A prebiotic effect of *Ecklonia cava* on the growth and mortality of olive flounder infected with pathogenic bacteria

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Abstract

Olive flounder (*Paralichthys olivaceus*), also known as the Japanese flounder in Japan, is one of the most important commercial marine finfish species cultured in Korea and Japan. The purpose of this study was to evaluate how a species of brown algae (*Ecklonia cava*) affects the growth rate of olive flounder and its immune response to pathogenic bacteria. First, the experimental fish were divided into four groups: the control group was fed the diet containing only 1.0% *Lactobacillus plantarum* (*L. plantarum*), group I was fed 1.0% *L. plantarum* and 1.0% *Ecklonia cava* (EC), group II was fed 1.0% *L. plantarum* and 0.1% ethanol extract of EC (EE), and group III was fed 1.0% *L. plantarum* and 0.5% EE. The diets were fed to the fish twice a day for 16 weeks. The results indicated that supplementation with 1.0% EC and 0.1% EE improved the growth and body weight of olive flounder, and decreased its mortality. This diet, however, did not significantly affect the biochemical profiles of the experimental flounder. The supplementation of 1.0% EC also enhanced the innate immune response of the fish, as evidenced by the high respiratory burst, and increased serum lysozyme and myeloperoxidase activity. The addition of 1.0% EC and either 0.1% or 0.5% EE also decreased the accumulative mortality of olive flounder infected by pathogenic bacteria (*Edwardsiella tarda*, *Streptococcus iniae*, and *Vibrio harveyi*). Overall, these results suggest that *E. cava* can act as a prebiotic by improving the innate immune response in fish infected with pathogenic bacteria as increased the growth of the probiotic.

Keywords

Prebiotic; *Ecklonia cava*; Olive flounder; *Lactobacillus plantarum*; Innate immune response

1. Introduction

During the last 15 years, cultured fish production has more than doubled, and marine

aquaculture industries in South Korea now produce a number of fish species [1,2,3,4]. In particular, the olive flounder is one of the most important commercial marine aquaculture species in Korea, and improvements to culturing techniques have increased its production [5]. Recently, however, outbreaks of infectious disease caused by viruses, bacteria, and parasites in aquaculture facilities have inflicted severe damage to fish production in Southeast Asia, which in turn has become a major economic problem [2]. Bacteria have been identified as being responsible for the majority of economic losses [6]. In cultured olive flounder, the most common pathogenic bacteria are *Edwardsiella tarda*, *Streptococcus sp.* and *Vibrio sp.* [7]. *Edwardsiella tarda* affects both freshwater and marine fish, causing septicemia, skin lesions, and diseases of the muscles and internal organs, including the liver, kidney, and spleen [8]. *Streptococcus sp.* infection in fish is considered as a re-emerging disease affecting a variety of wild and cultured fish. These infections are actually a complex of diseases caused by different genera and species, and affect the central nervous system, causing suppurative exophthalmia and meningoencephalitis [6]. Pathogenic *Vibrio sp.*, the etiological agent of classical vibriosis, is widely distributed, and it causes hemorrhagic septicemia in a variety of warm- and cold-water fish species of economic importance [6]. Researchers have studied the treatment of bacterial diseases using antibiotics and chemotherapeutics [9,10,11,12]. However, antibiotics use in aquaculture may be detrimental to the environment and human health, and it causes the development and transfer of resistance to other aquatic bacteria [13], fish pathogens, and human pathogens, and the accumulation of residual antibiotics in aquaculture products [13,14,15,16,17].

Probiotics, especially lactic acid bacteria (LAB), have been widely employed to protect fish against infectious diseases [18], including edwardsiellosis, furunculosis, and vibriosis [19,20,21]. In addition, LAB probiotics are safe to use in fish that will be consumed and they also protect against harmful pathogens by inhibiting the growth of *Aeromonas salmonicida*,

Vibrio anguillarum, and *Flavobacterium psychrophilum*, as shown in rainbow trout [19,21]. For these reasons, the use of probiotics as a strategy to control fish pathogens is increasing [22,23,24,25]. Interestingly, prebiotics can enhance the growth of probiotics and induce their production of bioactive secondary metabolites. Therefore, probiotics and prebiotics can be useful in the study of how such components can limit bacterial disease in olive flounder.

Recently, the biological roles of *Ecklonia cava* (*E. cava*), a brown seaweed contains polyphenol and polysaccharide compounds as major components have been reported [26, 27]. Especially, LBA-fermented *E. cava* increased the protein contents in comparison with the non-fermented *E. cava* [26,28]. However, until now, there is no report about effect of *E. cava* as a prebiotic in cultured olive flounder.

Therefore, the present study investigated the use of *E. cava* as a prebiotic in cultured olive flounder and evaluated its effect on the immunological response of fish infected with pathogenic bacteria.

2. Materials and Methods

2.1 Preparation of experimental diets containing *E. cava* and *L. plantarum*

E. cava was collected along the coast of Jeju Island in South Korea during the period from March to May 2011 and identified by Dr. Lee (Jeju National University, South Korea, voucher specimen: Jeju-C-47). It was washed with fresh water, freeze-dried, and then pulverized into powder with a grinder. A previous study indicated the effect of *E. cava* polysaccharide on the growth of probiotics was similar to that of the original *E. cava* powder [26]. Therefore, the original *E. cava* powder (EC) was used in this study. To prepare the ethanol extract of *E. cava* (EE), which contains rich polyphenols with antibacterial effects, the powder (20 kg) was homogenized in 2 L of 100% ethanol. After 24 h, the EE was evaporated, freeze-dried, and then kept at -20°C for later experiments. The experimental diets

were prepared by adding 0.1% EE, 0.5% EE, or 1.0% EC to the soft extruded pellets (Daebong susan, Jeju, Korea, Table 1). In addition, 1.0% of *L. plantarum* was added to all of the diets as a probiotic. The experimental diets were fed to all fish twice a day for 16 weeks.

2.2 Preparation of cultured olive flounder fish for the field trial

Olive flounder (mean body weights 300 to 350 g) were purchased from a private hatchery (Geumdeung, Jeju Island, South Korea) and acclimated for one week. The field trial was performed in Manhae susan (Pyoseon-ri, Pyeongyang, Jeju, South Korea) for 16 weeks (February 2013 to May 2013). Cylindrical polypropylene (PP) tanks (8 m in diameter and 1.5 m in height) were used to house the fish. Each tank was stocked with 1,800 olive flounders, and the water was changed 10–15 times a day before samples were taken during the feeding trial. Seawater temperature (16–18°C), dissolved O content (8.23–9.37 mg/L), salinity (27.68–31.36 ppt), and pH (7.5–8.7) were maintained in their respective ranges during the experimental feeding period. During the 16-week trial, the fish were fed their respective diets (as described in section 2.1.1.) twice a day at 06:00 h and 17:00 h.

The experimental fish were divided into four groups: the control group was fed the diet containing only 1.0% *L. plantarum*, group I was fed 1.0% *L. plantarum* and 1.0% EC, group II was fed 1.0% *L. plantarum* and 0.1% EE, and group III was fed 1.0% *L. plantarum* and 0.5% EE.

2.3 Measurement of body weight and mortality in olive flounder

During the experimental period, the body weights and mortality of the olive flounder were recorded every 4 weeks for 16 weeks.

2.4 Analysis of biological indices in the serum of olive flounder

To investigate the effects of EC and EE on the biochemical blood profiles of the experimental olive flounder, blood samples were collected from the fish by a heparin-coated needle at 0 and 16 weeks of the experimental period. Individual fish were sampled only once

to prevent multiple bleeds and stress from affecting the assays. The blood was allowed to clot for 30 min and then kept at 4°C for 3 hr. Then, the clotted samples were centrifuged at 3000 rpm for 10 min at 4°C and the serum was collected and used to analyze total protein (TP), triglycerides (TRIG), aspartate aminotransferase (AST), alanine aminotransferase (ALT), glucose (GLU), phosphorus (PHOS), cholesterol (CHOL), and hematocrit (HT) with biochemical analysis kits (STANBIO, TX, USA) and an automatic analyzer (Boehringer Mannheim, Mannheim, Germany).

2.5 Effects of EC and EE on the innate immunity of olive flounder

Normally, lysozyme can either act as an innate opsonin, or have lytic activity. To evaluate the effects of EC and EE on the innate immunity of olive flounder, we investigated respiratory burst activity by measuring neutrophils, lysozyme function, and myeloperoxidase (MPO) activity with slight modifications of previously suggested methods [1,29]. This testing was done using previously collected serum samples at 0 and 16 weeks into the experimental period.

2.6 Polymerase chain reaction analysis

To determine whether the olive flounder was successfully infected with the three administered bacteria (*E. tarda*, *S. iniae* and *V. harveyi*), samples of kidney and liver tissue from fish injected with the bacteria were subjected to polymerase chain reaction (PCR) analysis. The cDNA purified from the kidney and liver samples was used with the primers of the three bacteria (Bioneer, Daejeon, South Korea), shown in Table 2. Then PCR analysis was performed for 40 cycles with a 5 min denaturing step at 94°C, a 1 min annealing step at 55 to 60°C, and a 20 min extension phase at 72°C using the TaKaRa PCR machine (Takara Bio, Shiga, Japan). All PCR products were run on a 1.5% agarose gel, and visualized by UV transillumination.

2.7 Challenge test with bacterial infection

Samples of the pathogenic bacteria used in the study (*E. tarda*, *S. iniae*, and *V. harveyi*) were obtained from the KCTC (Korean Collection for Type Culture, Daejeon, Korea). First, *E. tarda* and *S. iniae* were incubated in BHIA broth and *V. harveyi* in TSA broth at 25°C until they were needed. Fish were divided into four groups (30 fish per group). Each group was given dietary supplements with 1.0% EC, 0.1% EE, or 0.5% EE. Another group of 30 fish was a control (uninfected) group that was fed a basal diet. Before the experimental feeding was initiated, all fish were respectively injected intraperitoneally with 100 µl PBS containing *E. tarda*, *S. iniae*, and *V. harveyi* at 3×10^5 ciliates mL⁻¹. One day after the bacterial challenge, six fish were randomly collected from each tank and anesthetized with MS-222 (NaHCO₃ and tricaine methanesulfonate; Sigma Aldrich Inc., St. Louis, MO) in dechlorinated water for 2 min. The cumulative mortality was calculated with the formula described by [30] every day for 2 weeks.

3. Results

3.1 EC enhanced the growth of olive flounder by increasing body weight

The body weights (g) of fish were recorded every 4 weeks for 16 weeks. The growth of olive flounder fed the experimental diet containing 1.0% *L. plantarum* for 16 weeks is shown in Fig. 1A and 1B. During this time, the body weights of all fish fed the 1.0% EC diets were higher than those of fish fed the other diets (Fig. 1A). However, both the 0.1% EE and 0.5% EE diets showed similar or lower body weights than the control group for all experimental periods (Fig. 1A). Also, as shown in Fig. 1B, we found that the final body weights of fish increased by 108% when they were fed the 1.0% EC diet for 16 weeks; in contrast, neither EE supplement had this effect. These results demonstrate that the supplementation of EC effectively improved the growth of olive flounder.

3.2 Both 1.0% EC and 0.1% EE decreased the mortality of olive flounder

To access the effects of 1.0% EC, 0.1% EE, and 0.5% EE on the mortality of olive flounder, the number of deceased fish was counted every 4 weeks for 16 weeks. The number of dead fish in the control group increased markedly (up to nearly 316% at 4 weeks), and then gradually decreased over the following 12 weeks (Fig. 1C). However, the supplementation of 1.0% EC and 0.1% EE resulted in lower mortality than that of the control group throughout the experimental period. Moreover, the accumulated mortality of the fish fed the control diet was markedly increased (a total of approximately 636 fish over 16 weeks), whereas the mortality decreased by 18% and 13% in fish supplemented with 1.0% EC and 0.1% EE, respectively (Fig. 1D and 1E).

3.3 The supplementation of 1.0% EC, 0.1% EE, and 0.5% EE did not significantly affect the biochemical profiles of olive flounder

To assess the effects of 1.0% EC, 0.1% EE, and 0.5% EE on the biochemical profiles of olive flounder, serum was collected from the fish at 0 and 16 weeks and TP, TRIG, AST, ALT, GLU, PHOS, CHOL, and HT values were measured. None of these levels significantly changed over the course of the experimental period in any of the groups (Table 2).

3.4 The supplementation of 1.0% EC improved the growth of olive flounder by enhancing innate immunity

We identified the effects of 1.0% EC, 0.1% EE, and 0.5% EE on the innate immunity of olive flounder by measuring respiratory burst activity, lysozyme activity, MPO activity, and growth rates. As shown in Fig. 2A, supplementation with 1.0% EC, 0.1% EE, and 0.5% EE considerably increased superoxide anion production compared to the control group. In particular, the supplementation of 1.0% EC led to the highest respiratory burst activity with enhanced superoxide formation. Figure 2B shows that lysozyme activity was significantly enhanced in fish fed the 1.0% EC, 0.1% EE, and 0.5% EE diets at 16 weeks. In particular, fish fed the 1.0% EC diet had the highest lysozyme activity compared to the others.

Furthermore, MPO activity was significantly enhanced in fish fed the 1.0% EC and 0.5% EE diets at 16 weeks, whereas there was no significant change in the fish supplemented with 0.1% EE (Fig. 2C). The diet with 1.0% EC resulted in the highest MPO activity out of all the groups. Moreover, Fig. 2D shows how supplementation with 1.0% EC improved the growth rate of fish, compared to the control group. These results indicate that the supplementation of 1.0% EC produced the highest growth rate of olive flounder by enhancing their innate immunity.

3.5 The supplementation of 1.0% EC, 0.1% EE, or 0.5% EE decreased the cumulative mortality of olive flounder infected by pathogenic bacteria

In the *in vivo* trial, deaths resulting from *E. tarda*, *S. iniae*, and *V. harveyi* in fish fed diets containing 1.0% EC, 0.1% EE, or 0.5% EE were recorded. First, PCR assay was performed to confirm that the bacteria were actually present in the fish after injection. The results showed mRNA expression levels of specific regions of *E. tarda*, *S. iniae*, and *V. harveyi* (Supplement Fig. 1), which confirmed bacterial infection in the fish.

As indicated in Fig. 3, the injection of *E. tarda*, *S. iniae*, and *V. harveyi* markedly increased the cumulative mortalities of the control group over 14 days. When *E. tarda* was injected into the fish, the fish with supplements in their diet all showed decreased mortality, in contrast to the control group in which mortality increased (Fig. 3A). Among the supplements, 1.0% EC decreased mortality the most in fish injected with *E. tarda* (Fig. 3A). In addition, flounder infected with *E. tarda* developed heavy viscous liquid in the body cavity, liver congestion, and abnormal growth of the kidney and spleen, all of which are clinical signs seen in naturally infected olive flounder (data not shown). Interestingly, these changes were inhibited by the supplementation of 1.0% EC, 0.1% EE, and 0.5% EE.

In the fish injected with *S. iniae*, the 1.0% EC and 0.1% EE supplements also significantly decreased cumulative mortality compared to the control group, whereas 0.5%

EE did not affect the number of deaths (Fig. 2B). Moreover, the supplementation of 1.0% EC and 0.1% EE improved the clinical manifestations of fish infected with *S. iniae*, including skin ulcers of the scale-covered parts of the body and diffuse petechiation or hemorrhage of internal organs (data not shown).

Similarly, in the fish infected with *V. harveyi*, supplementation of 1.0% EC and 0.1% EE significantly decreased the cumulative mortality of the infected flounder (Fig. 3C). However, the 0.5% EE group showed a similar result to that of the control population. As with the other bacteria, 1.0% EC and 0.1% EE improved the clinical manifestations of *V. harveyi* infection, including generalized septicemia with hemorrhage at the base of the fins, exophthalmia, corneal opacity, and edematous lesions predominantly at the hypodermis (data not shown). These results indicate that the supplementation of 1.0% EC, 0.1% EE, and 0.5% EE decrease the cumulative mortality of olive flounder infected with pathogenic bacteria.

4. Discussion

The present study demonstrates that the supplementation of *E. cava* with 1.0% *L. plantarum* (a probiotic) supported the growth of olive flounder by enhancing its prebiotic activity, resulting in increased body weight and decreased mortality. The supplementation of EC or EE in diets containing *L. plantarum* also increased the activities of both lysozyme and MPO, which reduced the cumulative mortality of olive flounder infected with pathogenic bacteria. These results show the potential value of *E. cava* as a beneficial prebiotic. This is similar to our previous work, in which we showed that *E. cava* stimulates the growth of probiotics and produces various useful components with anti-bacterial effects [26].

A number of supplements containing probiotics or beneficial bacteria have been used to control pathogenic bacteria in fish. Indeed, probiotics have been proposed as an alternative method of improving the growth and health of cultured organisms [31,32,33]. The ability of

probiotics to control disease in fish have been documented in grouper [34,35,36], tilapia [21], sea bass [37,38], and white shrimp [39,40,41]. Our results showed that the supplementation of 1.0% EC with *L. plantarum* improved the growth and decreased the mortality of olive flounder. The supplementation of 0.5% EE also decreased the mortality of olive flounder, but did not affect body weight.

We previously demonstrated that the prebiotic effects of *E. cava* enhanced the growth of LBA and its secondary metabolites by inhibiting the growth of pathogenic bacteria in fish [26]. In addition, [42] reported that the increase in growth rates can be attributed to the improvement of the microbial flora balance in the intestines. These results suggest that EC and LBA can act synergistically to improve the growth and reduce mortality in olive flounder. Overall, the results of this field trial suggest the potential benefits of using *E. cava* in olive flounder aquaculture.

The innate immune system of fish, while primitive, is the first line of defense against pathogenic bacteria in fish in an aquaculture system [2]. The most important components of the innate immune system include macrophages, monocytes, granulocytes, and humoral elements, such as lysozymes and the complement system [43,44]. Cross reported that the regulatory signals generated by probiotics enhance host immunity against pathogens [45]. Recently, prebiotics have also been found to affect innate immunity both *in vitro* and *in vivo* [46,47,48]. In addition, probiotic bacteria have been shown to induce proinflammatory cytokines, stimulate the activity of natural killer cells, increase mucosal and systemic antibody production, activate phagocytes, and increase lysozyme and complement activity [2,35,36,49]. The influx of phagocytes to the infection site is actually an innate defense mechanism of fish against pathogens [39]. During the respiratory burst, phagocytes produce toxic oxygen forms [40], which are proportional to the intensity of a respiratory burst [50]. In fish specifically, phagocytic cells, including monocytes, macrophages, and/or neutrophils can

generate superoxide anions, all components of respiratory burst activity, to kill pathogens [39,51,52]. In addition, phagocytic cells can induce lysozyme to act as an opsonin, with bactericidal activity and activate the complement system and other phagocytes [53,54,55]. Phagocytic cells can also induce MPO activity [56,57].

In our study, the supplementation of 1.0% EC and *L. plantarum* in the diets of olive flounder markedly enhanced the innate immune system by activating the respiratory burst and lysozyme and MPO activity, compared to the control group. These results were supported by the ability of LAB to enhance the activity of phagocytes, lysozyme, and complement, as also demonstrated in previous reports [36,58]. Furthermore, our research showed the beneficial effect of *E. cava* on the growth of LBA [26]. It is possible that these enhancements of the innate immune system protect against pathogenic bacterial infection. Interestingly, the supplements of EC and/or EE with *L. plantarum* reduced mortality in olive flounder for 2 weeks after being exposed to *E. tarda*, *S. iniae* and *V. harveyi*. Our previous report indicated that the secondary metabolites obtained from *E. cava* treated *L. plantarum*, including cyclohexasiloxane, dodecamethyl-, 2,4,6-cycloheptatrien-1-one, 3,5-bis-trimethylsilyl- and 9-anthracenyltrimethyl-, inhibited the growth of pathogenic bacteria [26]. Moreover, the polysaccharide components of *E. cava* and its ferments led to immune enhancement in immune cells and gamma ray-irradiated immune cells [27,28]. The previous study supports our results by indicating the potential capacity of *E. cava*-treated LAB to improve immunological and antibacterial activity in olive flounder infected with pathogenic bacteria [26,58]. Furthermore, diet supplements with seaweed extracts can influence select genera of gut-associated microbiota [59,60]. More specifically, supplements with fucoidan have been shown to stimulate the growth of lactobacilli in the intestine [60,61]. In addition, recent reports showed that novel dietary polysaccharides derived from seaweed species can affect the gut associated microbiota, which includes the stimulation of LAB such as lactobacilli

[60,61] and prohibitive effects on detrimental bacteria such *Enterobacteria* [60], including *Escherichia coli* [62]. In summary, these results suggest that *E. cava* can enhance the innate immune system, inhibit pathogenic bacteria growth, promote LAB, and produce secondary metabolites from LAB in the gut-related immune system, all of which contribute to lower mortality in fish infected with pathogenic bacteria.

5. Conclusions

In conclusion, diets supplemented with *E. cava* (a prebiotic) and *L. plantarum* (a probiotic) can enhance the innate immune system and gut-related microbiota of olive flounder, thereby improving the resistance of these fish to pathogenic bacteria. The evaluation of the commercial cost–benefit analysis of these supplements is an area for future study.

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Figure legends

Fig. 1. Effects of 1.0% EC, 0.1% EE, and 0.5% EE on body weight, mortality, and accumulated mortality of olive flounder. Supplements containing 1.0% EC, 0.1% EE, or 0.5% EE were added to the diet and 1.0% *L. plantarum* was added to all diets. The fish were fed twice a day for 16 weeks. The body weight (g/fish) (A) and mortality (C) of experimental olive flounder were recorded every 4 weeks for 16 weeks. The body weight (%) (B) and the accumulated mortality (D) in experimental olive flounder were measured at 4 weeks. The accumulated mortality (E) was calculated as a percentage, with the control group being 100%. Statistical evaluation was performed to compare the experimental and control groups. $p < 0.05$.

Fig. 2. Effects of 1.0% EC, 0.1% EE, and 0.5% EE on the respiratory burst activity of neutrophils, MPO activity, serum lysozyme activity, and growth rate of olive flounder. Supplements containing 1.0% EC, 0.1% EE, or 0.5% EE were added to the diet and 1.0% *L. plantarum* was added to all diets. The fish were fed twice a day for 16 weeks. The respiratory burst activity of neutrophils (A), MPO activity (B), serum lysozyme activity (C) and growth rate (D) were measured at 16 weeks. Statistical evaluation was performed to compare the experimental and control groups. $p < 0.05$.

Fig. 3. Effects of 1.0% EC, 0.1% EE, and 0.5% EE on the cumulative mortality of olive flounder infected by *E. tarda* (A), *S. iniae* (B), and *V. harveyi* (C). Supplements containing 1.0% EC, 0.1% EE, or 0.5% EE were added to the diet and 1.0% *L. plantarum* was added to all diets. The fish were fed twice a day for 2 weeks. Before feeding, all experimental fish were injected intraperitoneally with 100 μ l PBS containing *E. tarda*, *S. iniae*, and *V. harveyi* at 3×10^5 ciliates mL^{-1} . The accumulative mortality of infected olive flounder was recorded every day for 2 weeks. The accumulated mortality was calculated as a percentage, with day 0

being 100%. Statistical evaluation was performed to compare the experimental and control groups. $p < 0.05$.

Table 1. The composition of the experimental diets in all the groups.

Experimenal groups	Soft extruded pellets						EC (%)	EE (%)	<i>L. Plantarum</i> (%)
	CP	CF	Ca	Ash	Fi	P			
Control group	37	10	1.5	17.0	3.0	2.7	-	-	1.0
EC 1.0%-fed group	37	10	1.5	17.0	3.0	2.7	1.0	-	1.0
EE 0.1%-fed group	37	10	1.5	17.0	3.0	2.7	-	0.1	1.0
EE 0.5%-fed group	37	10	1.5	17.0	3.0	2.7	-	0.5	1.0

Table 2. The oligonucleotide sequences of primers used in this study.

Primer	Oligonucleotide sequence (5'-3')	Expected sizes	Target gene	Reference
VH-K1	CAAGGCACACTTGACGAGCT	638bp	<i>rpoB</i>	Oh (2008)
VH-K2	AAACCGCCTTCTTCAGTTAA			
etfD-F	GGTAACCTGATTTGGCGTTC	445bp	etfD	Castro et al. (2010)
etfD-R	CCTAATTCTGTAATCGCTCC			
LOX-1	AAGGGGAAATCGCAAGTGCC	870bp	<i>lctO</i>	Mata et al. (2004)
LOX-2	ATATCTGATTGGGCCGTCTAA			

Table 3. Effects of 1.0% EC, 0.1% EE and 0.5% EE on the serum biochemical profiles of olive flounder.

	Diet	TP ^a (g/dL)	TRIG ^b (mg/dL)	AST ^c (U/L)	ALT ^d (U/L)	GLU ^e (mg/dL)	PHOS ^f (mg/dL)	CHOL ^g (mg/dL)	HT ^h (%)
0 wk	Control	5.2 ± 1.4	70.0 ± 6.2	73.5 ± 14.8	<10	21.5 ± 8.0	11.1 ± 6.6	179.5 ± 11.2	22.2 ± 2.2
	EC 1.0%	5.0 ± 2.4	68.0 ± 8.0	69.2 ± 8.1	<10	19.0 ± 7.4	12.7 ± 4.3	169.3 ± 10.1	27.7 ± 1.7
	EE 0.1%	4.7 ± 6.3	66.0 ± 9.6	72.1±10.0	<10	19.0 ± 12.4	10.5 ± 6.4	156.6 ± 12.7	17.0 ± 6.5
	EE 0.5%	4.5 ± 3.1	66.0 ± 6.7	64 .6±7.6	<10	26.0 ± 10.8	10.6 ± 7.0	180.4 ± 24.7	15.4 ± 3.8
16 wks	Control	5.2 ± 0.3	111.7 ± 31.8	59.8 ± 15.0	16.3 ± 5.9	22.3 ± 3.6	9.7 ± 0.7	175.2 ± 34.4	18.0 ± 2.4
	EC 1.0%	5.3 ± 0.2	130.8 ± 21.3	52.0 ± 16.5	16.0 ± 5.2	18.3 ± 7.3	9.7 ± 1.5	189.2 ± 28.3	23.0 ± 2.0
	EE 0.1%	5.1 ± 0.3	128.8 ± 38.2	51.6 ± 9.7	15.8 ± 13.0	20.0 ± 3.0	10.5 ± 1.6	183.0 ± 25.4	21.7 ± 1.9
	EE 0.5%	5.1 ± 0.3	100.5 ± 41.1	55.7 ± 11.1	19.2 ± 6.8	16.3 ± 4.8	6.3 ± 0.6	146.3 ± 40.1	22.2 ± 2.8

^aTP: Total protein, ^bTRIG: Tryglyceride, ^cAST: Aspartate aminotransferase, ^dALT: Alanine aminotransferase, ^eGLU: Glucose, ^fPHOS: Phosphorus, ^gCHOL: Cholesterol, ^hHT: Hematocrit

Figure

Figure 1. Lee et al.

Figure 2. Lee et al.

Figure 3. Lee et al.