

## Research Article

# Effects of Dietary Inclusion of Canthaxanthin- and $\alpha$ -Tocopherol-Loaded Liposomes on Growth and Muscle Pigmentation of Rainbow Trout (*Oncorhynchus mykiss*)

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Dietary inclusion of canthaxanthin, a common carotenoid pigment, has been long practiced in aquaculture to give the favorable flesh color in farmed salmonids. However, carotenoids are associated with limited solubility and poor physicochemical stability, and their dose in fish feed is widely regulated. In this study, we included canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes into fish diets and evaluated the effects of supplemented fish feed on fish growth, color, nutrition, and canthaxanthin deposition in fillets of cultured rainbow trout (*Oncorhynchus mykiss*). The liposomes were fabricated using lecithin as phospholipids with the initial concentrations (IC =  $m_{\text{canthaxanthin}}/m_{\text{lipids}}$ , % wt/wt) of canthaxanthin at 0.1%, 0.5%, and 1.0%. Particle size characterization showed that liposome mean sizes were  $109.70 \pm 6.36$ ,  $105.10 \pm 8.41$ , and  $109.20 \pm 5.66$  nm (mean  $\pm$  SD;  $n = 3$ ), respectively, corresponding with liposomes synthesized at canthaxanthin IC = 0.1%, IC = 0.5%, and IC = 1%. The polydispersity index (PDI) of all samples remained lower than 0.2. There were no significant differences in the mean size and PDI between blank lecithin liposome and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes. The encapsulation efficiency of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes decreased when increasing the concentration of canthaxanthin in lecithin liposomes, with EE% values of IC = 0.1%, IC = 0.5%, and IC = 1% being  $85.3 \pm 2.1$ ,  $72.9 \pm 1.8$ , and  $55.3 \pm 2.6$ , respectively. For fish growth, at the end of the experiment, final weight was significantly higher in fish fed with diet supplemented with 1 g/kg canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%) in comparison to other experimental control groups. The difference in color of the salmon muscle was most apparent after two months of feeding. However, after three months, there was no noticeable change in the color score of the fish muscle, indicating saturation of color of the fish muscle. The above results suggest the potential of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes as the red pigment in fish aquaculture.

## 1. Introduction

In the market of farmed aquatics, reddish color is often perceived as indication of high quality fish flesh and is associated with consumer acceptance [1]. However, pigments responsible for the red coloration of fish flesh, comprising mostly carotenoids such as canthaxanthin and astaxanthin, can only be naturally synthesized in bacteria, algae, yeasts, molds, and some higher plants with *de novo* synthesis capacity [2, 3]. Therefore, supplementation of carotenoids in feeds has been commonly exercised in commercial farming of salmonids, crustaceans, and other aquatic organisms to give desirable coloration to the resulting products [4–6]. Carotenoids are a pigment class that is responsible for a wide range of colors in plant and animal species and figure prominently among other pigments as an important antioxidant primarily derivable from marine species [7]. It has been shown that dietary supplementation of canthaxanthin, a common carotenoid, in aquaculture salmon and trout could color the fillet with red-pink color [8, 9] and that pigmentation intensity of rainbow trout flesh was positively correlated with added canthaxanthin in fish feed [10].

Carotenoid feeding in aquaculture is associated with several issues. First, production of carotenoids largely depends on chemical processes and thus presents certain disadvantages including strict requirements on process control and generation of undesirable by-products that might be allergic to some parts of consumers [11]. In addition, high-dose consumption of canthaxanthin has been reported to result in health issues, most notably the development of retinal crystals in human eye [12] due to canthaxanthin-induced damage in lipid membranes of macula blood vessels [13]. As a result, canthaxanthin addition in commercial fish feed is widely regulated, and the allowable dose per kilogram of salmonid feed is 25 mg according to the regulation of European Union [14]. The second obstacle is that carotenoids such as astaxanthin and canthaxanthin, due to their unsaturated chemical structures, have low aqueous solubility and are sensitive to physicochemical conditions such as heat, oxygen, and light, resulting in low bioavailability of the substances when being used as nutraceutical ingredients [15, 16]. Indeed, Torrisen et al. [17] showed strong, inverse relationship between canthaxanthin dose in fish feed and apparent digestibility in rainbow trout, while increased dietary lipid levels increased its apparent digestibility [17]. Therefore, further measures in aiding the distribution of drugs into target sites and prevention of impacts of negative environmental factors on the carotenoids are essential.

In this study, we approached the aforementioned concerns by first formulating liposomes loaded with active substances derived from natural sources, followed by evaluation of the effect of dietary supplementation of liposomal canthaxanthin on growth, fillet color, muscle deposition, and nutrient composition of fish flesh of cultured rainbow trout (*Oncorhynchus mykiss*). The use of liposome has been regarded as an efficient strategy for delivery of drugs with

low bioavailability since it allows better oral adsorption of lipid insoluble polar compounds, in turn resulting in improved drug efficacy. Canthaxanthin was extracted from bacterium *Paracoccus carotinifaciens*, an emerging natural source that is known for accumulation of canthaxanthin at high yield, at around 0.4% [18]. In liposome formation,  $\alpha$ -tocopherol was coloaded along with canthaxanthin to maintain the quality and color of the fish flesh [19–22], and soybean lecithins were used as phospholipids owing to their continuous availability and advantages in terms of cost, emulsifying behavior, and sensorial characteristics [23, 24]. The inclusion of  $\alpha$ -tocopherol is justified by its role in filling the gaps left by the imperfect packing of phospholipids in liposomal formation and in maintaining fish flesh quality, including the color of salmon, by affecting the oxidative stability of lipids [25]. Current findings are expected to justify the use of liposome containing natural-derived colorants in intensive aquaculture practices and greatly enhance the value of natural canthaxanthin given that consumers are increasingly aware of negative health effects that synthetic colorants may bring.

## 2. Materials and Methods

**2.1. Materials.** Bacterium *Paracoccus carotinifaciens* VTP 20181 strain was isolated from a salt field land in Diem Dien town, Thai Binh province, Vietnam, and fermented for 64 hours. Methanol (MeOH), acetone, n-hexane, dichloromethane, tetrahydrofuran (THF), 2-propanol, and acetonitrile solvents were obtained from Sigma Aldrich (St. Louis, MO, USA). Canthaxanthin standard was purchased from TRC Canada. A commercial preparation of soybean lecithin phospholipids was used to synthesize liposomes.

**2.2. Extraction of Canthaxanthin.** Biomass of the bacterium *Paracoccus carotinifaciens* VTP20181 obtained after fermentation was provided by the Food Industry Research Institute (Hanoi, Vietnam). Biomass was refrigerated at  $-40^{\circ}\text{C}$  prior to extraction process. Wet biomass was collected by centrifugation and washed with water, followed by drying. Then dried, canthaxanthin-rich biomass was extracted with solvent (ethanol 96% + 0.5% glycerol monostearate) under ultrasound assistance. Afterwards, the solvent in the mixture was removed by rotary evaporation to afford the canthaxanthin extract. The extract is crystallized with urea to remove saturated fatty acids. Subsequently, column chromatography was used to purify the extract of canthaxanthin. Finally, emulsification was carried out from the liquid, which was then dried to collect canthaxanthin powder [26]. The final yield was approximately 1.5 mg of canthaxanthin, accounting for 0.15 % of dry biomass weight. The compounds were elucidated using spectroscopic methods (IR, 2D-NMR and MS).

Canthaxanthin (1.5 mg): orange powder,  $\text{C}_{40}\text{H}_{52}\text{O}_2$ , melting point  $211^{\circ}\text{C}$  (literature  $211\text{--}212^{\circ}\text{C}$  [25]).  $^1\text{H-NMR}$   $\text{d}(\text{CDCl}_3)$ : 1.20 (s, ca. 12H, 1,1'-gem-Me), 1.85 (m, J 6.7, 4H, 2,2'-H<sub>2</sub>), 1.87 (s, 6H, 18,18'-Me), 1.99 (s, 6H, 19,19'-Me),

2.00 (s, 6H, 20,20'-Me), 2.51 (mm, *J* 6.7, 4H, 3,3'-H<sub>2</sub>), 6.25 (mm, *J* 16, 2H, 7,7'-H), 6.27 (m, *J* 12, 2H, 10,10'-H), ca. 6.29 (m, 2H, 14,14'-H), 6.36 (d, *J* 16, 2H, 8,8'-H), 6.40 (d, *J* 14.8, 2H, 12,12'-H), ca. 6.65 (m, 2H, 15,15'-H), 6.68 (m, 4H, 11,11'-H). <sup>13</sup>C-NMR d(CDCl<sub>3</sub>): 35.8 (1, 1'), 37.5 (2, 2'), 34.3 (3, 3'), 203.9 (4, 4'), 129.9 (5, 5'), 160.9 (6, 6'), 124.2 (7, 7'), 141.3 (8, 8'), 134.5 (9, 9'), 134.3 (10, 10'), 124.7 (11, 11'), 139.3 (12, 12'), 136.6 (13, 13'), 133.6 (14, 14'), 130.5 (15, 15'), 27.7 (1, 1'-gem-Me), 14.1 (5, 5'-Me), 12.5 (9, 9'-Me), 13.9 (13, 13'-Me). *m/z*: 564 (M, 100%), 549 (M-15, 20.6%), 508 (M-56, 8.4%), 484 (M-80, 9.4%), 472 (M-92, 44.9%), 458 (M-106, 12.2%).

### 2.3. Liposome Synthesis and Characterization

**2.3.1. Synthesis of Liposome.** Canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes were prepared by thin-film evaporation method as described previously [27]. Briefly, a mixture of canthaxanthin and  $\alpha$ -tocopherol at the fixed mass ratio of 1:9 (mixture A) was dissolved in 2 mL of dichloromethane together with soybean lecithin. The initial concentrations ( $IC = m_{\text{canthaxanthin}}/m_{\text{lipids}}$ , % wt/wt) of canthaxanthin were selected at 0.1%, 0.5%, and 1.0%, respectively. After dissolution, the thin membrane was obtained by removing the organic solvent in the water bath at 30 °C under flask pressure of 250 mbar and rotational speed of level 2. Distilled water (4 mL) was added to peel off the membrane, forming canthaxanthin-loaded liposomes. In order to get a fixed size, liposome was passed through a 100 nm polycarbonate membrane in a mini extruder 50 times. The final sample was sealed in a new eppendorf and kept in the refrigerator (about 4°C in the dark). All other samples (blank liposomes, canthaxanthin-loaded liposomes, and  $\alpha$ -tocopherol-loaded liposomes) were prepared with the same procedure and respective ratios of materials. The structure of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes is shown in Figure 1.

**2.3.2. Size Measurement.** The *z*-average, mean size, and polydispersity index (PDI) of the liposome samples were monitored by using a SZ-100 nanoparticle size and zeta potential analyzer (Horiba, Japan) with a He/Ne laser ( $\lambda = 633$  nm) and scattering angle 90°. The obtained results are the average of at least three measurements.

**2.3.3. Canthaxanthin Loading.** The encapsulation efficiency (EE%) and drug loading content (DL%) were determined by UV-Vis spectrophotometer. Unencapsulated canthaxanthin was removed by dialysis (14000 Da, 10 cm × 10 cm). The suspension was dialyzed for 24 hours against 1 L of distilled water. The distilled water was changed every 2 hours from the start 4 times. The liposomal system was dissolved in 3.5 mL of hexane and sonicated in a bath for 2 min to destroy the liposomal suspensions. After that, 100  $\mu$ L of liposomes mixture was combined with 2900  $\mu$ L of hexane and vortexed for 30 seconds. The free amount of canthaxanthin was quantified spectrophotometrically by Hitachi U-2900

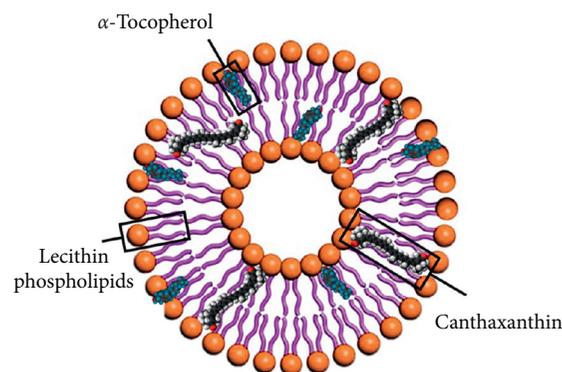


FIGURE 1: The structure of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposome.

spectrophotometer (Hitachi, Tokyo, Japan) at 474 nm with hexane as a blank. The EE % and DL % were determined, respectively, by the following equations:

$$EE\% = \frac{\text{the amount of canthaxanthin in the liposomes}}{\text{the total amount of canthaxanthin}} \times 100,$$

$$DL\% = \frac{\text{mass of canthaxanthin in the liposomes}}{\text{mass of canthaxanthin/liposomes}} \times 100. \quad (1)$$

**2.3.4. In Vitro Drug Release.** First, 1 mL of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes was placed into dialysis bags (molecular weight cutoff = 14000 Da), followed by incubation in 50 mL of phosphate buffered solution (PBS) (pH = 7.4) containing Tween 80 (0.5% wt) at 37°C with gentle shaking (100 rpm). After certain time period, 1 mL of sample was taken from the bag, and an identical volume of fresh buffer was added. Withdrawn media were extracted and analyzed by using UV-Vis spectrophotometer to calculate the cumulative amount of released canthaxanthin. Each experiment was carried out in triplicate and shown as mean  $\pm$  standard deviation (SD).

### 2.4. In Vivo Experiments

**2.4.1. Fish and Diet.** The effects of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes as diet supplemented for feeding rainbow trout were investigated at Sapa Research Center for Coldwater Aquaculture, Research Institute for Aquaculture (Lao Cai Province, Vietnam). A total of 900 fish were individually weighed and randomly distributed into 30 tanks (280 L each). Each tank contained 30 fish, and each replication comprised 10 tanks. One month prior to dietary supplementation, fish were raised on noncolored feeds to have their muscle color returned to original color. Afterwards, each tank in each replication group was fed with one of the ten experimental diets for another 3 months (Table 1). The dietary feeds used in the experiment were produced by Kinh Bac Feed Mill, Bac Ninh province, Vietnam, with the basic nutrient content as follows: total protein 40%, total fat 18%, crude fiber 5%, and energy of 3800 kcal/kg. Fish were

TABLE 1: Composition of artificially composed fish feeds used in the feeding trial.

Diet	Dietary treatments	Weight in diets (mg)		
		Lecithin	$\alpha$ -Tocopherol	Canthaxanthin
I	Commercial diet	—	—	—
II	Commercial diet supplemented with 1 g/kg lecithin	1000	—	—
III	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol	—	100	—
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	—	—	20
V	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol and 20 mg/kg canthaxanthin	—	100	20
VI	Commercial diet supplemented with 1 g/kg $\alpha$ -tocopherol-loaded liposomes	990	10	—
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	990	—	10
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.1%)	990	9	1
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%)	950	45	5
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 1%)	900	90	10

fed at 6 am, 10 am, 2 pm, and 6 pm every day until full. Tank water was continuously replaced with fresh water at the rate of 4 L/min. Water temperature and dissolved oxygen were maintained in the range from 6 to 17.5 °C and from 10 to 11 ppm, respectively. Air pumps were used for water aeration.

**2.4.2. Weight Measurement.** During the trial, fish were periodically weighed every month by taking the mean weight of three randomly caught fish from each treatment group. Growth indicators of fish were determined as follows:

$$\text{Weight gain (WG, \%)} = (W_f - W_i) / W_i \times 100$$

$$\text{Specific growth rate (SGR) } g \text{ day}^{-1} = 100 (\ln W_f - \ln W_i) / t$$

Here,  $W_f$  is the final body weight (g),  $W_i$  is the initial body weight (g), and  $t$  is the experimental duration (days).

**2.4.3. Muscle Canthaxanthin Extraction and Determination.** After three months of feeding trial, fish fillets were collected and measured for color using SalmoFan Lineal colorimeter (DSM Nutritional Products, Kaiseraugst, Switzerland) on a scale of 20 to 34, which is the accepted international benchmark for salmon color (Figure 2). Scores recorded for each animal were made in agreement with three researchers.

Canthaxanthin disposition in fish muscle tissue was determined as follows. First, muscle samples were collected on the fish dorsal and stored at  $-20^\circ\text{C}$ . Then, canthaxanthin was extracted following a previously described procedure [16]. Briefly, after crushing with an IKA basic ULTRA-TURRAX® homogenizer, the samples were extracted with organic solvents, then cleaned, and analyzed by a HPLC chromatograph with the standard canthaxanthin of Sigma Aldrich. The liquid gas chromatography system used the C-18 Hypersil Gold analysis column (5  $\mu\text{m}$ , 150 mm  $\times$  4.6 mm), with flow rate set to 1 mL/min. The retention time was 6 minutes. The process was carried out at the analysis room of the Institute of Chemistry and Natural Compounds.

**2.5. Statistical Analysis.** Experimental data was calculated with mean and standard error. Average data of the treatments was processed by ANOVA on Minitab 16. Duncan comparison was used to differentiate each treatment. The difference is considered significant when  $p < 0.05$ .

### 3. Results and Discussion

**3.1. Extraction of Canthaxanthin.** Canthaxanthin was isolated from extract as an orange powder with melting point 212–213°C. A total of 40 carbons were observed in the  $^{13}\text{C}$  NMR spectrum, and there is a signal of C=O group (C-4/4') at  $\delta$  203.9. The DEPT spectrum indicated ten methyls ( $\delta$  27.7 (C-16, 16'), 27.7 (C-17, 17'), 13.9 (C-20, 20'), 14.1 (C-18, 18'), 12.5 (C-19, 19')), four methylenes ( $\delta$  37.5 (C-2, 2'), 34.3 (C-3, 3')), fourteen methines ( $\delta$  124.2 (C-7, 7'), 141.3 (C-8, 8'), 134.3 (C-10, 10'), 124.7 (C-11, 11'), 139.3 (C-12, 12')), and twelve quaternary carbons ( $\delta$  203.9 (C-4, 4'), 160.9 (C-6, 6'), 136.6 (C-13, 13'), 134.5 (C-9, 9'), 129.9 (C-5, 5'), 35.7 (C-1, 1')) in the molecule. Further, a molecular ion peak was detected in the mass spectrum result at  $m/z$  564, confirming the molecular formula  $\text{C}_{40}\text{H}_{52}\text{O}_2$ .

From  $^1\text{H}$  NMR spectrum, five singlet signals at  $d$  1.20,  $d$  1.20,  $d$  1.87,  $d$  1.99, and  $d$  2.00 were assigned to H-16/16', H-17/17', H-18/18', H-19/19', and H-20/20', respectively. The canthaxanthin spectrum data are given in Table 2. Total amount of canthaxanthin from 1 g dry biomass was 1.5 mg. Yield =  $1.5/1000 \times 100 = 0.15\%$ .

**3.2. Synthesis and Characterization of Canthaxanthin- and  $\alpha$ -Tocopherol-Loaded Liposomes.** Liposomes were made by the established thin-film hydration method followed by extrusion. The physicochemical properties of the optimal blank lecithin liposome, canthaxanthin-loaded liposomes,  $\alpha$ -tocopherol-loaded liposomes, and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes formulation were studied. As shown in Figure 3, the mean size of blank lecithin liposome was  $105.53 \pm 9.02$  (mean  $\pm$  SD;  $n = 3$ ), while the mean sizes of canthaxanthin-loaded liposomes and  $\alpha$ -tocopherol-loaded liposomes were  $97.33 \pm 4.64$  and

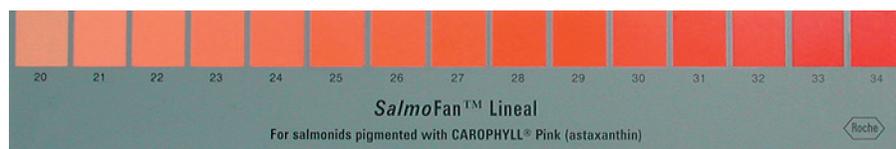


FIGURE 2: DSM SalmoFan Lineal colorimeter.

TABLE 2: NMR data for canthaxanthin in CDCl<sub>3</sub>; 500 MHz.

	<sup>1</sup> H (δ) (500 MHz, CDCl <sub>3</sub> )	<sup>13</sup> C (δ) (500 MHz, CDCl <sub>3</sub> )
1/1'	—	35.8
2/2'	1.85 (m)	37.5
3/3'	2.51 (m)	34.3
4/4'	—	203.9
5/5'	—	129.9
6/6'	—	160.9
7/7'	6.25 m	124.2
8/8'	6.36 d	141.3
9/9'	—	134.5
10/10'	6.27 m	134.3
11/11'	6.68 m	124.7
12/12'	6.40 d	139.3
13/13'	—	136.6
14/14'	6.29 m	133.6
15/15'	6.65 m	130.5
16/16'	1.20 (s)	27.7
17/17'	1.20 (s)	27.7
18/18'	1.87 (s)	14.1
19/19'	1.99 (s)	12.5
20/20'	2.00 (s)	13.9

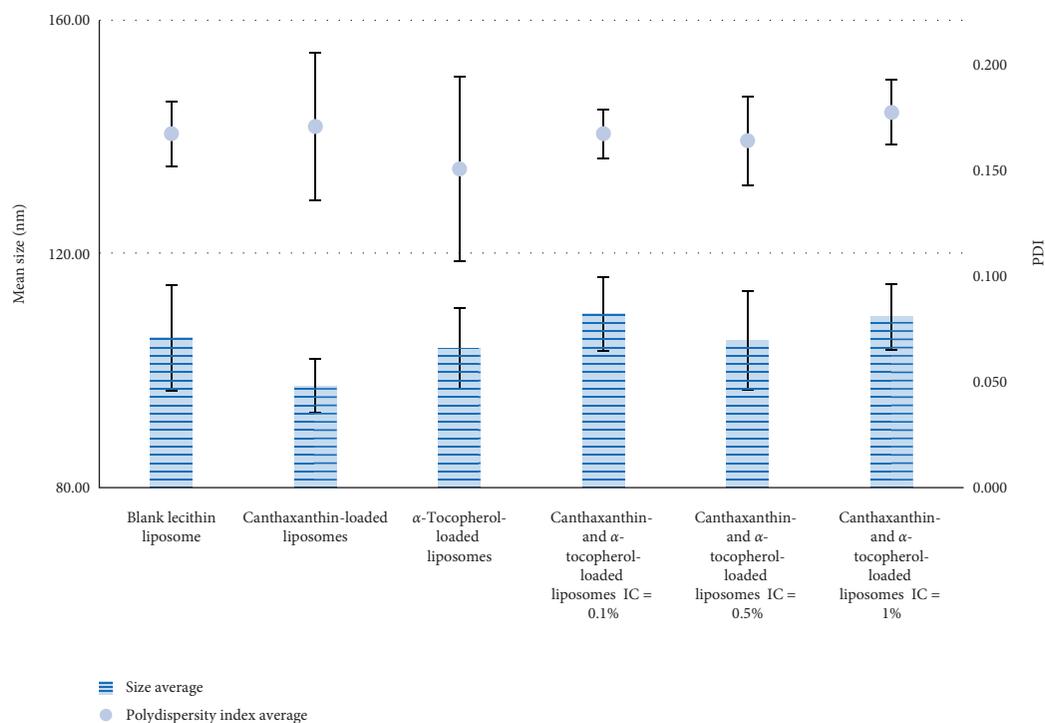


FIGURE 3: Mean size and PDI of blank lecithin liposome, canthaxanthin-loaded liposomes, α-tocopherol-loaded liposomes, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and α-tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and α-tocopherol-loaded liposomes with IC = 1%.

103.80 ± 6.95 (mean ± SD;  $n = 3$ ), respectively. In case of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes, the mean sizes of liposomes with canthaxanthin IC = 0.1%, IC = 0.5%, and IC = 1% were 109.70 ± 6.36, 105.10 ± 8.41, and 109.20 ± 5.66 (mean ± SD;  $n = 3$ ). Polydispersity index (PDI), an indicator of dispersion homogeneity, of all formulated liposomes ranged from 0 and 0.2, suggesting that active ingredients were homogeneously dispersed on the synthesized liposomes. PDI value of  $\alpha$ -tocopherol-loaded liposomes was lowest at 0.150 ± 0.044 (mean ± SD;  $n = 3$ ). All other liposomes have higher PDI values but remain lower than 0.2. There were no significant differences of the mean size and PDI between blank lecithin liposome and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes.

The encapsulation efficiency (EE%) and drug loading content (DL %) of canthaxanthin-loaded liposomes were 59.6 ± 2.3% (mean ± SD;  $n = 3$ ) and 2.39 ± 0.23% (mean ± SD;  $n = 3$ ), respectively (Table 3). The encapsulation efficiency of canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes decreased when increasing the concentration of canthaxanthin in lecithin liposome. EE% values of liposomes loaded with canthaxanthin at IC = 0.1%, IC = 0.5%, and IC = 1% were 85.3 ± 2.1, 72.9 ± 1.8, and 55.3 ± 2.6, respectively.

The role of lipid layer in protecting core materials of the liposome is accentuated by the fact that active ingredients such as carotenoids easily degrade under acidic microenvironments and by the influence of enzymes. *In vitro* release profiles of canthaxanthin from canthaxanthin-loaded liposomes, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 1% in PBS at pH 7.4 are shown in Figure 4. Within the first 4 hours, release of canthaxanthin from liposomes formed without  $\alpha$ -tocopherol seemed to be more rapid than other liposomes. By contrast, liposomes loaded with canthaxanthin at IC = 0.1% showed slower, sustained release rate, which reached 0.46 after 4 h. In addition, increasing the loaded canthaxanthin concentration apparently induced faster canthaxanthin release within the first 1 h. After 4 h of incubation, approximately 68% of the encapsulated canthaxanthin was released from the liposome containing 1% canthaxanthin. These data suggest that canthaxanthin can be well encapsulated in liposomes and released in an extended period.

**3.3. Fish Growth.** The culture of rainbow trout was carried out in the winter to ensure suitable growth conditions and water temperature [8]. Dissolved oxygen in experiment was controlled and ranged from 7.1 to 10.9 mg/l, with an average of 8.2 mg/L [28, 29].

Initially, weight of the fish was 309.43 ± 12.98 g before being raised with feeds, and there was no difference between groups. Mean wet weights of fish were recorded monthly during feeding trial and once before filleting. During the experiment, the fish remained in good health, and the mortality rate was 0%. At the end of the feeding trial, fish fed with diet supplemented with 1 g/kg canthaxanthin- and

$\alpha$ -tocopherol-loaded liposomes (IC = 0.5%) showed significantly higher average weight than those of other experimental control groups ( $p < 0.05$ ) (Figure 5).

Rainbow trout fed with diet supplemented with canthaxanthin at 20 mg/kg (diet IV) showed significantly lower wet weights ( $p < 0.05$ ) after two, three, and four months compared to other groups. This finding is in line with that reported by Torrisen et al. [17] where increased dose of fed canthaxanthin resulted in the lower weight gain in cultured rainbow trout. Final weights of fish in groups fed with diets supplemented with 1 g/kg lecithin or 100 mg/kg  $\alpha$ -tocopherol were higher, albeit not statistically significant ( $p > 0.05$ ), than those fed with unsupplemented commercial diet. In case of groups fed with diets supplemented with canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes (diets VII, IX, and X), their weight gain was higher when increasing the concentration of canthaxanthin from 0.1% to 0.5% but was stagnant when further increasing concentration of canthaxanthin from 0.5 to 1% (not significant difference).

Current results are similar to those of previous reports pointing out that inclusion of carotenoids such as canthaxanthin or astaxanthin in fish feed affected the growth rate and efficiency of food usage of freshwater salmon or parrot fish [30, 31]. In addition, several studies also reported that carotenoids could enhance growth by acting as fertilization hormones, but, up to date, biological functions of the carotenoids in fish have not been unconfirmed [32, 33].

Table 4 shows the results of the muscle proximate composition of rainbow trout after 3 months of feeding with various experimental diets. No significant differences were recognized among 10 treatment groups in all indicators (crude protein, crude lipid, ash, and moisture). These findings are not consistent with previous reports demonstrating that dietary canthaxanthin deficiency can affect digestion and absorption of lipid. To be specific, it was found that dietary supplementation of carotenoids did affect digestion and absorption of lipid in rats [34]. Similarly, for aquatic animals, Brizio et al. noted that canthaxanthin affected the digestion of lipid in fish [35]. Nevertheless, the effects of canthaxanthin and  $\alpha$ -tocopherol on fish muscle composition are still controversial and complex, and further studies are needed to reveal their mechanisms.

**3.4. Muscle Canthaxanthin.** At the beginning of the experiment, the color of fish muscle was uniform among experimental groups with the color score ranging from 20.1 to 20.9 (Table 5). After 1 month of feeding, the color began to show differences between treatments ( $p < 0.05$ ). The highest score (27.0) was recorded in fillets of fish fed with commercial diet supplemented with 100 mg/kg  $\alpha$ -tocopherol and 20 mg/kg canthaxanthin (diet V), closely followed by that of diet IV and diet IX (26.9). The lowest score of 23.1 was recorded in diet I, which is in absence of canthaxanthin. After 2 months, the difference in color of the muscle became more apparent (Figure 6). However, towards the end of the experiment, improvements in color score were less noticeable, which is consistent with previous studies. This result is similar to previous research

TABLE 3: EE and DL of blank lecithin liposome, canthaxanthin-loaded liposomes,  $\alpha$ -tocopherol-loaded liposomes, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 1%.

Liposomes	EE (%)	DL (%)
Blank lecithin liposome	—	—
Canthaxanthin-loaded liposomes	59.6 $\pm$ 2.3	2.39 $\pm$ 0.23
$\alpha$ -Tocopherol-loaded liposomes	—	—
Canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes, IC = 0.1%	85.3 $\pm$ 2.1	1.87 $\pm$ 0.35
Canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes, IC = 0.5%	72.9 $\pm$ 1.8	2.09 $\pm$ 0.13
Canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes, IC = 1%	55.3 $\pm$ 2.6	2.23 $\pm$ 0.21

Numbers resulted from triplicate measurements and are presented as mean value  $\pm$  standard deviation.

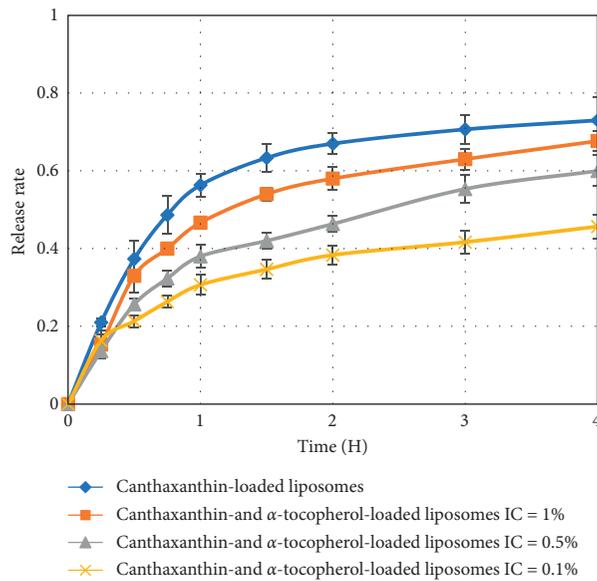


FIGURE 4: *In vitro* release profile of canthaxanthin from canthaxanthin-loaded liposomes, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.1%, canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 0.5%, and canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes with IC = 1% in PBS at pH 7.4.

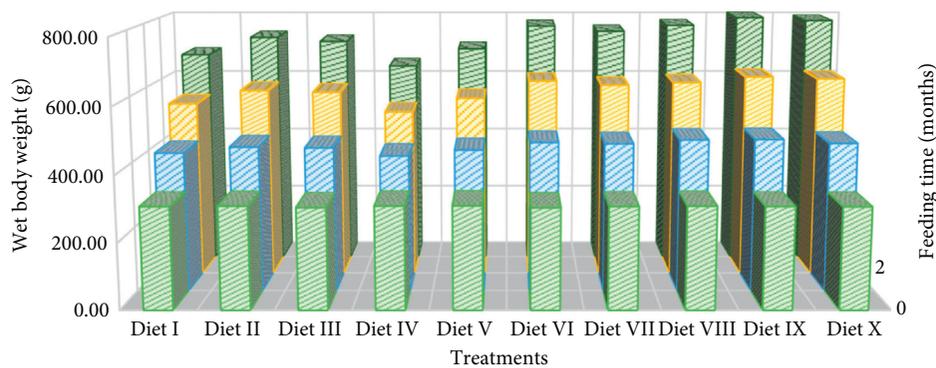


FIGURE 5: Weight variations of rainbow trout groups fed with different diet formulations. Feeding time of 0 indicates the initial time point where experimental feeding began.

with the astaxanthin and canthaxanthin supplementation at the ratio of 40:40 mg/kg of food [36]. In addition, Choubert et al. and Torrissen et al. also reported that the color of the fish muscle would gradually not increase and reach saturation, even with increased concentration or longer feeding [10, 17].

The concentration of canthaxanthin accumulated in fish muscle in different treatments after 90 days of culturing is shown in Figure 7. After 90 days of feeding, fish fed with different experimental diets showed varying concentrations of canthaxanthin accumulated in the fish muscle ( $p < 0.05$ ). Canthaxanthin content in muscle was highest (2.91 mg/kg)

TABLE 4: Proximate composition of the muscle of rainbow trout after 3 months of feeding with experimental diets ( $n = 3$ , unit: % wet weight).

Diet	Experimental diets	Crude protein	Crude lipid	Ash	Moisture
I	Commercial diet	20.19 ± 1.15	6.79 ± 1.10	1.83 ± 0.12	73.79 ± 1.73
II	Commercial diet supplemented with 1 g/kg lecithin	18.89 ± 1.12	7.43 ± 1.09	1.75 ± 0.37	73.25 ± 0.81
III	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol	19.22 ± 1.32	7.42 ± 1.04	1.57 ± 0.22	72.21 ± 0.64
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	20.07 ± 2.37	8.67 ± 2.33	1.72 ± 0.17	71.57 ± 1.12
V	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol and 20 mg/kg canthaxanthin	19.86 ± 1.86	7.51 ± 1.56	1.68 ± 0.34	73.13 ± 0.79
VI	Commercial diet supplemented with 1 g/kg $\alpha$ -tocopherol-loaded liposomes	20.25 ± 1.06	6.32 ± 1.18	1.23 ± 0.23	72.13 ± 1.46
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	19.29 ± 1.19	7.29 ± 1.24	1.97 ± 0.28	70.22 ± 0.91
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.1%)	19.57 ± 1.28	7.12 ± 1.06	1.72 ± 0.21	73.15 ± 0.94
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%)	20.25 ± 2.17	8.13 ± 2.43	1.65 ± 0.12	74.75 ± 1.02
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 1%)	19.26 ± 1.69	7.91 ± 1.96	1.83 ± 0.24	71.03 ± 0.94

Figures resulted from triplicate measurements and are presented as mean ± SE.

TABLE 5: Color scores of fillets taken from rainbow trout treated with different formulated diets at the initial time point and after one, two, and three months of experimental feeding ( $n = 3$ ).

Diet	Experimental diets	0 th month	1st month	2nd month	3rd month
I	Commercial diet	20.56 ± 0.88	23.11 ± 1.10	24.67 ± 1.10	25.00 ± 1.41
II	Commercial diet supplemented with 1 g/kg lecithin	20.33 ± 0.71	23.22 ± 1.20	24.78 ± 1.20	25.22 ± 1.39
III	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol	20.56 ± 1.13	23.22 ± 1.00	24.44 ± 1.00	25.11 ± 1.27
IV	Commercial diet supplemented with 20 mg/kg canthaxanthin	20.89 ± 0.78	26.67 ± 1.00	28.33 ± 1.00	29.78 ± 1.20
V	Commercial diet supplemented with 100 mg/kg $\alpha$ -tocopherol and 20 mg/kg canthaxanthin	20.11 ± 0.78	27.00 ± 1.1	29.11 ± 0.80	30.56 ± 1.01
VI	Commercial diet supplemented with 1 g/kg $\alpha$ -tocopherol-loaded liposomes	20.44 ± 1.01	23.33 ± 0.70	24.89 ± 1.10	25.11 ± 1.54
VII	Commercial diet supplemented with 1 g/kg canthaxanthin-loaded liposomes (IC = 1%)	20.33 ± 1.00	25.89 ± 0.80	28.11 ± 0.80	29.11 ± 0.78
VIII	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.1%)	20.33 ± 1.00	25.00 ± 1.40	27.11 ± 1.30	28.78 ± 1.30
IX	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%)	20.67 ± 1.00	26.67 ± 0.70	28.89 ± 0.80	30.00 ± 0.71
X	Commercial diet supplemented with 1 g/kg canthaxanthin- and $\alpha$ -tocopherol-loaded liposomes (IC = 1%)	20.44 ± 1.13	26.89 ± 0.80	29.33 ± 1.10	30.22 ± 1.39

Color values for each muscle were made in agreement with three researchers. Figures resulted from triplicate measurements and are presented as mean ± SE.

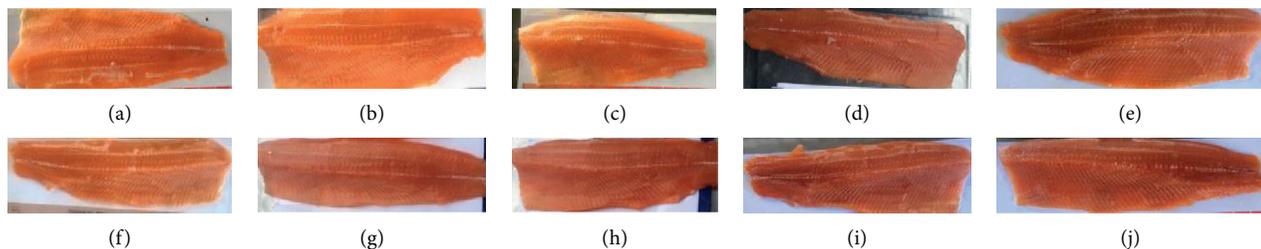


FIGURE 6: Muscle coloration of flesh at the end of experiments: (a) diet I; (b) diet II; (c) diet III; (d) diet IV; (e) diet V; (f) diet VI; (g) diet VII; (h) diet VIII; (i) diet IX; (j) diet X.

in the group fed with diet supplemented with 1 g/kg canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%), followed by diet supplemented with 1 g/kg canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes (IC = 1%) (2.90 mg/kg) and commercial diet supplemented with 100 mg/kg  $\alpha$ -tocopherol and 20 mg/kg canthaxanthin (2.75 mg/kg). The lowest canthaxanthin content in muscle

was found in the group fed with commercial diet supplemented with 1 g/kg lecithin (0.99 mg/kg). However, between diet IX and diet V, there is no statistically significant difference in canthaxanthin disposition.

Economic viability of diet IX (1 g/kg canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes, IC = 0.5%) is also justified by the prices of canthaxanthin and  $\alpha$ -tocopherol. To be

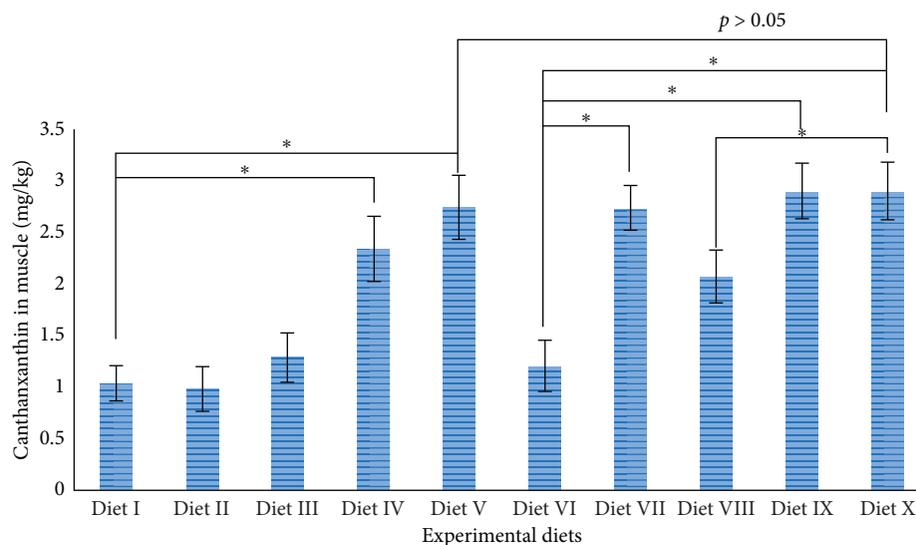


FIGURE 7: Canthaxanthin concentrations in muscle samples.

specific, while the price for canthaxanthin ranges between € 528 and € 4,278 per kilogram (based on commercial suppliers), the average global price of Vitamin E50 is negligible, at around \$ 8.5 per kilogram [37]. This suggests that the cost of canthaxanthin in the composition of diet IX is approximately four times lower than those of commercial fish feed supplemented with 20 mg/kg of unencapsulated canthaxanthin and that formulation of liposomal fish diet carries important implications for reducing carotenoid feeding while still meeting international standards.

According to Torrissen et al., canthaxanthin disposition in rainbow trout muscle was proportional to the ratio of additional canthaxanthin in food [17]. However, with the inclusion of canthaxanthin at a concentrations higher than 50 mg/kg, the accumulation of canthaxanthin in the rainbow trout muscle decreased gradually and stopped when reaching saturation.

Interestingly, canthaxanthin deposition in fish flesh increased in the presence of  $\alpha$ -tocopherol in the feed (diet V), which is in line with findings reported by Choubert et al. [10] proposing that, compared to feeding using bare canthaxanthin, the use of liposomes formed from lecithin and  $\alpha$ -tocopherol could reduce the required amount of canthaxanthin needed to achieve the same color score. Further experiments are needed to clarify this assertion.

#### 4. Conclusions

In the present study, liposomes containing  $\alpha$ -tocopherol and canthaxanthin as active ingredients were fabricated and included in fish feeds of cultured rainbow trout. There was no difference in growth rate, survival rate, or flesh nutritional composition between cultured rainbow trout fed with diets supplemented with bare lecithin,  $\alpha$ -tocopherol, and canthaxanthin and those fed with liposomes formed from lecithin,  $\alpha$ -tocopherol, and canthaxanthin. However, the color and canthaxanthin disposition in fish muscle of fish fed with diets containing 1 g/kg

canthaxanthin- and  $\alpha$ -tocopherol-loaded liposomes (IC = 0.5%) were similar to those of fish fed with commercial diet supplemented with 20 mg/kg of unencapsulated canthaxanthin. However, the experimental diet containing liposomal canthaxanthin and  $\alpha$ -tocopherol only required half of the amount of canthaxanthin compared to the diet supplemented with bare canthaxanthin. These results suggest the feasibility of using liposome formation techniques in manufacture of fish diet supplements. Further studies should focus on mechanisms of canthaxanthin pigmentation on fish flesh and evaluate industrial-scale utilization of supplemented fish diets.

#### Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

#### Conflicts of Interest

The authors declare no conflicts of interest.

#### Authors' Contributions

Quoc Toan Tran, Viet Anh Dang, Nguyen Thanh Duong, Phi Hung Nguyen, Thu Huong Trinh Thi, Thuy Ha Tran, Van Thinh Do, Van Khoi Le, Xuan Luong Ngo, Pham Tri Nhut, Hai Ha Pham Thi, and Manh Van Do carried out investigation; Pham Quoc Long supervised the work; and Quoc Toan Tran wrote the original draft.

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