ヒラメにおける紅藻Eucheuma denticulatumと褐藻Sargassum fulvellumの添加効果

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Comparative Effects of Dietary Supplementation Levels of *Eucheuma denticulatum* and *Sargassum fulvellum* in Diet of Juvenile Japanese Flounder *Paralichthys olivaceus*

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Abstract: The experiment was conducted to compare the effects of dietary supplementation of *Eucheuma denticulatum* and *Sargassum fulvellum* in diet of juvenile Japanese flounder, *Paralichthys olivaceus*. *S. fulvellum* contained 4.12% protein and 1.59% lipid while *E. denticulatum* contained 3.33% protein and 1.13% lipid. Isonitrogenous and isolipidic fishmeal (D1) and soy protein concentrate (SPC) based (D2) diets were prepared with *E. denticulatum* and *S. fulvellum* supplementation levels of 3% (D3) and 6% (D4), respectively. Triplicate groups of fifteen juveniles (3.23 ± 0.17 g, mean ± SD) were stocked in 100 *l* polypropylene tanks and fed diets twice daily to apparent satiation level for 56 days. The study demonstrated that fish fed D4 showed highest growth performance and feed utilization. Blood total cholesterol and triglycerides were significantly ($P < 0.05$) lowered in fish fed diets supplemented D3 and D4. Serum lysozyme activity was also higher in algae supplemented groups. *S. fulvellum* at 6% supplementation was also observed to result in improved carcass nutrient composition and enhanced health status than 3% *E. denticulatum* supplementation in diets for juvenile Japanese flounder.

Key words: *Sargassum fulvellum*; *Eucheuma denticulatum*; *Paralichthys olivaceus*; Fatty acid composition; Blood chemical parameters

Utilizing algae in feed formulation is increasingly trending in the aquaculture feeds industry today. Algae are incorporated in fish diets because they may provide good lipid and quality protein (Nozriah and Ching 2000), enhance immune system (Satoh et al. 1987), improve carcass quality (Morioka et al. 2008) and contribute other potential nutritional benefits. Algae are also rich sources of antioxidants, soluble dietary fibers, phtyochemicals and polyunsaturated fatty acids (Mohamed et al. 2012). Some algae contain compounds that serve biological functions against degenerative metabolic diseases (Mohamed et al. 2012). These properties make algae a candidate for use in feeds for marine fish (Kanazawa et al. 1979; Watanabe et al. 1983).

Lipids of high quality are important for fish growth. Polyunsaturated fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid are said to be more abundant in aquatic plants compared to terrestrial vegetation (Dantagnan et al. 2009). The mechanisms of synthesis and utilization of these essential fatty acids are also different and species-specific in fish (Dantagnan et al. 2009). Also, omega-3 fatty acids are known to induce lowering of triglycerides (Skulas-Ray et al. 2008). Thus, incorporation of algae in fish diets should produce positive effects on the carcass, nutritional quality, and health of fish.

Red algae *Eucheuma denticulatum* is classified

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as belonging to the family Solieriecеae, order Gigartinales, class Florideophyceae, division Rhodophyta. *E. denticulatum* has numerous hard cylindrical branches and a multi-axial structure (Al-Haj et al. 2009). The uses of *E. denticulatum* vary from being a major source of carrageenans, human food, to animal fodder. In addition, Al-Haj et al. (2009) confirmed in their study that one potential use of extract from *E. denticulatum* was as a source of antibacterial compounds.

Sargassum fulvellum is a type of perennial brown algae that grows in different coastlines around the globe. It is mainly used for human consumption, animal fodder, and biodiesel among others. Polysaccharides, lipids and extracts isolated from *S. fulvellum* were reported to exhibit several biological functions such as antitumor (Yamamoto et al. 1977), antioxidant (Kim et al. 2007), antipyretic, analgesic and anti-inflammatory in mice (Kang et al. 2008). Such biological functions were attributed to the presence of major functional compounds in brown algae like *S. fulvellum*. Compounds like fucoidan, fucoxanhin, tetrapeptides, alginates, laminarins and galactofucans among others, are naturally ubiquitous in brown algae.

In our previous studies, the optimal dietary supplementation level of *Eucheuma denticulatum*, a type of red algae was found to be at 3% for diets of both red sea bream *Pagrus major* juveniles (Ragaza et al. 2012) and Japanese flounder *Paralichthys olivaceus* fingerlings. *E. denticulatum* at 3% supplementation was efficiently absorbed and utilized by the fish yielding highest growth rates and enhanced feed utilization efficiencies. It also lowered serum total cholesterol and triglyceride levels. In a separate study, the optimal dietary supplementation level of *Sargassum fulvellum*, a type of brown algae was found to be at 6% for Japanese flounder fingerlings. Supplementation levels of 6% *S. fulvellum* resulted in increased feed intake, higher growth rates, and lowered hepatic, muscular and whole-body lipid contents in the flounders.

In this present study, the comparative effects of 3% *E. denticulatum* and 6% *S. fulvellum* supplementation on growth performance, feed utilization efficiency, carcass composition, blood chemistry, and serum lysozyme activities of juvenile Japanese flounder were investigated.

Materials and Methods

Red and brown algae

Red, *Eucheuma denticulatum* (supplied by Marine Science Co., Tokyo, Japan) and brown algae, *Sargassum fulvellum* (obtained from Andes Trading Company, Tokyo, Japan) used in the experiment were in 100% powdered form and 100% algal meal, respectively. Proximate composition and other characteristics of both algae are shown in Table 1.

Test diets

Three isonitrogenous and isolipidic experimental diets were prepared by adding 3% red algae (D3) and 6% brown algae (D4) in soy protein concentrate (SPC) based diet (D2). The supplementation levels used in this feeding trial were based on previous studies on use of *E. denticulatum* and *S. fulvellum* in diet of Japanese flounder fingerlings. Previous studies observed that optimal dietary inclusion levels for red and brown algae were 3% and 6%, respectively. D1 was served as positive control with brown fishmeal (FM) and activated gluten as the primary protein sources. D2, on the other hand, was served as negative control with FM, SPC and activated gluten as protein sources. D1 and D2 were prepared as controls for comparison to D3 and D4. The lipid sources used in the diets were Pollack liver oil (omega) and soybean lecithin. Alpha-cellulose was used

Table 1. Proximate composition and other characteristics of *E. denticulatum* and *S. fulvellum*

Parameters	*E. denticulatum*	*S. fulvellum*
Form	powder	powder
Protein (% in DM)	3.33	4.12
Lipid (% in DM)	1.13	1.59
Moisture	6.27	14.19
Ash (% in DM)	20.92	24.30
Fibre (% in DM)	10.85	8.30
Nitrogen free extracts (% in DM)	57.50	47.50
Total	100	100

Table 2. Ingredient and proximate composition of experimental diets

Ingredients (g/ 100 g)	D1	D2	D3	D4
Brown fish meal[1]	68	56	56	56
Soy protein concentrate[2]	0	15	15	15
E. denticulatum[3]	0	0	3	0
S. fulvellum[4]	0	0	0	6
Pollack liver oil[5]	6	6	6	6
Soybean lecithin[5]	4	4	4	4
Vitamin mix[6]	3	3	3	3
Mineral mix[7]	3	3	3	3
Vitamin C[8]	0.1	0.1	0.1	0.1
alpha-cellulose	9.9	6.9	3.9	0.9
Activated Gluten[9]	5	5	5	5
Attractant[10]	1	1	1	1
Total	100	100	100	100
Proximate Composition				
Crude Protein[11]	48.24	48.10	48.58	48.75
Moisture	11.95	11.53	11.30	11.98
Crude Lipid[11]	12.80	12.58	12.55	12.92
Ash[11]	14.72	14.45	14.53	14.10

[1] Nippon Suisan Co. Ltd., Tokyo, Japan.
[2] Fuji Protein Technologies, Inc., Tokyo, Japan.
[3] Andes Trading Co., Tokyo, Japan.
[4] Marine Science Co., Tokyo, Japan.
[5] Riken Vitamin, Tokyo, Japan.
[6] Vitamin mixture as reported by Kader et al. (2010).
[7] Mineral mixture as reported by Kader et al. (2010).
[8] Stay-C: L-ascorbyl-2-monophosphate-Na/Ca (DSM Nutrition Japan K.K.).
[9] Glico Foods Co. Ltd., Osaka, Japan; 85%, crude protein; 3.5% crude lipid.
[10] Attractant: betaine.
[11] Expressed in % dry matter.

Table 3. Total fatty acid (%) of experimental diets

Fatty acid	D1	D2	D3	D4
14:0	2.32	2.17	2.49	2.55
16:0	24.99	24.72	24.81	24.62
18:0	4.35	4.29	4.28	4.65
Σ Saturated	31.65	31.18	31.58	31.82
16:1	2.74	2.25	2.53	2.42
18:1	8.75	8.42	8.61	8.54
20:1	1.58	1.38	1.98	1.56
22:1	1.72	1.69	1.80	1.58
Σ Monoenes	14.78	13.74	15.33	14.09
18:2n-6	4.35	4.23	4.21	4.49
18:3n-6	0.04	0.04	0.03	0.04
20:2n-6	0.04	0.04	0.04	0.05
20:4n-6	0.22	0.17	0.26	0.40
22:3n-6	0.10	0.10	0.13	0.09
22:4n-6	1.38	1.31	1.52	1.64
22:5n-6	0.04	0.03	0.04	0.04
Σ n-6	6.14	5.92	6.22	6.53
18:3n-3	0.66	0.50	0.71	0.63
18:4n-3	0.46	0.41	0.49	0.49
20:4n-3	0.11	0.11	0.12	0.10
20:5n-3	2.81	2.76	4.32	4.99
22:5n-3	0.37	0.23	0.41	0.24
22:6n-3	5.43	5.03	5.36	6.22
Σ n-3	10.83	9.04	11.41	12.67

as carbohydrate or nitrogen-free extract source. The other ingredients were vitamins, minerals, attractant, etc (Table 2). The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 30 min. Lipid sources were then added and mixed for 15 min more. The mixture was then passed through a meat grinder with an appropriate diameter (1.3 to 1.5 mm) to prepare pellets and dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific Co., Ltd., Tokyo, Japan) at 60℃ for two hours. The dried pellets were then stored at -28℃ in a refrigerator until used.

Growth trial

Juvenile Japanese flounder used in the experiment were transferred from Matsumoto Suisan Co., Miyazaki, Japan to Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, where the feeding trial for 56 days was conducted. The juveniles were maintained on a commercial formulated diet (Higashimaru Food, Kagoshima, Japan) for two weeks prior to the feeding trial. Triplicate groups of 15 juveniles with a mean weight of 3.23 ± 0.17 g (mean ± SD) were randomly divided into twelve 100 *l* capacity polypropylene circular tanks, which were filled with 80 *l* of water and a flow-through seawater system (1.2-1.5 *l*/min). Each tank was supplied with continuous aeration to maintain dissolved oxygen level at saturation. Each tank was randomly assigned to one of the four dietary treatments. Daily ration size was divided into two equal feedings at 9.00 and 16.00 h. Diets were hand-fed to apparent satiation. Fecal matter was removed by siphoning the water from the bottom of each tank before giving the diet. Uneaten feed was removed 30 min after feeding and dried to quantify feed intake. The juveniles were counted and weighed every 14 days and the ration size was adjusted accordingly. In the duration of the feeding trial, monitored water

parameters were temperature (15° ± 1.5℃), pH (7.8 ± 0.3), dissolved oxygen (19.5 mg/*l* ± 1.2), and salinity (32.5 ± 0.8).

Sample collection and biochemical analysis

Twenty-five juveniles were kept in refrigerator at -20℃ at the beginning of the experiment as initial sample. At the end of the growth experiment, three juveniles from each tank were individually weighed and taken for whole body proximate analysis. The samples were freeze-dried and kept in freezer at -20℃ until analyzed. All fish were anesthetized with chilled water to ensure ease in handling during the blood collection. With heparinized syringes, blood was collected and pooled from the caudal vein of three fish in each replicate tank. A small portion of the heparinized blood was used to analyze the hematocrit (Ht) and hemoglobin (Hb) levels. Plasma samples were obtained at 3000 × *g* for 15 min using a high-speed refrigerated microcentrifuge (MX-160, Tomy Tech USA Inc., Tokyo, Japan) and kept at -80℃. Blood chemical parameters such as glutamyl oxaloacetic transaminase (GOT), total bilirubin (Tbil), glucose (GLU), blood urea nitrogen (BUN), glutamic pyruvate transaminase (GPT), total cholesterol (TCho) and triglycerides (TG) were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Liver was collected from three fish in each replicate tank, weighed individually to calculate hepatosomatic index (HSI), and finally pooled together and kept at -80℃. Feed and whole body total protein was analyzed by the Kjeldahl method (Kjeltec System 1002, Tecatore, Hoganas, Sweden), crude lipid was analyzed according to Bligh and Dyer (1959). Ash and moisture contents were determined according to the Association of Official Analytical Chemists methods (AOAC 1990). Crude lipid of dorsal muscle and liver of fish was also analyzed according to Bligh and Dyer (1959). Crude fibre content of algae was analyzed according to AOAC (1990) standards using the fibre-cap system.

Lysozyme activity

Serum lysozyme activity was analyzed by mixing 100 μl of test serum in 900 μl of *Micrococcus lysodeikticus* bacterial suspension in 0.05M sodium phosphate buffer of pH 6.2 and incubated for reaction at 25℃. Absorbance at 520 nm after 1 min and 5 min are then recorded using a spectrophotometer (DR/4000U, Hach Co., Loveland, CO, USA). One unit of lysozyme activity was defined as the amount of enzyme causing a reduction in absorbance of 0.001/min/*ml* serum.

Total fatty acid

Total lipid was extracted by homogenizing 0.2 g sample according to Bligh and Dyer (1959) method. Fatty acid composition was quantified according to Teshima et al. (1986), using extracted lipids from test diets and liver of fish. Calculated amounts of C23:0 were added in total lipid aliquots and were reacted with boron triflouride and dichloromethane in a heat block. Methylated fatty acid esters were then analyzed by a gas chromatograph (GC-17A, Shimadzu Co., Kyoto, Japan).

Statistical analysis

Statistical significance ($P < 0.05$) of difference among treatments was computed using one-way analysis of variance (Package Super-ANOVA, ver. 1.11, Abacus Concepts, Berkeley, CA, USA). Significant differences among treatments ($P < 0.05$) were evaluated by Tukey-Kramer test (Package Super-ANOVA, ver. 1.11, Abacus Concepts, Berkeley, CA, USA) as in Kramer (1956). Results were presented as mean ± SEM of three replicates. The survival rates presented in percentage were analyzed statistically by arcsine transformation.

Results

Proximate composition of S. fulvellum and E. denticulatum

S. fulvellum contained 4.12% protein and 1.59% lipid while *E. denticulatum* contained 3.33% protein and 1.13% lipid (Table 1).

Table 4. Survival, %WG, SGR, FER, FI and PER of juvenile Japanese flounder fed diets supplemented with levels of *E. denticulatum* and *S. fulvellum* algae for 56 days[1]

Diet	WG%	Survival[2]	SGR[3]	FER[4]	FI[5]	PER[6]
D1	457.23 ± 7.21[a]	100[a]	2.87 ± 0.01[b]	0.81 ± 0.01[ab]	0.32 ± 0.00[a]	1.68 ± 0.02[ab]
D2	422.32 ± 11.73[a]	100[a]	2.81 ± 0.01[a]	0.79 ± 0.00[a]	0.31 ± 0.00[a]	1.65 ± 0.01[a]
D3	472.73 ± 8.13[a]	100[a]	2.89 ± 0.01[b]	0.84 ± 0.01[b]	0.32 ± 0.00[a]	1.73 ± 0.02[b]
D4	590.22 ± 26.94[b]	100[a]	3.12 ± 0.01[c]	1.00 ± 0.00[c]	0.35 ± 0.00[b]	2.05 ± 0.01[c]

[1] Each value is the mean ± S.E.M. of data from triplicate groups. Within a column, means with the same letters are not significantly different ($P > 0.05$). Absence of letters also indicates no significant difference between treatments.
[2] Expressed in percentage (%)
[3] Specific growth rate = [100 × (ln final fish weight − ln initial fish weight)] / 56
[4] Feed efficiency ratio = wet weight gain (g) / dry feed intake (g)
[5] Expressed in g/fish/day
[6] Protein efficiency ratio = live weight gain (g) / protein intake (g)

Table 5. Proximate composition of whole body juvenile Japanese flounder fed diets with levels of *E. denticulatum* and *S. fulvellum* algae for 56 days[1]

Diet	Moisture	Protein[2]	Lipid[2]	Ash[2]
Initial[3]	75.10 ± 0.06	13.31 ± 0.12	3.72 ± 0.15	3.04 ± 0.18
D1	74.66 ± 0.15	13.32 ± 0.08[a]	4.13 ± 0.05	3.47 ± 0.11
D2	74.76 ± 0.11	13.29 ± 0.21[a]	3.95 ± 0.08	3.50 ± 0.09
D3	74.72 ± 0.14	13.41 ± 0.07[a]	4.05 ± 0.02	3.38 ± 0.14
D4	74.89 ± 0.95	14.57 ± 0.07[b]	3.98 ± 0.01	3.30 ± 0.28

[1] Each value is the mean ± S.E.M. of data from triplicate groups. Within a column, means with the same letters are not significantly different ($P > 0.05$). Absence of letters also indicates no significant difference between treatments.
[2] Expressed in wet weight basis
[3] Initial values were not included in statistical analysis.

Growth trial and proximate composition

Percent weight gain (%WG), specific growth rate (SGR), feed efficiency ratio (FER), protein efficiency ratio (PER), feed intake (FI), hepatosomatic index (HSI) and percent survival of Japanese flounder after 56-day feeding trial are summarized in Table 4. There was no mortality recorded in the 56-day feeding trial. Significantly ($P < 0.05$) highest growth performance and feed utilization was exhibited for fish fed D4 supplemented with *S. fulvellum*. Percent weight gain, SGR, FER and PER showed significantly ($P < 0.05$) highest values in fish fed D4. Percent weight gain of fish fed D1 to D3 were significantly lower than those of fish fed D4, but was not significantly ($P > 0.05$) different from each other. Also, SGR, FER, and PER of fish fed D1 and D3 were not significantly ($P > 0.05$) different from each other. Nonetheless, SGR, FER and PER of fish fed D1 was not significantly ($P > 0.05$) different from fish fed D2. Feed intake was higher for fish fed D4. Ash, lipid and moisture content of the whole body of juveniles in all treatments were found to be not significantly ($P > 0.05$) different, as shown in Table 5. Whole body crude protein content of fish fed D4 was found to be significantly highest ($P < 0.05$) among the treatments. On the other hand, crude lipid content of muscle and HSI were found to be not significantly different ($P > 0.05$) in all treatments (Table 6). However, crude lipid content of liver of fish fed *S. fulvellum* diet was found to be significantly lowest ($P < 0.05$) among the treatments.

Table 6. HSI and crude lipid of muscle and liver of juvenile Japanese flounder fed diets with levels of *E. denticulatum* and *S. fulvellum* algae for 56 days[1]

Diet	Muscle[2]	Liver[2]	HSI[3]
D1	14.15 ± 0.05	24.78 ± 0.22[b]	1.64 ± 0.06
D2	14.04 ± 0.03	24.01 ± 0.36[b]	1.82 ± 0.22
D3	14.07 ± 0.07	24.55 ± 0.16[b]	1.65 ± 0.14
D4	14.02 ± 0.05	21.87 ± 0.12[a]	1.71 ± 0.04

[1] Each value is the mean ± S.E.M. of data from triplicate groups. Within a column, means with the same letters are not significantly different ($P > 0.05$). Absence of letters also indicates no significant difference between treatments.
[2] Expressed in % crude lipid (dry matter)
[3] Hepatosomatic index = (100 × liver weight (g) / fish weight (g)

Table 7. Blood chemical parameters and serum lysozyme activities of juvenile Japanese flounder fed diets with levels of *E. denticulatum* and *S. fulvellum* algae for 56 days[1]

Diet	Ht[2]	Hb[3]	GOT[4]	Tbil[5]	Glu[6]	BUN[7]	GPT[8]	TCho[9]	TG[10]	Lys[11]
D1	21.33±3.79	3.03±0.03	103±2.00	3.80±0.04	59.1±0.90	8.9±0.10	45.6±2.20	199±2.00^{b}	322±4.00^{b}	12.96±0.15^{a}
D2	19.56±0.96	3.00±0.01	102±2.00	3.81±0.02	59.3±0.80	8.9±0.20	45.7±2.50	192±3.00^{b}	318±7.00^{b}	12.32±0.53^{a}
D3	20.00±0.87	3.00±0.00	105±6.00	3.81±0.20	58.0±0.10	8.8±0.20	44.8±2.30	173±2.00^{a}	211±2.00^{a}	19.91±0.11^{b}
D4	19.67±0.29	3.03±0.03	100±4.00	3.82±0.01	57.4±0.30	8.8±0.10	42.7±3.30	171±2.00^{a}	203±1.00^{a}	20.12±0.09^{b}

[1] Each value is the mean ± S.E.M. of data from triplicate groups. Within a column, means with the same letters are not significantly different ($P > 0.05$). Absence of letters also indicates no significant difference between treatments.
[2] Hematocrit; expressed in %
[3] Hemoglobin; expressed in g/*dl*
[4] Glutamyl oxaloacetic transaminase; expressed in IU/*l*
[5] Total bilirubin; expressed in mg/*dl*
[6] Glucose; expressed in mg/*dl*
[7] Blood urea nitrogen; expressed in mg/*dl*
[8] Glutamic pyruvate transaminase; expressed in IU/*l*
[9] Total cholesterol; expressed in mg/*dl*
[10] Triglycerides; expressed in mg/*dl*
[11] Lysozyme activity; expressed in U/*ml*

Table 8. Percent fatty acid composition on total fatty acid of liver of juvenile Japanese flounder fed diets with levels of *E. denticulatum* and *S. fulvellum* algae for 56 days[1]

Fatty acid	D1	D2	D3	D4
14:0	1.74±0.18	1.75±0.22	1.76±0.03	1.51±0.25
16:0	26.5±0.04	26.3±0.72	26.3±0.07	26.5±0.07
18:0	10.0±0.00	10.0±0.03	10.0±0.01	10.0±0.02
Σ Saturated	38.3±0.22	38.0±0.68	38.1±0.10	38.0±0.18
16:1	9.46±0.14	9.22±0.17	9.67±0.22	9.85±0.15
18:1	19.7±0.14	19.4±0.12	19.4±0.39	19.8±0.16
20:1	2.13±0.08	1.95±0.05	1.98±0.04	2.00±0.03
22:1	1.97±0.12	1.83±0.13	1.96±0.05	1.93±0.08
Σ Monoenes	33.3±0.02	32.4±0.27	33.0±0.69	33.6±0.08
18:2n-6	7.33±0.43	7.33±0.06	7.89±0.13	7.80±0.51
18:3n-6	0.20±0.00	0.21±0.03	0.23±0.01	0.24±0.01
20:2n-6	0.44±0.00	0.43±0.01	0.43±0.00	0.42±0.00
20:4n-6	0.26±0.06^{a}	0.22±0.02^{a}	0.24±0.02^{a}	0.68±0.02^{b}
22:3n-6	0.11±0.00	0.11±0.03	0.11±0.01	0.12±0.01
22:4n-6	1.34±0.04	1.28±0.21	1.76±0.09	1.54±0.12
22:5n-6	0.10±0.00	0.10±0.01	0.10±0.00	0.10±0.00
Σ n-6	9.81±0.45	9.68±0.21	10.8±0.24	10.9±0.36
18:3n-3	0.62±0.00	0.60±0.07	0.62±0.01	0.67±0.06
18:4n-3	0.39±0.00	0.39±0.05	0.42±0.02	0.41±0.02
20:4n-3	0.28±0.01	0.20±0.07	0.20±0.02	0.26±0.03
20:5n-3	2.28±0.00	2.21±0.32	2.39±0.24	2.38±0.26
22:5n-3	1.00±0.02	0.80±0.02	0.83±0.08	0.93±0.01
22:6n-3	7.77±0.16^{a}	7.62±0.06^{a}	7.89±0.13^{a}	11.5±0.08^{b}
Σ n-3	12.2±0.21^{a}	11.8±0.38^{a}	12.4±0.05^{a}	16.1±0.24^{b}
Σ PUFA[2]	22.0±0.65^{a}	21.5±0.59^{a}	23.1±0.29^{a}	27.0±0.56^{b}

[1] Values are expressed as mean ± SEM (n=3). Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters also indicates no significant difference between treatments.
[2] Total PUFA is expressed as sum of total n-3 fatty acids and total n-6 fatty acids.

Blood chemical parameters and serum lysozyme activity

Table 7 summarizes the blood chemical parameters and serum lyzoyme activity of juveniles. Blood chemical parameters, except for triglyceride (TG) and total cholesterol (TCho) showed no significant ($P > 0.05$) difference among the treatments. TG and TCho of fish fed D1 and D2 were significantly ($P < 0.05$) higher than TG and TCho of fish fed diets supplemented

with two types of algae. Nonetheless, TG and TCho of fish fed D3 and D4 were not significantly ($P > 0.05$) different from each other. Serum lysozyme activities (LYS) of fish fed diets supplemented with two types of algae were significantly ($P < 0.05$) higher than that of D1 and D2. LYS of juveniles fed D3 and D4 were not significantly ($P > 0.05$) different from one another.

Total fatty acid composition of diets and liver

Total fatty acid composition of experimental diets is shown in Table 3. D1, D3 and D4 contained higher levels of n-3 and n-6 polyunsaturated fatty acids (PUFA). D2 contained lowest PUFA.

Table 8 presents percent fatty acid composition on total fatty acid of liver of fish samples. DHA and arachidonic acid contents were found to be significantly highest ($P < 0.05$) in liver of fish fed *S. fulvellum*.

Discussion

S. fulvellum contained 4.12% protein and 1.59% lipid. Its protein content is within the range of brown algae (1–15% in dry weight) (Darcy-Vrillon 1993; Mabeau and Fleurence 1993). Its lipid content is also within the range of earlier studies on lipid content of brown algae (1–3% in dry weight) (Matanjun et al. 2009).

E. denticulatum contained 3.33% protein and 1.13% lipid. The lipid content of *E. denticulatum* is within the range of those previously reported on red algae (less than 4% in weight) (Matanjun et al., 2009) while its protein fraction is slightly lower compared to known ranges (10–47% in dry weight) (Darcy-Vrillon 1993; Mabeau and Fleurence 1993).

Variations in the protein (Fleurence 1999; Galland-Irmouli et al. 1999) and lipid (Colombo et al. 2006) contents of algae can occur due to difference in species, season and environmental conditions.

Ash contents in both algae are high, but are still within the known ranges reported (Mabeau and Fleurence 1993; Ortega-Calvo et al. 1993). The mineral content of algae is of higher quantity and quality than that of land plants (Ortega-Calvo et al. 1993).

Crude fibre contents of *S. fulvellum* and *E. denticulatum* were also within the range of other algae (Mabeau and Fleurence 1993). These fibres are mainly responsible for eliciting hypocholesterolemic and hypoglycaemic effects and decrease in digestive tract transit time (Matanjun et al. 2009). Both algae appear to be a good potential functional ingredient for lowering cholesterol and glycaemic index for prevention of metabolic diseases.

The present study investigated the effects of supplementation levels of 3% *E. denticulatum* and 6% *S. fulvellum* in the diets for juvenile Japanese flounder (*Paralichthys olivaceus*). The said supplementation levels were based on our previous works on the determination of optimal supplementation of *E. denticulatum* and of *S. fulvellum* in diets of juvenile red sea bream *Pagrus major* (Ragaza et al. 2012) and Japanese flounder *Paralichthys olivaceus* fingerlings. Our previous studies concluded that *E. denticulatum* and *S. fulvellum* can be efficiently used and absorbed by Japanese flounder fingerlings up to 3% and 6%, respectively without any significant negative or adverse effects on growth performance, feed utilization efficiency, and carcass quality and composition.

In the present study, supplementation of 6% *S. fulvellum* in diets of juvenile Japanese flounder yielded highest growth performance and feed utilization efficiency, significantly higher than that of control and basal diets and diet supplemented with 3% *E. denticulatum*. In the presence of algal substances through supplementation, the dietary nutrients are utilized effectively by delaying its absorption and improving carbohydrate and protein utilization (Yone et al. 1986). There were substances in algae that might activate metabolism and act as stimulants for growth (Nakajima 1991). Also, increased feed intake in fish fed *S. fulvellum* may also explain the higher growth and feed utilization. This might imply that *S. fulvellum* contains attractants. The polysaccharides known as alginates in algae have been shown to increase feed stability thereby improving

growth rate and feed utilization (Hashim and Mat Saat 1992). Moreover, supplementation of *S. fulvellum* in diet considerably improved feed efficiency, as shown in high FER values.

Supplementation of 6% *S. fulvellum* in diets increased whole body protein and lowered hepatic lipid content in juvenile Japanese flounder. Increased whole body protein (Xu et al. 1993; Davies et al. 1997; Soler-Vila et al. 2009) and lowered hepatic lipid (Xu et al. 1993) in fish fed algae supplemented diets were also shown in other studies. *S. fulvellum* supplementation might have caused activation of hormonal regulation of lipid metabolism that led to mobilization of reserved lipids to be used instead of muscle protein degradation (Nematipour et al. 1987, 1990). Lowered hepatic lipid levels might be explained by compounds, such as fucoxanthin (though not tested in this study) which are present in brown algae that have been found to decrease hepatic lipids in mice (Woo et al. 2009). The decrease in hepatic lipids might be attributed to the increase in DHA, which decreased the activity of enzymes (found in the liver) in fatty acid production and increased hepatic fatty acid β-oxidation in the liver (Maeda et al. 2008). This was also observed in the present study, given the significant elevated levels of DHA in the liver of flounder fed diet supplemented with 6% *S. fulvellum*. This is of significant importance as a fatty liver may be a risk factor linked to chronic liver disease. Natural substances that can prevent lipid accumulation in liver may be used as nutraceuticals (Dvir et al. 2009)

Higher levels of DHA, EPA, and arachidonic acid in the diet supplemented with *S. fulvellum* might have caused increased levels of DHA and arachidonic acid found in the liver of flounder fed diets supplemented with 6% *S. fulvellum*, but not in flounder fed diets supplemented with 3% *E. denticulatum*. Moreover, numerous studies conducted on mammals have also reported increased levels of hepatic DHA and arachidonic acid of mice fed fucoxanthin extracted from brown algae (Tsukui et al. 2007, 2009; Airanthi et al. 2011). The increase in the hepatic DHA and arachidonic acid levels of animals fed extracts from algae might be attributed to the upregulation of enzymatic activities associated with bioconversion of α-linolenic acid (C18:3n-3) to DHA and linoleic acid (C18:2n-6) to arachidonic acid (Tsukui et al. 2007, 2009). Also, through the Sprecher's shunt pathway, DHA can be synthesized from EPA via beta oxidation (Voss et al. 1991).

Supplementation of 6% *S. fulvellum* and 3% *E. denticulatum* in diets significantly reduced levels of triglycerides and total cholesterol in serum of flounder. Recent studies on a variety of animals have shown benefits of supplementing algae in diets by lowering plasma cholesterol (Nakajima, 1991; Kim et al. 2002; Dvir et al. 2009; Matanjun et al. 2010), triglyceride (Nakajima 1991; Dvir et al. 2009; Matanjun et al. 2010) and lipid (Nakagawa et al. 2000) levels. Both *S. fulvellum* and *E. denticulatum* used in the diets have high omega-3 fatty acids, which are said to have triglyceride-lowering ability (Skulas-Ray et al. 2008). Also, it is a generally accepted observation that plasma TGs are reduced by consuming soluble dietary fibers (Dvir et al. 2009), which are known to be present (but not tested) in both algae used in the present study. Other pigments (Nagaoka et al. 2005) and bioactive substances that are present in both algae might also provide a biological function of lowering cholesterol and triglycerides and affecting lipid metabolism. Carrageenan, which is commonly extracted from red algae such as *E. deniticulatum*, has also been clinically proven in humans to lower blood cholesterol (Panlasigui et al. 2003). Fucoxanthin from brown algae such as *S. fulvellum* might also impart hypocholesterolemic and triglyceride lowering effects. Thus, it is possible that high omega-3 fatty acids, rich fiber content and other specific biological substances present in both algae contributed to the lowering of serum TCho and TG levels.

Supplementation of 6% *S. fulvellum* and 3% *E. denticulatum* in diets also significantly increased serum lysozyme activity. Lysozyme is distributed widely in the tissues, body fluids and secretion of aquatic animals. It relates to the nonspecific immunological response of

aquatic animals against invasive microorganisms (Osserman et al. 1974). It lyses Gram-positive and kills Gram-negative bacteria after other enzymes have disrupted the polysaccharides in the outer cell walls (Qi et al. 2005). Polysaccharides and extracts from algae have been observed to show immune-stimulatory effects against pathogens in fish and shrimp (Qi et al. 2005; Yeh et al. 2008). These polysaccharides were suggested to increase both humoral and cellular immune responses (Cheng et al. 2008). The mechanism of how these algae polysaccharides enhance immune system of fish is unknown (Cheng et al. 2008). Nonetheless, β-glucan, which is a polysaccharide known to be present in algae enhances the innate immune system of the fish through the direct activation of macrophages (Robertsen 1994).

In conclusion, supplementation level of 6% *S. fulvellum* in the diet of juvenile Japanese flounder resulted in better growth performance and improved feed utilization efficiency and carcass nutrient composition compared to supplementation of 3% *E. denticulatum* in the diet. Nonetheless, both supplementation levels of *S. fulvellum* and *E. denticulatum* reduced serum triglyceride and total cholesterol and enhanced nonspecific defence system of flounder via increasing serum lysozyme activity. As bioactive substances might be present in algae, it is recommended for future studies to determine, quantify, and test the availability of such functional compounds in algae, and as to how these compounds might affect growth and overall health status of fish.

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ヒラメにおける紅藻 *Eucheuma denticulatum* と褐藻 *Sargassum fulvellum* の添加効果

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ヒラメにおける紅藻と褐藻の添加効果を明らかにするために，魚粉の一部を濃縮大豆タンパク（SPC）に代替し，紅藻 3%または褐藻 6%をそれぞれ添加した試験飼料を作製した。ヒラメ稚魚（平均体重3.2 g）を用いて，56日間の飼育実験を実施した結果，褐藻 6%添加区は他の試験区に比べ有意に高い成長を示した。試験終了後の血液化学性状では，海藻粉末添加区の血中総コレステロール量とトリグリセリド量が無添加区と比較して有意に低い値を示した。また，血清リゾチーム活性は海藻粉末添加により有意に増加することが示された。以上の結果から，紅藻及び褐藻添加はヒラメの健康状態を改善し，紅藻より褐藻の方が効果が高いことが明らかとなった。