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The Effect of Dietary Carotenoids of Different Forms: Microemulsified and Non-microemulsified on the Growth Performance, Pigmentation and Hematological Parameters in Hybrid Catfish *(Clarias Macrocephalus × Clarias Gariepinus)*

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Abstract

We tested the feasibility and effect of including dietary carotenoids of different forms, microemulsified (MY) and non-microemuslified (NMY), in diets for hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). The results showed that the abdominal skin and back muscle yellowness (b^*) of fish fed with 0.7 kg/t MY was higher than the other treatments (1.0 kg.t NMY and control) ($p<0.05$) with a score of 8.30 and 16.33 respectively. This group also gave the highest total carotenoid deposited in the muscle of 88.27 mg/kg. Carotenoid addition to the control diet at 1.0 kg/t NMY and 0.7 kg/t MY dosage have no adverse effect on the growth, in fact they helped to improve the body weight gain by 82.98 g and 84.17 g ($p<0.05$) with an FCR improvement of 13 points and 16 points ($p<0.05$) respectively. There was no adverse effect on the immune system after feeding carotenoid to the catfish and enhancement was observed in certain immune response indices when compared to the control. The findings in the present study were very significant because it supported that microemulsified yellow carotenoids (with smaller particle size of ~0.25 µm) and when used at lower inclusion rate of 30% could achieve better overall performance when compared to the regular size carotenoids and control due to its enhanced bioavailability.

Keywords: Microemulsified; Bioavailability; Carotenoids; Pigmentation; Hybrid catfish; Growth; Hematological parameters

Introduction

In aquaculture, maintenance of the natural skin pigmentation is of great importance from a commercial point of view, as it has a direct impact on consumer acceptance or rejection [1,2] as well as product market price. A variety of natural and synthetic carotenoids are available to enhance coloration in the flesh of salmonid fish [3] and in the skin of others such as European red porgy, *Pagrus pagrus* [4]. Both synthetically produced pigments, astaxanthin and canthaxanthin, either alone or in combination, have been efficiently used as dietary additives for muscle pigmentation in salmonids [5]. While synthetic carotenoid pigments are commercially available as feed additives, they are expensive and up-take levels are poor, estimated between 5% and 10% [6]. Moreover, there is increasing consumer awareness about safety of synthetic feed additives. This increases the interest in use of natural carotenoids for some fish and shrimp species of economic interest. Carotenoids, which are lipid-soluble pigments, are divided into two groups: (red) capsanthin and (yellow) xanthophylls. The carotenoids are also vital nutrients for healthy growth, metabolism and reproduction [7]. Since fish, like other animals, are not able to synthesize carotenoids [8,9], they have to obtain carotenoids from dietary sources. Fishes can modify alimentary carotenoids and store them in the integument as well as other tissues and organs such as skin, flesh, gonads, kidney, liver, intestines and only in very small amounts in the brain. Wild carnivore fish obtain most of their carotenoids by feeding on small crustaceans and other vertebrates previously fed on algae. However, when fish are deprived from their natural sources of food, under rearing conditions, fish depend entirely on added dietary carotenoid intake to achieve its natural coloration. Accordingly, feeding fish with carotenoid pigments is a universally accepted strategy in salmonids culture to produce the desired colour normally associated with these fish. In addition to their role in pigmentation, carotenoids may have various biological effects in fish such as supplying provitamin A, anti-oxidation (involving lipid peroxidation) or immune-enhancement [10].

To improve colour yield through increased bioavailability, various methods have been devised with the objective of reducing the particle size of the active ingredient. A common approach is through the use of microemulsions. Microemulsions are thermodynamically stable, transparent, low viscosity and isotropic dispersions consisting of oil and water, stabilized by an interfacial film of surfactant molecules, typically in conjunction with a co-surfactant. In particular, bicontinuous microemulsion [11] has been a widely studied system due to its unique structure that lends itself well to controlled release applications. Amphiphilic molecules form bicontinuous water and oil channels, where "bicontinuous" refers to two distinct (continuous, but non-intersecting) hydrophilic regions separated by bilayers. This allows for simultaneous incorporation of water- and oil-soluble active ingredients. The phase structure also provides a tortuous diffusion pathway for controlled release of the encapsulated ingredients. In our earlier work [12,13], we have showed the enhanced solubility and dissolution of carotenoids can be achieved in bicontinuous microemulsions (liquid system) containing polyethoxylated sorbitan ester (Tween 80), water, limonene, ethanol and glycerol. This system is able to prepare stable bicontinuous carotenoid microemulsions of droplet size ~0.25 µm upon mild agitation in liquid media. We hypothesized that the fine droplets of microemulsions have the advantage of presenting the

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carotenoids in a dissolved form, with a large interfacial surface area for absorption, which will result in an enhanced, more uniform and reproducible bioavailability. Indeed, we have been able to demonstrate the possibility of using the microemulsified carotenoids in enhancing the bioavailability over corresponding regular size carotenoid preparations, leading to greater yolk pigmentation at lower inclusion rate in layers. With this success, an attempt has been made to look at using the microemulsified carotenoids for skin and flesh pigmentation in aquaculture. In this present work, we would like to test the feasibility and effect of including dietary carotenoids of different forms, microemulsified (MY) and non-microemuslified (NMY), in diets for hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). This study was also designed to test the hypothesis that microemulsified carotenoids can give improved bioavailability over corresponding regular size carotenoid preparations, leading to improved growth, pigmentation and immune response indices of the hybrid catfishes at 30% lower inclusion rate.

Materials and Methods

Production of carotenoid products

The microemulsified yellow (MY) and non-microemulsified yellow (NMY) (marigold flower extract (*Tagetes erecta*) contains mainly the natural yellow pigments lutein/lutein esters (>80%) and zeaxanthin (~5%)) were produced using Kemin© Industries' (Asia) pilot production facility. Briefly, silica and wheat pollard were first added into a ribbon mixer. MY or NMY concentrate containing the saponified yellow concentrate was then sprayed into the mixer at a rate of 15Hz with constant mixing at 28rpm for 40 minutes until a homogenous mixture was obtained. The targeted total xanthophyll content for both MY and NMY products is at min 20.5 g/kg. Homogeneity of both the MY and NMY products were assessed by analysing 6 independent bags measured by HPLC following the official AOAC (Association of Analytical Communities) method number 970.64 (1990). For MY product, the average analysed total xanthophyll was 20.58 g/kg ± 0.242 while the average total xanthophyllanalysed for NMY product was 20.62 g/kg ± 0.733.

Particle size analysis and transmission electron microscopy observations of carotenoid products

The particle size analysis of the MY and MNY was carried out using a particle size analyzer (HORIBA SZ-100Z). To observe the morphology, the MY was directly deposited onto carbon film supported by copper grids, stained with a 1% aqueous solution of osmium tetroxide (OsO_4) and investigated using the transmission electron microscope (TEM) JEOL 1010. The morphology of the NMY was also determined for comparison.

Trial specifications

The trial was conducted in the Laboratory of Nutrition and Aquafeed, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. Hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*) produced under captivity, with an initial body weight of 45-55g, was randomly divided into nine groups of 15 fishes each (three treatments, three replicates). Each group was stocked in a 500L tank and the fishes were allowed to acclimatise one week prior to the start of the experiment. The fishes were hand-fed to apparent satiation, corresponding to 3-3.5% of the body weight, thrice daily (08:30, 12:30 and 16:30) for 12 weeks. During the feeding trial, the water temperature ranged from 27 to 30°C, pH and dissolved oxygen content of water was greater than 7.2 and 76mg/L respectively for the duration of the study.

Three treatment diets containing different levels of carotenoids (0 kg/t (C), 1.0 kg/t NMY (T1) and 0.7 kg/t MY (T2) were prepared. Ingredients and proximate composition of the experimental diets are given in Table 1. The experimental diets were formulated and pelletized using a 3-mm pellet press. The amount of feed consumed per tank and per treatment was recorded and monitored throughout the feeding trial. At the end of the feeding trial, fish of each tank were collectively weighed and the fish weight gain, feed conversion ratio and specific growth rate were determined.

Colorimetric and total carotenoids analysis

Colour analysis was performed every four weeks by reflective spectroscopy with a Minolta color reader CR-10 colorimeter in accordance with the system CIE L*a*b* (CIELAB) for lightness, redness, and yellowness, respectively [14]. The measurements were performed on skin and muscle areas of the fish's body. Total carotenoid content in the fish's muscle was determined after extraction with acetone. For carotenoid extraction, sample was weighted and 60 ml acetone and some sodium sulphate anhydrous were added. The mixture was ground and filtered through glass microfiber filters (GF/A, whatman paper) and rinsed with chloroform to increase the boiling point of the mixture. After mixing and phase separation between diethyl ether and water in separatory funnel, the upper layer was taken and placed in a round bottle flask to evaporate in a rotary evaporator at 35°C. The extract was concentrated and dissolved in benzene. Total carotenoids concentration in the muscle was determined spectrophotometerically in benzene using E (1%, 1 cm) = 2500 at 460 nm for yellow carotenoid.

Haematological assay

Blood was analysed with routine methods used in fish haematology [15,16]. In short, blood was collected from the caudal vein with 1 mL nonheparinized disposable syringes fitted with 0.55 × 25 mm disposable needles. Blood samples (approximately 1 mL/fish) were centrifuged at 300 × g, 25°C for 10 min. A volume of 500 uL of the serum was removed and vortexed with 1 mL of ethanol for 30s, then 2 mL of petroleum ether was added, and the mixture was vortexed for 1 min. The petroleum ether was separated by centrifuging at 300 × g, 25°C for 10 min. Red

Ingredients (%)	Experimental diets		
	C	T1	T2
Soybean	25	25	25
Poultry meal	10	10	10
Wheat gluten	3	3	3
Canola/rapeseed	5	5	5
Deoil-rice bran	5	5	5
Tapioca	26.67	26.62	26.57
Dehull full fat soybean meal	15	15	15
Soy protein concentrate	5	5	5
Crude fish oil	1	1	1
Choline	0.3	0.3	0.3
Vitamin C	0.2	0.2	0.2
Lysine	0.2	0.2	0.2
Methionine	0.23	0.23	0.23
Di-calcium phosphate	2.3	2.3	2.3
Limestone	0.1	0.1	0.1
Vitamin premix	1	1	1
NMY (non-microemulsified yellow)	0	0.1 (1.0 kg/t)	0
MY (microemulsified yellow)	0	0	0.07 (0.7 kg/t)

Table 1: Details of experimental treatments and dosages of test additives.

and white blood cell count was determined with chamber method using Neubauer's haemocytometer; haemoglobin concentration with cyanmethaemoglobin method [17] and haematocrit in capillary tubes of 75 µL volume, which were centrifuged in a microhaematocrit centrifuge and the haematocrit values were read with a reader. The total protein determination followed method [18].

Statistical analysis

Significant differences among treatment groups were tested by one-way analysis of variance (ANOVA) and the comparison of any values was made by Duncan's multiple range tests. A significance level of $p<0.05$ was used. The statistical analysis was performed using Statgraphics 5.1.

Results

Particle size analysis and transmission electron microscopy observations of carotenoid products

The MY carotenoids were found to be approximately 0.25 µm in size, as analyzed by electron microscopy and light-scattering diffraction study (Figure 1). This was a dramatic reduction from the approximately 20 m size measured for NMY. The results also suggested that the surfactant and oil phases used in this study not only influenced the formation of protective colloid responsible for establishing colloidal stability against agglomeration but also facilitating the uptake of carotenoids during the intestinal passage.

Growth performance and feed utilization

The effects of carotenoid diets on the growth parameters for the fishes throughout the experimental periods are given in Table 2. All fish grew normally, and no specific signs of disease were observed (Figure 2). No mortality occurred throughout the experiment. Feed intake among treatments showed no significant differences ($p<0.05$), so that total carotenoids intake was proportional to dietary carotenoid concentration. The results showed that carotenoid addition to the control diet at 1.0 kg/t of NMY and 0.7 kg/t of MY helped to improve body weight gain by 82.98g and 84.17g ($p<0.05$) with an FCR improvement of 13 points and 16 points ($p<0.05$) respectively.

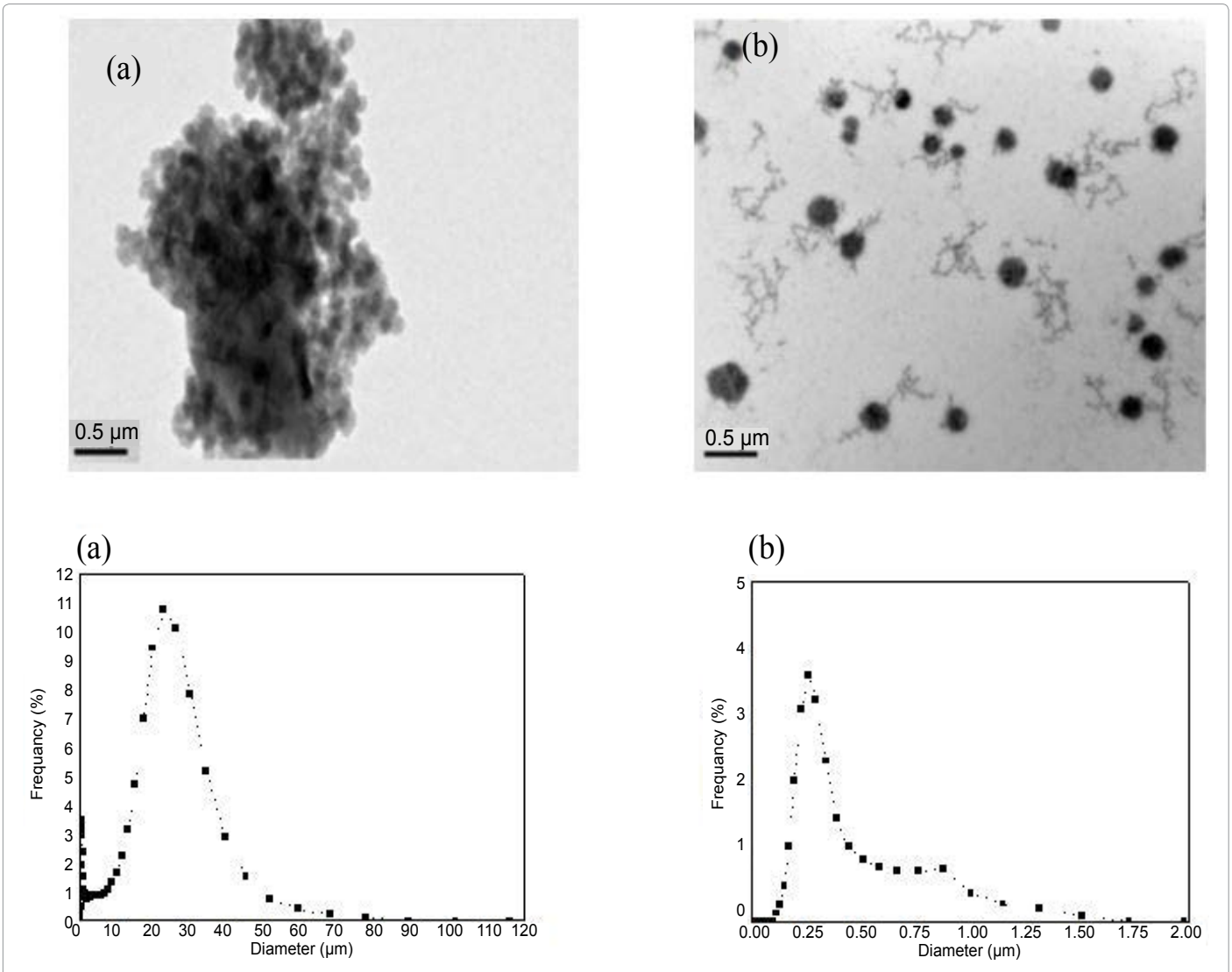

Figure 1: Particle size analysis by transmission electron microscopy and light scattering diffraction study of the (a) non-microemulsified (NMY) and (b) microemulsified yellow carotenoids (MY).

However, similar growth performance achieved even when dosage used for MY is at 30% lower than that of NMY ($p<0.05$).

Colorimetric and total carotenoids analysis

Colour intensity of hybrid catfish fed with experimental diets throughout the experimental periods is shown in Table 3. Lightness (L) was not affected by carotenoid supplementation ($p<0.05$), although the white skin of these groups changed slightly from a white hue to a yellow hue. There were, however, significant ($p<0.05$) differences in yellow (b^*) among the treatment groups. The group fed the control diet showed a weak redness and yellowness, which differed significantly from values found for group fed the other diets. Yellow tonality was best in fish fed the diet supplemented with 0.7 kg/t MY followed by 1.0 kg/t NMY and the control (Figure 3). The highest numerical values were obtained for the groups fed with diets containing 0.7 kg/t MY (with a score of 8.30 and 16.33 for abdominal skin and back muscle, respectively). The effect of different experimental diets on the total carotenoids in muscle was also determined (Table 3). Total carotenoids measured in fish fed with 0.7 kg/t MY is the highest compared to the other treatments ($p<0.05$).

Haematological assay

Fishes fed on carotenoid diets exhibited increasing RBCs counts for the fishes fed with 1.0 kg/t NMY and 0.7 kg/t MY, respectively ($p<0.05$; Table 4). The highest counts of WBC's were obtained for the 1.0 kg/t NMY treatment (1.28×10^5 cell/mL, $p<0.05$). Dietary carotenoids significantly affected the haemoglobin of fishes (7.19 and 7.23 g/dl for 1.0 kg/t NMY and 0.7 kg/t MY, respectively). Haematocrit percentage was higher for carotenoid fed fish compared to the control fish ($p<0.05$). Similarly, the total protein measured was also higher for the carotenoid fed fish ($p<0.05$) and the highest value was observed for 0.7 kg/t MY diet.

Discussion

We tested the feasibility and effect of including dietary carotenoids of different forms, microemulsified and non-microemulsified, in diets for hybrid catfish (*Clarias macrocephalus* × *Clarias gariepinus*). The data in this study showed neither the growth nor the feed conversion efficiency were affected significantly. As a micronutrient, the effects of carotenoids on fish are discreet and are not easily translated into somatic growth. However, its positive role in the intermediary metabolism of fish [19,20] could enhance nutrient utilization and may ultimately result in improved growth. A few studies have reported enhanced growth with carotenoid supplementation [21] whereas others claim reduced feed conversion [22].

Rearing parameter	Experimental diets		
	C	T1	T2
Weight gain (g)	120.20 ± 4.72^a	126.48 ± 3.50^{ab}	126.39 ± 6.18^{ab}
Average weight gain (g/fish/day)	75.79 ± 5.49^a	82.98 ± 3.16^{ab}	84.17 ± 5.0^{ab}
Specific growth rate (%/day)	1.17 ± 0.05^a	1.23 ± 0.08^{ab}	1.23 ± 0.03^{ab}
Feed conversion ratio (FCR)	1.58 ± 0.11^a	1.45 ± 0.04^{ab}	1.42 ± 0.08^{ab}
Daily feed consumed (g/fish/day)	1.42 ± 0.02^a	1.42 ± 0.02^a	1.42 ± 0.02^a
Survival rate (%)	100	100	100
Mean with different superscripts in the same rows are significantly different ($p<0.05$)			

Table 2: Growth performance parameters of hybrid catfish fed with experimental diets for 12 weeks.

Figure 2: Digital image of hybrid catfish fed with experimental diets over 12 weeks: (C) Control, (T1) 1.0 kg/t NMY and (T2) 0.7 kg/t MY.

Experimental diets	L	a*	b*
	Abdominal skin		
C	67.23 ± 3.16^a	0.07 ± 0.15^a	0.57 ± 0.42^a
T1	70.00 ± 1.7^b	-0.77 ± 0.38^b	7.73 ± 1.29^b
T2	70.53 ± 1.26^b	-0.73 ± 0.35^b	8.30 ± 0.78^b
	Back muscle		
C	45.13 ± 0.80^a	2.43 ± 0.45^a	10.57 ± 1.25^a
T1	49.20 ± 1.92^a	4.43 ± 1.00^b	16.47 ± 4.51^b
T2	49.30 ± 0.75^a	4.53 ± 0.85^b	16.33 ± 1.36^b
	Total carotenoid (mg/kg) in muscle		
C	22.80 ± 6.31^a		
T1	62.45 ± 11.08^{ab}		
T2	88.27 ± 21.18^b		
Mean with different superscripts in the same columns are significantly different ($p < 0.05$)			

Table 3: Body color intensity and total carotenoid of hybrid catfish fed with experimental diets over 12 weeks (L= Lightness, a* = Red and b* = Yellow).

In contrast to growth, addition of dietary carotenoid supplements significantly increased total carotenoid deposited in the catfish muscle and enhanced yellowness in skin and muscle colour. Skin lightness was the only color parameter not influenced positively by carotenoid enhancers, which is not surprising considering the generally negative correlation observed between this color variable and skin carotenoid concentration in other species [23,24]. From this study, bioavailability

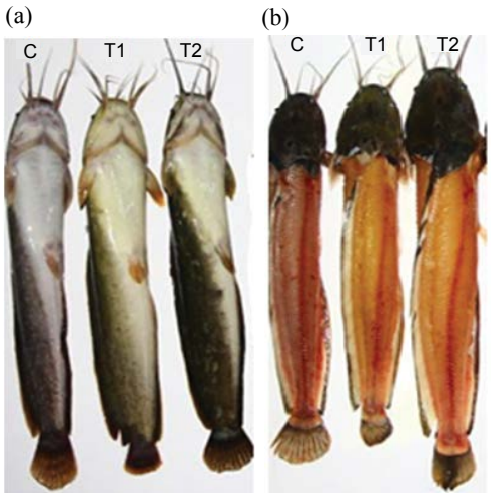

Figure 3: Digital image of (a) abdominal skin and (b) muscle for color measurement of hybrid catfish fed with experimental diets over 12 weeks: (C) Control, (T1) 1.0 kg/t NMY and (T2) 0.7 kg/t MY.

Hematological parameter	Experimental diets		
	C	T1	T2
Red blood cell (RBC) (x 10^6cell/ml)	1.59 ± 0.06^a	1.64 ± 0.05^a	1.64 ± 0.06^a
White blood cell (WBC) (x 10^5cell/ml)	1.20 ± 0.08^a	1.28 ± 0.07^a	1.25 ± 0.08^a
Hemoglobin (Hb) (g/dl)	6.64 ± 0.13^a	7.19 ± 0.35^b	7.23 ± 0.37^b
Hematocrit (HCT) (%)	36.00 ± 1.00^a	39.33 ± 1.00^b	39.33 ± 1.50^b
Total protein (mg/dl)	6.18 ± 0.13^a	6.32 ± 0.1^{ab}	6.44 ± 0.1^{ab}

Table 4: Hematological factors of hybrid catfish fed with experimental diets for 12 weeks.

of carotenoids prepared using microemulsion system was increased. There are a number of possible reasons for this increase. Firstly, microemulsified pigment molecules present a larger surface area, and may be acted on more quickly, physiologically. Secondly, reducing the size may enable carotenoid molecules to better penetrate the intestinal epithelium, increasing their residence time and enhancing absorption. Thirdly, smaller pigment particles may be more efficiently absorb through the intestinal mucosa [25], transport them through the blood via serum lipoproteins [26], metabolically oxidize them to other forms [27], and deposit them into specialized skin cells called chromatophores [28]. One thing is for sure, the ratio of surface area to volume of the smaller carotenoid structures was found to have increased dramatically after the emulsification, by approximately 80 orders of magnitude. As such, improved efficiency in carotenoid delivery, uptake and utilization was expected to be significant. Apart from an increase in pigment absorption, colour intensity of the fish skin and muscle is possibly dependent also on the pigment particle size. Perhaps at the nanometer range, the number of carotenoid molecules that can be packed at the surface has increased, leading to an increase in light absorption and scattering coefficient. The emulsifier used in the self-microemulsifying system and the presence of carotenoids in solubilized form in the digestive tract, will eventually enhance the uptake of carotenoids by intestinal. This may explain why a richer yellowness was observed for the skin and muscle obtained from fishes treated with the microemuslified carotenoids over the non-microemulsified carotenoids.

Natural antioxidants, including carotenoids, are important to animal health. They function to remove harmful free radicals produced through normal cellular activity and thus help to maintain the structural integrity of e.g. immune cells. Immuno-enhancement by dietary carotenoid manipulation may complement, if not offer an alternative to, the use of drugs in aquaculture. In fish, an increasing number of studies have demonstrated the role of various dietary carotenoids in immune responses [29,30]. The results of the present study showed that the exposure of hybrid catfish to carotenoids resulted in higher RBC and WBC counts.The haemoglobin values were also increased as compared with fish fed with the control diet. This increase could be due to the presence of lutein and zeaxanthin in the natural yellow pigment, which can help build the immunity capacity. These results showed the improvement of fish health when fed carotenoid-supplemented diets which increased the ability to fight off infections through the reduction of stress levels. The major functions of WBC are to fight infection; defend the body against foreign organisms and in immune response [31]. Feeding dietary carotenoids significantly ($p<0.05$) increased the levels of haematocrit and total protein content which contradicts the finding reported by [32] where the haematocrit value in treated fish was lower than that of control fish. In all, the haematological results showed some stimulation in the immune response for the carotenoid-fed fish compared to control fish and these effects could be attained with the microemulsified carotenoid when used at a reduced dosage of 30%. As indicated, carotenoid particle sizes exerted a significant influence on the relative bioavailability. It has been reported that lutein and zeaxanthin, is a poorly water-soluble lipophilic compound, that follows the same route of absorption as lipids. This implies the involvement of endogenous emulsifiers in promoting solubilization and absorption of carotenoids *in vivo*. Although the exact mechanism of the absorption is not yet fully understood, lutein and zeaxanthin have been thought to be absorbed through enterocytes by simple diffusion or receptor-mediated transport. Specifically, the lutein and zeaxanthin are emulsified into small lipid droplets in the stomach and further incorporated into mixed micelles by the action of bile salts and biliary phospholipids, after which mixed micelles are taken up by enterocytes.

Conclusion

Colour intensity, growth and immune resistance are important quality criteria and market value determinants for hybrid catfishes. The use of natural carotenoid may contribute to an enhanced image and quality of hybrid catfish. The findings as reported in this paper clearly demonstrated that the microemulsified yellow carotenoids when supplemented at a 30% reduced dosage can achieved on par growth performance, enhanced pigmentation and has no adverse effect in the immune response of the catfish when compared to the regular carotenoids and control. In summary, microemulsified yellow carotenoids can provide greater cost saving with its better bioavailability due to its characteristic size of ~0.25 µm, making it the carotenoid of choice in catfish aquaculture for control over pigmentation as well as maintaining fish growth and health.

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