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Dietary administration of the extract of *Rhodobacter sphaeroides* WL-APD911 enhances the growth performance and innate immune responses of seawater red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*)

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ABSTRACT

A commercial carotenoid product from the extract of probiotic *Rhodobacter sphaeroides* mutant strain WL-APD911 (Lycogen™) contains neurosporene, ξ-carotene, spheroidenone and methoxyneurosporene rather than lycopene. In the present study, Lycogen™ was used to improve the growth performance of seawater red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*) for 7 weeks. The results showed that dietary supplementation with 1.0% Lycogen™ in seawater tilapia did not cause changes in body length but significantly increased muscle weight, weight gain, the specific growth rate (SGR) and the feed conversion ratio (FCR). Because there were no intergroup differences in the proximate composition of the fish muscle, the increased body weight of Lycogen™-supplemented tilapia might have resulted from muscle growth instead of changes in muscle composition. An analysis of the expression levels of growth-associated genes, i.e., growth hormone receptor 1 (GHR1) and insulin growth factor-1 (IGF-1), showed that an alternative splicing form of GHR1 accumulated to a significant extent in the muscle of tilapia fed with a Lycogen™-supplemented diet. In addition, innate humoral responses, reflected by lysozyme activity and alternative complement activity (ACH_{50}), also significantly increased in the sera of Lycogen™-treated tilapia. The present study suggests a potential application of the biomaterial Lycogen™ in the aquaculture field: to enhance tilapia growth performance via immune regulation.

1. Introduction

Lycogen™ is a commercial carotenoid product extracted from the photobacterium *Rhodobacter sphaeroides* WL-APD911. The *crtC* gene of *R. sphaeroides* WL-APD911 has been altered by random chemical mutagenesis, leading to the production of a new carotenoid known as neurosporene (Wu and Liu, 2011). The carotenoid composition of Lycogen™ includes neurosporene and ξ-carotene rather than lycopene, a traditional carotenoid found in plants and photobacteria. In addition, gamma-aminobutyric acid (GABA) and ubiquinone (Q10) were also identified in Lycogen™. Previous studies have shown that Lycogen™ is able to decrease inflammation and dextran sodium sulfate (DSS)-induced colitis (W.S. Liu et al., 2012). Photobacteria typically serve as probiotics in the aquaculture industry. They are used as live microbial or feed supplements, and they beneficially affect the host by producing inhibitory compounds, competing for chemicals and adhesion sites, modulating and stimulating immune function and improving microbial balance (Balcazar et al., 2006; Das et al., 2008; Farzanfar, 2006; Munoz-Atienza et al., 2013; Ninawe and Selvin, 2009; Ravi et al., 2007; Sahu et al., 2008; Vine et al., 2004). For these reasons, we hypothesized that Lycogen™ could also be adopted as a feed supplement in aquaculture.

Tilapia is one of the most commonly cultured fish in the world and is a source of animal proteins and essential vitamins and minerals. As this fish can be easily cultured and is highly adaptable to environmental changes, tilapia is an important species in less-developed countries. In Taiwan, income from freshwater fishing is typically limited to rainy seasons, depending on the incidence of typhoons or summer monsoons. The availability of freshwater is not consistent over the course of the year and is occasionally reduced in other seasons. The occurrence of extreme weather, due to global changes in climate, frequently produces instability in the resources provided by freshwater, and this instability impacts the fisheries. Because Taiwan is surrounded by seas, it is an appropriate place to culture marine organisms such as seawater tilapia.

Tilapia is an omnivorous fish and feeds on a wide variety of dietary sources, including phytoplankton, zooplankton, larval fish and detritus. The present study used Lycogen™ as a feed additive and evaluated the

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use of this probiotic-derived biomaterial for improving the muscle growth and innate immune responses of seawater red tilapia (*Oreochromis mossambicus* × *Oreochromis niloticus*) fed with a diet low in fish meal.

2. Materials and methods

2.1. Bacterial strain, media and chemicals

R. sphaeroides WL-APD911 was isolated from mutants using chemical mutagenesis and deposited in the Bioresource Collection and Research Center (BCRC), Hsinchu, Taiwan. The strain was grown at 30 °C on an agar plate composed of Luria-Bertani (LB) medium. Dulbecco's modified Eagle's medium, FBS, penicillin and streptomycin were all obtained from Gibco BRL (Grand Island, NY, USA).

2.2. Preparation of R. sphaeroides WL-APD911 extracts (Lycogen™)

R. sphaeroides WL-APD911 was harvested and washed with saline buffer, followed by two extractions with methanol in the dark and at room temperature. The methanol extract was filtered. The solvent was then removed under reduced pressure in a rotary evaporator to yield dried crude total extracts.

2.3. Diets

The basal diet (32% crude protein, 8% moisture, 6.5% crude fat, 7% crude lipid, 1.2% calcium, 0.6% phosphorus and 10% ash) contained different levels of Lycogen™ (Asia-Pacific Biotech Developing, Inc., Kaohsiung, Taiwan) as a feed additive, ranging from 0.1 to 1% (L_0: 0% (control); L_1: 0.1%; L_2: 0.2%; L_3: 0.5%; L_4: 1.0%) (Table 1). Because Lycogen™ powder is lipophilic, this substance was dissolved in 10 mL corn oil and then mixed with other ingredients. In addition, we separated the experimental diets which were freshly supplemented with 100 mg/kg diet vitamin C (L-ascorbyl-2-monophosphate), and the fish was fed by the vitamin C-supplemented diets instead of the normal experimental diets once a week.

2.4. Animals

To avoid the variation in water quality that would have resulted from the use of different water sources, 30 individual cages were used in this study in conjunction with a 120-m^3 seawater flow-through rearing system. 15 fish were reared in each cage and 6 cages for each treatment group, thus a total of 450 fish were used for the experiment.

Table 1
Basal ingredient composition of the experiment diets per kilogram for red seawater tilapia.

Ingredients	Diets				
	L_0	L_1	L_2	L_3	L_4
Lycogen™ (g)	0	1	2	5	10
Fish meal (g)	70	70	70	70	70
Soybean (g)	280	280	280	280	280
Wheat gluten (g)	180	180	180	180	180
Corn gluten (g)	90	90	90	90	90
Corn starch (g)	110	110	110	110	110
α-starch (g)	120	120	120	120	120
Cellulose (g)	100	99	98	95	90
Mineral mix[a]	20	20	20	20	20
Vitamin mix[b]	20	20	20	20	20
Oil (mL)[c]	10	10	10	10	10

[a] Mineral mix contains per 100 g: $MgSO_4.7H_20$, 13.3 g; $AlCl_3.6H_2O$, 0.7 g; $ZnSO_4$, 2.0 g; KI, 0.005 g; $FeSO_4.7H_2O$, 3.5 g; $CuSO_4.5H_2O$, 1.0 g; NaCl, 5.0 g; cellulose, 74.5 g.
[b] Vitamin mix contains per kg: retinoic acid, 600,000 IU; Vit. D3, 1,000,000 IU; Vit. K3, 2.5 g; alpha-tocopherol acetate, 50.0 g; thiamin hydrochloride, 10.0 g; riboflavin, 15.0 g; pyridoxine hydrochloride, 7.5 g; cyanocobalamin, 250 mg; calcium pantothenate, 27.5 g; nicotinic acid, 60.0 g; biotin, 0.5 g; folic acid, 2.5 g; inositol, 100 g.
[c] Corn oil.

The supplied seawater (salinity of 30–32) was pretreated using sand filtration and ozone to remove microorganisms. The flow rate of sand-filtered, ozone-sterilized seawater was approximately 250 L/h, sufficient to replace 50% of the water in the 120-m^3 experimental tank. The bottom of the tank was cleaned every day, and the daily water temperature, dissolved oxygen (DO) concentration, salinity, pH, ammonia-N and nitrite-N were maintained at 31.3 ± 0.35 °C, 6.2 ± 0.05 ppm, 31.1 ± 1.11 ppt, 8.1 ± 0.09, 0.05 ± 0.01 ppm and 0.02 ± 0.01 ppm, respectively. The water quality was consistent and stable throughout the experiment.

The present study was conducted during the seawater red tilapia hatching season of 2012 (June and July). Red hybrid tilapia (*O. mossambicus* × *O. niloticus*) were obtained from a marine fish farm in Pingtung County (Taiwan), and the fish were acclimated for 2 weeks prior to the experiments. During the experiments, the different feeds were offered to the fish twice daily (at 09:00 and 17:00) at a daily feeding rate of 3–5% of the fish biomass in each diet group, and the feeding rate was reduced by 0.5% of the biomass every 2 weeks. The standard length and weight of the fish were measured every week. The experiment was carried out for 7 weeks. When the feeding experiments were complete, the fish from each dietary treatment were euthanized by an overdose of 2-phenoxyethanol. The proximate composition of the muscle samples was then analyzed (n = 3). In addition, muscle and liver samples were collected for gene expression analysis (n = 6), and serum samples were obtained for the evaluation of innate humoral immune parameters (n = 6).

2.5. Survival rate

The dead fish were removed daily and the number of dead individuals was calculated for measurement of the survival rate during the experimental period (7 weeks).

2.6. Growth performance

Growth performance was evaluated based on the specific growth rate (SGR) and the feed conversion ratio (FCR). These two values were calculated using the following formulas:

$SGR = (\mathrm{Ln}W_f - \mathrm{Ln}W_i) \times 100/t$ and $FCR = F/(W_f - W_i)$, where W_f and W_i are the final and initial fish body weights at stocking, respectively; t is the duration of the feeding trials (days); and F is the weight of the feed offered to the fish.

2.7. Proximate muscle composition

Muscle samples (n = 3) from each diet group were analyzed for moisture, crude protein, crude lipid and total ash content following the procedures outlined by the Association of Official Analytical Chemists (AOAC, 1984).

2.8. Growth hormone receptor 1 (GHR1) and insulin growth factor-1 (IGF-1) expression

Fish muscle and liver tissues (n = 6 for each treatment) were obtained and immediately stored in liquid nitrogen. The total RNA was extracted using Trizol reagents (Invitrogen) according to the

Table 2
Primer sequences used in the study.

Gene name	Forward primer (5′–3′)	Reverse primer (5′–3′)	Gene product size
GHR1	5′-tcccaacctgtcagtcaaca-3′	5′-tgctgtccaggagacaacac-3′	326 bp
IGF-1	5′-tcttcaagagtgcgatgtgc-3′	5′-tacgctctgtccccttgttc-3′	378 bp
β-actin	5′-atggtgggtatgggtcagaa-3′	5′-cagggcataaccctcgtaga-3′	384 bp

Table 3
Survival, initial and final body standard length, initial and final body weight, and growth performance of red tilapia fed dietary Lycogen™ for 7 weeks.

	Diet				
	L_0 (control)	L_1 (0.1%)	L_2 (0.2%)	L_3 (0.5%)	L_4 (1.0%)
Survival (%)	96.6 ± 0.19^a	96.6 ± 0.20^a	98.9 ± 0.11^a	100 ± 0.00^a	95.5 ± 0.11^a
Initial body standard length (cm)	4.6 ± 0.12^a	4.7 ± 0.14^a	4.9 ± 0.27^a	4.6 ± 0.16^a	4.8 ± 0.15^a
Final body standard length (cm)	12.5 ± 0.17^a	12.3 ± 0.14^a	12.8 ± 0.23^a	12.7 ± 0.21^a	12.9 ± 0.25^a
Initial body weight (g)	3.5 ± 0.28^a	3.6 ± 0.29^a	4.2 ± 0.65^a	3.92 ± 0.43^a	3.6 ± 0.34^a
Final body weight (g)	73.5 ± 3.54^a	84.3 ± 3.16^b	90.5 ± 4.59^{bc}	92.3 ± 1.97^{bc}	102.6 ± 6.63^c
Weight gain (g)	70.1 ± 3.67^a	80.8 ± 3.14^b	86.3 ± 4.55^b	88.4 ± 1.92^b	98.9 ± 6.78^{bc}
SGR (%)	7.3 ± 0.26^a	7.6 ± 0.21^{ab}	7.4 ± 0.39^{ab}	7.6 ± 0.25^{ab}	8.0 ± 0.30^b
FCR (%)	1.6 ± 0.11^a	1.5 ± 0.03^{ab}	1.4 ± 0.07^{ab}	1.3 ± 0.06^{ab}	1.2 ± 0.07^b

Values (mean ± SE) in the same row with different superscripts are significantly different from each other (n = 6) ($P < 0.05$).

manufacturer's manual. The total RNA (2 μg) was reverse-transcribed using an oligo-dT primer and Superscript III Reverse Transcriptase (Invitrogen, USA). The mRNA levels of tilapia GHR1 and IGF-1 were then determined using a quantitative real-time polymerase chain reaction (qRT-PCR) including iQ SYBR Green Supermix (Bio-Rad) and an iCycler and MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad). Primers were designed based on *O. niloticus* GHR1 mRNA, complete coding DNA sequence (CDS) (AY973232); *O. niloticus* IGF-1 mRNA, complete CDS (EU272149); and *O. niloticus* β-actin mRNA, partial CDS (EU887951). The primer sequences are listed in Table 2.

2.9. Innate humoral responses

The measurements of the innate humoral responses, including lysozyme activity and alternative complement activity (ACH_{50}), were based on the protocol reported by Cheng et al. (Cheng et al., 2007, 2009; Yeh et al., 2008). The serum lysozyme activity was defined as the amount of enzyme needed to produce a decrease in absorbance of 0.001/min/mL serum. The serum ACH_{50} was assayed based on the volume of serum complement producing 50% hemolysis of rabbit erythrocyte suspension (2×10^8 cells/mL), and the ACH_{50} was calculated from the degree of hemolysis as units/mL.

2.10. Statistical analysis

The data are presented as the mean ± standard error (SE). The results were analyzed using an analysis of variance (ANOVA), and the means were subsequently compared using the Tukey–Kramer multiple range test (significance level of $P < 0.05$).

3. Results and discussion

3.1. Water stability

During the experiment, from June to July 2012, the weather was stable from day to day. The study period did not include the rainy season, and no typhoons occurred during this period. Thus, the salinity of the natural seawater did not vary significantly. Freshwater resources are affected only by the supply of new freshwater produced during typhoons or rainy seasons. For this reason, the environment considered in this study had the potential to support seawater aquaculture. Thus, the stable salinity observed during the experiment was favorable for the potential application of Lycogen™ to seawater-cultured red tilapia in the future.

3.2. Growth performance

Seven weeks of Lycogen™ supplementation of the feed significantly increased the body weight but not the body length of the tilapia (Table 3) compared with the body weight of the fish fed with the control diet. In particular, the experimental doses of Lycogen™ had no effect on the standard body length of the tilapia (Table 3), whereas the feed supplemented with 0.2–1.0% Lycogen™ (L_2, L_3 and L_4 diets) significantly increased body weight after 1 week of supplementation. However, there was no difference between the 3rd and 4th weeks for any group, and only L_4 enhanced the tilapia body weights in the 7th week of dietary supplementation (Table 3).

After 7 weeks of feeding, compared to the fish fed with the control diet, both of the fish SGR and FCR increased with supplementation of 1.0% Lycogen™ (L_4 group), not in the other three Lycogen™-supplemented groups (Table 3), however, the final weight increased in all Lycogen™-supplemented groups. It might have resulted from the fish weight reflecting the accumulated changes, thus the application of Lycogen™ on fish growth should be a long-term treatment.

In addition, high SGR (7–8%) and FCR (1.2–1.6%) observed showing that the fish has the ability of high growth rate but low feed conversion efficiency. That means the fish need much more food and grow fast like other kinds of seawater carnivorous fish. Because the diversity of tilapia hybrid is high, the hybrid used in this study was selected and sorted from the offspring of live individuals that were transferred from freshwater to seawater environment. The offspring were suited to the seawater environment, and cannot live in the water below 12 ppt. Thus the fish might preserve some genes associated with salinity adaptation, and the growth was similar to the seawater carnivorous fish.

Because the availability of fish meal has gradually decreased worldwide and because of the high cost of adding fish meal to fish diets, developing diets low in fish meal is ecologically imperative. Therefore, it has ecological and economical meanings to culture the organisms which have the ability to utilize vegetable sources, e.g., soybean or seaweed products, as major protein sources in aquafeed.

Table 4
Effects of dietary supplementation of Lycogen™ on muscle chemical composition.

	Diet				
%	L_0 (control)	L_1	L_2	L_3	L_4
Moisture	76.73 ± 1.44^a	77.30 ± 0.4^a	78.63 ± 0.19^a	76.00 ± 0.85^a	77.43 ± 0.19^a
Crude protein	18.27 ± 0.43^a	15.43 ± 1.56^a	17.83 ± 0.29^a	18.27 ± 1.23^a	19.43 ± 0.15^a
Crude lipid	2.83 ± 0.75^a	2.53 ± 0.76^a	1.57 ± 0.23^a	2.93 ± 0.55^a	1.77 ± 0.22^a
Ash	1.40 ± 0.1^a	1.27 ± 0.03^a	1.30 ± 0.00^a	1.23 ± 0.03^a	1.17 ± 0.03^a

Values (mean ± SE) in the same row with same superscripts are not different from each other (n = 3).

However, the complete replacement of fish or animal meal in aquafeed is still difficult, for instance, Moharram and Raky (2007) found that the complete replacement of a protein source by soybean resulted in significantly lower growth performance and less efficient feed utilization in seawater red tilapia. Thus we still added some fish meal (7%) in the feed for red tilapia culture. Many studies have used feed additives, such as *Spirulina platensis* and amino acids, to improve the efficiency of replacing fish- and animal-based feed with vegetable sources (Mambrini et al., 1999; Nandeesha et al., 2001). In this study, the Lycogen™-improved growth in red seawater tilapia might be the result of an influence on the physiological responses of tilapia and that Lycogen™ may help to counteract the side effects of vegetable protein sources. In the current study, the basal diet contained 7% (w/w) fish meal and was developed based on our pilot study (data not shown), which yielded no significant difference in growth rates between the seawater red tilapia fed with the basal diet and those fed the commercial tilapia aquafeed. Accordingly, the aim of this investigation was to test the feed efficiency resulting from the addition of Lycogen™ to the diet containing 7% fish meal.

In our experiments, Lycogen™, a carotenoid-derived feed additive, increased the growth performance of seawater red tilapia. The disparity between this and previous findings may have resulted from 1) the interspecies difference in the carotenoid effect and/or 2) the carotenoids identified in Lycogen™, namely, neurosporene, ξ-carotene, spheroidenone and methoxyneurosporene, are different from the carotenoids examined in previous studies. In our investigations of the physiological mechanisms associated with Lycogen™-enhanced growth performance of seawater red tilapia fed with diets low in fish meal, we hypothesized that the weight gain resulted from increased muscle mass or altered muscle composition. Thus, we analyzed the expression levels of two growth-associated genes, encoding GHR1 and IGF-1, and the proximate composition of the muscle. In addition, we examined the innate humoral immune responses of red tilapia after 7 weeks of Lycogen™ supplementation.

3.3. Gene expression

Fig. 1 shows the gene expression levels of GHR1 and IGF-1 in tilapia muscle and liver tissues after 7 weeks of Lycogen™ administration. IGF-1 expression in the muscle and liver tissues did not differ between the groups. For GHR1 expression, two gene fragments were identified in the muscle. The shorter fragment (approximately 326 bp) was expected to be the GHR1 gene but was expressed only in tilapia muscle, and the larger fragment (approximately 400 bp) was expressed in the muscle and liver tissues. In addition, the expression of the larger fragment was decreased in the Lycogen™-supplemented L_3 and L_4 groups,

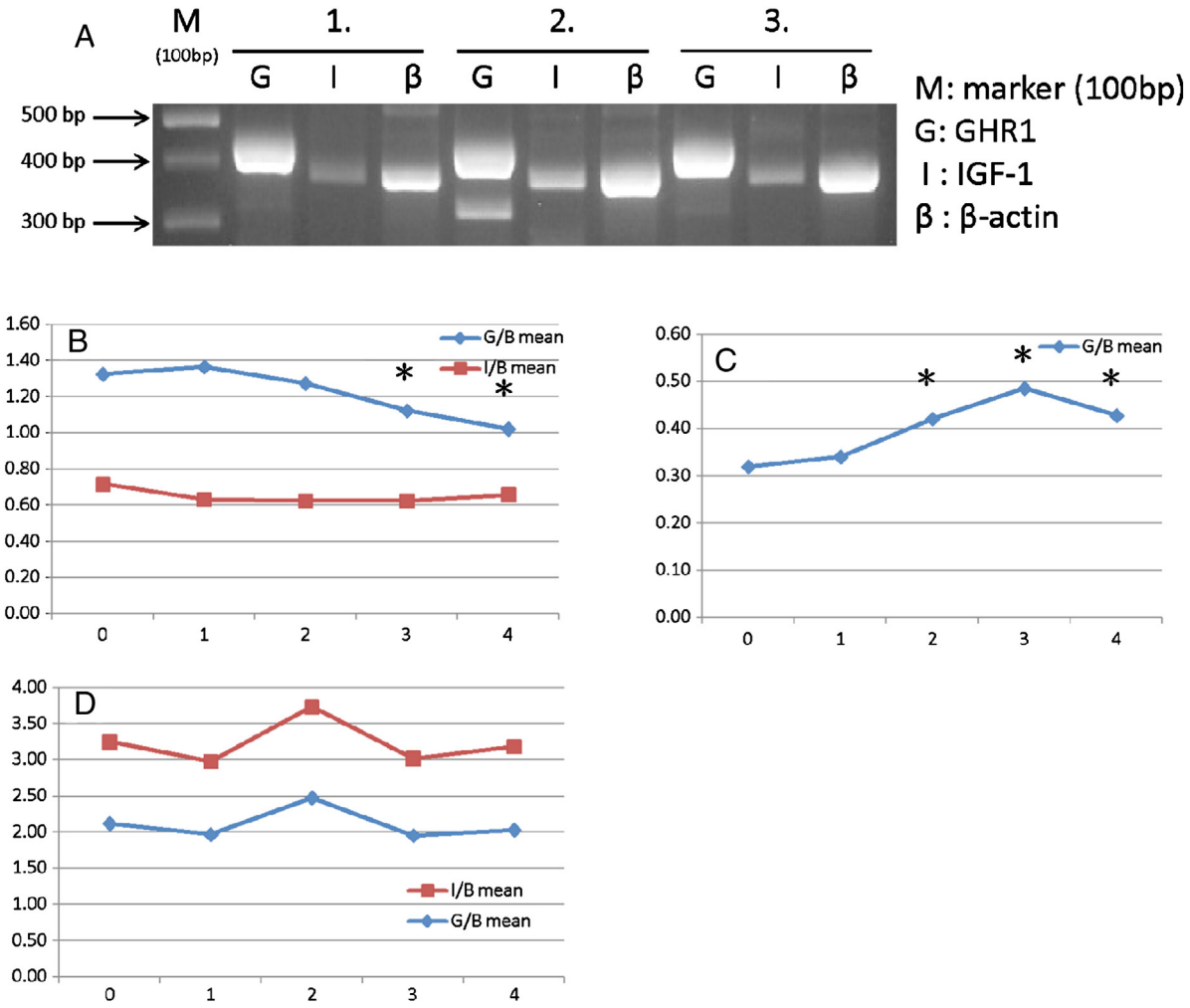

Fig. 1. Gene expression levels of GHR1 and IGF-1 in tilapia muscle and liver tissues after 7 weeks of Lycogen™ administration. The mRNA expression patterns of GHR1, IGF-1 and β-actin (A). The normalized expression levels of 400-bp GHR1 and IGF-1 in the muscle (B) and liver (D) tissues of seawater red tilapia. The normalized expression level of 326-bp GHR1 in the muscle tissues of seawater red tilapia (C). 0, 1, 2, 3, 4 on the *x* axis of (B), (C) and (D) are representative of L_0, L_1, L_2, L_3 and L_4 groups. "*" represents significant differences ($P < 0.05$) compared to the control group (L_0).

whereas the shorter fragment showed a higher expression in the L_2, L_3 and L_4 groups. It appears that these two fragments had complementary effects in the muscle tissue.

To study the divergence of the PCR fragments, both fragments were cloned, sequenced and then analyzed using the Basic Local Alignment Search Tool (BLAST). The results showed that the shorter fragment was the GHR1 transcript (AY973232), which has been published in the NCBI, and that the larger fragment partially aligned with the GHR1 transcript, with the insertion of a 99-nucleotide sequence. To test whether the inserted sequence may have resulted from alternative splicing, this 99-nucleotide fragment was analyzed by BLAST and identified as intron 8 of the GHR1 gene. Therefore, we concluded that the 99-nucleotide insertion was due to tissue-specific intron retention. To further study the effect of the retention of intron 8 in the GHR1 transcript, we translated the two PCR fragments into amino acid sequences. The results showed that the short fragment could be translated into a 109-peptide sequence identical to the sequence of the GHR1 protein, whereas the larger fragment generated a premature stop codon, which may produce a truncated GHR1 without a C-terminal domain. Similar phenomena were reported for the primate GHR gene (Pantel et al., 2000), suggesting that this gene expression is an evolutionarily conserved mechanism that plays an important role in the GHR-mediated signal pathway (Ebihara et al., 1996).

3.4. Muscle proximate composition

In addition to the upregulation of growth genes, we posited that weight gain may also have resulted from changes in muscle composition. Table 4 shows the muscle proximate composition of seawater red tilapia after 7 weeks of feeding with Lycogen™-supplemented diets. There was no significant difference between the fish groups fed with the control and Lycogen™-supplemented diets. In general, dietary carotenoids have been associated with skin coloration or pigmentation, and few studies have addressed the effects of dietary carotenoids on growth via compositional changes in muscle. In the present study, we did not find significant changes in muscle proximate composition after Lycogen™ supplementation. Thus, the Lycogen™-enhanced growth may have resulted from another physiological mechanism, e.g., the improvement of the immune response.

3.5. Effect of Lycogen™ on the humoral immune responses

The humoral immune parameters, ACH_{50} and lysozyme activity, of the tilapia fed with the diets containing Lycogen™ for 7 weeks were higher than the parameters of the fish fed with the control diets (Table 5). Specifically, compared with the control diet group, the fish fed diets supplemented with Lycogen™ yielded a higher level of lysozyme activity. The serum lysozyme activities were 2.27 ± 0.27, 4.86 ± 0.17, $4.24 \pm 4.78 \pm 0.18$ and 4.60 ± 0.14 units/mL for the red tilapia fed with the 0, 0.1, 0.2, 0.5 and 1.0% Lycogen™-supplemented diets, respectively. Moreover, the serum ACH_{50} values in the fish fed with the supplemented diets were also significantly higher than in the fish fed with the control diet: 430.15 ± 23.70, 627.54 ± 21.62, 545.10 ± 24.95, 613.82 ± 22.76 and 592.11 ± 17.83 for the red tilapia fed with the 0, 0.1, 0.2, 0.5 and 1.0% Lycogen™-supplemented diets, respectively.

In this study, two humoral immune parameters, i.e., serum lysozyme activity and ACH_{50}, were measured after 7 weeks of dietary Lycogen™ supplementation. ACH_{50} is considered to be a powerful nonspecific defense mechanism for protecting fish against many types of invasive organisms, including bacteria, fungi, viruses and parasites (Bjornson, 1990; Keusch, 1991; Kraiczy et al., 2002; Lardner, 2001; Mayilyan, 2012; Nakao and Yano, 1998; Schwaeble and Reid, 1999). Lysozymes are also a defensive factor in nonspecific immune responses against invasive microorganisms in vertebrates (Alvarez-Pellitero, 2008; Bjornson, 1990; Pratheepa et al., 2010; Sitja-Bobadilla, 2008; Virella et al., 1991; Watts et al., 2001). Thus, several studies have used the humoral immune response as an indicator of the effectiveness of protection against pathogenesis.

Multiple studies have used the dietary administration of a substance to enhance the innate immune response, increasing disease resistance and growth performance (C.H. Liu et al., 2012; Chiu et al., 2010; Harikrishnan et al., 2011; Lin et al., 2012; Martinez et al., 2012; Panicker et al., 2012; Soleimani et al., 2012; Son et al., 2009; Tukmechi et al., 2011). For instance, dietary sodium alginate administration has been adopted to enhance fingerling growth and resistance to bacteria and viruses. Moreover, this dietary supplementation can increase the juvenile ACH_{50} and lysozyme activity of the orange-spotted grouper (Cheng et al., 2007; Yeh et al., 2008). Previous studies have demonstrated that dietary carotenoids are also associated with immune responses and can play a role as antioxidants, protecting phagocytic cells from autooxidative damage, enhancing T and B lymphocyte proliferative responses and stimulating effector T cell functions (Bendich, 1989; Bendich and Olson, 1989; Butler and McGraw, 2012; Canfield et al., 1992; Chew, 1993; Horak et al., 2006; Perez-Rodriguez et al., 2008).

The carotenoids identified in Lycogen™ include neurosporene, ξ-carotene, spheroidenone and methoxyneurosporene, which are different from the carotenoids examined in previous studies of dietary supplementation. ξ-carotene is the precursor of neurosporene, and neurosporene is the precursor of lycopene (Albrecht et al., 1995, 1997). Large amounts of lycopene, obtained from tomatoes, have been widely used as an antioxidant for the prevention of the formation and action of reactive products, decreasing the risk of major diseases (Weisburger, 1999). In one study, Rodriguez et al. (2004) added mutant stains of the fungus *Mucor circinelloides*, which are enriched in lycopene or β-carotene, to the diet of gilthead seabream. The fish fed with a lycopene- or β-carotene-supplemented diet showed a higher specific growth rate than the fish fed with the control diet. Moreover, in the fish fed with a wildtype strain-supplemented diet, the serum lysozyme activity did not increase significantly, but the cellular responses significantly increased. Thus, in the current study, the increased immune response may have resulted from the effects of lycopene derived from the ζ-carotene and neurosporene contained in Lycogen™. However, as spheroidenone is one of the major carotenoids that accumulate in variable amounts in *R. sphaeroides* (Yeliseev and Kaplan, 1997), and as methoxyneurosporene is a substance involved in spheroidene biosynthesis (Scolnik et al., 1980), we cannot exclude the effects of these substances. The biological functions of these compounds remain to be clarified.

4. Conclusions

The purpose of this work was to evaluate Lycogen™, a methanol extract from *R. sphaeroides* WL-APD911. Our results showed that seawater red tilapia fed with Lycogen™-supplemented diets (0.1–1.0%

Table 5
Innate humoral responses of red tilapia fed dietary Lycogen™ for 7 weeks.

	Diet				
	L_0 (control)	L_1 (0.1%)	L_2 (0.2%)	L_3 (0.5%)	L_4 (1.0%)
ACH_{50}	430.15 ± 23.70^a	627.54 ± 21.62^b	545.10 ± 24.95^b	613.82 ± 22.76^b	592.11 ± 17.83^b
Lysozyme activity	2.27 ± 0.27^a	4.86 ± 0.17^b	4.24 ± 0.27^b	4.78 ± 0.18^b	4.60 ± 0.14^b

Values (mean ± SE) in the same row with different superscripts are significantly different from each other ($n = 6$) ($P < 0.05$).

Lycogen™) for 7 weeks exhibited significant increases in both their serum lysozyme activity and their ACH_{50} values compared with the fish fed with the control diet. Thus, Lycogen™ increased the innate humoral immunity and the expression of the alternative tissue-specific splicing form of GHR1 in seawater red tilapia. The increased growth performance of the seawater red tilapia might have been related to this enhanced immunity. In this study, we provide preliminary results showing the potential application of a naturally occurring source of carotenoids, marketed as Lycogen™, in seawater aquaculture.

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