Supranutrition of microalgal docosahexaenoic acid and calcidiol improved growth performance, tissue lipid profiles, and tibia characteristics of broiler chickens

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Abstract

Background: Enriching chicken with docosahexaenoic acid (DHA) and calcidiol may be used to improve public nutrition and health. It remains unclear if superanutritional levels of DHA and calcidiol impair growth or metabolism of broiler chickens. The aim of the study was to determine singular and combined effects of high levels of supplemental DHA-rich microalgal biomass or oil and calcidiol on growth performance, plasma and tissue lipid profiles, and bone characteristics of broiler chickens.

Methods: In Experiment 1, 144 day-old Cornish chicks were divided into 4 groups (6 cages/treatment, 6 birds/cage), and were fed a corn-soybean meal basal diet (BD), BD + 10000 IU calcidiol/kg (BD+Cal), BD + 1% DHA-rich *Aurantiochytrium*(1.2 g DHA/kg; BD+DHA), and BD+Cal+DHA for 6 wk. In Experiment 2, 180 day-old chicks were divided into 5 groups (6 cages/treatment, 6 birds/cage), and were fed: BD, BD+ DHA (oil, 1.5 to 3.0 g DHA/kg), BD + DHA + EPA (eicosapentaenoic acid, 0.3 to 0.6 g/kg), BD+DHA+ calcidiol (6,000 to 12000 IU/kg diet), and BD+DHA+EPA+Cal for 6 wk. Growth performance, concentrations of triglyceride, cholesterol, and nonesterifed fatty acids in plasma, liver, breast, and thigh, and biophysical properties of tibia were determined.

Results: Birds fed BD+Cal diet in Experiment 1 and BD+DHA+EPA diet in Experiment 2 had higher ($P < 0.05$ body weight gain (10-11%) and gain: feed ratio (7%), and lower ($P < 0.05$) total cholesterol and triglyceride concentrations in plasma (18-54%), liver (8-26%), breast (19-26%), and thigh (10-19%), respectively, over the controls. The two diets also improved ($P < 0.05$) tibial breaking strength (8-24%), total bone volume (2-13%), and(or) bone mineral density (3-19%) of chickens.

Conclusion: Superanutrition of dietary calcidiol and DHA alone or together did not produce adverse effects, but improved growth performance, lipid profiles of plasma and muscle, and bone health of broiler chickens.

Background

Biofortifications of chicken with omega-3 fatty acids in particular DHA, and calcidiol have been viewed as an effective strategy to produce health-promoting meat for human consumption [1, 2]. Earlier studies with relatively low to moderate inclusion levels of DHA-rich microalgal biomass or oil (0.55 to 2.55 g DHA/kg diet) and calcidiol (1600 to 2800 IU/kg diet) in broiler diets demonstrated no negative effects on growth performance, lipid profile of tissues, or bone strength [3–7]. In contrast, high inclusion levels of DHA-rich microalgal biomass (4.9 and 6.8 g DHA/kg diet) and calcidiol (27600 IU/kg diet) in broiler diets decreased growth performance by 19% and breast muscle weight by 21% [8–10]. Past studies were focused on fortifying chicken with DHA and calcidiol singularly [1, 4, 11]. Little research was attempted to enrich chicken simultaneously with these two nutrients or to look out for potential adverse effects of extremely high supplementations of these two nutrients together on growth performance, lipid metabolism, and bone integrity of chickens.

To fill in the gap of knowledge, we conducted two experiments to examine those effects of supplementing high levels of these two bioactive nutrients in broiler chickens. In the first experiment, DHA-rich *Aurantiochytrium* sp biomass was used as the source of DHA (Tolba et al., 2019). In the subsequent experiment, a DHA-rich microalgal oil was the source of DHA, along with EPA-rich *Nannochloropsis* sp CO18 biomass. In both experiments, a feed grade of synthetic calcidiol was used as the source of bioactive (OH)24 vitamin D3.

Materials And Methods

Animal, diets, and management

Our animal protocols were approved by the Cornell University Institutional Animal Care and Use Committee. DHA-rich microalgal *Aurantiochytrium* biomass and oil were provided by Heliae (Gilbert, AZ) and ADM (Decatur, IL), respectively. EPA-rich *Nannochloropsis* sp CO18 and calcidiol were provided by Duke University (Beaufort, NC) and DSM (Parsippany, NJ), respectively. In Experiment 1, Cornish male broiler chicks (day old, total = 144) were purchased from Moyer's Chicks (Quakertown, PA) and housed in a temperature-controlled unit at Cornell University Poultry Research Farm. Chicks were allotted into 4 treatment diets (6 replicates per diet, 6 birds per replicate). Birds were fed 1 of the 4 diets: a corn-soybean meal basal diet (BD), BD + 10000 IU calcidiol/kg of diet (BD + Cal), BD + 1% DHA-rich microalgal biomass (*Aurantiochytrium*, 1.2 g DHA/kg diet; BD + DHA), and BD + Cal + DHA.

In Experiment 2, 180 Cornish male broiler chicks were purchased from same supplier as in Experiment 1 and allotted into 5 treatment diets (6 cages/diet, 6 birds/cage): BD, BD + DHA-rich microalgal oil (1.5 g DHA/kg diet (0.33%) for 0–3 wk and 3.0 g DHA/kg (0.66%) for 4–6 wk; BD + DHA), BD + DHA + EPA rich *Nannochloropsis* sp CO18 (0.3 g EPA/kg diet (1.9%) for 0–3 wk and 0.6 g EPA/kg diet (3.8%) for 4–6 wk; BD + DHA + EPA), BD + DHA + Cal (6,000 IU/kg diet for 0–3 wk and 12,000 IU/kg diet for 4–6 wk; BD + DHA + Cal), and BD + DHA + EPA + Cal. All experimental diets were formulated according to nutrient requirements for broilers by the National Research Council [12]. Compositions of starter and finisher diets used in Experiment 1 and Experiment 2 are presented in Supplemental Tables 1–4. Both experiments lasted for 42 days, Birds had free access to feed and water and received a lighting schedule of 22 h light and 2 h dark throughout.

Growth Performance Measures And Sample Collections

During both experiments, body weights of individual birds were recorded at wk 3 and wk 6. Feed disappearance of cages were recorded weekly to calculate feed intakes. Chicken health and mortality were checked daily. At the end of wk 3 and wk 6, 2 birds per cage were euthanized via asphyxiation with carbon dioxide. Blood was drawn from heart puncture by using heparinized needles to prepare plasma samples that were stored at – 20°C until analysis. Liver, breast, thigh, and tibia samples were removed and stored at -20^{o}C for later analyses.

Laboratory Analyses

Concentrations of non-esterified fatty acids (NEFAs), total cholesterol (TC), triglycerides (TGs), and phospholipids (PL) in plasma, liver, breast, and thigh samples were determined using commercially available kits (Wako Chemicals, Richmond, VA) as described in previous studies [13, 14]. In Experiment 1, tibia bone (wk 6) characteristics were determined using an Instron machine following the protocol described previously by Manor et al. [15]. Briefly, before measuring tibia bone strength, the soft tissues were removed manually, and bone strength was measured at the center of the shaft for both tibias and averaged for each bird. Bone strength was measured on the right tibia with the use of an Instron 5965 (Instron Corp.) equipped with a 5-kN load cell and a cross-head speed of 20 mm/min. Bluehill 3 Testing Software (Instron Corp.) was used to perform a flexure test with a 38-mm supported length. Maximum slope, maximum load, and energy to maximum load were recorded for each tibia. In Experiment 2, characteristics of tibia (wk 6) bone were determined using Micro-CT as per the method described by Sharma et al. [16]. Briefly, tibia bones were thawed at 4^{o}C and cleaned of all tissues, and analyzed by Skyscan X-ray microtomography (Bruker MicroCT, Billerica, MA). The X-ray source was set at 75 kV and 133 µA. The pixel size was

fixed at 25 µm, the rotation angle of 0.4^{o} was applied at each step, and 4 images per rotation were captured. A series of 2D images were captured, which were later used to reconstruct a 3D image using N-Recon (Brucker MicroCT, Billerica, MA). Microtomography was performed on the distal epiphyses of the tibia, and a part of the distal supracondylar region was selected as a volume of interest wherein all bone sections (cortical bone and trabecular bone) were present. Percentage bone volume and bone mineral density (BMD) were measured from the whole total volume of interest, cortical bone, and trabecular bone sections. From trabecula bone, trabecular thickness, trabecular separation, and degree of anisotropy were also measured.

Statistical analysis

Data from Experiments 1 and 2 were analyzed by two-way (2 by 2 factorial arrangement of treatments) and one-way analysis of variance (ANOVA) using a completely randomized design, respectively. Data were presented as mean ± SEM and $P < 0.05$ was assumed to be statistically significant. The means of different experimental groups were compared using Duncan's multiple range test. Pen served as an experimental unit (n = 6).

Results

Growth performance

In Experiment 1, there was no difference in the body weight gain (BWG) or feed intake of chicks among the 4 treatment diets at wk 3 (Supplemental Table 5). Compared with those fed the BD, birds fed the BD + Cal diet had 11% higher ($P < 0.05$) BWG, and 7% higher ($P < 0.05$) gain: feed ratio at wk 6 (Table 1). Birds fed BD + DHA had 6% higher BWG and 8% higher feed intake (8%) compared to those fed the BD alone, but the differences were not statistically significant. Moreover, birds fed BD + Cal + DHA also showed non-significantly higher BWG (9%) and feed intake (12%) compared with birds fed BD alone at wk 6 (Table 1).

In Experiment 2, birds fed BD + DHA + EPA had 17–27% higher BWG and 6–25% higher gain: feed ratio than those fed the other diets at wk 3, but the differences were not statistically significant (Supplemental Table 5). Compared with birds fed the BD, birds fed the BD + DHA + EPA diet had 10% higher ($P < 0.05$) BWG and 14% higher ($P < 0.05$) feed intake at wk 6 (Table 1). Birds fed the BD + DHA diet had 3% higher BWG and gain: feed ratio, compared to birds fed BD alone, but the differences were not statistically significant (Table 1). Moreover, birds fed BD + DHA + EPA + Cal had 3% higher ($P > 0.05$) BWG and 15% higher ($P < 0.05$) feed intake, compared with those fed BD. Further, no difference in the BWG, feed intake, or gain: feed ratio was shown in birds fed BD + DHA + Cal, compared with those fed BD (Table 1).

Tibia Bone Health

In Experiment 1, tibia from birds fed BD + Cal had 8–24% greater ($P < 0.05$) breaking strength (energy) than that of birds fed the other diets at wk 6 (Table 5). However, there was no difference in other measured variables among the 4 treatment diets. In Experiment 2, birds fed BD + DHA + Cal had 3–19% higher ($P < 0.05$) BMD and 2–13% higher total bone volume compared with birds fed the other diets at wk 6 (Table 6). Diets produced no significant effects on other measured variables including cortical BMD, cortical percentage bone volume, trabecular BMD, trabecular percentage bone volume, trabecular thickness, trabecular separation, or degree of anisotropy.

Discussion

In Experiment 1, broilers fed the BD + Cal diet had a higher BWG and gain: feed ratio compared with birds fed the BD at wk 6. This result is consistent with previous studies in which supplemented calcidiol improved BWG and feed efficiency [17, 18]. Supplemental cholecalciferol in broiler diets also increased BWG [19]. However, we supplemented the broiler diet with a much higher level of calcidiol i.e. 10000 IU/kg compared with the NRC recommendation of 200 IU/kg. The supplementation-resultant improvements in the growth and feed efficiency indicate that a higher level of calcidiol was not only well tolerated by the broilers but also beneficial to their metabolism and growth of chickens. In contrast, Chou et al. [20] reported that calcidiol as a source of cholecalciferol did not improve BWG or feed efficiency in broilers. Furthermore, combined doses of calcidiol and DHA-rich microalgae biomass did not produce any negative effects on growth performance of chickens.

In Experiment 2, birds fed BD + DHA + EPA had better BWG, and feed intake compared with birds fed BD. These results agreed with Long et al. [5] and Ribeiro et al. [21] who found no negative effect but improved BWG and feed conversion ratio in broiler chickens after feeding them with a high dose of DHA-rich microalgae biomass. The positive growth performance in birds fed BD + DHA + EPA could be associated with the positive effect of EPA and DHA on feed consumption and muscle protein synthesis [22, 23]. Moreover, DHA-rich microalgal oil in a singular dose level or in a combined dose with calcidiol did not produce any negative effect on body weight gain or feed efficiency of broilers.

In this study, we observed metabolic effects of supplemental microalgal DHA and calcidiol on lipid profiles of plasma and tissues of broiler chickens. In Experiment 1, birds fed BD + Cal showed lower plasma cholesterol levels at wk 6 than those fed BD. Decreased cholesterol level was in the accordance with previous studies where calcidiol supplementation reduced plasma cholesterol levels by inhibiting activity of HMG-CoA reductase enzyme [24, 25]. In addition, DHA-rich microalgae biomass either fed alone or in combination with calcidiol did not produce any negative effect on bird's plasma lipid profile. In Experiment 2, the addition of DHA-rich microalgal oil did not cause any negative effect on plasma and tissue lipid profile. Instead, a combined supplementation of DHA and EPA reduced TC, TG, and (or) NEFAs in the plasma liver, breast, and(or) thigh tissues. These results are consistent with previous findings where DHA supplementation reduced cholesterol biosynthesis and TGs levels by inhibiting squalene epoxidase enzyme [26, 27] and by reducing very low-density lipoprotein (VLDL) synthesis and secretion [5, 28, 29], respectively.

In Experiment 1, birds fed BD + Cal diet, showed high energy at maximum load for tibia bone, which indicated greater tibia bone strength. These results are consistent with earlier reports where higher doses of calcidiol in the poultry diet had a positive effect on tibia bone development and integrity in birds [3, 30]. In addition, DHA-rich microalgae biomass either fed alone or in combination with calcidiol did not negatively affect tibia bone parameters. These results are consistent with previous reports in which DHA supplementation did not cause any change in bone structural integrity and strength [31–33]. Likewise, in Experiment 2, a high level of DHA-rich microalgal oil did not cause any negative effects on bone measures. Instead, the combined supplementation of DHA and calcidiol increased total bone volume and total BMD in birds. A larger bone volume would allow more space for mineral deposition and a low BMD is usually associated with a high risk of bone fracture [34]. The improvement in the bone volume and BMD with a combined supplementation of DHA and calcidiol may imply synergistic effects of DHA and calcidiol on calcium and bone metabolism [35, 36]. Fast-growing broiler chickens are susceptible to tibial dyschondroplasia (TD) which reduces the stability of leg bones and deteriorates the quality of meat from the legs [3]. Dietary supplementation of calcidiol as a source of cholecalciferol may be effective in

reducing the severity of TD. This is because the high dose of calcidiol in our study improved tibia bone strength, but did not produce any negative effects on growth performance.

Conclusions

In the present study, we determined singular and combined effects of super highly levels of supplemental DHA and calcidiol on growth performance, plasma and tissue lipid profiles, and biophysical characteristics of tibia in broiler chickens. Feeding chickens with supernutritional levels of these supplements, much beyond nutrient requirements of broiler chickens, led to no negative effects on growth performance, tissue lipid profile, or bone health. Instead, some of the supplementations resulted in moderate beneficial responses of the measures, suggesting that it is safe to use high levels of dietary DHA and calcidiol supplementation for biofortifying chicken with these bioactive nutrients.

Abbreviations

BD, basal diet; BMD, bone mineral density; BW, body weight; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; NEFA, non-esterified fatty acid; PL, phospholipids; TC, total cholesterol; TD, tibial dyschondroplasia; TGs, triglyceride.

Declarations

Acknowledgments

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Author Contributions

XL designed the research from project conception to study oversight and edited the paper. SK, AM, TS, and GL conducted the animal trial and collected data. SK performed statistical analyses and wrote the paper. WK supervised the tibia analysis. ZJ supervised the cultivation of EPA-rich *Nannochloropsis* sp CO18. All authors have read and approved this submission. We thank Dr. Nelson Ward of DSM for providing calcidiol and Dr. John Less of ADM for providing DHA oil.

Ethics approval

The current study was conducted at Cornell University Poultry Research Farm. Animal research protocols were approved by the Cornell University Institutional Animal Care and Use Committee

Conflict of interest statement

All the authors declared no conflict of interest.

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Tables

Table 1 Effects of supplementation of calcidiol, DHA-rich microalgal biomass or oil, and EPA-rich microalgal biomass on body weight, feed intake, and gain: feed ratio in broiler chickens in Experiments 1 and 2 (0-6 wk)

Experiment 1							
Parameters	**BD**	**BD+Cal**	**BD+DHA**	**BD+Cal+DHA**	**SEM**	***P*-value**	
BW 0 day (g/chick)	41.85	41.77	41.80	41.65	0.17	0.85	
BW 0-6 wk (g/chick)	2913.20^{a}	3232.62^{b}	3084.55^{ab}	3160.82^{ab}	51.20	<0.01	
BWG 0-6 wk (g/chick)	2871.34^{a}	3190.84^{b}	3042.75^{ab}	3119.17^{ab}	57.25	<0.01	
FI 0-6 wk (g/chick)	4860.10^{a}	5050.36^{ab}	5240.20^{ab}	5450.56^{b}	115.4	<0.01	
Gain: Feed	0.59^{a}	0.63^{b}	0.58^{a}	0.57^{a}	0.008	<0.01	
Experiment 2							
	BD	**BD+DHA**	**BD+DHA+EPA**	**BD+DHA +Cal**	**BD+DHA+EPA+Cal**	**SEM**	***P*-value**
BW 0 day (g/chick)	42.25	42.32	42.39	42.43	42.36	0.07	0.64
BW 0-6 wk (g/chick)	2522.33^{a}	2598.92^{ab}	2774.17^{b}	2533.25^{ab}	2592.92^{ab}	62.82	0.04
BWG 0-6 wk (g/chick)	2480.08^{a}	2556.60^{ab}	2731.78^{b}	2490.82^{ab}	2550.56^{ab}	62.84	0.04
FI 0-6 wk (g/chick)	3875.52^{a}	3867.97^{a}	4413.40^{b}	4075.90^{ab}	4474.10^{b}	121.8	<0.01
Gain: Feed	0.64^{ab}	0.66^{b}	0.62^{ab}	0.61^{ab}	0.57^{a}	0.02	0.02

BW: Body weight; BWG: Body weight gain; FI: Feed intake.

Experiment 1; BD = Corn-soybean basal diet; BD+Cal = BD + 10000 IU calcidiol/Kg of diet; BD+DHA = BD + 1% microalgal biomass; BD+Cal+DHA = BD+Cal + 1% microalgal biomass.

Experiment 2; 0-3 Wk: BD = Corn-soybean basal diet; BD+DHA= BD + 1.5 g/kg DHA oil; BD+DHA+EPA = BD+DHA + 0.3 g/kg *Nannochloropsis* sp CO18; BD+DHA+Cal = BD+DHA + 6000 IU/kg calcidiol; BD+DHA+EPA+Cal = BD+DHA+EPA + 6000 IU/kg calcidiol.

4-6 Wk: BD = Corn-soybean basal diet; BD+DHA= BD + 3.0 g/kg DHA oil; BD+DHA+EPA = BD+DHA + 0.6 g/kg *Nannochloropsis* sp CO18; BD+DHA+Cal = BD+DHA + 12000 IU/kg calcidiol; BD+DHA+EPA+Cal = BD+DHA+EPA + 12000 IU/kg calcidiol.

Means bearing the different superscripts ($^{a, b}$) in a row differ significantly (P <0.05).

Data are expressed as means (n = 6 cages and 6 birds/cage) and were analyzed by one-way ANOVA.

Table 2 Effects of supplementation of calcidiol and DHA-rich microalgal biomass on plasma lipid profile of broiler chickens in Experiment 1

	BD	BD+Cal	BD+DHA	BD+Cal+DHA	SEM	*P*-value
Wk 3						
TG (mg/dL)	22.48	22.21	25.27	28.07	3.24	0.65
TC (mg/dL)	133.17	136.11	139.29	129.68	5.03	0.62
NEFA (μmol/mL)	0.21	0.18	0.17	0.16	0.02	0.27
Wk 6						
TG (mg/dL)	20.26	23.49	21.84	22.52	1.97	0.55
TC (mg/dL)	101.16^{b}	77.96^{a}	97.76^{b}	100.53^{b}	4.42	<0.01
NEFA (μmol/mL)	0.24	0.24	0.24	0.28	0.03	0.69

NEFA: Non-esterified fatty acid; TC: Total cholesterol; TG: Triglyceride.

BD = Corn-soybean basal diet; BD+Cal = BD + 10000 IU calcidiol/kg of diet; BD+DHA = BD + 1% microalgal biomass; BD+Cal+DHA = BD+Cal + 1% microalgal biomass.

Means bearing the different superscripts ($^{a, b}$) in a row differ significantly (P <0.05).

Values are expressed as means of 6 birds/treatment and data were analyzed using one-way ANOVA.

Table 3 Effects of supplementation of DHA-rich microalgal oil, EPA-rich microalgal biomass, and calcidiol on plasma and tissue lipid profile of broiler chickens at wk 3 in Experiment 2

	BD	BD+DHA	BD+DHA +EPA	BD+DHA +Cal	BD+DHA +EPA+Cal	SEM	*P*-value
Plasma							
PL (mg/dL)	102.28	90.22	91.54	100.57	104.44	6.26	0.48
TG (mg/dL)	31.64	25.51	33.74	25.23	34.95	2.88	0.08
TC (mg/dL)	100.3	103.29	90.64	105.5	106.76	2.50	0.41
NEFA (μmol/mL)	219.42	203.65	202.14	229.52	212.39	15.60	0.72
Liver							
PL (mg/g tissue)	16.72	14.74	15.20	16.14	15.80	2.42	0.16
TG (mg/g protein)	66.76	70.12	64.81	65.08	70.04	5.17	0.95
TC (mg/g protein)	15.28	14.12	15.02	14.80	14.61	1.14	0.81
NEFA (μmol/g protein)	45.34	46.80	41.12	41.95	43.44	3.80	0.65
Breast							
PL (mg/g tissue)	6.01	4.16	6.12	5.76	6.09	0.44	0.06
TG (mg/g protein)	21.44	21.16	19.30	21.05	20.82	1.36	0.73
TC (mg/g protein)	6.36	6.10	6.53	6.69	6.80	1.24	0.80
NEFA (μmol/g protein)	5.27	5.12	4.85	4.96	4.79	0.70	0.56
Thigh							
PL (mg/g tissue)	3.75	3.25	3.43	3.64	4.34	0.52	0.64
TG (mg/g protein)	23.50	22.14	21.60	23.44	22.40	1.22	0.87
TC (mg/g protein)	4.56	4.60	4.53	4.37	4.32	0.51	0.64
NEFA (μmol/g protein)	5.50	5.33	5.06	5.53	5.24	0.83	0.71

BD = Corn-soybean basal diet; BD+DHA= BD + 1.5 g/kg DHA oil; BD+DHA+EPA= BD+DHA + 0.3 g/kg *Nannochloropsis* sp CO18; BD+DHA+Cal = BD+DHA + 6000 IU/kg calcidiol; BD+DHA+EPA+Cal = BD+DHA+EPA + 6000 IU/kg calcidiol.

Means bearing no superscripts did not differ significantly (P >0.05).

Values are expressed as means of 6 birds/treatment and data were analyzed using one-way ANOVA.

Table 4 Effects of supplementation of DHA-rich microalgal oil, EPA-rich microalgal biomass, and calcidiol on plasma and tissue lipid profile of broiler chickens at wk 6 in Experiment 2

	BD	BD+DHA	BD+DHA +EPA	BD+DHA +Cal	BD+DHA +EPA+Cal	SEM	*P*-value
Plasma							
PL (mg/dL)	78.92	61.70	67.09	76.26	70.74	4.50	0.08
TG (mg/dL)	40.37^{b}	27.84^{ab}	18.72^{a}	39.23^{b}	28.77^{ab}	3.76	<0.01
TC (mg/dL)	101.78^{b}	89.57^{ab}	83.76^{a}	90.29^{ab}	79.84^{a}	3.53	<0.01
NEFA (μmol/mL)	87.31^{b}	62.01^{a}	76.64^{ab}	85.62^{b}	78.77^{ab}	4.21	<0.01
Liver							
PL (mg/g tissue)	14.60	12.31	12.48	14.66	13.65	1.74	0.22
TG (mg/g protein)	71.40^{b}	68.16^{b}	58.72^{a}	70.85^{b}	66.10^{ab}	6.12	<0.01
TC (mg/g protein)	14.70	13.30	13.55	14.06	13.57	1.37	0.95
NEFA (μmol/g protein)	45.98	41.51	34.05	42.14	38.82	3.10	0.15
Breast							
PL (mg/g tissue)	3.60	2.89	3.09	3.38	2.76	0.19	0.32
TG (mg/g protein)	23.77^{b}	22.92^{b}	18.07^{a}	23.06^{b}	22.88^{b}	2.11	0.04
TC (mg/g protein)	5.82	4.61	4.69	5.41	5.59	0.30	0.05
NEFA (μmol/g protein)	5.08^{b}	4.67^{ab}	3.78^{a}	4.50^{ab}	4.44^{ab}	0.91	0.03
Thigh							
PL (mg/g tissue)	3.34	2.47	2.92	2.83	2.56	0.32	0.12
TG (mg/g protein)	26.62^{b}	24.47^{ab}	21.52^{a}	24.16^{ab}	23.96^{ab}	2.16	0.01
TC (mg/g protein)	4.00	3.93	3.63	3.88	3.78	0.11	0.21
NEFA (μmol/g protein)	5.18	4.99	4.58	5.07	4.53	1.01	0.90

BD = Corn-soybean basal diet; BD+DHA= BD + 3.0 g/kg DHA oil; BD+DHA+EPA = BD+DHA + 0.6 g/kg *Nannochloropsis* sp CO18; BD+DHA+Cal = BD+DHA + 12000 IU/kg calcidiol; BD+DHA+EPA+Cal = BD+DHA+EPA + 12000 IU/kg calcidiol.

Means bearing the different superscripts ($^{a, b}$) in a row differ significantly (P <0.05).

Values are expressed as means of 6 birds/treatment and data were analyzed using one-way ANOVA.

Table 5 Effects of supplementation of calcidiol and DHA-rich microalgal biomass on tibia bone properties of broiler chickens at wk 6 in Experiment 1

	BD	BD+Cal	BD+DHA	BD+Cal+DHA	SEM	*P*-value
Extension at Maximum Load (mm)	2.13	2.12	2.06	2.21	0.10	0.79
Energy at Maximum Load (J)	0.47^{a}	0.56^{b}	0.44^{a}	0.49^{ab}	0.04	0.02
Maximum Slope (mm/N)	0.09	0.05	0.05	0.04	0.01	0.06
Maximum Load (N)	500.19	515.46	514.46	519.89	31.48	0.97
Maximum Extension (mm)	2.37	2.49	2.44	2.30	0.17	0.14

BD = Corn-soybean basal diet; BD+Cal = BD + 10000 IU calcidiol/kg of diet; BD+DHA = BD + 1% microalgal biomass; BD+Cal+DHA = BD+Cal + 1% microalgal biomass.

Means bearing the different superscripts ($^{a, b}$) in a row differ significantly (P <0.05).

Values are expressed as means of 6 birds/treatment and data were analyzed using one-way ANOVA.

Table 6 Effects of supplementation of DHA-rich microalgal oil, EPA-rich microalgal biomass, and calcidiol on tibia bone properties of broiler chickens at wk 6 in Experiment 2

	BD	BD+DHA	BD+DHA +EPA	BD+DHA +Cal	BD+DHA +EPA+Cal	SEM	*P*-value
Total BMD (g/cm^3)	0.27^{ab}	0.26^{a}	0.29^{ab}	0.31^{b}	0.30^{ab}	0.01	0.04
Total Bone Volume (%)	34.09^{a}	33.75^{a}	37.40^{ab}	38.21^{b}	36.17^{ab}	1.50	0.04
Cortical BMD (g/cm^3)	0.60	0.58	0.61	0.66	0.65	0.03	0.38
Cortical Bone Volume (%)	99.999	99.999	100.000	99.992	99.999	0.0014	0.20
Trabecular BMD (g/cm^3)	0.10	0.10	0.12	0.10	0.10	0.02	0.94
Trabecular Bone Volume (%)	9.97	10.11	11.36	10.40	9.99	1.44	0.97
Trabecular Thickness (mm)	0.14	0.14	0.15	0.16	0.15	0.01	0.19
Trabecular Separation (mm)	1.20	1.31	1.22	1.24	1.36	0.11	0.52
Degree of Anisotropy	1.70	1.72	1.65	1.66	1.63	0.05	0.72

BD = Corn-soybean basal diet; BD+DHA= BD + 3.0 g/kg DHA oil; BD+DHA+EPA = BD+DHA + 0.6 g/kg *Nannochloropsis* sp CO18; BD+DHA+Cal = BD+DHA + 12000 IU/kg calcidiol; BD+DHA+EPA+Cal = BD+DHA+EPA + 12000 IU/kg calcidiol.

BMD = Bone mineral density.

Means bearing the different superscripts ($^{a, b}$) in a row differ significantly ($P<0.05$).

Values are expressed as means of 6 birds/treatment and data were analyzed using one-way ANOVA.

Supplementary Files

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- Supplementaltable29152022.docx