, Nicholasville, KY, USA). AURA provided 0, 0.85, 2.55 and 4.25 g DHA /kg for the respective treatment diets.

The analytical composition of AURA was determined using standardized and validated procedures (Association of Analytical Chemists, AOAC, USA) prior to the start of the study at Eurofins Nutrition Analysis Centre (Des Moines, USA): crude protein (AOAC 990.03), crude fat (AOAC 954.02), moisture (AOAC 930.15) and ash (AOAC 942.05). The fatty acid composition (AOAC 996.06) of AURA was determined at Mérieux NutriSciences (Burnaby, BC, Canada) in compliance with ISO 17025. In brief, fat and fatty acids were extracted from the algae biomass powder by acidic hydrolytic methods and subsequently into ether followed by methylation resulting in Fatty Acid Methyl Esters (FAMEs) using boron trifluoride (BF_3) in methanol. The FAME reference solution (GLC-85) and triundecanoin (C11:0) used as an internal standard for sample extraction were purchased from Nu-Chek-Prep Inc. (Minnesota, USA). The FAMEs were quantitively measured by gas chromatography equipped with a hydrogen flame ionization detector as described in Section 2.4.

2.3. Performance

Performance observations included live weight (d0, 10, 24, 35 and 41), feed intake (pen) and daily mortality. The feed conversion ratio was calculated and corrected for mortality. On day 41, five birds per treatment (20 birds total) were euthanized by cervical dislocation, coded, weighed individually, abdominal cavity opened and representative samples of the thigh and breast muscles (skinless), liver, kidney and skin (with adhering fat) were removed and stored in polyethylene bags frozen at −20˚C immediately.

Table 1. Basic broiler control diets for the three phases of growth.

	Starter % 0 - 10 days	Grower % 10 - 24 days	Finisher % 24 - 41 days
Wheat	59.84	64.17	65.79
Soybean meal 48	31.80	27.90	25.50
Sunflower oil	3.60	3.46	4.50
Monocalcium phosphate	1.60	1.50	1.40
Limestone	1.65	1.55	1.48
NaCl	0.40	0.40	0.40
Mineral premix*	0.20	0.20	0.20
Vitamin premix**	0.20	0.20	0.20
Methionine	0.26	0.23	0.21
L-Lysine HCl	0.36	0.31	0.25
Threonine	0.09	0.08	0.07
Total	100	100	100
MJ/kg	12.42	12.55	12.92
CP g/kg	220.97	206.13	195.54

[a]Contents of the mineral premix: calcium 296.8 g/kg, iron 12.5 g/kg, copper 4 g/kg, manganese 25 g/kg, zinc 32.5 g/kg, iodine 0.225 g/kg, selenium 0.1 g/kg. [b]Contents of the vitamin premix: calcium 331.3 g/kg, vitamin A 6,000,000 IU, vitamin D3 2,250,000 IU, vitamin E 30000, tocopherol 27,270 mg/kg, vitamin K3 1505 mg/kg, vitamin B1 1257.3 mg/kg, vitamin B2 3000 mg/kg, vitamin B6 2009.7 mg/kg, vitamin B12 12.5 mg/kg, biotin 75 mg/kg, folic acid 504 mg/kg, niacin 20,072 mg/kg, pantothenic acid 7506.8 mg/kg

2.4. Broiler Fatty Acid Profile

Fatty acid profiles of tissue and organ samples were determined at Mérieux NutriSciences (Burnaby, BC, Canada) in compliance with ISO 17025. Recently, a GC-FID method (AOAC 996.06) was validated and verified to demonstrate the fitness for purpose in analyzing seven fatty acids in five different chicken tissues; i.e. breast, thigh, skin, kidney and liver [29]. In this method, fat and fatty acids are extracted from the breast tissue by hydrolytic methods. Samples of chicken tissues (liver, kidney, breast and thigh) were defrosted overnight in a refrigerator (4˚C) and ground thoroughly using a mortar and pestle. The ground samples were left in a freezer at < −16˚C for 12 hours prior to freeze drying. Frozen samples were freeze dried with VirTis Benchtop Pro Freeze Dryer (SP Scientific, Pennsylvania, USA) for 48 hours. Fat was extracted from 0.5 g sample of the homogenized freeze-dried tissue into ether, pyrogallic acid was added to minimize oxidative degradation of fatty acids during analysis, followed by methylation resulting in Fatty Acid Methyl Esters (FAMEs) using boron trifluoride (BF_3) in methanol.

The following fatty acid methyl ester standards were sourced from Nu-Chek-Prep Inc. (Minnesota, USA); methyl 4,7,10,13,16,19-docosahexaenoate; methyl hexadecanoate; methyl 9-octadecenoate; methyl trans 9-octadecenoate; 9,12-methyl octadecadienoate;

methyl trans 9,12-octadecadienoate, methyl 5,8,11,14,17 eicosapentaenoate. The internal standard for sample extraction, 1,2,3-triundecanoylglycerol (common name: triundecanoin), the internal standard for calibration curve and QCs, methyl undecanoate and docosahexaenoic acid (DHA) were also all purchased from Nu-Chek-Prep Inc. (Minnesota, USA). The Omega-3 Concentrate (Standard Reference Material 3275) was purchased from NIST (Gaithersburg, USA). FAMEs were quantitatively measured on an Agilent 6890 N gas chromatograph (Agilent, Ontario, Canada) equipped with a SP2560 100 cm long capillary column, a hydrogen flame ionization detector (FID) set at 250˚C temperature and an Agilent 7683 autosampler (Agilent, Ontario, Canada). Total fat was calculated as the sum of the individual fatty acids expressed as triglyceride equivalents and calculated back to wet tissue weight.

2.5. Statistical Analysis

Performance and tissue data were analyzed by the general linear model procedure of Minitab® (Minitab® version 18, State College, USA) with Dunnett's (2-sided) or Tukey's post-hoc tests used to determine the differences between the mean values for each treatment. For the ANOVA model, $p < 0.05$ indicated a significant difference. Regression analysis was used to determine the relationship between DHA intake and the DHA content of the broiler tissues or organs.

3. Results and Discussion

3.1. Test Article Analysis and Zootechnical Performance

The test article, AURA, used in this study primarily consisted of 70.2 g crude fat/100 g DM biomass and was composed of a significant level of palmitic acid and DHA, 35.97% and 17.04% respectively (**Table 2**). Additionally, the AURA contained 13.1% crude protein, 3.2% ash and 2.2% moisture. The eicosapentaenoic acid (EPA; C20:5 n-3) content was low at 0.21%. The total omega 3 and omega 6 isomers measured in AURA were 17.6% and 4.5%, respectively. Supplementation with AURA did not affect the various performance parameters measured, with no difference between the groups observed in health, mortality, weight, feed intake or feed conversion ratio observed over the course of the experiment (data not presented). The average final weight of the broiler chickens on day 41, across treatment, was 3.59 kg with a FCR of 1.66.

3.2. Enrichment of Meat and Organ Tissues

The mean DHA content detected in the breast, thigh, liver and kidney and skin with adhering fat for each treatment group is presented in **Table 3**. In line with previous studies, supplementation with AURA resulted in an increased DHA content in all tissues [15] [20] [30] [31] [32] [33]. Each increase in AURA inclusion level resulted in a significant increase in tissue DHA content and with fatty tissues incorporating higher amounts of DHA. It is well documented that the fatty acid composition of the carcass reflects the fatty acid composition of the

Table 2. Fatty acid composition (g/100 g dry matter biomass) of the unextracted *Aurantiochytrium limacinum* biomass used to supplement the experimental diets.

Fatty Acid	g/100 g	Fatty Acid	g/100 g
C4:0 (Butyric acid)	<0.01	C18:4 (Octadecatetraenoic acid)	0.05
C6:0 (Caproic acid)	<0.01	C20:0 (Arachidic acid)	0.15
C8:0 (Caprylic acid)	<0.01	C20:1 (Gadoleic acid + isomers)	<0.01
Total Short Chain Fatty Acids	<0.01	C20:2 (Eicosadienoic acid)	<0.01
		C20:3 (Eicosatrienoic acid – Omega 3)	<0.01
C10:0 (Capric acid)	<0.01	C20:3 (di-homo-gamma-linolenic acid – Omega 6)	0.10
C11:0 (Undecanoic acid)	<0.01	C20:4 (Eicosatetraenoic acid – Omega 3)	0.31
C12:0 (Lauric acid)	0.10	C20:4 (Arachidonic acid – Omega 6)	0.05
C14:0 (Myristic acid)	3.31	C20:5 (Eicosapentaenoic acid – Omega 3)	0.21
C14:1 (Myristoleic acid)	<0.01	C21:5 (Heneicosapentaenoic acid – Omega 3)	<0.01
C15:0 (Pentadecanoic acid)	0.24	C22:0 (Behenic acid)	0.08
C15:1 (Pentadecenoic acid)	<0.01	C22:1 (Erucic + isomers)	<0.01
Total Medium Chain Fatty Acids	3.65	C22:2 (Docosadienoic acid – Omega 6)	<0.01
		C22:3 (Docosatrienoic acid – Omega 3)	<0.01
C16:0 (Palmitic acid)	35.97	C22:4 (Docosatetraenoic acid – Omega 6)	<0.01
C16:1 Total (Palmitoleic acid + isomers)	0.18	C22:5 (Docosapentaenoic acid – Omega 3)	0.04
C17:0 (Margaric acid)	0.08	C22:5 (Docosapentaenoic acid – Omega 6)	4.22
C17:1 (Heptadecenoic acid)	<0.01	C22:6 (Docosahexaenoic acid – Omega 3)	17.04
C18:0 (Stearic acid)	0.99	Total Long chain fatty acids	59.63
C18:2 (Linoleic acid + isomers)	0.11		
C18:3 (alpha linolenic acid – Omega 3)	0.02		
C18:3 (gamma linolenic acid – Omega6)	0.03	Total Fatty Acids	63.68

Table 3. The mean docosahexaenoic-acid (DHA) content (mg/100 g) of breast, thigh, liver and kidney, following dietary supplementation of broilers for 41 days with 0%, 0. %5, 1.0% or 2.5% unextracted *Aurantiochytrium limacinum* biomass.

	0.0%	0.50%	1.00%	2.50%	SEM	P-value
Breast	15.2^{d}	188.6^{c}	323.8^{b}	445.4^{a}	20.3	<0.001
Thigh	16.2^{d}	201.6^{c}	469.2^{b}	835.8^{a}	26.7	<0.001
Liver	62.1^{d}	514.0^{c}	835.0^{b}	1100.0^{a}	37.1	<0.001
Kidney	42.7^{d}	196.5^{c}	290.0^{b}	423.8^{a}	10.4	<0.001
Skin with fat	15.5^{c}	204.8^{c}	560.2^{b}	1208.6^{a}	49.0	<0.001

diet [15] [18] [20] [32] [34] [35]. Furthermore, the greater the concentration of fat in the tissue, the more total DHA was found as previously known from the literature [20] [32]. The Control breast meat (white) averaged 15.2 mg/100 g fresh edible tissue and in agreement with previous findings, 14 - 16.2 mg/100 g

fresh edible tissue [15] [31] and lower than 24 mg/100 g fresh edible tissue reported by Rymer *et al.* [20]. The dietary addition of 0.5% AURA, providing 0.85 g DHA/kg diet, increased the DHA of breast meat 12.4X to 188.6 mg/100 g fresh meat. At the highest inclusion level in our study, 2.5% AURA providing 4.25 g DHA/kg diet, improved the DHA content of the breast meat to 445.4 mg/100 g fresh edible meat. However, the literature is inconsistent at higher inclusion levels, Mooney and co-workers [30] reported a significantly lower concentration, 109 mg DHA/100 g fresh breast meat, after feeding a 2.8% *Schizochytrium* sp. biomass for the 4-week grower phase of the production cycle. Rymer and co-workers provided 7.5 g DHA/kg diet over the grower period and reported 187 mg/100 g fresh breast meat [20] whilst Ribeiro and co-workers reported a 12.9X increase in breast DHA over the control [32]. Long and coworkers [33] found the opposite in their study, where a 2% microalgae supplementation resulted in a level of 5710 mg/100 g fresh breast meat.

The Control thigh meat (dark) averaged 16.2 mg/100 g fresh edible tissue and in general agreement with previous findings, 9 - 24 mg/100 g fresh edible tissue [15] [20] and [31]. The dietary addition of 0.5% AURA, providing 0.85 g DHA/kg diet, resulted in a 12.4X increase in DHA content of thigh meat to 201.6 mg/100 g fresh meat. Again, results at the upper inclusion levels, 1% AURA and higher proved to be difficult to compare to those values found in the literature [33]. At the higher inclusion level, providing 7.5 g DHA/kg diet, the dietary supplementation of a related *Schizochytrium* sp. microalgae biomass fed over the grower period resulted in 203 mg/100 g fresh thigh meat was reported following [20] and Ribeiro and co-workers reported a 14.3X increase in thigh DHA over the control [32].

A strong relationship was found between the dietary concentration of DHA and the DHA concentration in all tissues (**Figure 1**). Skin with adhering fat was enriched to the highest degree, with a linear increase ($R^2 = 0.9325$, $p < 0.001$, $Y = 27.48 + 1.587X$) in skin DHA content observed with increasing algae intake. The liver was the second most enriched, with a quadratic relationship observed ($R^2 = 0.9435$, $p < 0.001$, $Y = 108.8 + 2.436X - 0.001511X^2$) between intake and tissue content. As the liver plays an important role in lipid metabolism, this high concentration could be expected [36]. This quadratic relationship has been described by other authors [37] [38]. Thigh was the third most enriched with a linear relationship ($R^2 = 0.9657$, $p < 0.001$, $Y = 21.70 + 1.086X$) found between DHA intake and thigh DHA content. Breast, with less fat than thigh meat was enriched to a lesser degree, with a quadratic relationship observed ($R^2 = 0.9227$, $p < 0.001$, $Y = 32.68 + 0.9349X - 0.000520X^2$) between DHA intake and breast DHA content. Kidney was the least enriched with DHA content increasing linearly ($R^2 = 0.9380$, $p < 0.001$, $Y = 75.57 + 0.4843X$) with increasing DHA intake.

The incorporation of dietary DHA from fish or marine algae sources into poultry meat tissue has a long history, going back to the 1960's [15] [34] [35]. In

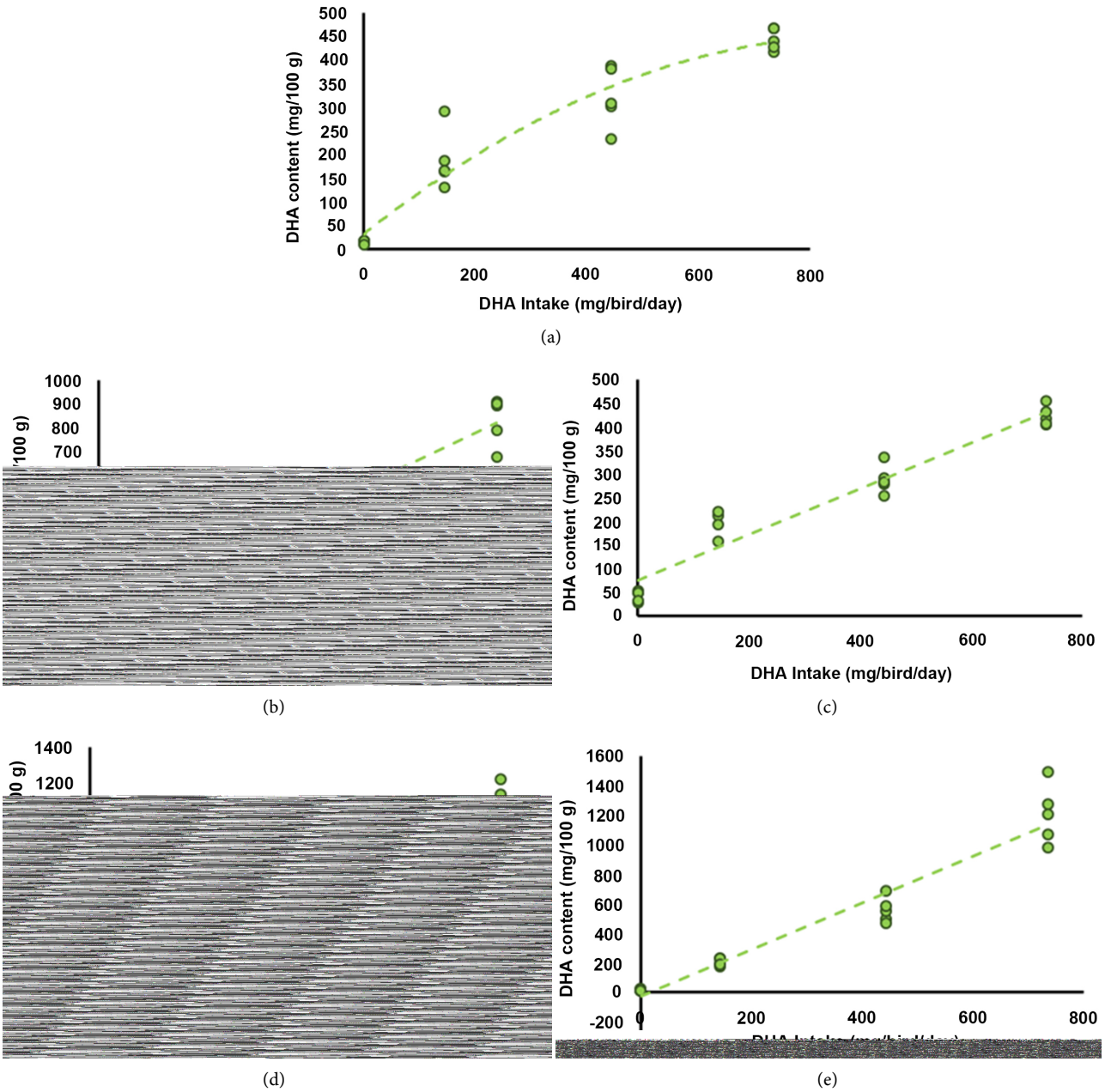

Figure 1. Scatterplot with regression lines for DHA intake (mg/bird/day) against the DHA content (mg/100 g) detected in the breast (a), thigh (b), kidney (c), liver (d) and skin with adhering fat (e).

contrast to our study findings, in a review of the literature, Rymer and Givens [15] found a poor relationship between DHA concentration in the diet and DHA content in the edible tissues of poultry; white meat, $R^2 = 0.093$ and dark meat, $R^2 = 0.143$. The difference in results between studies may be due to a range of factors: importantly the total quantity of DHA fed to the broiler and the level of fat in the tissue at slaughter; *i.e.* a fattier carcass will have greater deposition of DHA. However, other factors are unknown or have a limited role in DHA transfer from feed to tissue: the duration of feeding (entire production cycle versus grower phase), the presentation of the DHA (protected from processing condi-