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RESEARCH PAPER

Effects of Dietary Inclusion of Fermented Soybean Meal with *Phaffia rhodozyma* on Growth, Muscle Pigmentation, and Antioxidant Activity of Juvenile Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

This study was designed to test the effects of dietary supplementation of fermented soybean meal (FSM) with *Phaffia rhodozyma* on growth, muscle pigmentation, and antioxidant activities of juvenile rainbow trout (*Oncorhynchus mykiss*). Solid fermented soybean meal with *P. rhodozyma* (FSPH) contained 500 ppm astaxanthin was used as the natural source of astaxanthin in the present study. Four isonitrogenous and isocaloric diets were formulated to contain 0, 50, 75, and 100 ppm astaxanthin (designated as FSPH0, FSPH50, FSPH75 and FSPH100, respectively). Each experimental diet was fed to triplicate groups of fish (18.5 g/fish) to visual satiation twice daily for 10 weeks. Growth performance and proximate composition of muscle were not affected by dietary treatments. Total carotenoid and astaxanthin concentrations in muscle of fish were not affected by dietary treatments. Redness (a*) values of muscle from fish fed the FSPH50, FSPH75, and FSPH100 diets were significantly higher than those fed the FSPH0 diet. Plasma and liver DPPH, hydroxyl, and alkyl radical scavenging activities were not affected by dietary treatments. Plasma total antioxidant capacity of fish fed the FSPH50 diet was significantly higher than that of the FSPH0 group. Fish fed the supplemented diets showed significantly lower catalase activity compare to those fed the unsupplemented control diet. Plasma superoxide dismutase activity in fish fed the FSPH100 diet was significantly lower than that of fish fed the FSPH75 diet. These results suggest that the combination of FSM and *P. rhodozyma* is a good source of astaxanthin, and that a diet containing 10% of this mixture (equal to 50 ppm astaxanthin) could successfully improve muscle pigmentation and antioxidant status of juvenile rainbow trout.

Keywords: Rainbow trout, fermented soybean meal, *Phaffia rhodozyma*, muscle red color.

Introduction

Carotenoids are natural fat-soluble compounds that are widespread and structurally quite diverse pigmenting agents (Amar *et al*., 2001). Fish muscle color is dependent on tissue carotenoid concentration. However, fish are unable to synthesize carotenoids *de novo*. Thus, an exogenous source is required to achieve natural pigmentation (Yuangsoi *et al*., 2010). Although synthetic carotenoids are used in the diet to improve pigmentation of fish skin and muscle (Lee *et al*., 2010), use of synthetic carotenoids increases feed production expense and reduces aquaculture operation profits (Pham *et al*., 2014). Carotenoids are used for salmonid pigmentation and account for 15–20% of total feed cost (Torrissen, 1995). Many studies have been conducted to identify potential alternative carotenoids sources for fish feeds (Hancz *et al*., 2003; Choubert *et al*., 2006; Büyüçapar *et al*., 2007; Kalinowski *et al*., 2007; Lee and Lee, 2008; Hynes *et al*., 2009; Pham *et al*., 2014).

Phaffia rhodozyma is a red color yeast and a natural source of carotenoids including astaxanthin. It is also a rich source of essential nutrients including proteins, lipids, and vitamin B (Storebakken *et al*., 2004). *P. rhodozyma* accumulates a considerable amount of astaxanthin and is the basis for many commercial products used to pigment salmonid fish (Bjerkeng *et al*., 2007). It has been reported that diets containing *P. rhodozyma* reduce oxidative stress and enhance liver function in rainbow trout (*Oncorhynchus mykiss*) (Nakano *et al*., 1995, Nakano *et al*., 1999). *P. rhodozyma* is an economically feasible feed component for coloring aquatic animal muscle, and many studies have focused on its muscle pigmentation effects (Choubert *et al*., 1995, Bjerkeng *et al*., 2007; March and MacMillan, 1996; Nakano *et al*., 1999). Liu and Wu (2006) reported that *P. rhodozyma* is an outstanding astaxanthin-producing yeast and a potential source of dietary astaxanthin.

Rainbow trout is an important cultured freshwater fish species. The majority of farmed trout

is used as sashimi and smoked fillets for human consumption. Muscle color is one of the most important quality indicators and substantially influences fish acceptability and market price. This study was conducted to find out if the combination of FSM and *P. rhodozyma* at various dietary concentrations would affect growth performance, feed utilization, muscle pigmentation, and antioxidant capacity of juvenile rainbow trout, *Oncorhynchus mykiss*.

Materials and Methods

Strains and Cultivation Conditions

P. rhodozyma (CJ-001) was used in this study. The yeast was maintained for long periods lyophilized on YPD glycerol. An inoculum was prepared in YPR medium containing (per liter): 20g raw sugar, 8g yeast extract, and 2g peptone. Baffle flasks (250 mL) containing 27mL YPD medium were inoculated with 3mL of stock. The flasks were incubated on an orbital shaker at 200 rpm and 20°C for 48 h. Next, an intermediate fermenter was inoculated with 5% (v/v) of the inoculum for vegetative growth (Table 1). The vegetative stage was incubated at 20°C for 48 h with sufficient agitation to at least 50% dissolved oxygen, aeration at 1.5 v/v/min, and pH was controlled at 4.5 with 15% ammonia solution. The production phase was carried out in a 5L borosilicate glass tank fermenter with 1.6L of production-phase medium (Table 2). The fermenter was inoculated with 400 mL of the vegetative culture, and the conditions were adjusted for vegetative culture. A 70% raw sugar solution was injected after 26 h to supplement the carbon source at time 0–25 h: 0g/L, 26–52 h: 3.5g/L, and 53–120 h: 3.2g/L.

The *P. rhodozyma* fermented soybean meal preparation was as follows: soybean meal (CJ CheilJedang, Korea) was mixed with distilled water, and the mixture was heated to 100 °C for 30 min in a commercial autoclave. Then, they were cooled to room temperature inoculated of *P. rhodozyma* production phase culture, and incubated for 48 h at 20 °C and 95% humidity in a constant temperature humidity chamber. After solid-fermentation, samples were air dried at 60 °C for 8 h and stored at 4 °C.

Experimental Diets

The ingredients and proximate compositions of the experimental diets are presented in Table 3. Solid fermented FSM with *P. rhodozyma* (FSPH) contained 500 ppm astaxanthin was used as the natural source of astaxanthin in the present study. Four isonitrogenous and isocaloric diets were formulated to contain 0, 50, 75, and 100 ppm astaxanthin (designated as FSPH0, FSPH50, FSPH75 and FSPH100, respectively). Fish meal and dehulled soybean meal were used as dietary protein sources, and fish oil and linseed oil were used as lipid sources. All ingredients were thoroughly mixed with 30% distilled water, and pellets were prepared using a laboratory moist pelleting machine. The pellets were dried at room temperature for 48 h and ground into desirable particle sizes. All diets were stored at −25°C until used.

Fish and Feeding Trial

Juvenile rainbow trout were transported from a local hatchery (Pyeongchang, Korea) to the Gangneung-Wonju National University (Gangneung, Korea). The fish were acclimated to laboratory conditions for 2 weeks and fed commercial pellets. After this conditioning period, the juvenile rainbow trout (average body weight, 18.5 ± 0.57 g) were randomly distributed to 50 L glass rectangular tanks at a density of 10 fish per tank. Each experimental diet was fed to three replicate groups of fish to visual satiation twice per day (9:00 and 17:00) for 10 weeks. Uneaten feed and dead fish were removed and weighed daily. Freshwater was supplied to the re-circulating system at a flow rate of 3 $Lmin^{-1}$, and aeration was provided continuously to each tank. Photoperiod was natural, and water temperature was maintained at 13.0 ± 0.2°C.

Sampling Procedure and Growth Parameters Evaluation

At the end of the feeding trial, all fish in each tank were fasted for 24 h and collectively weighed after

Table 1. Composition of the vegetative phase medium

Compounds	(g/L)
Raw sugar	100
Yeast extract	7.5
$(NH_4)_2SO_4$	5
KH_2PO_4	2
K_2HPO_4	0.4
$MgSO_4.7H_2O$	1.5
NaCl	0.2
$CaCl_2$	0.4
Thiamine	0.001
Antifoam (10%)	1
Soybean meal	5

Table 2. Composition of the production phase medium

Compounds	(g/L)
Raw sugar	100
Yeast extract	7.5
$(NH_4)_2SO_4$	5
KH_2PO_4	2
K_2HPO_4	0.4
$MgSO_4.7H_2O$	1.5
NaCl	0.2
$CaCl_2$	0.2
Trace element	2
Thiamine	0.001
Ca-Pantothenate	0.004
Biotin(1g)	0.1
Antifoam (10%)	1
Inositol	0.065
Pyridoxine	0.035
Soybean meal	5

Table 3. Ingredients and chemical composition of the experimental diets

	Diets			
	FSPH0	FSPH50	FSPH75	FSPH100
Ingredients (%)				
Fish meal	49.0	49.0	49.0	49.0
Dehulled soybean meal	25.0	15.0	10.0	5.0
Fermented soybean meal with *Phaffia rhodozyma* (500 ppm astaxanthin)	-	10.0	15.0	20.0
Wheat flour	10.0	10.0	10.0	10.0
Potato-starch	5.0	5.0	5.0	5.0
Fish oil	4.0	4.0	4.0	4.0
Linseed oil	4.0	4.0	4.0	4.0
Cellulose	0.1	0.1	0.1	0.1
Vitamin premix[a]	1.2	1.2	1.2	1.2
Mineral premix[b]	1.0	1.0	1.0	1.0
Vitamin C (50%)	0.3	0.3	0.3	0.3
Vitamin E (25%)	0.1	0.1	0.1	0.1
Choline salt (50%)	0.3	0.3	0.3	0.3
Proximate composition (% dry matter)				
Crude protein	52.6	52.2	52.3	51.8
Crude lipid	13.1	12.7	12.9	13.7
Ash	11.9	12.7	10.7	14.5

[a]Vitamin premix contained the following amounts diluted in cellulose (g / kg premix): DL-α-tocopheryl acetate, 18.8; thiamine hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-D-pantothenate, 12.7; myo-inositol, 181.8; D-biotin, 0.27; folic acid, 0.68; p-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.

[b]Mineral premix contained the following ingredients (g / kg premix): $MgSO_4 \cdot 7H_2O$, 80.0; $NaH_2PO_4 \cdot 2H_2O$, 370.0; KCl, 130.0; ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 20.0; Ca-lactate, 356.5; CuCl, 0.2; $AlCl_3 \cdot 6H_2O$, 0.15; KI, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4 \cdot H_2O$, 2.0; $CoCl_2 \cdot 6H_2O$, 1.0.

anesthetizing them in100 ppm tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO, USA) to calculate growth performance and feed utilization parameters according to the following equations.

Weight gain = (final body wt. − initial body wt.)× 100/initial body wt.

Specific growth rate = [(ln (final fish wt.) − ln (initial fish wt.)] × 100/days of feeding.

Feed efficiency = wet weight gain × 100/feed intake.

Daily feed intake = feed intake − 100/[(initial fish wt. + final fish wt. + dead fish wt.)/2 × days reared].

Daily protein intake = protein intake × 100/[(initial fish wt. + final fish wt. + dead fish wt.)/2× days reared].

Protein efficiency ratio = wet weight gain/protein intake.

Chemical Analysis

Seven fish from each tank were sampled randomly at the end of the feeding trial, and stored at −75°C for analysis of muscle color and radical scavenging and antioxidant enzyme activities. Proximate composition of the fish muscle was analyzed according to standard methods (AOAC, 1995). Experimental diet samples were dried to constant weight at 105°C in a drying oven to determine moisture content. Crude protein (N× 6.25) was determined using the Kjeldahl method. Crude lipid was determined by Soxhlet extraction, and ash content was measured after combustion at 550°C for 4 h.

Total Carotenoid Analysis

Freeze dried flesh samples (200 mg) were suspended in 1 mL dimethyl sulfoxide (DMSO), and 1 mg of 0.5 mm glass beads was added. After 1 min of shaking, the mixture was centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to a clean test tube, and 2 mL of DMSO was added. After 1 min of shaking, the mixture was centrifuged at 10,000 rpm for 5 min, and absorbance was measured at 474 nm using Tean F-200 multiwell plate reader (Tecan Mannedorf; Zurich, Switzerland).

Astaxanthin Analysis

Astaxanthin concentrations were determined using an Agilent 1200 Series high performance liquid chromatography system (Palo Alto, CA, USA) equipped with a UV-visible detector and a YMC carotenoid column (250 nm ×4.6 mm, 5 µm). A guard column operated at 25 °C. The mobile phase was a mixture of methanol: tert-butyl tert-butyl ethyl ether: water (81:15:4; v/v/v). Solvent flow rate was 1.0 mL/min. A calibration curve was prepared using a 98% pure astaxanthin standard (Sigma, St. Louis, MO, USA) to determine astaxanthin content.

Color Analysis

Muscle color was measured on tissue taken from the dorsal surface of fish from each tank at the end of the feeding period. Three replicate measurements were taken using a chroma meter CR-400 (Konica Minolta, Osaka, Japan). The color parameters were L^* for lightness, ranging from 0 for black to 100 for white; a^* for red/green, and b^* values for yellow/blue. A standard white tile with reflectance values of L^* =95.91, a =+ 0.09, and b = + 2.02 was used as the reference.

Radical Scavenging Activities

At the end of the feeding trial, plasma samples were collected and extracted and liver samples were homogenized (Wiggenhauser, Berlin, Germany) with extraction buffer in 5mMTris-HCl and 35mM glycine (pH 8.4) followed by centrifugation (13,000 ×g for 10 min at 4°C). The supernatant was collected and analyzed for radical scavenging activities.

1,1-Diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging activity was evaluated using the method described by Nanjo *et al*. (1995). A 30 µL peptide solution (or ethanol as the control) was added to 30 µL DPPH (60 µM) in ethanol. After mixing vigorously for 10 sec, the solution was moved to a 100 µL quartz capillary tube, and the scavenging activity of the peptide for the DPPH radical was determined using a spectrometer (JEOL Ltd., Tokyo, Japan). After 2 min, the spin adduct was determined on an ESR spectrometer. The measurement conditions were: magnetic field, 336.5±5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude; 1 × 1000; sweep time; 30 s.

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber–Weiss reaction and were reacted rapidly with a 5, 5-dimethyl-1-pyrroline-*N*-oxide (DMPO) electron spin trap (Rosen and Rauckman, 1984). The resulting DMPO-OH adducts were monitored with an ESR spectrometer. The peptide solution (20 µL) was blended with DMPO (0.3 M, 20 µl), $FeSO_4$ (10 mM, 20 µL), and H_2O_2 (10 mM, 20 µL) in a phosphate buffer solution (pH 7.4) and was transferred to a 100 µL quartz capillary tube. The ESR spectrum was recorded after 2.5 min using the ESR spectrometer. The experimental conditions were: magnetic field, 336.5±5 mT; power, 1 mW,

modulation frequency, 9.41 GHz; amplitude; 1 × 200, sweep time; 4 min.

Alkyl radicals were determined using 2, 2-azobiz-(2-amidinopropane)-hydrochloride (AAPH). The phosphate buffered saline (pH 7.4) reaction mixtures included 10 mM AAPH, 10 mM4-POBN, and a known concentration of sample (100 µg/mL). The mixtures were incubated at 37°C in a water bath for 30 min (Hiramoto *et al*., 1993) and then moved to a capillary tube. Spin adducts were recorded using a spectrometer (JEOL Ltd., Tokyo, Japan). The measurement conditions were: modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9,441 MHz; magnetic field, 336.5±5 mT and sweep time, 30 s. DPPH, hydroxyl, and alkyl radical scavenging activities were calculated using the following equation:

$$\text{RSA (\%)} = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100$$

Where RSA is radical scavenging activity, A_{sample} is the relative peak height of the sample radical signal, and $A_{control}$ is the relative peak height of the control radical signal.

Antioxidant Enzymes Activities

Blood was taken from the caudal vein of fish from each tank using heparinized syringes. Plasma was collected after centrifugation at 5000 rpm and4°C for 10 min, and the plasma was separated and stored −70°C for chemical analysis. Plasma catalase (CAT), total antioxidant capacity (TAC), superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities were determined in 1.5g fish muscle. The muscle samples were homogenized in nine volumes of 5 mM Tris and 35mM glycine (pH 7.6). The homogenate was centrifuged at 10000×g for 30 min to remove debris. The resulting supernatants were used for the CAT, TAC, SOD, and GPx assays. Protein content in the supernatants was measured using the Bradford (1976) method.

The CAT Assay Kit (Bio assasy Systems Hayward, CA, USA) was used in the following method. Briefly, 10 µL samples or a 10 µL positive control was added to 90µL of 50 µM H_2O_2. After 30 min incubation, 100µL of detection reagent was added to the reaction mixture. After 10 min, absorbance was measured at 530nm using Tean F-200 multi well plate reader. CAT activity was calculated as U/L.

The TAC Assay Kit (Bioassay Systems) used the following method. A 20 µL sample was added to 100 µL of working reagent and 20 µL H_2O was added as the blank. After 10 min incubation, absorbance was measured at 570nm using the Tean F-200 multi well plate reader. TAC activity was calculated as U/mL.

SOD activity was measured by inhibition of xanthine oxidase using WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, mono-sodium salt). Sample supernatant (20 µL) and 200µL of WST-1 solution were added to 20µL of enzyme solution and incubated at 37°C for 20 min. Distilled water was used as the blank. Optical density (OD) was measured at 450 nm using a UV spectrophotometer (Jasco V-550, Tokyo, Japan). One unit of SOD activity was defined as the amount of enzyme required to inhibit xanthine oxidation by 50%. SOD activity was calculated as U/mL.

The GPx Assay Kit (Bio assasy Systems) was used in the following method. Cumene hydroperoxide was used as the peroxide substrate. Glutathione reductase and NADPH-R were included in the reaction mixture. Therefore, the change in A_{340} due to NADPH oxidation was monitored and was indicative of GPx activity. Briefly, 10 µL of sample with 10µL of a positive control were added to 90 µL of working solution and then 100µl cumenehydroperoxide was added and $OD_1$was read at 340 nm. After 4 min incubation, OD_2 was read at 340 nm using a Tecan Sunrise enzyme-linked immunosorbent assay reader (Mannedorf, Zurich, Switzerland). GPx activity was calculated as U/L.

Statistical Analysis

Data were subjected to one-way analysis of variance to test the effects of dietary astaxanthin level on growth performance, feed utilization, antioxidant activities, and chemical composition of the fish. Duncan's multiple range test (Duncan, 1955) was applied when significant differences ($P<0.05$) were found. All statistical analyses were performed using SPSS program ver. 20.0 (SPSS, Inc., Chicago, IL, USA). Data are presented as mean ± standard error of three replications.

Results

Growth performance and feed utilization of fish fed the experimental diets for 10 weeks are presented in Table 4. No significant differences were identified for weight gain, specific growth rate, feed efficiency, daily feed intake, daily protein intake, protein efficiency ratio and survival among the groups. Muscle proximate compositions (Table 5) of juvenile rainbow trout fed the experimental diets were not affected by dietary treatments.

Total carotenoid and astaxanthin concentrations in the muscle of juvenile rainbow trout fed the experimental diets are presented in Table 6. Total carotenoid and astaxanthin concentrations in muscle of fish fed diets containing FSPH were not different compared to that of the control group. Changes in chroma values and color of fish muscle are presented in Table 7 and Figure 1. Lightness (L^*) of the muscle from fish fed the FSPH100 diet was not different from

Table 4.Growth performance and feed utilization of juvenile rainbow trout fed the experimental diets for 10 weeks

Diets	FSPH0	FSPH50	FSPH75	FSPH100
IBW (g)[1]	18.4±0.31	18.7±0.13	18.4 ±0.20	18.4 ±0.12
WG (%)[2]	234 ± 12.2	224 ± 6.6	226 ± 6.2	219 ± 5.1
SGR (%)[3]	1.7 ± 0.09	1.6 ± 0.03	1.6 ± 0.02	1.6 ± 0.02
FE (%)[4]	109 ± 5.5	101 ± 2.9	105 ± 6.8	104 ± 5.2
DFI (%)[5]	1.4 ± 0.01	1.3 ± 0.07	1.4 ± 0.07	1.4 ± 0.05
DPI (%)[6]	0.7± 0.01	0.7 ± 0.04	0.7 ± 0.04	0.7 ± 0.03
PER[7]	2.1 ± 0.11	1.9 ± 0.05	2.0 ± 0.13	2.0 ± 0.10
Survival (%)	77 ± 6.7	80 ± 11.5	85 ± 15.0	83 ± 8.8

Values are mean of triplicate groups and presented as mean ± SE. The lack of superscript letter indicates no significant differences among treatments.
[1]IBW (g): Initial body weight.
[2]Weight gain = (final fish wt.− initial fish wt.) × 100/initial fish wt.
[3]Specific growth rate = [(ln (final fish wt.) − ln (initial fish wt.)] × 100/days of feeding.
[4]Feed efficiency = wet weight gain × 100/feed intake.
[5]Daily feed intake = feed intake × 100/[(initial fish wt. + final fish wt. + dead fish wt.)/2 × days reared].
[6]Daily protein intake = protein intake × 100/[(initial fish wt. + final fish wt. + dead fish wt.)/2 × days reared].
[7]Protein efficiency ratio = (wet weight gain/protein intake).

Table 5. Proximate composition (% wet weight) of muscle in juvenile rainbow trout fed the experimental diets for 10 weeks

	Proximate composition			
Diets	Moisture	Crude protein	Crude lipid	Ash
FSPH0	76.9±0.92	25.7±0.29	1.9±0.38	1.7±1.41
FSPH50	76.6±0.11	23.8±0.60	3.0±0.38	1.6±1.40
FSPH75	76.2±0.40	24.4±1.86	2.5±0.28	1.7±0.03
FSPH100	76.2±0.26	24.1±2.43	3.3±0.59	1.6±0.01

Values are mean of triplicate groups and presented as mean ± SE. The lack of superscript letter indicates no significant differences among treatments.

Table 6.Total carotenoid and astaxanthin concentrations in the muscle of juvenile rainbow trout fed the experimental diets for 10 weeks

Diets	Total carotenoids (μg/g, DM)	Astaxanthin (μg/g, DM)
FSPH0	2.1± 0.61	0.1± 0.04
FSPH50	3.8± 0.23	2.4± 0.61
FSPH75	4.2± 1.48	3.0± 1.38
FSPH100	4.0± 0.88	2.2± 0.23

Values are mean of triplicate groups and presented as mean ± SE. The lack of superscript letter indicates no significant differences among treatments.

that of fish fed the FSPH0 diet but fish fed the FSPH50 and FSPH75 diets had lower L^* values than that of the FSPH0 diet. Redness (a*)values of muscle of fish fed the FSPH50, FSPH75, and FSPH100 diets were higher than that of the FSPH0 diet but fish fed the FSPH100 diet had lower a* values than those of fish fed the FSPH50 and FSPH75 diets. Yellowness (b*) values of the muscle of fish fed the FSPH50, FSPH75, and FSPH100 diets were higher than that of the FSPH0 group but lower in fish fed the FSPH100 diet compared to that of fish fed the FSPH75 diet.

Plasma and liver radical scavenging activity results are presented in Table 8. DPPH, hydroxyl, and alkyl radical scavenging activities in the plasma of fish were not different among treatments. DPPH, hydroxyl, and alkyl radical scavenging activities in the liver of fish were not affected by dietary treatments.

Plasma CAT, TAC, GPx, and SOD activities in juvenile rainbow trout fed the experimental diets are presented in Table 9. Plasma CAT activities in fish fed the FSPH50, FSPH75, and FSPH100 diets were lower than that of the FSPH0 group. Plasma TAC in fish fed the FSPH75 and FSPH100 diets was not different compared to that of the FSPH0 group but fish fed the FSPH50 diet had higher plasma TAC than that of the FSPH0 group. Plasma SOD activities in fish fed the FSPH50, FSPH75, and FSPH100 diets

Table 7. Changes in muscle chroma values of juvenile rainbow trout fed the experimental diets for 10 weeks

	Chroma values		
Diets	*L**	*a**	*b**
FSPH0	54.6±1.55^{b}	2.8±0.26^{a}	6.1±0.20^{a}
FSPH50	46.3±2.54^{a}	14.8±0.98^{c}	12.5±1.30^{bc}
FSPH75	46.1±1.05^{a}	15.4±1.46^{c}	14.4±1.41^{c}
FSPH100	50.2±0.21^{ab}	11.5±0.55^{b}	10.1±0.10^{b}

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different (P<0.05).

Figure 1. Muscle color of juvenile rainbow trout fed the experimental diets for 10 weeks.

Table 8. Plasma and liver radical scavenging activities of juvenile rainbow trout fed the experimental diets for 10 weeks

	Radical (%)		
Diets	DPPH	Hydroxyl	Alkyl
Plasma			
FSPH0	60.4±0.88	57.9±4.47	39.3±6.50
FSPH50	60.9±4.90	41.9±5.60	51.7±6.72
FSPH75	58.1±3.55	49.6±6.00	45.4±7.88
FSPH100	61.4±2.57	47.8±5.16	45.3±3.57
Liver			
FSPH0	60.1±2.93	64.7±3.55	60.8±2.72
FSPH50	68.0±2.74	68.4±2.17	55.3±1.41
FSPH75	64.3±3.70	64.4±8.50	61.1±6.87
FSPH100	65.0±2.82	66.1±1.52	64.2±6.01

Values are mean of triplicate groups and presented as mean ± SE. The lack of superscript letter indicates no significant differences among treatments.

were not different compared to that of the FSPH0 group but fish fed the FSPH100 diet had lower SOD activity than that of fish fed the FSPH75 diet. Plasma GPx activity was not affected by dietary treatments.

Discussion

FSM with *P. rhodozyma* in this study had no effect on growth performance, feed utilization and proximate composition of muscle in juvenile rainbow trout. The observed results in this study agree with those reported in many studies used diets containing astaxanthin for rainbow trout (Amar *et al*., 2001, 2004, 2012; Rehulka, 2000; Yanar *et al*., 2007), characins (Wang *et al*., 2006), Atlantic salmon (Bell *et al*., 2000), olive flounder (Pham *et al*., 2014) and Pacific white shrimp, *Litopenaeus vannamei* (Zhang *et al*., 2013).

P. rhodozyma has been investigated because of its high carotenoid content, and it appears to be good

Table 9. Plasma catalase (CAT), total antioxidant capacity (TAC), superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities of juvenile rainbow trout fed the experimental diets for10 weeks

	Antioxidant enzyme activity			
Diets	CAT (U/L)	TAC (U/ml)	SOD (U/ml)	GPx (U/L)
FSPH0	1.3±0.32^{b}	279.4±2.04^{a}	68.0±2.35^{ab}	2.3±0.26
FSPH50	0.7±0.25^{a}	291.8±0.57^{b}	71.6±29.46^{ab}	1.6±0.33
FSPH75	0.7±0.16^{a}	288.4±3.50^{ab}	74.3±27.66^{b}	1.9±0.12
FSPH100	0.7±0.04^{a}	290.5±4.48^{ab}	66.4±17.84^{a}	1.3±0.40

Values are mean of triplicate groups and presented as mean ± SE. Values in the same column having different superscript letters are significantly different (P <0.05). The lack of superscript letter indicates no significant differences among treatments.

natural pigment source for rainbow trout (Rehulka, 2000; Moretti *et al*., 2006). Amar *et al*. (2004) reported that muscle carotenoid content of fish fed *P. rhodozyma* was higher than that of fish fed *Dunaliella salina*. Bjerkeng *et al*. (2007) reported that astaxanthin and total carotenoid concentrations were significantly higher in salmon fed a diet supplemented with *P. rhodozyma* than those of salmon fed a diet supplemented with Lucantin pink. *P. rhodozyma* astaxanthin extracts can be utilized for muscle pigmentation in rainbow trout (Storebakken *et al*., 2004) and Atlantic salmon (Bjerkeng *et al*., 2007; Tangeras and Slinde, 1994). *P. rhodozyma* is a better astaxanthin source for Atlantic salmon muscle pigmentation than a synthetic compound (Whyte and Sherry, 2001). Higgs *et al*. (1995) reported that a commercial Japanese product containing *P. rhodozyma* was a more efficient astaxanthin source than that of Carophyll Pink. We found no differences between total carotenoid and astaxanthin concentrations in the muscle of fish fed diets containing FSM and *P. rhodozyma*, indicating that FSM with *P. rhodozyma* may be utilized efficiently as astaxanthin by juvenile rainbow trout. The total carotenoid and astaxanthin concentrations in the muscle of juvenile rainbow trout fed diets containing FSM with *P. rhodozyma* suggest that a rate up to 50 ppm dietary astaxanthin formulated with 10% FSM could be adequate for proper astaxanthin deposition in fish.

Redness (a^* value) and yellowness (b^* value) of muscle from rainbow trout fed the diets containing astaxanthin increased compared to those in the FSPH0 group but redness (a^* value) and yellowness (b^* value) of rainbow trout fed the FSPH100 diet was lower than those fed the FSPH50 or FSPH75 diets. This result may be due to the rigid cell walls of *P. rhodozyma*, which reduce astaxanthin availability (Tangerås and Slinde, 1994) and hamper astaxanthin absorption by salmonid fish (Storebakken *et al*., 2004). The lightness (L^* value) of the treated rainbow trout decreased compared to that in the control group. Some researchers have reported that lightness decreases and redness and yellowness increase with increasing flesh astaxanthin concentrations in Atlantic salmon (Skrede and Storebakken, 1986; Christiansen *et al*., 1995) and coho salmon (*O. kisutch*) (Smith *et al*., 1992). Storebakken *et al*. (2004) found that redness and yellowness values of rainbow trout increased significantly after feeding diets containing red yeast (*Xanthophyllomyces dendrorhous*, formerly *P. rhodozyma*).

Free radicals can be evaluated in the form of reactive oxygen species (ROS), which include superoxide anion radicals (O^{2-}•), hydroxyl radicals (HO•), hydrogen peroxide (H_2O_2), and singlet oxygen (O_2). These ROS are necessary for cellular functions, such as killing phagocytes, suppressing bacterial ingestion, and redox regulation of signal transduction. Carotenoids scavenge and deactivate free radicals both *in vitro* and *in vivo* (Kiokias and Oreopoulou, 2006). Many studies have indicated that carotenoids have the ability to interact with free radicals including peroxyl radicals (Kiokias *et al*., 2008). Pavlidis *et al*. (2006) reported that about 40% of carotenoids present in the skin of wild red porgy are a digested form of astaxanthin. Pham *et al*. (2014) found that plasma and liver from fish fed various carotenoid sources and levels show strong scavenging activities against hydroxyl and superoxide radicals. Our results here indicate that plasma and liver radical scavenging activities were not affected by dietary astaxanthin level. Our results suggest that dietary astaxanthin level did not influence plasma and liver radical scavenging activities in the fish and could be a promising astaxanthin resource for aqua feeds to improve color and scavenge free radicals.

CAT is an antioxidant enzyme that catalyzes the breakdown of H_2O_2, which has significant oxidant potential for cell membranes (Mansour *et al*., 2006). CAT activity has been detected in several tissues of teleost fish (OzcanOruc *et al*., 2004) and is enhanced after hatching in rainbow trout fry (Aceto *et al*., 1994). Zhang *et al*. (2013) reported that dietary astaxanthin significantly reduce serum CAT activity in rainbow trout. In our study, plasma CAT activity in fish fed dietary astaxanthin decreased significantly compared to that in the control group. This result may have been caused by excess accumulation of free radicals, such as superoxide anions and hydrogen peroxide, in rainbow trout plasma.

TAC is useful to evaluate the antioxidant capacity of all antioxidants (Kusano and Ferrari, 2008).

Previous studies have demonstrated that dietary astaxanthin could increase the TAC in Pacific white shrimp, *Litopenaeus vannamei* (Zhang *et al*., 2013) and black tiger shrimp, *Penaeus monodon* (Pan *et al*; 2003; Chien *et al*., 2003) fed diets supplemented with astaxanthin. Increased TAC may protect the organism from oxidative damage and reduce lipid hydroperoxides (Liu *et al*., 2010). In the present study, dietary astaxanthin generally enhanced plasma TAC activity, indicating that supplemental astaxanthin enhances antioxidant ability.

SOD is a cytosolic enzyme unique for scavenging superoxide radicals. Supplementation with dietary vitamin E or carotenoids has significant effects on liver antioxidant enzyme activities in sea bream (*Sparus aurata* L.) (Mourente *et al*., 2002), salmon (Sakai *et al*., 1994), characin (Wang *et al*., 2006) and juvenile tiger prawn (*Penaeus monodon*) (Chien *et al*., 2003) fed diets with carotenoids have lower SOD activities than those of fish fed a control diet. Yang *et al*. (2010) reported that dietary carotenoids result in lower muscle SOD activity in white shrimp (*Litopenaeus vannamei*) than that in shrimp fed the control diet. Higher SOD activity indicates that more superoxide radicals need to be reacted. Our previous study reported that fish fed diets containing different carotenoid sources and levels had lower plasma SOD activity than that of fish fed the control diet (Pham *et al*., 2014). In this study, plasma SOD activity of fish fed dietary astaxanthin was not different from that of the control group.

Astaxanthin did not affect plasma GPx activities in this study. Similarly, Pham *et al*. (2014) found no differences in plasma GPx activities of juvenile olive flounder fed different carotenoid sources and levels. Wang *et al*. (2006) reported that dietary carotenoid supplementation significantly reduces serum GPx activity in the characin (*Hyphessobrycon callistus*). The present results indicate that dietary astaxanthin did not reduce peroxide in cells or GPx activity.

Our results suggest that a diet containing 10% of this mixture (equal to 50 ppm astaxanthin) could successfully improve muscle pigmentation and antioxidant status of juvenile rainbow trout. Our results also indicate that FSM with *P. rhodozyma* is a promising natural source of astaxanthin.

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