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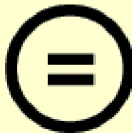

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A THESIS
FOR THE DEGREE OF MASTER OF SCIENCE

Effects of different dietary levels of Piperine supplementation on growth, feed utilization, intestinal morphology, innate immunity and IGF-I expression in olive flounder (*Paralichthys olivaceus)* and red sea bream (*Pagrus major*)

G H T Malintha

Department of Marine Life Science

GRADUATE SCHOOL

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2016. 08.

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Effects of different dietary levels of Piperine supplementation on growth, feed utilization, intestinal morphology, innate immunity and IGF-1 expression in olive flounder (*Paralichthys olivaceus)* and red sea bream (*Pagrus major*)

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(Supervised by Professor Kyeong-Jun Lee)

A thesis submitted in partial fulfilment of the requirement for the degree of
MASTER OF SCIENCE

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요약문

양어사료 내 piperine 과 black pepper 가루의 첨가 효능을 평가하기 위해 참돔(실험-I)과 넙치(실험-II) 치어를 대상으로 총 2 개의 사양실험이 수행되었다. 실험 목적을 달성하기 위해 어류의 성장률, 사료효율, 외관상소화율, 비특이적 면역력, 장 조직 변화, IGF-I 유전자발현 및 공격실험에 의한 질병저항성을 조사하였다.

실험-I 에서는 어분을 기초로 한 대조구(control), piperine 을 각각 0.025, 0.05, 0.1, 0.2% (T1, T2, T3, T4) 첨가한 실험구 및 black pepper 를 0.1% (T5) 첨가한 실험구로 총 6 개의 실험사료로 구성하였다. 참돔 치어(초기평균무게: 7.6 ± 0.1 g)는 총 3 반복구로 배치되었으며 1 일 3 회에 나누어 13 주 동안 만복공급 하였다. 13 주간 실시된 사양실험 결과, 사료전환효율에서 사료 내 piperine 이 0.05% 이상 첨가된 실험구(T2, T3, T4)가 대조구에 비해 유의적으로 낮았다. 비특이적 면역 분석 결과, SOD 에서 모든 첨가실험구가 대조구에 비해 유의적으로 높은 값을 보였다. NBT 를 제외한 MPO, SOD, total immunoglobulin, lysozyme, antiprotease 분석결과, piperine 첨가 실험구가 대조구에 비해 유의적으로 높은 결과 값을 보였다. 참돔의 형태학적 및 장 조직학적 변화 결과, 비만도(CF)는 첨가실험구들이 대조구와 비교하여 유의적으로 높았으며, T1 실험구가 가장 높은 값을 보였다. 또한, 융모길이(VL)는 사료 내 piperine 을 첨가한 실험구(T1, T2, T3)가 대조구에 비해 유의적으로 높은 값을 보였다. 배상세포(GC)의 수에서도 piperine 첨가실험구가 대조구와

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비교하여 높은 경향을 보였다. 간 내 IGF-I mRNA 분석 결과에서는 실험구간에 유의적인 차이는 없었지만, 저농도의 piperine 을 첨가한 실험구에서 대조구에 비해 증가하는 경향을 보였다.

실험-II 는 실험-I 과 동일하게 어분을 기초로 한 대조구(control), piperine 을 각각 0.025, 0.05, 0.075, 0.1, 0.2% (T1, T2, T3, T4, T5) 첨가한 사료구 및 black pepper 를 0.1% (T6) 첨가한 사료실험구로 총 7 개의 실험사료로 구성되었다. 넙치 치어(초기평균무게: 27.6 ± 0.4 g)는 총 3 반복구로 배치되었으며 1 일 3 회에 나누어 8 주 동안 실험사료를 만복공급 하였다. 8 주간 사양실험이 종료 된 후 성장지표와 사료효율 측정결과 유의적인 차이는 없었으나, 성장율과 일간성장율에서 T2 실험구가 가장 높은 값을 보였으며, 사료전환효율에서 T4 실험구가 가장 낮은 값을 보였다. Total immunoglobulin, lysozyme 활성 분석 결과, 모든 실험구에서 대조구와 비교하여 유의적으로 높은 값을 보였다. T6 실험구는 Ig 분석에서, T4 실험구는 lysozyme 활성 분석에서 가장 높은 결과를 보였다. 외관상소화율 분석결과, 건물소화율은 첨가실험구에서 유의적으로 높은 값을 보였다. T1, T2, T3, T6 실험구는 대조구와 비교하여 유의적으로 높은 소화율 값을 보였으며, T2 실험구가 가장 높은 소화율 값을 보였다. 단백질소화율 결과, T1, T2 실험구가 다른 실험구에 비교하여 유의적으로 높은 값을 보였다. 간 IGF-I mRNA 분석 결과, 첨가실험구에서 증가하는 경향을 보였지만 유의적인 차이는 없었다. E.*tarda* 를 이용한 공격실험 결과, 모든 실험구에서 대조구와 비교하여 높은 생존율을 보였으며, T1, T2, T6 실험구가 가장 높은 생존율을 보였다.

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비만도 측정 결과 수치상으로 증가하는 경향을 보였으며, 융모길이 측정 결과, piperine 첨가실험구가 대조구와 비교하여 유의적으로 높은 값을 보였다.

결론적으로, 사료 내 적당량의 piperine 첨가는 참돔과 넙치의 성장을 촉진시킬 수 있으며, 사료 내 영양소의 소화, 흡수를 도와 사료의 이용효율을 높일 수 있을 것으로 보인다. 또한 참돔과 넙치의 비특이적 면역력을 높일 수 있으며, 소장 내 융모의 길이와 표면적을 높이고 배상세포의 증식에도 긍정적인 영향을 미칠 것으로 사료된다. 참돔과 넙치 사료 내 piperine 적정 첨가함량은 약 0.05% 내외가 될 것으로 추측된다. Piperine 은 IGF-I 의 유전자 발현에도 긍정적인 영향이 있을 것으로 추측되지만 보충 연구가 요구된다. 또한 양어사료 내 piperine 의 첨가효과와 piperine 의 체내 이용 및 대사에 관한 보다 많은 보충연구가 요구된다.

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ABSTRACT

This study was carried out in two sets of experiments to evaluate the effects of dietary supplementation of piperine and black pepper for red sea bream (*Pagrus major*) (Exp-I) and olive flounder (*Paralichthys olivaceus*) (Exp-II). In this study, fish growth performance, feed utilization, diet digestibility, gene expression level of liver insulin-like growth factor I (IGF-I), intestinal histology, innate immunity and disease resistance were examined.

In Exp I, a fish meal based diet was regarded as a control diet and five other experimental diets were formulated by dietary inclusion of piperine at different inclusion levels of 0.025, 0.05, 0.1 and 0.2% (T1,T2,T3 and T4, respectively) or black pepper at a level of 0.1% (T5). Triplicate groups of fish (initial mean body weight, 7.6 ± 0.1 g) were fed one of the experimental diets to apparent satiation for 13 weeks. At the end of the feeding trial, all the treatment groups exhibited significantly higher growth compared to the control group. Specific growth rate (SGR) and protein efficiency ratio (PER) have followed similar trends to weight gain. Feed conversion ratio was significantly reduced with piperine and pepper supplementation while 0.05% piperine supplemented group showed the best performance. Plasma hemoglobin level was increased significantly, while hematocrit, glucose and protein levels were increased numerically by piperine. Total cholesterol level in plasma was significantly reduced with piperine supplementation in diets. Analysis of innate immunity parameters revealed significant enhancements with the supplementation. The highest Superoxide dismutase (SOD) value was observed in T2 group and the highest lysozyme and total immunoglobulin were observed in T5 group while all treatment groups showed significantly higher immune response for myeloperoxidase (MPO), SOD, total immunoglobulin, lysozyme and antiprotease activities compared to the control group. Results of whole-body composition showed significant reduction

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in dry matter of fish fed piperine compared to the control group. Ash content was significantly increased with the supplementation except for T5 group. The highest protein content was found in T2 group. Liver Insulin like growth factor-I (IGF-I) mRNA level was influenced by dietary inclusion of piperine and pepper even though it was not significant. Conditional factor and goblet cell count increased significantly with the dietary supplementation and the highest values were observed in T1 group. Also, villi length increased significantly in piperine supplemented groups in which all the treatment groups showed significantly higher values compared to the control while T1 has shown the highest.

In Exp II, a fish meal based diet was regarded as a control diet and six other experimental diets were formulated by dietary inclusion of piperine at different inclusion levels of 0.025, 0.05, 0.075, 0.1 and 0.2% (T1,T2,T3,T4 and T5 respectively) or black pepper at a level of 0.1% (T6). Triplicate groups of fish (initial mean body weight, 27.6 ± 0.4 g) were fed one of the experimental diets to apparent satiation for 8 weeks. At the end of the feeding trial, growth performance and feed utilization of the fish groups were significantly improved with dietary supplementation of piperine. Weight gain and specific growth rate were observed the highest in T2 group. Lowest feed conversion ratio was given by T4 group even though it was not affected significantly. All treatment groups showed significantly higher PER compared to the control while T3 group showed the highest. T6 group showed the highest Immunoglobulin and antiprotease levels whereas T4 represented the highest lysozyme activity. T5 exhibited the highest NBT value among the treatments. Other immunity parameters were increased with the dietary supplementation but were not significantly affected. Significantly higher whole-body protein level was observed in T2 group compared with the control group while dry matter and lipid levels were found to be the highest in T1 group. T1, T2, T3 and T6 groups showed

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significantly higher dry matter digestibility compared to the control group while T2 was the best. T1 and T2 groups showed significantly higher protein digestibility than other treatments. Liver IGF-I mRNA expression level was increased significantly with the dietary supplementation of piperine. During the *E. tarda* challenge test, all treatment groups showed higher survival rates compared to the control group. T1, T2 and T6 groups showed the highest survival rate. Conditional factor of fish was increased by the dietary treatment numerically while villi length and goblet cell count were increased significantly. All piperine supplemented groups exhibited significantly higher villi length values compared to the control while T2 group was the highest.

In conclusions, dietary piperine supplementation could improve the growth rate of red sea bream and olive flounder. Dietary piperine supplementation might assist the digestion and absorption of dietary nutrients to both fishes and thereby increase the diet utilization efficiency. Dietary piperine supplementation could improve the innate immunity of the fishes and increase disease resistance. Dietary piperine supplementation would have beneficial effects on the length and surface area of villi, and on the goblet cells which help to excrete mucosa, enzymes and hormones to facilitate a better digestibility in both fishes. Further studies are necessary for the IGF-1 expression even though piperine showed some positive effects in both fishes. Therefore, optimum level of piperine in diets would be approximately 0.05% for both red sea bream and olive flounder.

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1. INTRODUCTION

1.1 Antibiotics use in Aquaculture

Aquaculture industry is one of the fastest growing animal protein producing sectors, currently accounting for roughly 46% of the global food fish production. In the past decade, there has been a relatively stagnant fish supply from capture fisheries while aquaculture has emerged as the fastest-growing animal food producing sector (FAO, 2011). With the increasing intensification and commercialization of aquaculture production, infectious diseases are the major problem causing heavy losses to the fish farming industry (Bondad-Reantaso et al., 2005).

The recent expansion of intensive aquaculture practices has led to high interest in understanding various fish diseases so that they can be treated or prevented. It is widely demonstrated that the occurrence of diseases in fish farms is due to several factors concerned with the rearing methods, environmental conditions and variations. Consequently, cultivated fish can become more susceptible not only to pathogenic but also to opportunistic bacteria (Woo and Bruno, 1998).In intensive aquaculture, the use of antibiotics and chemotherapeutics for treatment and prophylaxis has been broadly criticized for its negative impact and thereby research on eco-friendly alternatives to antibiotics that may keep fish healthy, such as probiotics and plant based immune-stimulants, has been continuously increased (Sahu et al., 2007). In addition, the global demand for safe food has prompted the search for natural alternative growth promoters to be used in aquatic feeds. There has been heightened research in developing new dietary supplementation strategies in which various health and growth promoting compounds as

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probiotics, prebiotics, synbiotics, phytobiotics and other functional dietary supplements have been assessed (Denev, 2008).

1.2 Phytobiotics

Phytobiotics can be defined as plant derived products added to feed in order to improve the performance of animal. Phytobiotics have a wide variety of properties such as, antioxidant, antimicrobial, anticarcinogenic, analgesic, insecticidal, antiparasitic, anticoccidial, growth promoters, appetite enhancement, stimulant of secretion of bile and digestive enzyme etc. The evaluation of phytobiotics in aquaculture is a relatively new area of research showing promising results. Addition of different single herbal extracts (*Massa medicata*, *Crataegi fructus, Artemisia capillaries*, *Cnidium officinale*) or a mixture of all the herbs promoted growth and enhanced some non-specific immunity indicators of fish (Yin et al., 2009).

Wenk (2003) reported that herbs, spices and their extracts can stimulate appetite and endogenous secretions such as enzymes or have antimicrobial, coccidiostatic or anthelmintic activities in monogastric animals. Oligosaccharides and polysaccharides such as inulin (fructan), fructo– oligosaccharides (FOS) and arabinogalactans, which are extracted from plants, and sulfated fucans, which are extracted from seaweeds, are potential substitutes for currently used antibiotic growth promoting compounds (Tringali, 1997).

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1.3 Present status of phytobiotics in Aquaculture

In aquaculture, one of the most promising methods in strengthening defense mechanism and disease management is through prophylactic administration of immune-stimulants (Raa et al., 1992). Recent advancement in immuno-nutrition studies revealed that some nutrients are linked to the immunological status of fish (Kumar et al., 2005). This has drawn the attention of fish nutritionists to the immune-protection of fish besides the growth. Yuan et al. (2007), fed common carp (*Cyprinus carpio*) diets containing a mixture of *Astragalus membranaceus* (root and stem), *Polygonum multiflorum*, *Isatis tinctoria* and *Glycyrrhiza glabra* (0.5 and 1%) for 30 days and observed that both concentrations significantly increased macrophage phagocytic activity, respiratory burst and levels of total protein, albumin, globulin and nitric oxide synthetase activity in the serum; no significant difference was found in superoxide dismutase, lysozyme activities and triglyceride level. Root extracts of the Chinese herb *Astragalus* contain polysaccharides, organic acids, alkaloids, glucosides and volatile oil as major active components that have been found to enhance immune function in fish (Jeney et al., 2009). The *Astragalus* polysaccharide (APS) from *A. membranaceus* is reported to halt reactive oxygen species (ROS) production, stimulate humoral and cellular immunity, and thus possess anticancer and immune-stimulating effects (Yuan et al., 2008). The oriental medicinal herb *G. glabra* (liquorice) comprises flavonoids and pentacyclic triterpene saponin, including liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin and glycyrrhizic acid as major constituents and is reported to have anti-oxidant effects (Yin et al., 2011). Kim et al. (2007), demonstrated 80% higher lysozyme activity and 66% higher leukocyte phagocytic activity in olive flounder (*Paralichthys olivaceus*) fed a diet containing a mixed culture of extracts from the mushrooms

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Phellinus linteus and *Coriolus versicolor*. Kelp grouper, *Epinephelus bruneus*, fed a diet supplemented with ethanol extract of the mushroom *P. linteus* for 30 days showed significantly higher alternative complement activity, serum lysozyme activity, phagocytic activity, phagocytic index, respiratory burst activity, superoxide dismutase activity and glutathione peroxidase activity compared with fish fed the control diet without mushroom extract (Harikrishnan et al., 2011). This edible mushroom contains a large number of biologically active compounds, such as polysaccharides and tri-terpenes that may exhibit immune-modulating properties and stimulate proliferation of T lymphocytes, polyclonal activation of B cells.

1.4 Piperine and its potential as a phytobiotic

Piperine (1-piperoyl-piperidine) is a major alkaloid component of black (*Piper nigrum* Linn.) and long (*Piper longum* Linn.) pepper with pungent and biting tastes (Dogra et al., 2004). These pepper species have been used as seasoning in cookery and as an ingredient in alternative medicine for the treatment of several illnesses, such as bronchitis and asthma (Singh, 1992). Among its chemical-biological activities, piperine exhibits antimicrobial (Reddy et al., 2004), anti-inflammatory (Pradeep & Kuttan, 2004) and antioxidant (Mittal & Gupta, 2000) properties. It also increases the bioavailability of certain drugs in the organism (Karan et al., 1999) and acts as a chemo-preventive factor against pro-carcinogens activated by cytochrome P-450 (Reen et al., 1997). According to Kohlert et al. (2000), the active principles of phytogenic additives are absorbed in the intestine by enterocytes and are quickly metabolized by the body. Piperine induces alterations in membrane dynamics and permeation characteristics, as well as the synthesis of proteins associated with cytoskeletal function resulting in an increase in the small

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intestine absorptive surface (Khajuria et al., 2002). The rapid metabolism and the short-life of piperine indicate a low risk of accumulation in the tissue. Besides being a natural compound that does not produce detected residues in the animal or in their derived products, piperine is easily isolated in great amounts and has shown interesting biological effects in studies developed in mammals.

1.5 Objective of the study

Therefore, the objective of this research work was to determine the effect of piperine as a phytogenic additive in diets for both red sea bream and olive flounder. For this purpose, growth, feed utilization, innate immunity, digestibility, gut histology and IGF-1 expression were examined.

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[EXPERIMENT I]

2.1 MATERIALS AND METHODS

2.1.1 Experimental diets and design

Six experimental diets were formulated to be isonitrogenous (45% crude protein) and isocaloric (18.6 kJ g^{-1}) (Table I-1). A FM-based diet was regarded as a control diet and five other experimental diets were prepared by dietary inclusion of piperine at different inclusion levels of 0.025, 0.05, 0.1 and 0.2% (T1, T2, T3 and T4 respectively) or black pepper at a level of 0.1% (T5). All dry ingredients were thoroughly mixed and, after addition of fish oil and double-distilled water, the dough was extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) in ideal size, dried at 24 °C for 24 h and stored at −20 °C until use.

Figure I-1. Diet formulation

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Table I-1. Formulation and chemical composition of the experimental diets.

Ingredient	Experimental diets					
	Control	T1	T2	T3	T4	T5
Brown fish meal	38.00	38.00	38.00	38.00	38.00	38.00
Soybean meal	10.00	10.00	10.00	10.00	10.00	10.00
Corn gluten meal	10.00	10.00	10.00	10.00	10.00	10.00
Wheat flour	27.50	27.475	27.45	27.40	27.30	27.40
Fish oil	10.00	10.00	10.00	10.00	10.00	10.00
CMC	1.00	1.00	1.00	1.00	1.00	1.00
[1]Mineral Mix	1.00	1.00	1.00	1.00	1.00	1.00
[2]Vitamin Mix	0.50	0.50	0.50	0.50	0.50	0.50
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50
Di calcium phosphate	1.50	1.50	1.50	1.50	1.50	1.50
Piperine	0.00	0.025	0.05	0.10	0.20	0.00
Black pepper	0.00	0.00	0.00	0.00	0.00	0.10
Chemical composition						
Moisture	7.1	7.0	6.9	6.8	7.1	7.0
Protein	45.0	45.3	44.4	44.1	44.1	43.7
Lipid	15.4	15.0	14.5	13.7	13.8	14.4
Ash	9.2	9.3	9.2	7.4	9.4	9.8

[1]Mineral premix (g/kg mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3$. $6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[2]Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL-a tocopheryl acetate, 18.8; thiamin 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

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2.1.2 Feeding trial and sample collection

Juvenile red sea bream (*Pagrus major*) were transported from a private hatchery to Marine and Environmental Research Institute, Jeju National University, Jeju, Korea. All the fish were fed a commercial diet for two weeks to be acclimatized to the experimental condition and facilities. At the end of the acclimation period, the fish (initial mean body weight, 7.6 ± 0.1 g) were randomly distributed into eighteen tanks of 120 L capacity polyvinyl circular tanks at a density of 30 fish per tank. The tanks were supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were fed one of the experimental diets to apparent satiation (three times a day, 08:00, 13:00 and 18:00 h) for thirteen weeks.

Figure I-2. Growth measurement and sample collection

Water temperature was fluctuated at 23.1±2 ℃ range for the whole duration. Growth measurement was carried out at every 2 weeks. All the fish in each tank was bulk-weighed and counted for calculation of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival by the following formulas: Weight gain (%) = 100 × (final mean body weight – initial mean body weight) / initial mean body weight; Specific growth rate (%) = [(log_e final body weight – log_e initial body weight) / days] × 100; Feed

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conversion ratio = dry feed fed / wet weight gain; Protein efficiency ratio = wet weight gain / total protein given.

At the end of the feeding trial, all the fish in each tank were counted and bulk weighted for calculation of growth parameters. Three fish per tank (nine fish per dietary treatment) were collected for whole-body proximate composition analysis. Also, four fish per tank were randomly captured, anaesthetized with 2-phenoxyethanol solution (200 ppm) and blood samples were taken from the caudal vein with heparinized syringes. After analysis of hematocrit, hemoglobin and respiratory burst activity in whole blood samples, plasma were separated and used for determination of immunoglobulin level and biochemical parameters. Another set of blood samples were taken from caudal vein of four fish from each tank using non-heparinized syringes and allowed to clot at room temperature for 30 min. Then the serum was separated by centrifugation for 10 min at 5000×g and stored at −70 °C for the analysis of innate immune parameters including lysozyme, superoxide dismutase (SOD), myeloperoxidase (MPO) and anti-protease activities. Feeding was stopped 24 h prior to weighing or blood sampling to minimize the stress of fish.

Figure I-3. Sample analysis

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2.1.3 Analyses

Analysis of moisture and ash content were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FOSS, Sweden) and crude lipid was determined using the method described by Folch et al. (1957).

Hematocrit was determined by microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of glucose, total protein, cholesterol and triglyceride were determined by an automated blood analyzer (SLIM, SEAC Inc, Florence, Italy).

The oxidative radical production by phagocytes during respiratory burst was measured through NBT assay described by Anderson and Siwicki (1995). Briefly, blood and nitro-blue-tetrazolium (0.2%) (NBT; Sigma, USA) were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 μl was taken out and dispensed into glass tubes. Then, 1 ml of dimethyl formamide (Sigma, USA) was added and centrifuged at 2000×*g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using spectrophotometer. Dimethyl formamide was used as blank.

Plasma immunoglobulin (Ig) levels were determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein content was measured using a micro protein determination method (C-690; Sigma), prior to and after precipitating down the immunoglobulin molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

A turbidometric assay was used for determination of serum lysozyme level through the method described by Hultmark (1980) with slight modifications. Briefly, *Micrococcus*

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lysodeikticus (0.75 mg ml^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4) then 200 µl of suspension was placed in each well of 96-well plates and 20 µl of serum was added subsequently. The reduction in absorbance of samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader. Hen egg white lysozyme (Sigma) was used as a standard. The values were expressed as µg ml^{-1}.

Serum myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997). Briefly, twenty microliter of serum was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma, USA) in 96-well plates. Then, 35 µl of 3,3',5,5'-tetramethylbenzidine hydrochloride (TMB, 20 mM) (Sigma, USA) and H_2O_2 (5 mM) were added. The color change reaction was stopped after 2 min by adding 35 µl of 4 M sulfuric acid. Finally, the optical density was read at 450 nm in a microplate reader.

Serum superoxide dismutase (SOD) activity was measured by the percentage reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using a SOD Assay Kit (Sigma, 19160) according to the manufacturer's instructions. Each endpoint assay was monitored by absorbance at 450 nm (the absorbance wavelength for the coloured product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The per cent inhibition was normalized by mg protein and presented as SOD activity units.

The serum anti-protease activity was measured according to the method described by Ellis (1990), with slight modifications (Magnadóttir et al., 1999). Briefly, twenty microliter of serum was incubated with 20 µl of standard trypsin solution (Type II-S, from porcine pancreas, 5 mg ml^{-1}, Sigma-Aldrich) for 10 min at 22 °C. Then, 200 µl of phosphate buffer (0.1 M, pH 7.0) and 250 µl azocasein (2%) (Sigma-Aldrich) were added and incubated for 1 h at 22 °C. Five

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hundred microliter of tri-chloro acetic acid (10%) (TCA) was added and further incubated for 30 min at 22 °C. The mixture was centrifuged at 6000×*g* for 5 min and 100 µl of the supernatant was transferred to the wells of a 96 well flat bottomed microplate containing 100 µl of NaOH (1 N). Optical density was read at 430 nm. For a 100% positive control, buffer was replaced for serum, while for the negative control buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

$$\text{Trypsin inhibition (\%)} = (A_1 - A_2/A_1) \times 100$$

Where A_1 = control trypsin activity (without serum); A_2 = activity of trypsin remained after serum addition.

2.1.4 Intestinal morphology

Another set of two fish per tank collected and the whole intestine removed and sampled for its histology. The intestine samples will be fixed in Bouins' solution, dehydrated in graded series of ethanol, embedded in paraffin and then sectioned in 5µm sagittal serial sections.

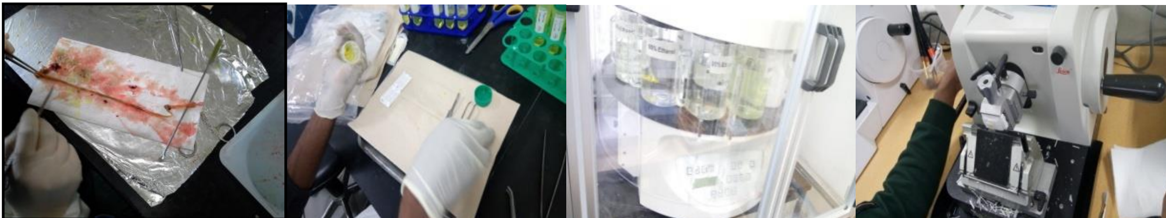

Figure I-4. Sampling and analysis of intestine samples

Slides were stained with Harris hematoxylin and 0.5% eosin for general histological observation and the Alcian blue (AB) at pH 2.5 and periodic acid Schiff (PAS) for observation of the mucus secreting goblet cells. The morphometric measurement of villus length (VL) was observed using the ImageJ 1.44 analysis software.

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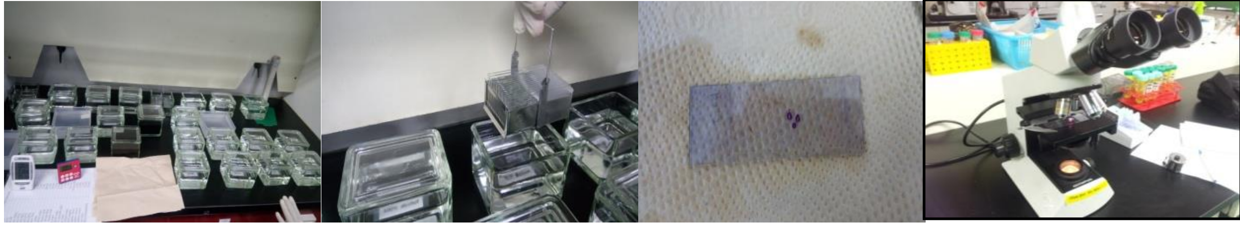

Figure I-5. Slide staining and observation

2.1.5 Expression levels of liver IGF-I mRNA

Liver total RNA was isolated using E.Z.N.A.™ Total RNA Kit (Omega Bio-Tek, Norcross, GA, USA) and treated with RNase-free DNase (Omega Bio-Tek), following the manufacturer´s protocol. The quantity of the RNA was calculated using the absorbance at 260 nm. The integrity and relative quantity of RNA was checked by electrophoresis. PrimeScript RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa Code. DRR047) was used to remove genomic DNA and reverse transcription. One microgram of total RNA was reverse-transcribed into cDNA in a volume of 10 μL, containing 1 μL of gDNA Eraser and 2 μL of 5·× gDNA Eraser Buffer. This mix was heated at 42 ºC for 2 min. and then 4 μL of 5 ×·Prime Script Buffer (for Real Time), 1 μL of Prime Script RT Enzyme Mix I, 1 μL of RT Primer Mix and 4 μL of RNase-free dH2O were added to a final volume of 20 μL. After incubation at 37 ºC for 30 min, the reaction was stopped by heating to 85 ºC for 5 s.

Figure I-6. Liver sampling and analysis for IGF-I expression

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Levels of IGF-I transcript were measured by real-time PCR (SYBR Green I), using 18S rRNA as a housekeeping gene. Primers for real-time PCR were designed based on the previously cloned sequence for IGF-I (NCBI Genbank accession no: AF061278) and 18S rRNA (NCBI Genbank accession no: EF126037) in *P. Major* and *P. olivaceus*. Real-time RT-PCR was conducted by amplifying 2.0 μL of cDNA with TaKaRa SYBR Premix Ex Taq (Perfect Real Time) (TaKaRa Code.DRR041A) on a TaKaRa PCR Thermal Cycler Dice Real Time System (TaKaRa Code.TP800). Amplification conditions were as follows: 30 s at 95 ºC; 40 cycles of 5 s at 95 ºC, 30 s at 60 ºC. Melting curve analysis of amplification products was performed at the end of each PCR to confirm that a single PCR product was detected. Each sample was run in triplicate, and PCRs without the addition of the template were used as negative controls. Relative expression ratio of IGF-I was calculated based on the PCR efficiency (E) and the C_t of a sample versus the control and expressed in comparison with the reference gene (18S rRNA); according to Pfaffl's mathematical model (Pfaffl, 2001).

$$Ratio = [(E_{IGF\text{-}I})^{\Delta Ct(control\text{-}sample)}]/[(E_{ref})^{Ct(control\text{-}sample)}]$$

2.1.6 Statistical analysis

All experimental diets were assigned using a completely randomized design. Data were analysed by one-way analysis of variance (ANOVA) in SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using Tukey’s HSD test at the 5% level of significance (P <0.05). Data are presented as mean ± SD. Percentage data were arcsine transformed before analysis.

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3.1 RESULTS

All the experimental diets were readily accepted by the juvenile red sea bream from the start of the feeding trial and they fed aggressively during the 13 weeks of the feeding trial. Growth and survival results at the end of the 13 weeks feeding trial are provided in Table I-2.

Growth was significantly affected by dietary treatments. All the piperine fed fish showed significantly higher growth compared to that of fish fed the control diet except for T5 group which was only numerically higher than the control. Also, specific growth rate followed a similar trend to weight gain. Feed conversion ratio was significantly reduced with the piperine and pepper supplementation while 0.05% piperine supplemented group showed the best performance. Accordingly, the highest protein efficiency ratio was observed in the T2 group as PER was increased significantly in all treatment groups.

Hemoglobin level was significantly increased by 0.025% inclusion of piperine compared to the control group. Hematocrit, plasma glucose and cholesterol levels were increased numerically by T1 compared to the control. Other hematological and hemochemical parameters were not increased significantly with the dietary supplementation of piperine (Table I-3).

Analyses of innate immunity parameters revealed significant enhancement with the piperine supplementation (Table I-4). Highest SOD value was observed in T2 group and highest lysozyme and total immunoglobulin levels were observed in T5 group while all treatment groups showed significantly higher immune responses for MPO, SOD, total immunoglobulin, lysozyme and antiprotease activities compared to the control group.

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Results of whole-body composition showed a significant reduction of dry matter content compared to the control except T1 group which expressed the highest. Ash content was significantly increased with the supplementation except T5 group. Highest whole-body protein level was seen in T2 group (Table I-5).

As shown in Figure I-7, liver IGF-I mRNA level was influenced by dietary inclusion of the piperine and pepper even though it was not significant.

Condition factor and goblet cell counts were increased significantly with the dietary piperine supplementation and the highest values were observed in T1 group (Table I-6). Also, villi length was increased significantly with the supplementation of piperine. All the treatment groups showed significantly higher values compared to the control while T1 showed the highest one.

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Table I-2. Growth performance and feed utilization of red sea bream fed the six experimental diets for 13 weeks.

	Experimental diets					
	Control	**T1**	**T2**	**T3**	**T4**	**T5**
FBW[1] (g)	45.3±2.2^{a}	51.4±2.6^{b}	53.6±0.9^{b}	53.8±1.7^{b}	53.5±3.9^{b}	46.8±1.8^{a}
WG[2] (%)	496±30^{a}	577±48^{b}	600±18^{b}	601±21^{c}	603±36^{b}	509±28^{a}
SGR[3] (%)	1.98±0.06^{a}	2.12±0.08^{b}	2.16±0.03^{b}	2.16±0.03^{b}	2.17±0.06^{b}	2.01±0.05^{a}
FCR[4]	1.44±0.09^{b}	1.25±0.15^{ab}	1.06±0.04^{a}	1.10±0.18^{a}	1.09±0.17^{a}	1.24±0.09^{ab}
PER[5]	1.55±0.09^{a}	1.78±0.22^{ab}	2.13±0.09^{b}	2.10±0.38^{b}	2.12±0.32^{b}	1.85±0.13^{ab}
Survival (%)	83.3±5.8	85.6±6.9	88.9±1.9	82.2±9.6	87.8±5.1	82.2±5.1

Values are mean of triplicate groups and presented as mean ± S.D. Values with different superscripts in the same row are significantly different ($P < 0.05$). The *lack of superscript letter* indicates *no significant* differences among treatments.

[1]FBW: final body weight (g)

[2]Weight gain (%) = 100 x (final mean body weight – initial mean body weight)/initial mean body weight

[3] Specific growth ratio (% day^{-1}) = [(loge final body weight - loge initial body weight)/days] x 100

[4]Feed conversion ratio = dry feed fed (g) / wet weight gain (g)

[5]Protein efficiency ratio = wet weight gain /total protein given

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Table I-3. Hematological parameters of red sea bream fed the six experimental diets for 13 weeks.

	Experimental diets					
	Control	**T1**	**T2**	**T3**	**T4**	**T5**
Ht[1]	34.8±2.0[b]	36.2±3.2[b]	28.6±4.9[ab]	30.6±6.6[ab]	30.6±2.9[ab]	25.2±1.5[a]
Hb[2]	5.07±0.85[ab]	6.33±0.94[b]	5.3±0.5[ab]	4.92±1.03[ab]	5.26±0.81[ab]	4.18±0.21[a]
Glucose[3]	117±11[b]	132±13[b]	72±16[a]	121±12[b]	118±28[b]	122±13[b]
TP[4]	4.02±1.08	4.24±0.19	3.97±0.29	4.4±60.27	4.09±0.63	4.63±0.84
TC[5]	263±14[b]	236±10[b]	165±18[a]	169±22[a]	169±14[a]	165±12[a]

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

[1]Hematocrit (%)

[2]Hemoglobin (g dL^{-1})

[3]Glucose (mg dL^{-1})

[4]Total protein (g dL^{-1})

[5]Total cholesterol (mg dL^{-1})

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Table I-4. Non-specific immune response of red sea bream fed the six experimental diets for 13 weeks.

	Experimental diets					
	Control	**T1**	**T2**	**T3**	**T4**	**T5**
NBT[1]	0.82±0.11	0.82±0.13	0.78±0.12	0.71±0.03	0.77±0.05	0.79±0.09
MPO[2]	1.19±0.03[a]	1.51±0.08[bc]	1.41±0.27[ab]	1.67±0.06[c]	1.42±0.1[ab]	1.4±0.07[ab]
SOD[3]	55.1±5.0[a]	61.7±8.9[c]	86.4±3.5[e]	62.2±3.7[b]	72.0±4.7[d]	73.0±5.6[d]
Ig[4]	30.7±4.2[a]	26.2±7.0[a]	34.1±9.4[b]	33.9±3.4[b]	24.2±3.6[a]	67.2±7.2[c]
Lysozyme[5]	15.3±0.7[a]	16.9±0.1[a]	22.4±1.7[b]	21.7±0.9[b]	22.4±0.4[b]	27.4±2.0[c]
Antiprotease[6]	19.3±2.6[a]	20.7±2.1[a]	20.4±4.9[a]	43.0±4.8[b]	19.1±4.2[a]	17.6±3.6[a]

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

[1]Nitro blue tetrazolium activity

[2]Myeloperoxidase level

[3]Superoxide dismutase (% inhibition)

[4]Total immunoglobulin (mg mL^{-1})

[5]Lysozyme activity (μg mL^{-1})

[6] Antiprotease activity (μg mL^{-1})

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Table I-5. Whole-body composition of red sea bream fed the six experimental diets for 13 weeks (%DM).

	Experimental diets					
	Control	**T1**	**T2**	**T3**	**T4**	**T5**
Dry matter	34.0±0.1^{d}	34.9±0.6^{e}	32.4±0.1^{b}	31.7±0.4^{a}	33.2±0.1^{c}	33.7±0.1^{cd}
Protein	49.2±4.5^{a}	46.3±1.5^{a}	54.6±0.5^{b}	47.2±1.8^{a}	47.9±0.8^{a}	47.4±0.5^{a}
Lipid	10.3±0.3	9.8±0.8	10.5±1.7	9.5±0.1	11.1±1.3	9.5±1.4
Ash	13.0±0.2^{a}	15.6±0.9^{b}	16.2±0.2^{b}	20.1±1.3^{c}	16.5±0.2^{b}	12.1±0.8^{a}

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

Table I-6. Morphometric and visceral parameters of red sea bream fed the six experimental diets for 13 weeks.

	Experimental diets					
	Control	**T1**	**T2**	**T3**	**T4**	**T5**
GC[1]	571±344^{a}	2069±458^{c}	1038±458^{ab}	957±27^{ab}	725±120^{a}	1311±189^{b}
CF[2]	1.52±0.20^{a}	1.92±0.28^{c}	1.84±0.13^{bc}	1.66±0.12^{ab}	1.79±0.12^{bc}	1.77±0.26^{bc}
VL[3]	722±33^{a}	1543±277^{d}	1144±111^{c}	1041±138^{bc}	944±101^{abc}	811±40^{ab}
HSI[4]	2.03±0.85^{b}	1.55±0.64^{ab}	1.59±0.56^{ab}	1.44±0.38^{ab}	1.50±0.67^{ab}	1.16±0.33^{a}

Values are mean of triplicate groups and presented as mean ± S.D. Values with different superscripts in the same row are significantly different ($P < 0.05$).

[1]Goblet Cell count

[2]Conditional factor; CF= (Fish weight/ Fish length^{3})*100 (gcm^{-3})

[3]Villi Length (μm)

[4]Hepatosomatic index; HSI= (Liver weight/ Fish weight)*100

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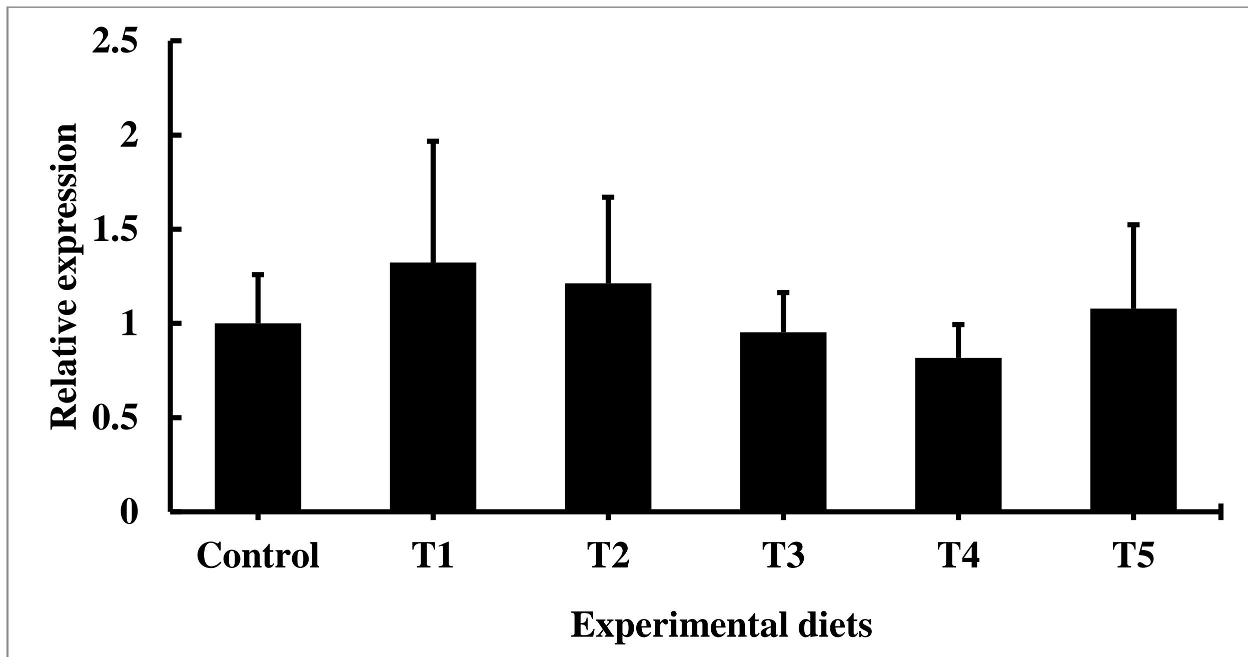

Figure I-7. Liver insulin-like growth factors I mRNA expression for each diet group expressed as a ratio to control diet values.

Data are presented as mean ± SD from three replicate tanks. Different letters above the bars denote significant differences between diet groups at the $P < 0.05$ level.

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[EXPERIMENT II]

2.2 MATERIALS AND METHODS

2.2.1 Experimental diets and design

Seven experimental diets were formulated to be isonitrogenous (49% crude protein) and isocaloric (17.5 kJ g^{-1}) (Table II-1). A FM-based diet was regarded as a control diet and six other experimental diets were prepared by dietary inclusion of piperine at different inclusion levels of 0.025, 0.05, 0.075, 0.1 and 0.2% (T1, T2, T3, T4 and T5 respectively) or black pepper at a level of 0.1% (T6). All dry ingredients were thoroughly mixed and, after addition of fish oil and double-distilled water, the dough was extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) to ideal size, dried at 24 °C for 24 h and stored at −20 °C until use.

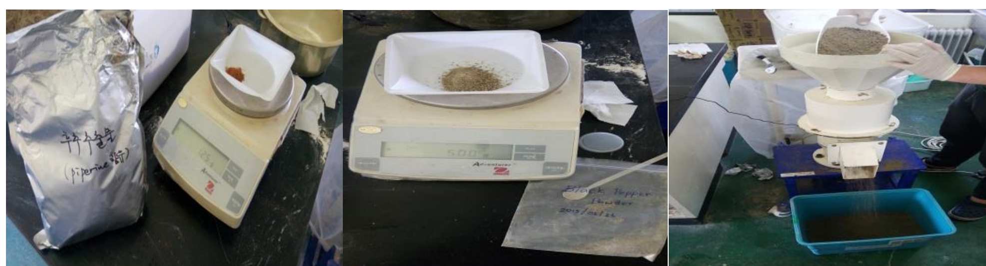

Figure II-1. Diet formulation

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Table II-1. Formulation and chemical composition of the experimental diets.

Ingredient	Experimental diets						
	Control	T1	T2	T3	T4	T5	T6
Chile fish meal	48.00	48.00	48.00	48.00	48.00	48.00	48.00
Soybean meal	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Corn gluten meal	8.00	8.00	8.00	8.00	8.00	8.00	8.00
Wheat flour	27.50	27.50	27.50	27.50	27.50	27.50	27.50
Fish oil	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Cellulose	1.00	0.975	0.950	0.925	0.90	0.80	0.90
[1]Mineral Mix	1.00	1.00	1.00	1.00	1.00	1.00	1.00
[2]Vitamin Mix	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Piperine	0.00	0.025	0.05	0.075	0.10	0.20	0.00
Black pepper	0.00	0.00	0.00	0.00	0.00	0.00	0.10
Chemical composition (% dry matter)							
Moisture	6.2	6.1	6.1	5.9	6.1	6.1	6.0
Protein	49.9	49.1	49.1	49.2	49.1	49.4	48.8
Lipid	11.2	11.1	11.0	10.5	11.3	11.7	11.7
Ash	10.1	9.9	9.8	10.0	10.1	10.0	9.7

[1]Mineral premix (g/kg mixture): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[2]Vitamin premix (g/kg mixture): L-ascorbic acid, 121.2; DL-a tocopheryl acetate, 18.8; thiamin 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-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

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2.2.2 Feeding trial and sample collection

Juvenile olive flounder (*Paralichthys olivaceus*) were transported from a private hatchery to the Marine and Environmental Research Institute, Jeju National University, Jeju, Korea. All the fish were fed a commercial diet for a week to be acclimatized to the experimental condition and facilities. At the end of the acclimation period, the fish (initial mean body weight, 27.6 ± 0.4 g) were randomly distributed into twenty one tanks of 150 L capacity polyvinyl circular tanks at a density of 30 fish per tank. The tanks were supplied with filtered seawater at a flow rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. Triplicate groups of fish were fed one of the experimental diets to apparent satiation (three times a day, 08:00, 13:00 and 18:00 h) for eight weeks.

Figure II-2. Growth measurements and sampling

Water temperature was fluctuated at 17.1±2 ℃ range for the whole duration. Growth measurement was carried out at every 2 weeks. All the fish in each tank were bulk-weighed and counted for calculation of weight gain (WG), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival by the following formulas: Weight gain (%) = 100 × (final mean body weight – initial mean body weight) / initial mean body weight; Specific growth rate (%) = [(log_e final body weight – log_e initial body weight) / days] × 100; Feed

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conversion ratio = dry feed fed / wet weight gain; Protein efficiency ratio = wet weight gain / total protein given.

At the end of the feeding trial, all the fish in each tank were counted and bulk weighted for calculation of growth parameters. Three fish per tank (nine fish per dietary treatment) were collected for whole-body proximate composition analysis. Also, four fish per tank were randomly captured, anaesthetized with 2-phenoxyethanol solution (200 ppm) and blood samples were taken from the caudal vein with heparinized syringes. After analysis of hematocrit, hemoglobin and respiratory burst activity in whole blood samples, plasma were separated and used for determination of immunoglobulin level and biochemical parameters. Another set of blood samples were taken from caudal vein of four fish from each tank using non-heparinized syringes and allowed to clot at room temperature for 30 min. Then the serum was separated by centrifugation for 10 min at 5000×*g* and stored at −70 °C for the analysis of innate immune parameters including lysozyme, superoxide dismutase (SOD), myeloperoxidase (MPO) and anti-protease activities. Feeding was stopped 24 h prior to weighing or blood sampling to minimize the stress of fish.

2.2.3 Estimation of apparent digestibility coefficients

For the estimation of apparent digestibility coefficients of the experimental diets, chromic oxide (Cr_2O_3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 0.5%. All dry ingredients were thoroughly mixed and extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) to ideal size after addition of fish oil and 20% double distilled water. The pellets were dried with electric fans at room temperature and stored at -20 °C until used.

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New sets of olive flounder (initial body weight, 70 g) were distributed into 400 L capacity Guelph system (fecal collection system) tanks at a density of 80 fish per tank. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. Fish were hand-fed one of the test diets to apparent satiation once daily at 17:00h. One hour after feeding, the rearing tanks were washed out to remove uneaten feed and fecal residues. On the next day, feces were collected from the fecal collection columns at 09:00h. A secondary fecal collection tube, under the collection tank, was additionally installed to collect all the possible feces. The collected feces in the tube was separated from supernatant water using a disposable paper filter and frozen at -40 °C. Then the fecal samples were stored at -20 °C until analyses.

Figure II-3. Fecal sample collection

Chromium oxide content of diet and feces samples were analyzed by the method described by Divakaran et al. (2002). Briefly, a known weight (5-10 mg) of ash samples of either diet or feces containing chromium oxide was placed in glass test tubes. Then 4 ml of perchloric reagent was added along the sides of the test tube to wash down any adhering ash. Perchloric reagent was prepared as follows: two hundred millilitre of concentrated nitric acid was added to 100 ml of distilled water, cooled down and then 200 ml perchloric acid (70%) was added. The test tubes were set in a heating block and heated at 300 °C for 20 min, for oxidation of chromium oxide to monochromate (CrO_4^{2-}). Then the tubes were cooled down to room temperature and their contents were quantitatively transferred and made up to 25 ml in a volumetric flask by

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rinsing repeatedly with distilled water. The absorbance of samples was read at 350 nm using a spectrophotometer (Beckman DU-730, USA). A known weight (2 – 4 mg) of chromium oxide was similarly treated and used as standard.

Figure II-4. Fecal samples analysis

The apparent digestibility coefficients for dry matter and protein of the experimental diets were calculated by the following formulas:

ADC of dry matter (%) = 100 - 100 × (% Cr_2O_3 in diet / % Cr_2O_3 in feces)

ADC of protein (%) = 100 - 100 × (% Cr_2O_3 in diet / % Cr_2O_3 in feces) × (%protein in feces /%protein in diet)

2.2.4 Analyses

The same protocols as in the first experiment were used.

2.2.5 Intestinal morphology

The same protocols as in the first experiment were used.

2.2.6 Expression levels of liver IGF-I mRNA

The same protocols as in the first experiment were used.

2.2.7 Challenge test

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At the end of the feeding trial, twelve flounders from each tank (36 fish per treatment) were intraperitoneally injected with *Edwardsiella tarda* (ATCC 15947, Korea Collection for Type Cultures) suspension (1×10^{8} CFU ml^{-1}). *E. tarda* was provided by the Marine Applied Microbes and Aquatic Organism Disease Control Laboratory at the Department of Aquatic Biomedical Sciences, Jeju National University. Injected fish were distributed into twenty one 60 L plastic tanks and their behavior and mortality were monitored and recorded for 8 days.

Figure II-5. Conducting challenge test

2.2.8 Statistical analysis

All experimental diets were assigned using a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were compared using Tukey's HSD test at the 5% level of significance ($P < 0.05$). Data are presented as mean ± SD. Percentage data were arcsine transformed before analysis.

3.2 RESULTS

The results of growth performance, feed utilizations and survival of fish fed the experimental diets are provided in Table II-2. Growth performance and feed utilization of the

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fish groups were improved significantly with the dietary supplementation. Weight gain and specific growth rate were observed the highest in T2 group while the lowest feed conversion ratio was given by T4 group. Protein efficiency ratio was affected significantly with the treatment. All treatment groups showed significantly higher PER compared to the control while T3 group showed the highest.

T3 group showed the highest plasma cholesterol level while T5 group showed the lowest (Table II-3). The highest triglyceride level was seen in T1 group while the lowest value was given by T6 group. Other hematological and hemochemical parameters were not influenced significantly by treatments.

Total immunoglobulin, NBT, antiprotease and lysozyme activity levels were significantly increased by the dietary piperine supplementation (Table II-4). T6 group showed the highest Ig and antiprotease level whereas T4 showed the highest lysozyme activity and T5 group showed the highest NBT value. Other innate immune parameters were increased with the dietary piperine supplementation but were not significantly affected.

The highest whole-body protein level was observed in T2 group while dry matter and lipid levels were found to be highest in T1 group (Table II-5).

Dry matter digestibility was increased significantly by the dietary treatments. T1, T2, T3 and T6 groups showed significantly higher digestibility compared to the control group while T2 represented the best. Protein digestibility was also elevated significantly by the dietary treatments. T1 and T2 groups exhibited significantly higher values than the others in protein digestibility (Table II-6).

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As shown in Figure II-6, liver IGF-I mRNA expression level was increased significantly with the dietary treatments compared to the control while T2 showed the highest.

Survival of fish after the challenge with *E. tarda* is shown in Figure II-7. During the *E. tarda* challenge test, first dramatic mortality was observed on 4th day after the injection. All treatment groups showed higher survival rates compared to the control group during the period. T1, T2 and T6 groups had the highest survival rates at whole duration.

Morphometric characteristics of the fish fed experimental diets are shown in Table II-7. Condition factor was increased by the dietary treatment numerically while villi length and goblet cells were increased significantly. All piperine supplemented groups showed significantly higher villi length values compared to the control. T2 group had the highest villi lengths while T5 showed the highest goblet cell count.

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Table II-2. Growth performance and feed utilization of olive flounder fed the seven experimental diets for 8 weeks.

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
FBW[1] (g)	50.6±1.4^{a}	51.5±1.3^{a}	58.7±5.2^{b}	57.2±1.4^{ab}	54.5±5.3^{ab}	51.2±2.3^{a}	57.1±5.5^{ab}
WG[2] (%)	80.6±5.5^{a}	86.7±7.3^{ab}	109.8±19.2^{b}	106.3±4.2^{ab}	99.3±14.8^{ab}	86.6±11.5^{ab}	106.1±21.4^{ab}
SGR[3] (%)	1.06±0.05^{a}	1.11±0.07^{ab}	1.32±0.16^{b}	1.29±0.04^{b}	1.23±0.13^{ab}	1.11±0.11^{ab}	1.29±0.18^{b}
FCR[4]	1.90±0.34	1.79±0.11	1.97±0.14	1.93±0.13	1.66±0.29	1.81±0.10	2.02±0.70
PER[5]	0.87±0.13^{a}	1.07±0.04^{ab}	1.29±0.16^{b}	1.32±0.15^{b}	1.22±0.08^{ab}	1.06±0.02^{ab}	1.21±0.2^{ab}
Survival (%)	88.9±10.7	96.7±3.3	94.4±5.1	100	98.9±1.9	96.7±3.3	95.6±7.7

Values are mean of triplicate groups and presented as mean ± S.D. Values with different superscripts in the same row are significantly different (*P* < 0.05). The *lack of superscript letter indicates no significant* differences among treatments.

[1]FBW: final body weight (g)

[2]Weight gain (%) = 100 x (final mean body weight – initial mean body weight)/initial mean body weight

[3] Specific growth ratio (% day^{-1}) = [(loge final body weight - loge initial body weight)/days] x 100

[4]Feed conversion ratio = dry feed fed (g) / wet weight gain (g)

[5]Protein efficiency ratio = wet weight gain /total protein given

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Table II-3. Hematological parameters of olive flounder fed the seven experimental diets for 8 weeks.

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Ht[1]	18.5±0.9	21.7±2.5	19.8±1.3	19.6±1.4	20.2±2.0	20.2±2.9	21.4±3.0
Hb[2]	4.25±1.55	3.92±1.37	5.19±1.3	5.66±1.16	4.60±1.16	5.69±1.62	5.30±1.54
Glucose[3]	49.6±4.0	50.9±3.3	47.0±6.7	48.0±1.9	50.0±4.3	48.2±2.0	47.0±3.4
Total protein[4]	3.36±0.71	3.71±0.32	3.92±0.32	3.90±0.30	3.73±0.39	3.73±0.38	3.47±0.37
Total cholesterol[5]	232±4[ab]	234±10[ab]	247±13[b]	257±22[b]	246±10[b]	205±28[a]	235±20[ab]
Triglycerides[6]	526±21[c]	542±27[c]	468±16[b]	460±27[b]	541±29[c]	536±17[c]	308±27[a]

[1]Hematocrit (%)

[2]Hemoglobin (g dL^{-1})

[3]Glucose (mg dL^{-1})

[4]Total protein (g dL^{-1})

[5]Total cholesterol (mg dL^{-1})

[6]Triglycerides (mg dL^{-1})

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Table II-4. Non-specific immune response of olive flounder fed the seven experimental diets for 8 weeks.

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
NBT[1]	0.36±0.06[a]	0.41±0.04[ab]	0.47±0.02[ab]	0.45±0.05[ab]	0.49±0.11[b]	0.50±0.07[b]	0.49±0.05[b]
MPO[2]	1.73±0.06	1.89±0.30	1.92±0.1	1.84±0.12	1.85±0.20	1.75±0.08	1.55±0.03
SOD[3]	73.6±1.1	64.6±0.4	62.7±9.6	65.8±7.2	67.5±3.2	66.8±9.6	60.2±9.8
Ig[4]	28.1±4.1[a]	55.7±5.1[b]	56.4±8.7[b]	56.1±2.2[b]	53.8±6.4[b]	55.8±6.8[b]	67.9±8.2[c]
Lysozyme[5]	18.9±0.3[a]	22.8±1.2[ab]	27.8±1.3[cd]	29.6±1.1[cd]	34.3±2.0[e]	26.6±4.0[bc]	31.8±3.5[de]
Antiprotease[6]	14.8±2.4[a]	16.1±1.7[ab]	17.5±2.2[ab]	18.0±1.2[ab]	15.9±2.5[ab]	14.7±1.6[a]	18.7±1.1[b]

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

[1]Nitro blue tetrazolium activity

[2]Myeloperoxidase level

[3]Superoxide dismutase (% inhibition)

[4]Total immunoglobulin (mg mL^{-1})

[5]Lysozyme activity (µg mL^{-1})

[6] Antiprotease activity (µg mL^{-1})

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Table II-5. Whole-body composition of olive flounder fed the seven experimental diets for 8 weeks. (%DM).

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
Dry matter	34.0±0.6[c]	35.1±0.1[d]	30.0±1.1[a]	32.1±0.7[b]	32.4±0.4[b]	32.5±0.2[b]	33.6±0.1[c]
Protein	52.4±4.3[a]	52.2±1.4[a]	60.4±0.9[b]	55.7±2.7[a]	52.6±0.2[a]	53.3[a]	55.4±0.3[a]
Lipid	11.1±1.1[b]	11.3±0.5[b]	9.2±0.8[a]	10.8±0.5[b]	10.7±0.8[b]	11.0±0.8[b]	10.7±0.7[b]
Ash	13.4±1.2[a]	14.5±0.9[a]	19.5±1.1[c]	20.1±1.2[c]	17.2±1.1[b]	18.2±1.0[bc]	20.1±0.9[c]

Values are mean of triplicate groups and presented as mean ± S.D.

Table II-6. Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for olive flounder.

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
ADCd (%)[1]	70.1±0.5[ab]	72.6±0.6[c]	74.9±0.5[d]	72.2±0.3[c]	70.0±1.8[ab]	68.0±0.8[a]	74.6±1.5[d]
ADCp (%)[2]	92.1±0.4[d]	94.0[e]	94.4±0.1[e]	91.2±0.3[c]	86.6±0.3[b]	83.1±0.4[a]	91.4±0.1[c]

Values are mean of triplicate groups and presented as mean ± S.D. Values with different superscripts in the same row are significantly different ($P < 0.05$). The *lack of superscript letter* indicates *no significant* differences among treatments.

[1] Apparent digestibility coefficient of dry matter

[2] Apparent digestibility coefficient of protein

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Table II-7. Morphometric and visceral parameters of olive flounder fed the seven experimental diets for 8 weeks.

	Experimental diets						
	Control	**T1**	**T2**	**T3**	**T4**	**T5**	**T6**
GC[1]	794±231[ab]	828±44[ab]	688±181[a]	639±115[a]	80±646[ab]	100±344[b]	641±202[a]
CF[2]	1.04±0.09	1.03±0.12	1.02±0.15	1.14±0.12	1.08±0.08	1.02±0.07	1.04±0.08
VL[3]	792±20[a]	915±27[ab]	1142±67[c]	1024±38[b]	937±74[b]	973±109[b]	797±86[a]
HSI[4]	1.64±0.27	1.59±0.25	1.53±0.31	1.50±0.13	1.43±0.39	1.57±0.64	1.59±0.50

Values are mean of triplicate groups and presented as mean ± S.D. Values with different superscripts in the same row are significantly different ($P < 0.05$).

[1]Goblet Cell count

[2]Conditional factor; CF= (Fish weight/ Fish length^3)*100 (gcm^{-3})

[3]Villi Length (μm)

[4]Hepatosomatic index; HSI= (Liver weight/ Fish weight)*100

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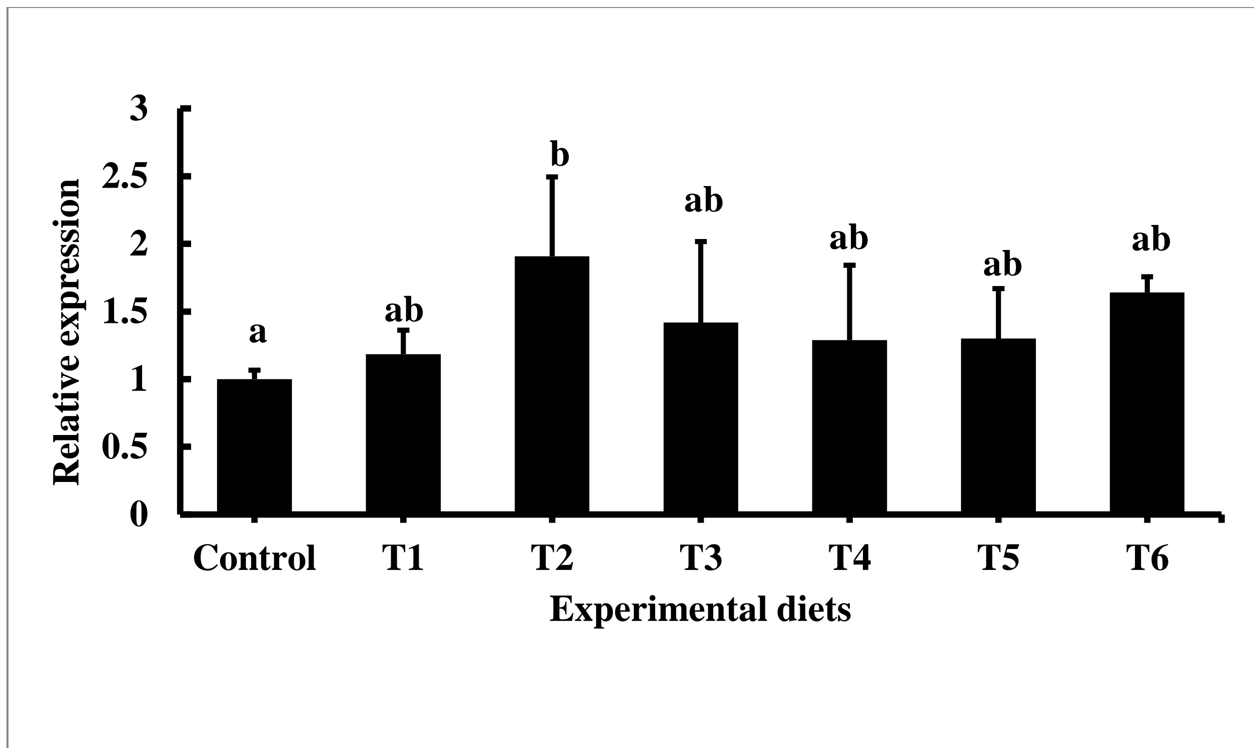

Figure II-6. Liver insulin-like growth factors I (IGF-I) mRNA expression for each diet group expressed as a ratio to control diet values. Data are presented as mean ± SD from three replicate tanks. Different letters above the bars denote significant differences between diet groups at the $P < 0.05$ level.

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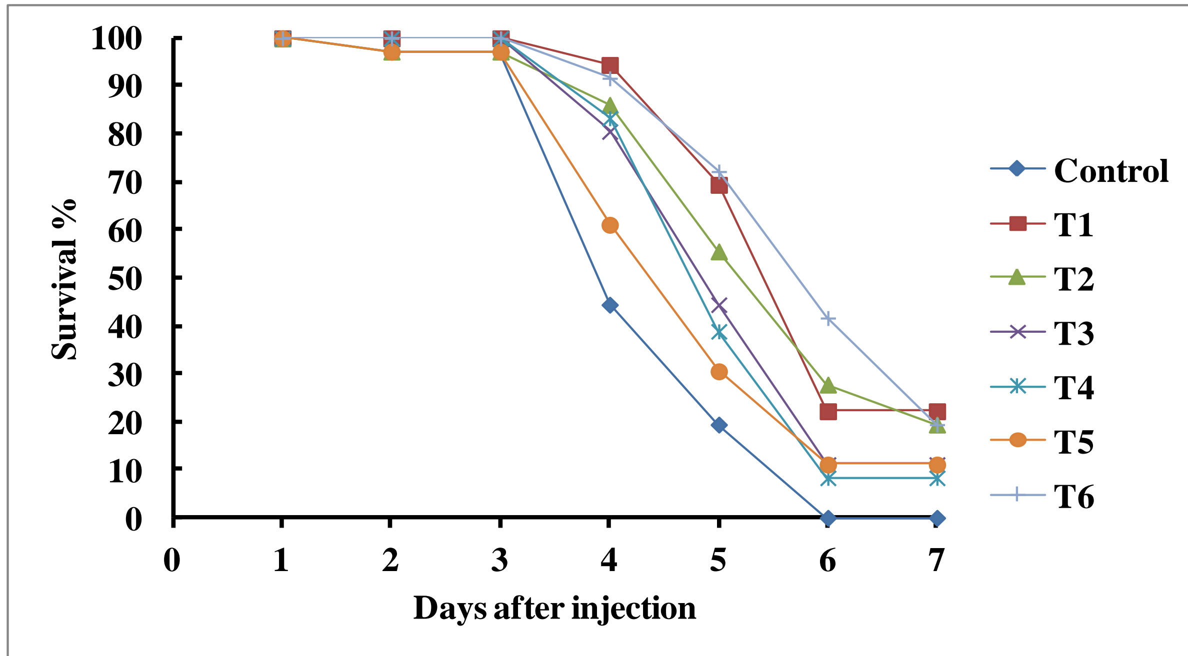

Figure II-7. Survival rate of olive flounder after challenge with *E. tarda*. .

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4. DISCUSSION

Results of the present study showed that the supplementation of piperine had a significant effect in growth performance and feed utilization in both species. Similarly, in a study conducted with broiler chicken, Cardoso et al. (2012) obtained higher growth in birds fed with 0.06% piperine. Feed utilization would be improved if piperine supplementation can enhance feed digestion. In order, it can either enlarge the absorption surface or modify the biochemistry. In our case, results showed significantly decreased FCR with the supplementation of piperine while PER has been increased significantly. Khajuria et al. (2002) related piperine (10 mg kg^{-1} of body weight) to alterations in membrane dynamics and permeation characteristics, and to the induction in the synthesis of proteins associated with cytoskeleton function, which increased the small intestine absorptive surface in a time-dependent manner up to 2 hours in rats.

Addition of phytobiotics seems to enhance growth performance in many species in fact. Possible mode of action of phytogenic bioactive compounds on growth performance of farm animals could be their effects on the activities of digestive enzymes. Xu et al. (2003) reported that dietary supplementation of fructo-oligosaccharides improved daily body weight gain of animals by increasing the activities of amylase and proteases. Furthermore, another study indicated that feeding a diet containing a commercial blend of essential oils (CRINAR) in combination with lactic acid induced a significant increase in activities of digestive enzymes of the pancreas and intestinal mucosa of animals, leading to a significant increase in growth (Jang et al., 2004).

Ji et al. (2007) showed that the addition of different single herbal extracts (*Massa medicata*, *Crataegi fructus*, *Artemisia capillaries*, *Cnidium officinale*) or a mixture of all the herbs promoted growth and enhanced some non-specific immunity indicators of red sea bream

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Pagrus major. Among a wide variety of herbs tested against *A. hydrophila* infection in tilapia (*Oreochromis niloticus*), the ethanol extract of *Psidium guajava* was found to have the highest antimicrobial activity (Pachanavan et al., 2008).

In the present study, innate immunity of both red sea bream and olive flounder were increased significantly by the supplementation of both piperine and powdered black pepper. Phytobiotics can act as immune-stimulants in many ways and piperine as its biochemical properties, is helping that too. Vijaykumar and Nalini (2006) reported that piperine can elevate erythrocyte antioxidant enzymes like SOD, Catalase (CAT) and glutathione peroxidase (GPx) significantly in rats. According to Selvendiran et al. (2003), simultaneous supplementation of piperine elevated the antioxidant enzyme activities (SOD, CAT and GPx) in rats. This may be because piperine is highly lipophilic in nature and helps in inhibiting lipid peroxidation initiated by free radicals, thus preventing or delaying the damage to cells. Increasing evidence has shown that piperine supplementation in the diet reduces the risk of oxidative damage by augmenting antioxidant enzymes.

There are many studies conducted using different kinds of phytobiotic compounds from many sources to supplement in aquaculture. And their results are promising and evidencing that these can enhance the innate immunity of many fish species. Yuan et al. (2007) fed common carp (*Cyprinus carpio*) diets containing a mixture of *Astragalus membranaceus* (root and stem), *Polygonum multiflorum*, *Isatis tinctoria* and *Glycyrrhiza glabra* (0.5 and 1%) for 30 days and observed that both concentrations significantly increased macrophage phagocytic activity, respiratory burst and levels of total protein, albumin, globulin and nitric oxide synthetase activity in the serum; no significant difference was found in SOD, lysozyme activities and triglyceride level. Root extracts of the Chinese herb *Astragalus* contain polysaccharides, organic acids,

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alkaloids, glucosides and volatile oil as major active components that have been found to enhance immune function in fish (Jeney et al., 2009). The *Astragalus* polysaccharide (APS) from *A. membranaceus* is reported to halt ROS production, stimulate humoral and cellular immunity, and thus possess anti-cancer and immune-stimulating effects (Yuan et al., 2008). The oriental medicinal herb *G. glabra* (liquorice) comprises flavonoids and pentacyclic triterpene saponin, including liquiritin, liquiritigenin, isoliquiritigenin, liquiritin apioside, glycyrrhizin and glycyrrhizic acid as major constituents and is reported to have anti-oxidant effects (Yin et al., 2011).

Other major area of interest in our study was to see if piperine made any modifications in fish intestines. In order we conducted a histological analysis to check the goblet cell count and villi length. Our results showed many positive effects of the supplementation in both fishes. Those are comparable with the results of different studies conducted with different animals. The gastrointestinal system is affected by black pepper and piperine, which have a definite effect on intestinal motility, anti -diarrhea property, and on the ultrastructure of intestinal villi, improving the absorbability of nutrients (Takaki et al., 1990; Bajad et al., 2001). According to Samanya and Yamauchi (2002), increased villus height is paralleled by an increased digestive and absorptive function of the intestine due to the increased absorptive surface area, the expression of brush border enzymes, and the nutrient transport systems.

In the present study, IGF-I mRNA expression was increased with the supplementation compared to the control group. In red sea bream it was found not to be a significant change of effect even the growth rate was affected significantly. Similar kind of results were obtained by Hossain et al. (2016) in which red sea bream showed a significant effect of the feed supplementation in growth but showed a marginal elevation of IGF-I levels. In a similar study to

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supplement seaweed extracts to olive flounder feed, Choi et al. (2014) resulted in significantly higher growths as well as significantly increased IGF-I levels with increased supplementation levels. Experiment-II in the present study also showed some similarities with those findings. Both IGF-I expression and growth performance were increased with the supplementation.

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5 CONCLUSION

The conclusions are as follows:

1. Dietary piperine supplementation could improve the growth rate of red sea bream and olive flounder.
2. Dietary piperine supplementation might assist the digestion and absorption of dietary nutrients to the fish and thereby increase the dietary utilization efficiency.
3. Dietary piperine supplementation could improve the innate immunity and increase disease resistance of both fishes.
4. Dietary piperine supplementation would have beneficial effects on the length and surface area of villi, and on the goblet cells which help to excrete hormones for better digestibility of both fishes.
5. Further studies are necessary for the IGF-1 expression even though piperine showed some positive effects on the IGF-1 in both fishes.
6. The optimum level of piperine in diets would be approximately 0.05% for both red sea bream and olive flounder.
7. Further studies are necessary on the effects of dietary piperine in terms of its availability and metabolism in fish.

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