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Dietary Encapsulated Butyric Acid (Butipearl™) and Microemulsified Carotenoids (Quantum GLO™ Y) on the Growth, Immune Parameters and their Synergistic Effect on Pigmentation of Hybrid Catfish (*Clarias macrocephalus* × *Clarias gariepinus*)

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Abstract

A 12-weeks feeding trial was conducted to evaluate the effects of encapsulated butyric acid (ButiPEARL™) and microemulsified yellow carotenoid (Quantum GLO™ Y) on growth, immune parameters and their synergistic effect on pigmentation of hybrid catfish. In the experiment, the catfish was randomly divided into 12 groups of 15 fishes and then fed with four experimental diets containing 0.5 kg/t ButiPEARL™, 0.7 kg/t Quantum GLO™ Y, 0.5 kg/t ButiPEARL™ + 0.7 kg/t Quantum GLO™ Y, or none of these supplements (control diet). The results showed that the ButiPEARL™ + Quantum GLO™ Y fed group gave the highest yellowness (b*) score of 18.43 in the back muscle and almost double the total carotenoid measured in the fish muscle (151.35 mg/kg) compared to the Quantum™ Y fed group. This suggests that butyric acid in the diet had a synergistic effect on carotenoid absorption and pigmentation performance of catfish. The body weight of all treatment groups was significantly different from the control group and the catfish fed ButiPEARL™ alone had the highest body weight gained followed by the ButiPEARL™ + Quantum GLO™ Y fed group with an FCR improvement of 25 points and 13 points respectively over the control. There was no adverse effect on the immune system after feeding both butyric acid and carotenoid to the catfish and the immune parameters (number of leucocytes, erythrocyte, percent of hematocrit, haemoglobin and total protein) of ButiPEARL™ + Quantum GLO™ Y fed group were improved compared with the other groups. In conclusion, ButiPEARL™, Quantum GLO™ Y and the combination have positive effects on performance and pigmentation in catfish aquaculture.

Keywords: Encapsulated butyric acid; Microemulsified carotenoids; Hybrid catfish; Synergistic; Growth; Immune parameters; Pigmentation

Introduction

Hybrid catfish (Female *Clarias macrocephalus* × Male *Clarias gariepinus*) have become one of the most important protein sources for Thai people because of its low cost, rapid growth, high availability, and high nutritional value. To match the increasing demand, the production of hybrid catfish in Thailand has dramatically increased every year for more than ten years. However, hybrid catfish grown in intensive aquaculture are often exposed to stressful conditions which have a negative impact on their growth and immunity. Therefore, hybrid catfish in such environments usually have low growth rate and high tendency to develop diseases, especially bacterial infectious diseases. Currently, bacterial infections in aquaculture are mainly controlled by antibiotics. However, recently, the use of antibiotics in aquaculture has received considerable attention because their use can lead to the development of drug resistant bacteria, thereby reducing drug efficacy. Because the usage of antibiotics in aquaculture is discouraged, it is necessary to find an alternative solution to prevent bacterial infection. Organic acids are among the most promising substances as they have been reported to increase survival rate of catfish. Carotenoids can also improve survival rate, enhance resistance to several stress conditions as well as pigmentation. Therefore, both organic acids and carotenoids have the potential to be used in catfish farming as feed additives [1].

Butyric acid has been shown to have bactericidal activity on some enteric bacteria as well as to stimulate villi growth [2]. Researchers have shown that butyrate is quickly absorbed in the upper digestive tract, which makes it less ideal as a feed additive [3]. However, butyrate efficacy has been shown to increase when it is fed in a protected form such as encapsulation [4]. Researchers have shown that encapsulation can effectively deliver butyric acid throughout the intestinal system, providing energy for intestinal proliferation and differentiation and thereby ensuring that absorption of important nutrients is optimized [5]. 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 [6] as well as product market price. A variety of natural and/ or synthetic carotenoids are available to enhance coloration in the flesh of salmonid fish and in the skin of others such as European red porgy (Pagrus pagrus). Both synthetically produced pigments, astaxanthin and canthaxanthin, either alone or in combination, have been efficiently used as dietary additives for muscle pigmentation in salmonids. While synthetic carotenoid pigments are commercially available as feed additives, they are expensive and up-take levels are poor, estimated between 5% and 10% [7]. Moreover, there is increasing consumer awareness about synthetic feed additives and safety because these substances may exert effects within the body. This has recently promoted increase interest in the use of natural carotenoid sources for some fish and shrimp species

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of economic interest. In our earlier works [8,9], 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. These fine droplets of microemulsions have the advantage of presenting the carotenoids in a dissolved form, with a large interfacial surface area for absorption, which will result in an enhanced, more uniform and reproducible absorption. Indeed, we have been able to demonstrate the possibility of using the microemulsified carotenoids in enhancing the bioavailability over corresponding regular 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 evaluated the effects of encapsulated butyric acid (ButiPEARL™) and microemulsified yellow carotenoid (Quantum GLO™ Y) on growth, immune parameters and their synergistic effect on pigmentation of hybrid catfish under stress-free condition.

Materials and Methods

Production of products

The encapsulated source of butyric acid (ButiPEARL™; Kemin Cavriago, Italy used in the experiment consisted of min 45% butyrate salt. The microemulsified source of yellow carotenoid (Quantum GLO™ Y; Kemin Industries (Asia) Pte Limited, Singapore) used in the experiment consisted of min 20 g/kg total xanthophyll. The carotenoids were found to be approximately 0.25 µm in size, as analyzed by electron microscopy and light-scattering diffraction study [9]. The dosages for each product were expressed in kg/t of feed.

Trial specifications

The experimental trial was conducted in the Laboratory of Nutrition and Aqua feed, Department of Aquaculture, Faculty of Fisheries, Kasetsart University, Bangkok, Thailand. Hybrid catfish was produced under captivity, with an initial body weight of 45-55 g, were randomly divided into twelve groups of 15 fishes each (four treatments, three replicates). Each group was stocked in a 500 L 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 thrice daily, corresponding to 3%-3.5% of the body weight, (08:30, 12:30 and 16:30) for 12 weeks. During the feeding trial, the water temperature ranged from 27°C to 30°C, pH and dissolved oxygen content of water was greater than 7.2 mg/L and 76 mg/L respectively for the duration of the study. These water quality parameters are crucial for normal fish mortality and will have an impact on the growth and pigmentation. All use of experimental animals was in compliance with guidelines from the Kasetsart University, animal care and use committee.

Four treatment diets containing 0.5 kg/t ButiPEARL™, 0.7 kg/t Quantum GLO™ Y, 0.5 kg/t ButiPEARL™ + 0.7 kg/t Quantum GLO™ Y, or none of these supplements (control diet) 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. No mortality was registered during the experiment. 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.

Ingredients (%)	Experimental diets			
	Control	T1	T2	T3
Soybean	25	25	25	25
Poultry meal	10	10	10	10
Wheat gluten	3	3	3	3
Canola/rapeseed	5	5	5	5
Deoil-rice bran	5	5	5	5
Tapioca	26.67	26.62	26.62	26.57
Dehull full fat SB	15	15	15	15
Soy protein concentrate	5	5	5	5
Crude fish oil	1	1	1	1
Choline	0.3	0.3	0.3	0.3
Vitamin C	0.2	0.2	0.2	0.2
Lysine	0.2	0.2	0.2	0.2
Methionine	0.23	0.23	0.23	0.23
Di-calcium phosphate	2.3	2.3	2.3	2.3
Limestone	0.1	0.1	0.1	0.1
Vitamin premix	1	1	1	1
ButiPEARL	0	0.05 (0.5 kg/t)	0	0.05 (0.5 kg/t)
Quantum GLO Y	0	0	0.07 (0.7 kg/t)	0.07 (0.7 kg/t)

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

Colorimetric and total carotenoids analysis

Colour analysis was performed every four weeks by reflective spectroscopy with a Minolta colour reader CR-10 colorimeter in accordance with the system CIE Lab (CIELAB) for lightness, redness, and yellowness, respectively [10]. 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.

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Haematological assay

Blood was analysed with routine methods used in fish haematology [11,12]. In short, blood was collected from the caudal vein with 1 mL non-heparinized 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 µL of the serum was removed and vortexed with 1 mL of ethanol for 30 s, 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 and white blood cell count was determined with chamber method using Neubauer's haemocytometer; haemoglobin concentration with cyanmethemoglobin method [13] and haematocrit in capillary tubes of 75 µL volume, which were centrifuged in a microhematocrit centrifuge and the haematocrit values were read with a reader. The total protein was determined using the following method [14].

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 by Stat graphics 5.1.

Results

Growth performance and feed utilization

The effects of both encapsulated butyric acid and microemulsified carotenoid diets on the growth parameters for the fishes throughout the experimental periods are given in Table 2. There were significant differences among the fish growth parameters measured (Figure 1). All fish grew normally and no specific signs of disease were observed. No mortality occurred throughout the experiment. Feed intake among treatments showed no significant differences ($p>0.05$) except for the ButiPEARL™ fed group. The results showed that ButiPEARL™ addition to the control diet at 0.5 kg/t significantly improved the body weight gain by 95.81 g ($p<0.05$) with an FCR improvement of 25 points ($p<0.05$). This group also gave the best specific growth rate among other groups. However, growth performance of the ButiPEARL™ + Quantum GLO™ Y fed group was not significant different from the Quantum GLO™ Y fed group ($p>0.05$).

Figure 1: Digital image of hybrid catfish fed with experimental diets over 12 weeks: (C) Control, (T1) 0.5 kg/t ButiPEARL, (T2) 0.7 kg/t Quantum GLO Y and (T3) 0.5 kg/t ButiPEARL + 0.7 kg/t Quantum GLO Y.

Rearing parameter	Experimental diets			
	C	T1	T2	T3
Weight gain (g)	120.20 ± 4.72^{a}	139.96 ± 1.22^{c}	126.39 ± 6.18^{ab}	133.65 ± 4.62^{ab}
Average weight gain (g/fish/day)	75.79 ± 5.49^{a}	95.81 ± 1.35^{c}	84.17 ± 5.0^{ab}	87.65 ± 4.03^{ab}
Specific growth rate (%/day)	1.17 ± 0.05^{a}	1.35 ± 0.01^{bc}	1.23 ± 0.03^{ab}	1.30 ± 0.04^{b}
Feed conversion ratio (FCR)	1.58 ± 0.11^{a}	1.33 ± 0.01^{c}	1.42 ± 0.08^{ab}	1.45 ± 0.06^{ab}
Daily feed consumed (g/fish/day)	1.42 ± 0.02^{a}	1.52 ± 0.01^{b}	1.42 ± 0.02^{a}	1.52 ± 0.01^{b}
Survival rate (%)	100	100	100	100

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

Colorimetric and total carotenoids analysis

Colour intensity of hybrid catfish fed with experimental diets throughout the experimental periods is shown in Table 3 and Figure 2. 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 groups, fed the other diets. Yellow tonality for abdominal skin was observed to be the best for fish fed the diet supplemented with Quantum GLO™ Y followed by ButiPEARL™ + Quantum GLO™ Y. However, for the back muscle

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colour score, the ButiPEARL™ + Quantum GLO™ Y combination gave the highest numerical value of 18.43 which is significantly different from the other treatments. This group also gave the highest total carotenoid measured in the back muscle (151.35 mg/kg) which is almost two times higher than what was measured for the Quantum GLO™ Y fed fish.

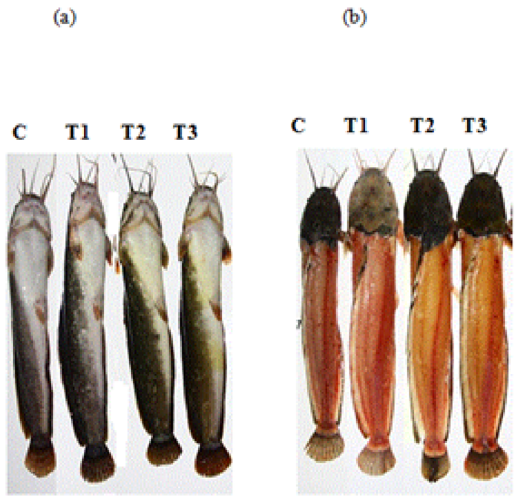

Figure 2: Digital image of (a) abdominal skin and (b) muscle for colour measurement of hybrid catfish fed with experimental diets over 12 weeks: (C) Control , (T1) 0.5 kg/t ButiPEARL, (T2) 0.7 kg/t Quantum GLO Y and (T3) 0.5 kg/t ButiPEARL + 0.7 kg/t Quantum GLO Y.

Experimental diets	L	a	b
	Abdominal skin		
C	67.23 ± 3.16^{a}	0.07 ± 0.15^{ab}	0.57 ± 0.42^{a}
T1	66.67 ± 3.07^{a}	0.37 ± 0.21^{b}	1.60 ± 0.72^{a}
T2	70.53 ± 1.26^{a}	-0.73 ± 0.35^{a}	8.30 ± 0.78^{bc}
T3	70.08 ± 1.74^{a}	-0.10 ± 0.36^{ab}	7.20 ± 0.75^{b}
	Back muscle		
C	45.13 ± 0.80^{a}	2.43 ± 0.45^{a}	10.57 ± 1.25^{a}
T1	48.63 ± 1.40^{a}	3.57 ± 0.42^{ab}	13.30 ± 0.70^{bc}
T2	49.30 ± 0.75^{a}	4.53 ± 0.85^{b}	16.33 ± 1.37^{bc}
T3	49.47 ± 0.85^{a}	4.47 ± 0.93^{ab}	18.43 ± 1.11^{c}
	Total carotenoid (mg/kg) in muscle		
C	22.80 ± 6.31^{a}		
T1	35.00 ± 4.71^{a}		
T2	88.27 ± 21.18^{b}		
T3	151.35 ± 31.06^{c}		

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). Mean with different superscripts in the same column are significantly different (p<0.05).

Haematological analysis

Fishes fed on carotenoid, butyric acid or combination diets exhibited numerically increased RBC and WBC counts ($p<0.05$; Table 4) when compared to the control. Dietary carotenoids and butyric acid significantly affected the haemoglobin and hematocrit of fishes as compared to the control. Haemoglobin varied from 7.23 g/dl to 7.33 g/dl, hematocrit from 39.33% to 41.67%. The total protein was significantly increased for the ButiPEARL™ + Quantum GLO™ Y fed fish compared to the control while numerically increased for the other treatments.

Hematological parameter	Experimental diets			
	C	T1	T2	T3
Red blood cell (RBC) ($\times 10^{6}$ cell/ml)	1.59 ± 0.06^{a}	1.67 ± 0.04^{a}	1.64 ± 0.06^{a}	1.67 ± 0.13^{a}
White blood cell (WBC) ($\times 10^{5}$ cell/ml)	1.20 ± 0.08^{a}	1.26 ± 0.09^{a}	1.25 ± 0.08^{a}	1.27 ± 0.12^{a}
Hemoglobin (Hb) (g/dl)	6.64 ± 0.13^{a}	7.28 ± 0.17^{b}	7.23 ± 0.37^{b}	7.33 ± 0.18^{b}
Hematocrit (HCT) (%)	36.00 ± 1.00^{a}	40.67 ± 0.58^{b}	39.33 ± 1.50^{b}	41.67 ± 0.58^{b}
Total protein (mg/dl)	6.18 ± 0.13^{a}	6.48 ± 0.13^{ab}	6.44 ± 0.10^{ab}	6.50 ± 0.07^{b}

Table 4: Haematological factors of hybrid catfish fed with experimental diets for 12 weeks. Mean with different superscripts in the same rows are significantly different (p<0.05).

Discussions

Organic acids are mainly used as feed additives for improving growth performance of pigs and poultry [15-17]. There are also reports on the benefit of organic acids in aquatic animals, including red hybrid tilapia [18], yellowtail [19] and Pacific white shrimp [20]. The organic acids and salts used are formic acid/calcium formate, acetic acid/ sodium acetate, propionic acid/ calcium propionate or butyric acid/ sodium butyrate that are in the free form and they can enhance the growth performance and health status of fish. However, in this study, we would like to demonstrate the additional beneficial effect of feeding an encapsulated source of organic acid. ButiPEARL™ used in this study is an encapsulated butyric acid where the encapsulation can lead to a slow release of the butyric acid alongside the gastro-intestinal tract. The encapsulation of the butyric acid is beneficial because it is a method to prevent leaching. This is especially important in animals that do not swallow whole feed particles but masticate their feed. Furthermore, the encapsulation leads to a slow release effect of free butyric acid throughout the gut. The result obtained from this study showed that fish fed ButiPEARL™ has significant increase in body weight gain and FCR improvement by 25 points compared to fish fed with other treatments. This indicated that ButiPEARL™ had a positive effect on performance and the encapsulation prevented the butyrate from being absorbed too fast. As a result, the butyrate would have been more available in the small intestine to enhance the villi growth for better nutrient digestibility and growth performance.

In addition to growth, addition of dietary ButiPEARL™ also helped to significantly increase the carotenoid deposition in the catfish muscle and enhance yellowness in muscle colour when compared to using Quantum GLO™ Y alone. Quantum GLO™ Y is a microemuslified pigment that belongs to the xanthophyll class and from our earlier studies [8,9], the bioavailability of the carotenoids prepared using this system was significantly better than other carotenoid preparation due to the increased ratio of surface area to volume of the smaller carotenoid structures after the emulsification (size reduction from 20 µm to 0.25 µm that is 80 order of magnitude smaller). This enabled the carotenoid molecules to better penetrate the intestinal epithelium, increasing their residence time and enhancing the absorption. Also, ButiPEARL™ has shown to have diverse modes of action, such as increased villi height and crypt depth, leading to increased absorptive surface of the small intestine and resulting in better nutrient utilization [5]. Based on our results, ButiPEARL™ might have activated the intestinal function and allowed increased intestinal absorption of the microemulsified carotenoids, resulting in richer yellowness observed for the muscle and almost doubling the amount of carotenoid in the muscle from the fishes treated with the ButiPEARL™ + Quantum GLO™ Y combination. This further suggests that dietary butyric acid had a synergistic effect on the carotenoid absorption and pigmentation performance of catfish.

In fact, many immune parameters of Quantum GLO™ Y, ButiPEARL™ or combination-fed fish were improved as shown in Table 4, including the RBC, WBC, haemoglobin, haematocrit and total protein. These outcomes suggest that carotenoid had an immunostimulatory property preventing disease infection in catfish. Antioxidant activity of carotenoids may be involved in the immunomodulatory effect; by quenching singlet oxygen and free radicals, carotenoids can protect white blood cells from oxidative damage [21]. Given that carotenoids also possess an antioxidant property this suggests that such mechanism can take part in immunomodulation. Furthermore, the effects of carotenoids on enhancing cell-mediated and humoral immune responses of vertebrates are also documented [21,22]. Several studies have reported that dietary carotenoids can increase the immune parameters, enhance the survival rate, or act as a prophylactic to pathogens for many aquatic animals such as common carp [23,24] and rainbow trout [25]. Even if butyric acid and carotenoid have different modes of action to the catfish, both had positive effects on their immune responses. Also, our results showed that dietary ButiPEARL™ and Quantum GLO™ Y synergistically enhance the immune parameters of catfish.

Conclusions

Disease resistance, growth improvement and colour intensity are important quality criteria and market value determinants for hybrid fishes. The use of butyric acid source alone may contribute to stimulate villi growth and enhance growth but the butyrate efficacy can be increased when it is fed in encapsulated form (ButiPEARL™). The findings as reported in this paper clearly demonstrated and proved that ButiPEARL can synergistically enhance the microemulsified carotenoids (Quantum GLO™ Y) absorption and significantly improve the pigmentation in catfish when used in combination. In addition, ButiPEARL + Quantum GLO™ Y fed fish also showed improvement in many immune parameters compared to other treatments. In conclusion, ButiPEARL™, Quantum GLO™ Y and their combination have positive effects on performance and pigmentation in catfish aquaculture.

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