A DISSERTATION

FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Nutritional, physiological and immunological studies of olive flounder (*Paralichthys olivaceus*) and red sea bream (*Pagrus major*) fed marine protein hydrolysates

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ABSTRACT

Marine protein hydrolysates are potential feed ingredients that are used in aquaculture feeds mainly as functional supplements, attractants and palatability enhancers. The use of protein hydrolysates produced by pre-processing the raw material with hydrolytic enzymes in diets has shown to improve growth and feed utilization. Also, there have been reports on biologically active peptides with immunostimulant and antibacterial properties. Recently, dietary supplementation of some protein hydrolysates has gained a huge interest of aquafeed nutritionists because of the functional properties and potential benefits of the hydrolysates. However, there is limited information on their nutritional value as potential dietary ingredients in diets for olive flounder and red sea bream which are important marine finfish species cultured in Korea. Therefore, a series of experiments was conducted to determine the insights of the utilization of the different protein hydrolysates, including krill, shrimp, tilapia and tuna hydrolysates, in diets for both fish species by examining overall performance and health status of the fish.

In the first feeding trial, the results indicated that dietary supplementation of the protein hydrolystes particularly krill hydrolysate, even in high fish meal (FM) diet is a efficient strategy to improve growth performance, feed utilization, innate immunity and disease resistance of juvenile olive flounder.

Based on the results obtained from the second and third experiments, 50% of FM protein could be substituted by soy protein concentrate (SPC) with dietary supplementation of the protein hydrolysates, without any adverse impact on juvenile olive flounder and red sea bream growth performance and health/welfare condition, when compared to the fish fed the high FM diet. These findings also demonstrated that dietary inclusion of the tested protein hydrolysates, improved the innate immune responses of fish which is most probably responsible for the enhanced disease resistance of fish against bacterial infection caused by *E. tarda*, which was depressed in fish fed the low FM diet. Moreover, these results suggested that krill hydrolysate, apparently, is the most effective dietary supplement for juvenile olive flounder while shrimp hydrolysate seemed to be the best choice for juvenile red sea bream.

In view of finding an appropriate inclusion level for the hydrolysate products that showed the best results in terms of growth performance, a study was conducted to verify the effects of different dietary levels of shrimp and tilapia hydrolysates in low FM diet at the

levels of 1.5, 3.0 and 4.5% on growth performance, feed utilization, intestine histology, digestibility, innate immunity and disease resistance against *Edwardsiella tarda* for olive flounder. The results showed that the highest growth performance and feed utilization efficiency were observed in fish fed 4.5% dietary shrimp hydrolysate which were comparable to those of the high FM group. Moreover, the significant improvement of liver IGF-I mRNA expression by increment of dietary shrimp hydrolysate level in this study further confirmed that supplementation of appropriate level of shrimp hydrolysate in diets for olive flounder would enhance IGF-I production, which is fundamentally involved in growth regulation. In contrast, fish fed the highest tilapia hydrolysate level exhibited lower growth performance and feed utilization efficiency than other inclusion levels, even though the difference was not significant. Nevertheless, it was found that tilapia hydrolysate can induce the innate immune response causing enhanced disease resistance.

In the last study, two feeding trials were carried out for the two species to investigate the effects of dietary supplementation of krill and tuna hydrolysates on growth performance, feed utilization, digestibility, innate immunity and disease resistance against *E. tarda*. In both experiments a basal FM-based diet was regarded as a control and two other diets were prepared by top-coating 2% of the tested hydrolysates. Results of this study indicated that supplementation of 2% krill hydrolysate in diets for red sea bream and olive flounder can enhance growth performances and feed efficiency, and that non-specific immune response can be positively affected by both hydrolysates.

To My Dearest FATHER

And

My Loving Husband, SAMAD

If I had to choose between loving you and breathing…

I would use my last breath to tell you…

I LOVE YOU

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CHAPTER 1

INTRODUCTION

INTRODUCTION

1.1. Fish meal as ingredient in aquafeed

Aquaculture, the farming of aquatic plants and animals, is no different from any other terrestrial farming activity in that production is totally dependent upon the provision and supply of nutrient inputs (Tacon and Metian, 2008). These nutrients are either supplied through the consumption of natural food organisms produced within the culture system for the target species or through the direct external application of feed inputs.

Clearly, if the finfish and crustacean aquaculture sector is to sustain its current growth rate, then it follows that the supply of feed inputs will also have to grow at similar rates so as to meet demand. Nowhere is this supply more critical than with the current dependency of the export oriented fish and crustacean aquaculture sector upon capture fisheries for sourcing feed inputs, including fish meal (FM) and fish oil.

The supply of marine raw materials such as fishmeal, the predominant sources of protein for carnivorous fish feeds, has become a limiting factor for expansion of aquaculture due to the pressure on feed-grade fisheries (Naylor et al., 2009). Production of these marine raw materials cannot increase above current levels and, coupled with the increasing demand driving prices upwards, it is no longer feasible to use FM at the current inclusion levels (FAO, 2009). This has led to the investigation of new, cost-efficient protein sources as alternatives to FM in aquafeeds (Turchini et al., 2009; Hardy, 2010). Previous studies indicated that replacement of FM with protein sources from terrestrial plant and animal sources would be possible provided amino acid and fatty acid requirements are met (Glencross et al., 2007; Webster et al., 2007).

1.2. Soybean as an alternative protein source

Several land animal products including poultry by-products, meat, bone and blood meals have been investigated as substitutes for FM in fish diets (Smith et al., 1995; Robaina et al., 1997; Webster et al., 1999). However, plant products potentially offer more sustainable protein sources for aquafeeds although they often contain antinutritional factors, which can affect growth performance and fish health (Francis et al., 2001; Gatlin et al., 2007). Thus rapeseed, soybean and sunflower meals as well as various legumes (beans and peas) are less expensive and readily available in high quantities, although they have variable desirable and undesirable characteristics that both support and limit their use (Francis et al., 2001; Gill et al., 2006; Krogdahl et al., 2010).

Among plant proteins, soybean meal is the good candidate for partial or total replacement of fish meal protein in diets because of higher protein content, balanced essential amino acids, availability of sources and low cost, but its full nutritional value is obtained only after inactivation of soy anti-nutritional factors (ANFs), such as protease inhibitor, anti-vitamin and lectin, which decrease the nutritional value of food through the mineral bioavailability and digestibility of nutrients. Different soybean products, such as soy protein

concentrate (SPC), extracted and toasted (defatted) soybean meal, full-fat soybean meal, or low oligosaccharides soybean meal, tended to produce contrasting fish growth because of the varied quantities of antinutrient compounds and/or lowly digestible carbohydrates present in these products.

SPC is produced through aqueous ethanol or methanol extraction of defatted soy flakes, which typically contains 65–70% crude protein (Lusas and Riaz, 1995). This additional extraction removes or deactivates ANFs, soluble carbohydrates and fiber, but not phytic acid (Bureau et al., 1998; Storebakken et al., 2000). Further, the extraction by alcohol can eliminate bitter off-flavor (Morr and Ha, 1991). Many studies have been carried out during the past few years on the use of SPC in fish diets and the results are contradictory (Kaushik et al., 1995; Médale et al., 1998; Mambrini et al., 1999; Kissil et al., 2000). Although SPC has a relatively balanced amino acid profile for fish, it is low in some essential amino acids, especially methionine (Storebakken et al., 2000). Thus, more attention has been focused on the beneficial effect of amino acid supplementation in SPC-based diets on growth performance for several fish species (Mambrini et al., 1999; Kissil et al., 2000).

However, it has recently been demonstrated that the complementary effect of different ingredients is often more effective compared to crystalline amino acid supplementation to combat the nutritional deficiencies in formulating alternative protein based diets. Hence, blend of different ingredients is often recommended in achieving balanced nutritional composition, complementing amino acid profiles and masking the unpalatable substances present in feed ingredients (Yamamoto et al., 1995; Tidwell et al., 2005; Guo et al., 2007; Kader et al., 2011, 2012). Therefore, it might also be possible to replace FM, at high levels or completely from feed by combining different ingredients in appropriate proportion. Indeed, it has been indicated that animal proteins, especially marine by-products contain comparatively balanced amino acids and high amount of free amino acids which could complement the deficiencies in high plant protein based diets for marine carnivorous fish (Kader, 2008; Kolkovski et al., 2000; Kousoulaki et al., 2009; Mai et al., 2006).

1.3. Marine by-products

The fish industry is a major economic source for a number of countries worldwide. Fish protein is an essential source of nutrients for many people, especially in developing countries. It is estimated that worldwide, one billion people depend on producing, processing and trading fish for their livelihood (Oosterveer, 2008). The fish processing industry produces more than 60% byproducts as waste, which includes head, skin, trimmings, fins, frames, viscera and roes, and only 40% fish products for human consumption (Dekkers et al., 2011). These large quantities of fish by-product waste from fisheries would create serious pollution and disposal problems in both developed and developing countries. These by-product wastes contain good amount of protein rich material that are normally processed into low market-value products, such as animal feed, FM and fertilizer (Hsu, 2010). In view of utilizing these protein rich fish processing by-product wastes, several biotechniques have been developed to recover the essential nutrients and bioactive compounds.

The biotechniques which are currently employed to recover the nutritional and physiological important peptides are hydrolysis of fish proteins that results in the production of biologically active protein hydrolysates from these commercially low value fish processing by-products and underutilized fish species.

1.4. Protein hydrolysate

Protein hydrolysates are breakdown products of the conversion of proteins into smaller peptides. Generally, protein hydrolysates are small fragments of peptides that contain 2–20 amino acids. These protein hydrolysates are produced by the hydrolysis of native proteins. Protein hydrolysis decreases the peptide size, and thereby making hydrolysates the most available amino acid source for various physiological functions of human body. Protein hydrolysates are used as readily available sources of protein for humans and animals due to their good functional properties (Neklyudov et al., 2000).

1.4.1. Production of protein hydrolysate

The first commercial fish protein hydrolysates were produced in the 1940s in Sweden using a chemical process (Kristinsson and Rasco, 2000). In that process, fish protein is cleaved into peptides of variable molecular weights by either acidic or alkaline treatment using high temperature (121 °C) and high pressure (100 kPa) (Sanmartin et al., 2009). It was popular due to the high protein recovery, fast processing and low cost. However, it operated with little ability to control the quality of fish protein hydrolysates, resulting in weak aforementioned functionalities, both physicochemical and bioactive. These disadvantages significantly limited the high value-applications of these protein hydrolysates for food and drug. They are currently used for low-value products such as fertilizer (Kristinsson and Rasco, 2000) with aprofit of only US50 cent/ton, or as a nitrogen source for the growth of lactic acid bacteria (Gao et al., 2006).

With the aim of using protein hydrolysates in high value-added products, enzymatic processing was employed to produce well-defined protein hydrolysates. The proteins can be easily deactivated in mild conditions of temperature and pH. The availability of various enzymes from different sources enables the manufacturer to choose the best one based on the desired final product (Pasupuleti and Braun, 2010). For example, enzymes can be employed to systematically remove amino acids from either the N-or C-terminus (Sanmartin et al., 2009). The enzymatic processes have a number of advantages and hold the most promise for the future of protein hydrolysate production.

1.4.2. Properties of protein hydrolysate

Protein hydrolysates have good functional properties and can contribute to water-holding, texture, gelling, foaming and emulsification properties in different food systems (Kristinsson, 2007). In addition, hydrolysates have high nutritional properties (Shahidi et al., 1995; Slizyte et al., 2005a,b) and exhibit bioactive properties such as antioxidative, antihypertensive, antithrombotic and immunomodulatory activities (Kim and Mendis, 2006).

Several reviews on the functional properties of protein hydrolysates have been published (Rustad et al., 2011; Chalamaiah et al., 2012; He et al., 2013).

1.4.3. Potential applications of protein hydrolysate in aquafeed

Protein hydrolysates have been used in aquaculture feeds in order to enhance the growth and survival of fish (Kotzamanis et al., 2007). Kotzamanis et al. (2007) incorporated two fish protein hydrolysates, from *Sardina pilchardus*, into four diets prepared for start-feeding sea bass larvae, at two different levels (10% and 19% of total ingredients), and reported that the peptides in the protein hydrolysates affected the growth performance and immunological status of sea bass larvae. In another study, a pollock protein hydrolysate was used for enrichment of the live feed offered to halibut larvae from the onset of exogenous feeding and studied the effects of treatment on selected innate immune parameters and concluded that feeding peptide-enriched live feed to larvae stimulated the production of lysozyme and C3 during the first weeks in feeding (Hermannsdottir et al., 2009). Nguyen et al. (2012) conducted a feeding trial to evaluate the effect of the supplementation of hydrolysates from tuna head on the survival and growth of shrimp *Penaeus vannamei* and reported the hydrolysates of tuna head improved significantly both growth and the survival rates of shrimps.

Accordingly, in exchange of FM, fish hydrolysate generally shows a beneficial effect on growth performance and feed utilization at low inclusion levels, but decreased performance when exceeding a specific dietary level. This performance is postulated to be due to the balance of free amino acids, peptides and proteins in digestion, absorption and utilization from the plasma pool (Espe et al., 1999; Hevrøy et al., 2005).

Moreover, the stimulatory effects of some protein hydrolysates on fish leucocytes in vitro suggests they may also be effective in reducing disease-related losses among farmed fishes. If given prior to pathogen exposure, possibly as feed supplements, one or more of these compounds might work to stimulate the fish's immune system sufficiently to resist infection by pathogenic microorganisms. Despite promising results in vitro, however, the prophylactic benefits of protein hydrolysates for protecting fishes in laboratory challenges have been disappointing. For example, no protective effect was observed among Atlantic salmon fry (Gildberg et al., 1995) or Atlantic cod fry (Gildberg and Mikkelsen, 1998) fed a commercial feed supplemented with hydrolyzed fish protein and challenged with *Aeromonas salmonicida* or *Vibrio anguillarum*, respectively. A more complete understanding of the humoral and cellular processes involved in disease resistance may eventually lead to more efficacious results with protein hydrolysates.

However, to our knowledge, no studies have examined the growth performance and immune functions of olive flounder and red sea bream, as two key marine finfish species cultured in Korea, fed diets supplemented with protein hydrolysates.

1.5. Chapter justification

The studies presented in this dissertation are aimed to evaluate the effect of dietary protein hydrolysates on nutritional and physiological performance of juvenile olive flounder and red sea bream, and to estabilish their optimum dietary level as a supplement and/or fish meal replacement based on short-term feeding trial for juvenile olive flounder, as the most important marine fish cultured in Korea. In addition, the findings of these research works will encourage feed manufactures to utilize plant proteins more efficiently in generating low-cost and sustainable aquafeed. The short justification of each chapter is as follows:

Chapter 2 is about the dietary supplementation of three different protein hydrolysates including krill, shrimp and tilapia hydrolysates in FM based diets for juvenile olive flounder. In this study, we demonstrated that the tested protein hydrolysates can be used as a valuable protein source by at least 10% of fish meal protein repralcement in juvenile flounder diet. The study revealed that dietary inclusion of the krill hydrolysate can positively affect the fish growth performance and feed utilization efficiency as well as diet digestibility. Also, immune function of the fish was significantly improved in goups offered the krill hydrolysate. In addition, chapter 2 documents that dietary treatment has no significant effect on fish disease resistance against bacterial infection caused by *Edwardsiella tarda*.

Chapter 3 examines the three protein hydrolysates as feed ingredients in high plant protein diets for juvenile olive flounder, as inclusion of the palatability enhancer or attractants is recommended in high SPC diets. The results in the current chapter may offer convincing evidence that 50% of FM protein could be substituted by SPC and protein hydrolysate blend without any adverse impact on juvenile olive flounder growth performance and health/welfare condition, when compared to the fish fed the high FM diet. Dietary inclusion of the tested protein hydrolysates, in exchange for FM, also improved the innate immune responses of fish which is most probably responsible for the enhanced disease resistance of fish against bacterial infection caused by *E. tarda*, which was depressed in fish fed the low FM diet. In addition, these results suggest that krill hydrolysate, apparently, is the most effective dietary supplement for this species than two other tested protein hydrolysates.

Similarly, the aim of the study presented in chapter 4 is to describe the supplemental effects of the three protein hydrolysates in low FM diets, and to compare their efficiency with that of a high FM diet for juvenile red sea bream. This chapter showed that dietary inclusion of the tested protein hydrolysates in low FM diets for red sea bream improves the feed nutritional value resulting in enhanced growth performance, non-specific immune response and disease resistance. Also, it is suggested that SPC could replace up to 50% of the FM by the supplementation of the tested protein hydrolysates, particularly shrimp hydrolysate.

Chapter 5 focuses on the determination of the appropriate inclusion level for the hydrolysate products that showed the best results in terms of growth performance for juvenile olive flounder. Therefore, a study was desgined to verify the effects of different dietary levels of shrimp and tilapia hydrolysates in low FM diet on growth performance and health status of the juvenile olive flounder. Based on the study, the highest growth performance and feed utilization efficiency were observed in fish fed 4.5% dietary shrimp hydrolysate which were comparable to those of the high FM group. Moreover, the significant improvement of liver

insulin-like growth factor I (IGF-I) mRNA expression by increment of dietary shrimp hydrolysate level in this study further confirmed that supplementation of appropriate level of shrimp hydrolysate in diets for olive flounder would enhance IGF-I production, which is fundamentally involved in growth regulation. In contrast, fish fed the highest tilapia hydrolysate level exhibited lower growth performance and feed utilization efficiency than other inclusion levels, even though the difference was not significant. Therefore, based on the fish growth performance, shrimp hydrolysate seemed to be the most effective dietary supplement for juvenile olive flounder whereas tilapia hydrolysate, apparently, might be the best choice for induction of the innate immune response causing enhanced disease resistance.

In chapter 6, we discusse the possible effect of tuna and krill hydrolysate as a feed attractant in both fish species fed FM based diets. This chapter indicates that coating the FM-based diets with krill hydrolysate can improve growth performance of both red sea bream and olive flounder juveniles. Moreover, tuna and krill hydrolysates can be used as potential stimulator of innate immunity in both fish species.

CHAPTER 2

Effect of Dietary Hydrolysate Supplementation on Growth Performance, Non-specific Immune Response and Disease Resistance of Olive Flounder (*Paralichthys olivaceus*)

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Abstract

A 9-week feeding trial was conducted to investigate the effects of dietary supplementation with protein hydrolysates on growth, innate immunity and disease resistance of olive flounder. A fishmeal (FM)-based diet was regarded as a control, and three diets were prepared by partial replacement of FM with krill hydrolysate, shrimp hydrolysate or tilapia hydrolysate (designated as Con, KH, SH and TH, respectively). Triplicate groups of fish (24.5 ± 0.3 g) were fed one of the diets to apparent satiation twice daily for 9 weeks and then challenged by *Edwarsellia tarda*. Fish-fed KH diet showed significantly ($P<0.05$) higher growth performance and feed utilization compared with the Con diet. Dry matter digestibility of the diets was significantly increased by KH and TH supplementation. All the examined innate immune responses were significantly increased in fish fed KH diet. Significantly, higher respiratory burst and superoxide dismutase (SOD) activities were found in fish-fed SH diet. Lysozyme and SOD activities were significantly increased in fish-fed TH diet. However, no significant effect was found on fish disease resistance. This study indicates that dietary supplementation of the hydrolysates, particularly KH, can improve growth performance, feed utilization and innate immunity of olive flounder.

Introduction

Aquaculture is one of the fastest growing animal-producing sectors now supplying nearly 50% of the world's food fish. Intensive aquaculture is highly dependent to fishmeal (FM) and fish oil supply from wild fisheries. FM is regarded as the best dietary protein source because it is very palatable and provides an excellent balance of essential amino acids, essential fatty acids and other substances as well as highly digestible energy and vitamins (Hardy, 2010). The rapid growth of the aquaculture industry has resulted in higher prices of FM over the past three decades, and it is expected to rise further with continued growth in demand (Hardy and Tacon, 2002; Hardy, 2010). Moreover, the sustainability of employing wild forage fish for FM production has been questioned (Naylor et al., 2000; Tacon and Metian, 2008). During the past decades, numerous studies have been conducted aiming at finding appropriate protein sources including both plant and animal origin for aquafeed industry. However, high replacement levels of FM by plant protein has generally resulted in inferior fish growth performance and feed utilization (Aksnes et al., 2006a; Gaylord et al., 2006; Hernandez et al., 2007; Lim and Lee, 2008).

One of the most promising ways of meeting the future demands for protein sources is the efficient utilization of marine by-products including fisheries by-catch and seafood processing leftovers (Hardy et al., 2001). Furthermore, utilization of these products in feed industry would prevent the environmental pollution from their disposal. Estimates show that the amount of FM that can be produced globally from by-products exceeds the whole forage fish landings used for FM production (New, 1996; Hardy and Tacon, 2002). Some

preliminary studies have revealed the efficiency of by-product meals as potential substitutes for FM in aquafeeds (Hardy et al., 2005; Lee et al., 2010).

Fish-processing by-products are generally regarded as low-value resources with negligible market value (Hsu, 2010). Hydrolysis process is regarded as one of the promising ways for the conversion of fish by-products into acceptable forms by improvement of their quality and functional characteristics (Gildberg, 1993; Shahidi, 1994; Chalamaiah et al., 2012). Enzymatic hydrolysis of fish protein results in the formation of a mixture of free amino acids, di-, tri- and oligo-peptides and enhances the occurrence of polar groups and solubility of hydrolysate compounds. Such changes lead to improvement of nutritional value and digestibility and reduced bitterness of by-products offering interesting opportunities for food applications (Liceaga-Gesualdo and Li-Chan, 1999; Kristinsson and Rasco, 2000a,b; Spellman et al., 2003).

Crustacean protein hydrolysates have been used in aquafeeds as potential protein sources (Plascencia-Jatomea et al., 2002) or as dietary supplements in small amounts for improvement of diet palatability (Kolkovski et al., 2000). Processing of shrimp for human consumption results in the production of a large amount of by-products accounting for 50–70% of the processed materials. These waste materials contain valuable components such as protein, chitin and astaxanthin (Bataille and Bataille, 1983; Shahidi and Synowiecki, 1991; Tacon et al., 2006) making them as potential ingredient for aquafeeds. Recovery of the shrimp waste by enzymatic hydrolysis results in the formation of biologically active peptides with pharmaceutical and growth-stimulating properties (Gildberg and Stenberg, 2001). Krill is regarded as one of the most promising marine protein resources and has been examined as a potential dietary ingredient in aquafeeds for a long time (Storebakken, 1988; Tacon et al., 2006). Krill is distinguished as a rich source of high-quality protein compared with the other animal protein sources due to its low-fat content and being rich in omega-3 fatty acids (Nicol et al., 2000; Tou et al., 2007). In the same way, tilapia processing industries generate a huge quantity of by-product having potentially high nutritional and functional values. As for other hydrolysate manufactured from fish by-products, strong antioxidative activities were identified from tilapia hydrolysate (Fan et al., 2012; Zhang et al., 2012). Such biological activities could find application in new development of fish feed for higher performances.

Olive flounder has been one of the most important fish species for marine aquaculture in Asian countries and has been successfully cultured in Korea, Japan and China (Kang et al., 2008; Castano-Sanchez et al., 2010). In the present study, the effects of partial replacement of FM by krill, shrimp or tilapia hydrolysates were examined on growth, feed utilization, diet digestibility, non-specific immune response and disease resistance of olive flounder.

Material and methods

Experimental diets and design

Marine protein hydrolysates were all obtained from AQUATIV (Aquaculture division of DIANA Group), Elven, France. Briefly, whole Antarctic krill (*Euphausia superba*) was

enzyme hydrolyzed and defatted to produce krill hydrolysate concentrate. Farmed white shrimp (*Litopenaeus vannamei*) head co-products were enzyme hydrolyzed and spray-dried to produce shrimp hydrolysate powder. Farmed tilapia co-product, consisting in heads, bones, trims and viscera, was enzyme-processed and spray-dried to produce tilapia hydrolysate powder. Proximate composition and peptide profiles of marine protein hydrolysates are respectively introduced by Tables 1 & 2. Peptide profiles of hydrolysates were determinated using size exclusion chromatography method (Guerard et al., 2001).

Four experimental diets were formulated to be isonitrogenous (440 g kg^{-1} crude protein) and isocaloric (18 MJ kg^{-1}) (Table 3). A basal FM-based diet was regarded as a control, and three other experimental diets were prepared by partial replacement of FM with krill hydrolysate concentrate (KH), shrimp hydrolysate powder (SH) and tilapia hydrolysate powder (TH) (designated as Con, KH, SH and TH, respectively). Dosages of hydrolysates, expressed on a dry matter basis, were fixed to supplement equivalent levels of crude protein. All dry ingredients were thoroughly mixed and pelleted through a meat chopper machine (SMC-12; Kuposlice, Busan, Korea) in 3 mm diameter after addition of squid liver oil, soybean oil and double-distilled water, freeze-dried at 40 °C for 24 h and stored at 20°C until use

Feeding trial

The feeding trial was conducted at the Marine and Environmental Research Institute of the Jeju National University (Jeju, South Korea). Juvenile olive flounder was obtained from a private hatchery (Jeju, South Korea). All the fish were fed a commercial diet for 2 weeks to be acclimatized to the experimental conditions and facilities. At the end of the acclimation period, the fish (initial mean body weight, 24.5 ± 0.3 g) were randomly distributed into twelve polyvinyl circular tanks of 150 L capacity at a density of 35 fish per tank and 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 hand-fed with one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 9 weeks. The water temperature during the feeding trial ranged from 16 to 23°C according to the seasonal change. The photoperiod was maintained on a 12:12 h (light/dark) schedule. The uneaten food was collected and weighed to determine the feed intake. The insides of the tanks were routinely cleaned by a sponge to prevent the growth of microflora. Growth of fish was measured with 3-week interval. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish.

Sample collection and analyses

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for the calculation of growth parameters and survival. Three intact fish per tank were randomly selected and kept at 20 °C for whole-body proximate composition analysis. Three fish per tank (nine fish per dietary treatment) were randomly captured, anaesthetized with 2-phenoxyethanol (200 mg L^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for the determination of haematocrit and haemoglobin concentrations,

and respiratory burst activity. After the above-mentioned measurements with whole blood, plasma was separated by centrifugation at 5000 *g* for 10 min and stored at 70 °C for the determination of immunoglobulin (Ig) level and blood biochemical parameters including glucose, total protein, total cholesterol and triglyceride levels. Another set of blood samples (three fish per tank, nine fish per dietary treatment) was taken without heparin 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 non-specific immune responses including lysozyme, superoxide dismutase (SOD), myeloperoxidase (MPO) and antiprotease activities. Haematocrit was determined by a microhaematocrit technique (Brown, 1980). Haemoglobin and plasma levels of glucose, total protein, total cholesterol and triglyceride were determined by using an automated blood analyser (SLIM; SEAC Inc, Florence, Italy).

Analysis of moisture and ash contents of diets and whole-body samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Hoganas, Sweden), and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Seoul, Korea).

Monitoring of non-specific immune responses

The oxidative radical production by phagocytes during respiratory burst was measured by the NBT (nitro-blue-tetrazolium; Sigma, St. Louis, MO, USA) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, and then, 50 µL was taken out and dispensed into glass tubes. Then, 1 mL of dimethylformamide (Sigma) was added and centrifuged at 2000 *g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as blank.

Plasma immunoglobulin (Ig) level was determined according to the method described by Siwicki and Anderson (1993). Briefly, plasma total protein content was measured using a microprotein 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 the determination of serum lysozyme level by the method described by Hultmark et al. (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg mL^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), and then, 200 µL of suspension was placed in each well of 96-well plates, and 20 µL serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340; Biochrom, Cambridge, UK). Hen egg white lysozyme (Sigma) was used for the standard curve. Values are expressed as µg mL^{-1}.

Serum MPO activity was measured according to Quade and Roth (1997). Briefly, serum (20 µL) was diluted with HBSS (Hanks Balanced Salt Solution without Ca^{2+} or Mg^{2+}; Sigma) in 96-well plates. Then, 35 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 5 mM H_2O_2 were added. The colour change reaction was stopped after 2 min by adding 35 µL of 4 M sulphuric acid. Finally, the optical density was read at 450 nm in the 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 percent inhibition was normalized by mg protein and presented as SOD activity units.

The serum antiprotease activity was measured according to the method described by Ellis (1990), with some modifications (Magnadóttir et al., 1999). Briefly, 20 µL of serum was incubated with 20 µL of standard trypsin solution (Type Π-S, from porcine pancreas, 5 mg mL^{-1}, Sigma) for 10 min at 22 °C. Then, 200 µL of phosphate buffer (0.1 M, pH 7.0) and 250 µL azocasein (2%; Sigma) were added and incubated for 1 h at 22 °C. Five-hundred microlitres of 10% trichloro acetic acid (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 1 N NaOH. Optical density was read at 430 nm. For a 100% positive control, buffer replaced the serum, while for the negative control, buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated as follows:

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

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

Digestibility test

For the estimation of apparent digestibility coefficient of the experimental diets, chromic oxide (Cr_2O_3) (Sigma Aldrich) was included in the diets as an inert indicator at a concentration of 10 g kg^{-1} diet. The digestibility trial was performed in another water circulation system equipped with four fibreglass tanks of 300 L capacity, designed according to Cho et al. (1982). The faeces settling column was connected to the outlet of each tank. New sets of olive flounder with mean weight of 50 g were stocked into each tank at a density of 40 fish per tank, and each group of fish was fed one of the test diets. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. The digestibility trial consisted of three periods of 10 days. In each 10-day period, the fish were allowed to become acclimatized to the feed for the first 3 days, and faeces were collected over the next 7 days. Then, diets were randomly changed between

tanks, and the procedure repeated for two more times, giving a total of three faecal samples for each diet. On the faecal collection days, the faeces from each tank were collected directly into 50-mL centrifuge tubes. All faeces collected from each tank in each period were pooled, centrifuged for 30 min at 2100 *g*, and the supernatant was discarded, and faeces were frozen at 20 °C until analysed. After feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed waste and faecal residues. Chromium oxide content of diet and faeces samples were analysed by the method described by Divakaran et al. (2002). The apparent digestibility coefficients of 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) × (%drymatter in feces / %dry matter in diet)

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

Challenge test

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly selected and subjected to a bacterial challenge. *Edwarsellia tarda* was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The fish were injected intraperitoneally with *E. tarda* suspension containing 1×10^3 CFU mL^{-1} of bacterium. The pathogenic dose of bacterium had previously been determined in a preliminary test using fish of a similar size. After injection, the fish were distributed into twelve 60-L plastic tanks, and their mortality was monitored and recorded for 10 days.

Statistical analysis

All experimental diets were assigned by a completely randomized design. Data were subjected to one-wayANOVA in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). The significant differences ($P<0.05$) between group means were compared using Tukey's test. Data are presented as mean ± SD. Percentage data were arcsine transformed before analysis.

Results

The results of growth performance, feed utilizations and survival of fish fed the experimental diets are provided in Table 4. Fish fed the KH diet showed significantly ($P < 0.05$) higher growth performance than fish fed the control diet. Significantly higher protein efficiency ratio and lower feed conversion ratio were obtained in fish fed the KH diet compared with those fed the control diet. Fish survival varied from 84 to 89% without significant differences among dietary treatments.

Apparent digestibility coefficients for dry matter and protein of the experimental diets are provided in Table 5. Inclusion of KH and TH in diets significantly affected the

digestibility of dry matter and higher coefficients were found compared with the control group.

The results of whole-body proximate composition analysis revealed no significant differences among treatments (Table 6). However, slightly higher lipid content was found in fish fed the KH diet.

The results of analysis of haematological parameters are shown in Table 7. Among the examined parameters, only the plasma total protein level was significantly affected by dietary treatments and higher values were obtained in groups fed the KH and SH diets.

Inclusion of KH in diet resulted in significant improvement of all the examined immune response parameters compared with the control diet (Table 8). Fish-fed SH diet exhibited significantly higher NBT and SOD activities. Significant improvements in lysozyme and SOD activities were obtained in fish-fed TH diet.

In the challenge test with *E. tarda*, all the fish in the control group were dead by 6 days, while the other fish groups fed the tested hydrolysates showed higher survival rate even 10 days after the challenge (Fig. 1). However, the differences were not significant.

Discussion

In this study, dietary supplementation of KH significantly improved growth performance and feed utilization. KH used in the present study mainly consists of small peptides and free amino acids with molecular weight below 5000 Da (Table 2). Similar results were achieved by Zheng et al. (2012a) with the same species where the best growth and feed efficiency were obtained with ultrafiltered fraction of the fish hydrolysate. They suggested that the presence of some small molecular weight compounds in fish protein hydrolysate might be essential for maximum growth performance and feed utilization in olive flounder. It has also been emphasized that the positive effect of fish protein hydrolysates could potentially be influenced by their peptide size distribution and inclusion level (Aksnes et al., 2006a,b; Kotzamanis et al., 2007; Zheng et al., 2012b). Zheng et al. (2012b) found that FM replacement with ultrafiltered fish hydrolysate containing a larger proportion of small molecular weight compounds in diets for juvenile turbot resulted in better growth performance and feed efficiency compared with the use of non-ultrafiltered fish hydrolysate at the same level. Similar results have also been reported in previous studies regarding the beneficial effect of the small molecular weight compounds from fish protein hydrolysate on growth performance and feed utilization (Aksnes et al., 2006a,b; Zheng et al., 2012a). Moreover, higher growth responses along with increased feed intake in KH group compared with that of the control group may suggest that KH contains some attractants that improve the palatability of the KH diet. In this regard, Kolkovski et al. (2000) found that coating a commercial diet with KH resulted in significant improvements in ingestion rates of the diet in yellow perch (*Perca flavescens*) and lake whitefish (*Coregonus clupeaformis*). It has been indicated that dietary protein hydrolysate at certain inclusion levels can positively affect diet palatability (Refstie et al., 2004; Hevrøy et al., 2005). Although such increase in feed intake

was also observed in fish fed the SH diet, it did not lead to significant improvement of growth performance. This phenomenon can be justified by the notion that if dietary protein is not in the appropriate form for physiological requirements, it cannot be completely assimilated (Arredondo-Figueroa et al., 2013). Also, the higher growth performance obtained in KH group compared with SH group might be related to higher solubility of KH (see Table 1). It has been suggested that protein solubility affects the absorption capacity and rate of diet passage through the gastrointestinal tract (Espe et al., 1999). The present study may provide more evidence for the previous finding as a significant improvement in protein efficiency ratio was obtained by dietary inclusion of KH.

In the current study, significantly higher digestibility of dry matter was found in fish-fed KH and TH-containing diets compared with the control diet (Table 5). Also, slightly higher ADC of protein was found in fish fed the same two protein hydrolysate sources. Similarly, Refstie et al., (2004) reported no significant effect of fish protein hydrolysate on digestibility of protein for Atlantic salmon, although the lowest digestibility was observed in fish-fed diet with no protein hydrolysate supplementation. Aksnes et al., (2006a) found no significant differences in digestibility of dry matter and protein for rainbow trout fed FM or fish hydrolysate–containing diets. There are a few available studies on the effects of protein hydrolysates on digestive enzymes in aquatic organisms (Cordova-Murueta and Garcıa-Carreno, 2002; Cahu et al., 2004; Zambonino Infante and Cahu, 2007), and the results have shown the variations in the effects of protein hydrolysates on digestive enzymes activity from species to species. Even though further studies are required to define the exact mechanism for enhanced dry matter digestibility by dietary hydrolysates, it could be hypothesized that the protein molecular form of hydrolysate could positively influence the dietary protein assimilation via an enhancement in intestinal peptide and/or amino acids transporter gene expression as shown in chicken by Gilbert et al. (2010) and in fish by Bakke et al. (2010).

Whole-body composition of fish was not significantly affected by protein hydrolysates in this study which is in accordance with the results of Oliva-Teles et al. (1999). Aksnes et al. (2006b) found no beneficial effect of fish hydrolysate on whole-body protein and ash in rainbow trout fed high plant protein based diets. Zheng et al. (2012a) reported that dietary inclusion of higher dose of ultrafiltered fraction of fish hydrolysate was able to effectively increase the whole-body protein content in Japanese flounder whereas a lower dose failed. Similar results were reported by the same authors for juvenile turbot (*Scophthalmus maximus* L.) in a study with a similar design (Zheng et al., 2012b). These findings show that proper amount of protein hydrolysate should be applied to observe any significant effects on whole-body composition. Therefore, the results of the present study indicate that the inclusion level of the low-molecular-weight hydrolysates, KH and SH, was not enough to cause any significant impact on the whole-body composition of olive flounder.

Haematological parameters of juvenile olive flounder did not differ significantly among the dietary treatments in this study. However, total protein level of fish-fed KH and SH diets were significantly higher than that of fish fed the control diet. Plasma total protein level has been considered as an indicator of physiological and health condition in fish

nutrition studies (Watanabe et al., 2001; Dugenci et al., 2003; Harikrishnan et al., 2003; Congleton and Wagner, 2006). Increment of plasma protein level in this study may indicate a better absorption of the dietary protein hydrolysate fraction and improvement of the overall health condition of fish.

The enzymatic hydrolysis of proteins may results in the formation of biologically active peptides with immunostimulating and antibacterial properties (Bøgwald et al., 1996; Kotzamanis et al., 2007). Immunomodulatory activity of peptides has been demonstrated trough the stimulation of lymphocyte proliferation, natural killer (NK) cell activity, antibody synthesis and cytokine regulation (Horiguchi et al., 2005; Fitzgerald and Murray, 2006; Hartmann and Meisel, 2007). It has been reported that the immune enhancement effect of hydrolysates is dependent to both their size and concentration. Small and medium size peptides from fish protein hydrolysate can stimulate the non-specific defence system in fish (Bøgwald et al., 1996; Gildberg et al., 1996). The use of peptides with sizes ranging from 500 to 3000 Da stimulated superoxide anion production in Atlantic salmon (Gildberg et al., 1996). The results in the present study clearly indicate that moderate replacement of FM with KH can induce a significant enhancement of all the studied nonspecific immune responses. The best immune function was obtained in fish provided with KH diet; however, the use of SH and TH resulted in significant increase in NBT, lysozyme and SOD activities. Such difference in response to the administered hydrolyste sources can be due to the variations in origin and/or molecular weight distribution of hydrolysates (Liang et al., 2006). These findings are in line with the observed enhancement in lysozyme and complement activity of Japanese sea bass (*Lateolabrax japonicus*) fed a diet containing 15% fish protein hydrolysate (Liang et al., 2006). Also, the most recent study in our laboratory (Bui et al., 2013) revealed the significant enhancement of SOD and antiprotease activities as well as Ig level in red sea bream juveniles fed the same protein hydrolysates. In contrast, Murray et al. (2003) could not find any significant effect of fish protein hydrolysate on innate immune function of coho salmon (*Oncorhynchus kisutch*). Similar results were also reported by Zheng et al. (2012b) for juvenile turbot.

The improvement of immune function, either cellular or humoral, accompanied with enhanced disease resistance has already been demonstrated for various fishes (Murray et al., 2003; Liang et al., 2006; Kotzamanis et al., 2007). The immunostimulatory effects of protein hydrolysates in fish may suggest them as potential dietary ingredients for increasing disease resistance of farmed fish (Murray et al., 2003). However, the results of previous studies show that laboratory challenges did not confirm such effects in Atlantic salmon fry (Gildberg et al., 1996) or Atlantic cod fry (Gildberg and Mikkelsen, 1998). Liang et al. (2006) reported that no significant effect was detected on disease resistance of fish against *Vibrio anguillarumal* though dietary inclusion of fish protein hydrolysate significantly increased complement, lysozyme and phagocytic activities of Japanese sea bass. They suggested that such enhancement in innate immune function of fish may not have been biologically significant. In the present study, after 10 days of challenge with *E. tarda*, no significant effect was found even though fish fed the protein hydrolysates had numerically higher survivals compared with fish fed the control diet. Gildberg and Mikkelsen (1998) found a higher survival rate of

Atlantic cod fry fed dietary protein hydrolysate during the first 12 days of *V. anguillarum* challenge. But after 24 days of challenge, no significant difference was observed in terms of mortality for all the fish groups. As suggested earlier by Liang et al. (2006), in the present study, challenge dosage seemed too high to detect differences from the dietary supplementation of the protein hydrolysates.

In conclusion, the findings in this study indicate that dietary FM can be successfully replaced with either of the tested protein hydrolysate at least up to 10%. Also, beneficial effects of dietary inclusion of KH are demonstrated on growth performance, feed utilization and non-specific immune response of olive flounder.

Table 2.1. Proximate compositions of marine protein hydrolysates (from product technical data sheets).

	KH	SH	TH
Dry matter (%)	56.1	96.0	95.0
Protein (% DM)	44.1	63.4	71.2
Lipid (% DM)	2.3	11.2	15.4
Ash (% DM)	10.5	10.5	4.8
Soluble Nitrogen (% N)	96.3	88.8	91.5
Essential Amino Acids (% products)			
Arg	2.94	3.71	4.06
His	0.50	1.35	1.36
Ile	1.39	2.32	2.45
Leu	2.32	3.76	4.38
Lys	2.85	3.54	4.23
Met	0.87	1.15	1.58
Phe	1.25	2.60	2.42
Thr	1.24	2.32	2.77
Val	1.57	2.91	2.94

KH, Krill Hydrolysate concentrate; SH, Shrimp Hydrolysate powder; TH, Tilapia Hydrolysate powder.

Table 2.2. The molecular weight of krill hydrolysate (KH), shrimp hydrolysate (SH) and tilapia hydrolysate (TH) (%).

Molecular weight (Da)	KH	SH	TH
>30000	<0.1	2.0	<0.1
20000-30000	<0.1	<0.1	<0.1
10000-20000	<0.1	<0.1	1.0
5000-10000	2.0	<0.1	2.0
1000-5000	18	12	27
500-1000	16	18	18
<500	64	68	52

Table 2.3. Formulation and proximate composition of the experimental diets for olive flounder (g kg^{-1}).

Ingredients	Experimental diets			
	Con	KH	SH	TH
White fishmeal	500	460	452	452
KH	0	40.3	0	0
SH	0	0	48	0
TH	0	0	0	42.3
Soybean meal	70	70	70	70
Corn gluten meal	70	70	70	70
Wheat flour	245	245	245	250
Squid liver oil	40	40	40	40
Soybean oil	40	40	40	40
Mineral Mix[1]	10	10	10	10
Vitamin Mix[2]	10	10	10	10
CMC	10	10	10	10
Choline chloride	5	5	5	5
Chromic oxide	10	10	10	10
Proximate composition (g kg^{-1} in dry matter)				
Dry matter	912	914	916	918
Crude protein	444	451	451	449
Crude lipid	130	122	137	140
Ash	183	153	158	146

[1] Mineral premix (g kg^{-1} of 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, 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^{-1} of mixture): L-ascorbic acid, 121.2; DL-α 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.

Con, Control; KH, Krill Hydrolysate concentrate; SH, Shrimp Hydrolysate powder; TH, Tilapia Hydrolysate powder; CMC, CarboxyMethyl Cellulose.

Table 2.4. Growth performance and feed utilization of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	Diets			
	Con	KH	SH	TH
Initial body weight (g)	24.1±0.3	24.9±0.6	24.4±0.5	24.6±0.3
Final body weight (g)	74.8±4.1^{a}	93.1±2.4^{b}	84.1±4.8^{ab}	81.6±2.5^{ab}
Specific growth rate (%)	1.89±0.1^{a}	2.20±0.1^{b}	2.06±0.1^{ab}	2.00±0.1^{ab}
Feed intake (g fish^{-1})	81.4±2.4	87.5±1.2	87.6±4.0	81.1±2.2
Feed conversion ratio	1.52±0.0^{b}	1.25±0.0^{a}	1.36±0.1^{ab}	1.37±0.1^{ab}
Protein efficiency ratio	1.48±0.0^{a}	1.78±0.1^{b}	1.63±0.1^{ab}	1.64±0.1^{ab}
Survival rate (%)	87.6±6.6	89.5±1.7	84.8±11.6	85.7±5.0

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.

Table 2.5. Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for olive flounder (*Paralichthys olivaceus*).

	Diets			
	Con	KH	SH	TH
ADCd (%) [1]	75.2±1.5[a]	81.3±0.6[b]	77.0±3.1[ab]	79.9±1.3[b]
ADCp (%) [2]	93.6±0.7	94.9±0.2	93.5±0.9	94.7±1.1

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] Apparent digestibility coefficient of dry matter

[2] Apparent digestibility coefficient of protein

Table 2.6. Whole-body composition of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	Diets			
	Con	KH	SH	TH
Dry matter (%)	26.2±0.8	26.5±0.4	26.5±0.4	26.7±0.6
Protein (% DM)	70.3±1.6	70.7±0.2	70.3±0.8	70.0±2.6
Lipid (% DM)	15.3±1.2	16.8±1.0	14.9±1.4	15.5±2.4
Ash (% DM)	13.6±0.5	12.8±0.9	13.5±1.2	13.7±0.7

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

Table 2.7. Hematological parameters of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	Diets			
	Con	KH	SH	TH
Haematocrit (%)	25.9±3.8	29.3±3.3	30.1±4.4	28.8±2.9
Haemoglobin (g dL^{-1})	4.21±0.7	4.40±0.4	4.54±0.2	4.42±0.4
Glucose (mg dL^{-1})	42.4±6.5	36.7±3.7	41.1±2.6	35.4±2.0
Total protein (g dL^{-1})	$3.41±0.2^{a}$	$4.09±0.3^{b}$	$4.06±0.3^{b}$	$3.85±0.3^{ab}$
Total cholesterol (mg dL^{-1})	271±61	259±11	229±33	241±27
Triglyceride (mg dL^{-1})	412±53	337±54	416±38	337±74

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.

Table 2.8. Non-specific immune parameters of olive flounder (*Paralichthys olivaceus*) fed the experimental diets for 9 weeks.

	Diets			
	Con	KH	SH	TH
NBT[1]	0.47±0.0[b]	0.61±0.1[a]	0.60±0.1[a]	0.55±0.0[ab]
Ig[2]	12.5±0.5[b]	16.2±1.5[a]	15.2±1.6[ab]	15.7±0.6[ab]
Lysozyme[3]	18.4±2.0[b]	30.6±3.3[a]	24.9±1.0[ab]	29.5±1.9[a]
MPO[4]	1.71±0.2[b]	2.68±0.3[a]	2.25±0.1[ab]	2.16±0.2[ab]
SOD[5]	42.5±4.4[b]	54.1±1.3[a]	52.7±1.0[a]	54.1±5.1[a]
Antiprotease[6]	16.1±2.7[b]	26.1±5.9[a]	24.4±1.7[ab]	22.0±1.7[ab]

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

[3]Lysozyme activity (μg mL^{-1})

[4]Myeloperoxidase level

[5]Superoxide dismutase (% inhibition)

[6]Antiprotease (% inhibition)

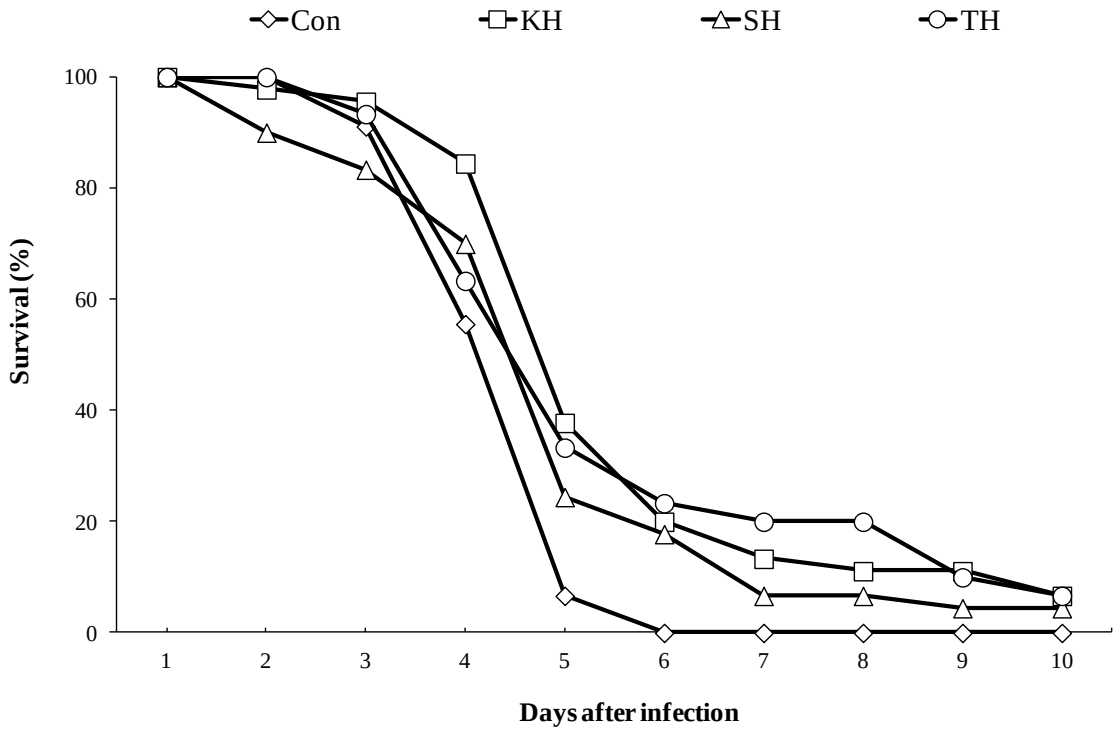


Fig. 2.1. Survival rate of olive flounder fed the experimental diets after challenge with *E. tarda*. Con, Control; KH, Krill Hydrolysate concentrate; SH, Shrimp Hydrolysate powder; TH, Tilapia Hydrolysate powder.

CHAPTER 3

Effect of Dietary Protein Hydrolysate Inclusion on Growth Performance, Intestinal Morphology, Innate Immunity and Disease Resistance of Juvenile Olive Flounder (*Paralichthys olivaceus*) Fed High Plant Protein Diets

Effect of Dietary Protein Hydrolysate Inclusion on Growth Performance, Intestinal Morphology, Innate Immunity and Disease Resistance of Juvenile Olive Flounder (*Paralichthys olivaceus*) Fed High Plant Protein Diets

Abstract

An 11-week feeding trial was performed to evaluate three different protein hydrolysates (PHs) as feed ingredients in high plant protein diets for juvenile olive flounder. Five experimental diets were fed to juvenile flounder to examine the effect of the PHs on growth performance, innate immunity and disease resistance against bacterial infection. A basal fish meal-based diet was regarded as a high fish meal diet (HFM) and a diet containing soy protein concentrate (SPC), as a substitute for fish meal (FM), at replacement level of 50% was considered as low fish meal diet (LFM). Three other experimental diets contained three different source of PH including shrimp, tilapia and krill hydrolysates (designated as SH, TH and KH) in exchange for fish meal. All diets were formulated to be isonitrogenous and isocaloric. Triplicate groups of fish (15.1 ± 0.1 g) were hand-fed one of the diets to apparent satiation twice daily for 11 weeks and subsequently challenged in vitro with infamous *Edwardsiella tarda*. As a result, growth performance and feed utilization of the fish fed the hydrolysate supplemented diets were significantly improved compare to those of fish fed the LFM diet and even better results were achieved in comparison with the corresponding values in HFM group. Dietary inclusion of the PHs significantly enhanced the apparent digestibility of dry matter and protein of the diets. In the proximal intestine, histological alterations were observed in the fish fed the LFM diet. In this respect, in fish fed the hydrolysate diets, the mucosal fold and enterocytes were significantly longer and exhibited a greater number of goblet cells compared to those of fish fed the LFM group. Respiratory burst activity was significantly higher in fish fed the TH and KH diets than fish fed the LFM diet. Significantly higher total immunoglobuline levels were found in fish groups fed SH and KH diets compared to those fed the LFM diet. Dietary inclusion of the PHs in SPC-based diets exhibited the highest lysozyme activity. Significantly higher superoxide activity was observed in groups of fish fed the KH diet. The effect of the tested PHs on fish disease resistance has revealed that fish offered the PH diets were more resistant to the bacterial infection caused by *E. tarda*. The findings in this study indicated that SPC supplemented with PHs could effectively replace 50% of FM protein in the diets for juvenile olive flounder without any adverse effects on fish well-being and overall performance.

Introduction

Olive flounder is one of the most important marine finfish species culture in Korea (Kang et al., 2008; Castano-Sanchez et al., 2010) which is traditionally feeding with trash fish or commercially manufactured aquafeeds with high FM content (Kim et al., 2007). As reported by FAO (2011), its production sharply increased from 21 368 metric tons in 1999 to 54 674 metric tons in 2009 (Okorie et al., 2013), placing further demands on FM supply as the main component of commercial flounder feeds. However, limiting supply and increasing cost of FM has led to the investigation of cost-efficient alternative protein sources (Turchini

et al., 2009; Hardy, 2010). Obviously, this has to be done by supporting both the optimum growth and at the same time not compromising the health of the farmed fish.

Over the past decades, numerous studies have been performed to find an appropriate substitute for FM in the diets of several fish species (Gomes et al., 1995; Sánchez-Lozano et al., 2009). In this regard, plant protein sources have been shown to have potential for inclusion in carnivorous fish feeds (Storebakken et al., 2000). However, compared to FM these products are believed to be deficient in one or more essential amino acids or nutrients that make them inferior to FM (Francis et al., 2001). In fact, it is now clear that very few available raw materials have a balanced amino acid profile suitable to serve as the sole protein source for FM. Yet, when the FM is substituted by a blending of different alternative protein sources, amino acid profiles and essential nutrients can be adjust to overcome the nutrient limitations of single protein source (Pratoomyot et al., 2011). Indeed, this topic has received most attention over the past years as it leads to more reliable results (Kaushik et al., 2004; Martínez-Llorens et al., 2008). In this regard, recent studies by Kader et al. (2012a,b) proved that inclusion of different crude ingredients, especially marine by-products, in high plant protein based diets are even more effective compared to crystalline amino acid supplementation (Kader et al., 2010) to achieve similar performances of olive flounder and red sea bream (*Pagrus major*) as with FM-based control group.

Although this interest for the substitution of FM by more sustainable and renewable protein sources were initiated as far back as the late 1970s (Alliot et al., 1979), most of these studies have focused on fish growth performance as the main criteria while only limited information is available today on the effect of these alternative protein sources on fish immune system and their disease resistance. Early research by Wedemeyer and Ross (1973) indicated that feeding juvenile coho salmon (*Oncorhynchus kisutch*) with dry diet containing either corn gluten or cottonseed meal had no effect on the susceptibility of fish to bacterial kidney disease. Later, Maita et al. (1998) found that yellow tail (*Seriola quinqueradiata*) fed with isonitrogenous diets, but differing in their protein source was subject to changes in their immune response. In that case, mortality due to a natural infection with *Pasteurella piscicida* occurred among the groups fed the non-FM diet, but not among control fish which offered standard FM diet during 55 days of feeding trial. In contrast, comparing the possible impact of FM replacement by a mixture of plant protein sources on immune status of gilthead sea bream (*Sparus aurata*), Sitja-Bobadilla et al. (2005) reported a significant enhancement in some of the immune response parameters. Hence, it is reasonable to assume that the efficiency of diet should be evaluated not only by growth but also by the impact on fish health.

Processing of fisheries by-catch and seafood leftovers with modern biological technology helps to improve their digestibility and recover the essential nutrients and bioactive compounds with functional properties (Kim and Wijesekara, 2010). Compared with intact protein, protein hydrolysates (PHs) obtained by limited enzymatic hydrolysis of by-products has improved functional and nutritional properties (Slizyte et al., 2005a,b), thus are considered as a high quality protein sources in aquafeeds. In exchange of fish meal, protein hydrolysate generally show a beneficial effect on growth performance and feed utilization at low inclusion levels, but decreased performance exceeding a specific dietary level (Refstie et

al., 2004; Hevrøy et al., 2005). Furthermore, PHs contains several low-molecular weight bioactive peptides with diverse health functions, including anti-oxidative, anti-microbial, and immunomodulatory activities (Kim and Mendis, 2006). So far, very few studies (Bøgwald et al., 1996; Gildberg et al., 1996) investigated the immunological potential of the PH products although it is widely known that they can act as immunostimulants that enhance the fish innate immune system and provide improved protection against pathogens (Kotzamanis et al., 2007; Bui et al., 2014). However, to the best of our knowledge, no studies have been performed to examine the immune function of olive flounder fed high plant protein diets supplemented with PHs. Therefore, current study was designed to evaluate the possible impact of dietary supplementation of three different types of PHs in high plant protein diets, and to compare the efficiency of the diets with a conventional FM based diet in juvenile olive flounder.

Material and methods

Experimental diets and design

The three PH sources tested in this study were provided by AQUATIV Company (Aquaculture Division of DIANA Group), Elven, France. Briefly, whole Antarctic krill (*Euphausia superba*) was enzymatically hydrolyzed and defatted to produce krill hydrolysate (KH). Farmed white shrimp (*Litopenaeus vannamei*) head co-products were enzymatically hydrolyzed and spray-dried to produce shrimp hydrolysate (SH). Farmed tilapia co-products containing heads, bones, trims and viscera were enzymatically processed and spray-dried to produce tilapia hydrolysate (TH). Proximate composition and peptide profile of the protein hydrolysates have been provided in Tables 1 & 2, respectively. Peptide profile of the hydrolysates was determined using size exclusion chromatography method (Guérard et al., 2001).

Five experimental diets were formulated to be isonitrogenous (500 g kg^{-1} crude protein) and isocaloric (18 MJ kg^{-1}) (Table 3). A basal FM-based diet was regarded as a high FM diet (HFM) and a diet containing soy protein concentrate (SPC), as a substitute for FM, at replacement level of 50% was considered as low FM diet (LFM). Three other experimental diets were prepared by partial replacement of FM with either SH, TH or KH in low FM diet (designated as SH, TH and KH, respectively). Doses of hydrolysates, expressed on a dry matter basis, were fixed to supplement equivalent levels of crude protein. All dry ingredients were thoroughly mixed and after addition of squid liver oil, soybean oil and double-distilled water pelleted through a meat chopper machine (SMC-12; Kuposlice, Busan, Korea) in 3 mm diameter. Then the diets were freeze-dried at -40 °C for 24 h, crushed into desirable particle sizes, and stored at -24 °C until used.

Fish and feeding trial

The feeding trial was conducted at the Marine and Environmental Research Institute of the Jeju National University (Jeju, South Korea). Juvenile red sea breams were transported from a private hatchery (Jeju, South Korea). The health status of the fish was checked upon arrival, and the fish were immediately treated with 100 mg L^{-1} formalin for 20 min. All the fish were fed a commercial diet for 2 weeks to be acclimated to the experimental conditions and facilities. At the end of the acclimation period, the fish (initial mean body weight, 15.1 ±

0.1 g) were randomly distributed into fifteen polyvinyl circular tanks of 150 L capacity at a density of 35 fish per tank and 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 hand-fed with one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 11 weeks. The average water temperature during the feeding trial ranged from 16 to 28 °C according to the seasonal change. The photoperiod was maintained on a 12:12 h (light/dark) schedule. The uneaten feed was collected and weighed to determine the feed intake. The insides of the tanks were routinely cleaned by a sponge to prevent the growth of microflora. Growth of fish was measured with 3-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish.

Sample collection and analyses

At the end of the feeding trial, all fish in each tank were counted and bulk-weighed for calculation of growth parameters and survival. Three intact fish per tank were randomly selected and kept at -24 °C for whole-body proximate composition analyses. Three fish per tank (nine fish per dietary treatment) were randomly captured, anaesthetized with 2-phenoxyethanol (200 mg L^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for the determination of hematocrit and hemoglobin concentrations, and respiratory burst activity. After the above-mentioned measurements with whole blood, plasma was separated by centrifugation at 5000 × *g* for 10 min using a high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) and stored at -70 °C for the determination of total immunoglobulin (Ig) level and blood biochemical parameters including glucose, total protein, triglyceride, total cholesterol, aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Another set of blood samples (three fish per tank, nine fish per dietary treatment) was taken 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 non-specific immune responses including lysozyme, myeloperoxidase (MPO), antiprotease, superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities.

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

Analyses of moisture and ash contents of diets and whole-body samples were performed by the standard procedures (AOAC, 1995). Crude protein was measured by using an automatic Kjeltec Analyzer Unit 2300 (Foss Tecator, Höganäs, Sweden), and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

Monitoring of non-specific immune responses

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Sigma, St. Louis, MO, 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. One milliliter of dimethylformamide (Sigma) was

added and centrifuged at 2000 × *g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as the blank.

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

A turbidometric assay was used for the determination of serum lysozyme level by the method described by Hultmark et al. (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg mL^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), and then, 200 µL of suspension was placed in each well of 96-well plates, and 20 µL serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340; Biochrom, Cambridge, UK). Hen egg white lysozyme (Sigma) was used for the standard curve. Values are expressed as µg mL^{-1}.

MPO activity was measured according to Quade and Roth (1997). Briefly, serum (20 µL) was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma) in 96-well plates. Then, 35 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 5 mM H_2O_2 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 the microplate reader.

Antiprotease activity was measured according to the method described by Ellis (1990), with some modifications (Magnadóttir et al., 1999). Briefly, 20 µL of serum was incubated with 20 µL of standard trypsin solution (Type Π-S, from porcine pancreas, 5 mg mL^{-1}, Sigma) for 10 min at 22 °C. Then, 200 µL of phosphate buffer (0.1 M, pH 7.0) and 250 µL azocasein (2%; Sigma) were added and incubated for 1 h at 22 °C. Five-hundred microlitres of 10% trichloro acetic acid (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-wellflat-bottomed microplate containing 100 µL of 1N NaOH. Optical density was read at 430 nm. For a 100% positive control, buffer replaced the serum, while for the negative control, buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

Trypsin inhibition % = $(A_1 – A_2 / A_1) \times 100$

where A_1= control trypsin activity (without serum); A_2= trypsin activity remained after adding serum.

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 colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

GPx activity was measured using a GPx Assay Kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and NADPH (b-nicotinamide adenine denucleotide phosphate, reduced) were included in the reaction mixture. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to $NADP^+$. The change in 340 nm due to NADPH oxidation was monitored for GPX activity. Briefly, 50 µl of serum was added to the reaction mixture and incubated for 15 min and then 10 µL of cumene hydroperoxide was added. The optical density of NADPH was measured at 340 nm and 25 °C, and the rate of the reaction was estimated from the absorbance readings in the first 5 min after adding cumene hydroperoxide. Specific activity was expressed as GPx mU ml^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25 °C.

Digestibility test

For estimation of apparent digestibility coefficient of the experimental diets, chromic oxide (Cr_2O_3) (Sigma) was included in the diets as an inert indicator at a concentration of 10 g kg^{-1} diet. The digestibility trial was conducted in another water circulation system equipped with four fiberglass tanks of 300 L capacity, designed according to Cho et al. (1982). Thirty olive flounder with mean body weight of 80 g were stocked into each tank, and each group of fish was fed one of the test diets. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. The digestibility trial consisted of three periods of 10 days. In each 10-day period, the fish were allowed to become acclimatized to the feed for the first 3 days, and feces were collected over the next 7 days. Then, diets were randomly changed between tanks, and the procedure repeated for two more times, giving a total of three fecal samples for each diet. All feces collected from each tank in each period were pooled and frozen at -20 °C until analysis. After feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed waste and fecal residues. Chromium oxide content of diet and feces samples were analyzed by the method described by Divakaran et al. (2002). The apparent digestibility coefficients of dry matter, protein and lipid of the experimental diets were calculated through the following formula:

$ADC = 1 - ((Cr_2O_3 \text{ in feed} / Cr_2O_3 \text{ in feces}) \times (\text{nutrient content of feces} / \text{nutrient content of feed}))$.

Challenge trial

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly selected and subjected to a bacterial challenge. *Edwardsiellia tarda* was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The bacterium, originally isolated from diseased olive flounder, was cultured in 10 mL BHI broth (Difco, Detroit, MI, USA) with 1.5% NaCl and incubated with shaking for 24 h at 37 °C. Bacterial growth was measured at an optical density of 700 nm followed by plate counting in BHI-NaCl. The isolated bacteria were identified using the API 20E commercial identification kit (bioMérieux, Marcy l'Etoile, France). The fish were injected intraperitoneally with *E. tarda* suspension containing 1×10^3 CFU mL^{-1}. The pathogenic dose

of bacterium had previously been determined in a preliminary test using fish of a similar size. After injection, the fish were distributed into fifteen 65 L plastic tanks, and their mortality was monitored and recorded for 17 days.

Intestinal histology

Three individual fish from each tank (9 per treatment) were euthanized with an overdose of 2-phenoxyethanol. Subsequently, the body cavity was opened and the entire gastrointestinal tract was dissected for histological examination of the anterior intestine for light microscopical analysis (Hur et al., 2013). The fragments of anterior intestine were fixed in Bouin's solution, dehydrated in graded ethanol concentrations and embedded in paraffin according to routine procedures. For each specimen, multiple sets of cross-sections (5 µm thick) were stained with Alcian blue (AB) and periodic acid-Schiff (PAS).

The villus height, enterocyte height and the number of goblet cells in the epithelium (defined as the region between the lamina propria and the microvilli brush border), across the whole cross-sectional epithelial area, were monitored and, then, calculated by averaging the values from all specimens.

These measures were made using the Image J 1.32j image analysis software (National Institutes of Health, USA). Images were obtained with Olympus CKX41 (Tokyo, Japan) microscopy equipped with a JUJAK 5.5 digital camera (DIXI Optics, Daejeon, South Korea) and eXcope V 5.0.1 software. Results were expressed as mean values with their standard deviation.

Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range tests. Statistical significance was determined at $P < 0.05$. Data are presented as mean± SD. Percentage data were arcsine transformed before analysis.

Results

All the experimental diets were readily accepted by the juvenile olive flounder at the start of the feeding trial and they fed aggressively during the 11 weeks of the feeding trial. Growth performance and feed utilization of the fish fed the hydrolysate supplemented diets were significantly improved compared to those of fish fed the LFM diet and even better results were achieved in comparison with the corresponding values in HFM group (Table 4). In fact, dietary inclusion of the KH to the LFM diet resulted in significant enhancement of protein efficiency ratio (PER) while fish fed the TH and KH diets showed the lowest feed conversion ratio compared to those of fish fed the LFM diet. Also, significantly higher feed intake (FI) values were observed in fish fed the HFM and hydrolysate supplemented diets compared to those of fish fed the LFM diet, however no significant differences were found among the hydrolysate treated groups. Fish survival rate was not significantly affected by dietary treatments.

Dietary inclusion of the PHs significantly improved the apparent digestibility of the diet dry matter and protein and makes it comparable to the corresponding values in HFM group (Table 5).

Fish fed the KH diet showed significantly lower ALT level than the LFM group. However, other fish hematological parameters were not significantly influenced by dietary treatments (Table 6).

No significant differences were found in whole body proximate composition of fish fed the experimental diets (Table 7).

Dietary inclusion of the tested PHs significantly improved the morphological indices of the fish anterior intestine in terms of villus height (Vh), enterocyte height (Eh) and number of goblet cells (GC) compared to those of the LFM group (Table 8).

Fish fed the TH and KH diets indicated significantly higher NBT activity than the groups of fish fed the LFM diet. Significantly higher Ig levels were found in groups of fish fed the SH and KH diets compared to those of fish fed the LFM diet. Dietary inclusion of the PHs resulted in significantly higher lysozyme activity compared to LFM group. Dietary supplementation of the SH to the experimental diet significantly enhanced the SOD activity compared to the HFM and LFM groups. Even though, numerically higher MPO, antiprotease and GPx activities were observed in fish fed the hydrolysate supplemented diets, the differences were not significant (Table 9).

During the challenge test, the first dramatic mortality was observed on the third day after injection where the fish fed the LFM diet showed the lowest disease resistance compared to all of the other groups (Fig. 1). At the end of the challenge test, significantly higher survival rate was found in KH group compared to the HFM and LFM groups, however, no significant difference was found among hydrolysate treated groups.

Discussion

Results of the current study revealed that SPC could replace up to 50% of FM protein when supplemented with PHs in olive flounder diets. Nevertheless, growth and feed utilization efficiency of juvenile flounder fed SPC-based diet without hydrolysate supplementation resulted in significantly reduced growth performance, lower FI and decreased feed efficiency. There is convincing evidence that decreased diet palatability is associated with a reduction in FI, which could in turn cause poor growth performance. Hence, the addition of the potential attractants and/or palatability enhancers to diets containing high plant protein is often recommended in achieving balanced nutritional composition, complementing amino acid profiles and, subsequently, counteracting the depressed FI (Kikuchi, 1999; Choi et al., 2004; Deng et al., 2006). Thus, it can be considered that the PHs used in the current study were act as natural feed attractants and improved the diet palatability. It is, however, interesting to note that the current replacement level is far higher than those values cited in previous studies for this species (Kikuchi, 1999; Choi et al., 2004;

Uyan et al., 2006; Kader et al., 2012a). An early study by Kikuchi, 1999 indicated that dietary inclusion of the defatted soybean meal (SBM) in combination with blood meal or corn gluten meal and blue mussel meat as substitutes for fish meal up to 45%, provides both good growth and feed utilization, by increasing the feed intake of juvenile flounder. Choi et al. (2004) found that dehulled SBM could replace up to 30% of FM from the diet of same species with amino acid and/or attractant supplementation. In a recent study, Kader et al. (2012) reported that 36% of FM protein could be substituted by fermented SBM and squid by-product blend without any negative effects on growth performance and health/welfare of fish. Indeed, improved palatability is often used to explain observed enhances in FI and growth performance whenever PHs is exchanged with fish meal (Refstie et al., 2004; Hevrøy et al., 2005) associated with the presence of the small molecular weight compounds including free amino acids and free nucleotide (Aksnes et al., 2006). In addition, it has been suggested that the higher availabilities of several amino acids in the hydrolysate containing diets could result in more efficient utilization of the protein (Refstie et al., 2004). This is in line with the present results where the apparent digestibility of hydrolysate supplemented diets was higher than LFM diet in terms of dry matter and protein.

Blood parameters are being increasingly used to assess the physiological changes and health condition of fish due to the effects of nutrient. The results of the present study demonstrate that supplementation of the tested hydrolysate products in high SPC-based diets had no significant effect on the heamatolgical indices of fish. Yet, these values are within a normal range reported for this species (Kikuchi, 1999; Kader et al, 2012a; Khosravi et al., 2015). Likewise, Kader et al. (2012a) found that dietary replacement of FM with fermented SBM and squid by-product blend did not alter most of the plasma metabolites, while some of the health parameters were improved in the replacement groups. Moreover, fish fed the KH diet in the current study showed significantly lower ALT activity compared to those fed the LFM group, even though no significant difference was found among all the other dietary groups. It is well known that increases in AST and ALT activities indicate hepatocellular injury caused by various chemicals and lipid peroxidation (Hyder et al., 2013). Song et al. (2014) suggested that the significant reduction in serum AST and ALT activities in juvenile starry flounders (*Platichthys stellatus*) in response to moderate soy protein hydrolysate inclusion replacing FM protein, demonstrating a protective mechanism of this hydrolysate product to the liver. However, the reason for the decreasing activity of ALT in the present experiment is not fully understood.

Partial FM replacement by SPC, in the current study, is indeed feasible as it had no significant effect on the non-specific immune response and health status of fish. Similarly, Bransden et al. (2001) found that partial substitution of FM by dehulled lupin meal had no adverse effect on different indicators of the innate immunity in Atlantic salmon (*Salmo salar* L.). Conversely, several papers have reported that several cellular and humoral components of the innate immune response were enhanced in fish fed the plant protein based diets, which have been considered as inflammatory or immunostimulating effects (Krogdahl et al., 2000; Sitja-Bobadilla et al., 2005). Exceeding a specific level, however, may cause immunosuppression (Sitjà-Bobadilla et al., 2005; Lin and Luo, 2011). Hence, the suitability of the replacement of FM by variety of plant protein sources in terms of immune response has

resulted to be highly variable among fish species and experimental conditions. Moreover, the results of the current study revealed that dietary inclusion of the tested PHs, at the expense of FM, significantly increased the various indicators of the cellular and humoral innate immunity, which is believed to be of great importance before the mounting of a specific immune response in fish (Dalmo et al., 1997). In exchange of FM, fish PHs generally show a potential beneficial effect on fish immune response and disease resistance at low inclusion levels (Liang et al., 2006; Tang et al, 2008; Bui et al., 2014; Khosravi et al., 2015). These beneficial health effects has been attributed to the presence of numerous known peptide sequences exhibiting, e.g., antimicrobial, antioxidative and immunomodulatory activities (Korhonen and Pihlanto, 2003; Kim and Wijesekara, 2010; Harnedy and Fitzgerald, 2012) which is related to their inherent amino acid composition and sequence (Korhonen and Pihlanto, 2006).

In the present study, the serum respiratory burst activity of fish fed with TH and KH diets significantly increased compare to that of the LFM group. Also, low inclusion levels of fish protein hydrolysates have been shown to induce phagocytic index of Japanese sea bass (*Lateolabrax japonicus*) (Liang et al., 2006). In fish, the respiratory burst, which generates reactive oxygen species, is well characterized in macrophages and neutrophils (Secombes and Fletcher, 1992). Phagocytes produce large quantities of superoxide anion (O^-_2), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH^-), during phagocytosis or upon stimulation which is believed to be important in defence against pathogens (Anderson and Siwicki, 1995). Indeed, both in vivo and in vitro studies have shown that low molecular weight peptide fractions from fish PHs stimulate oxidative burst reactions (e.g., superoxide anion production) in Atlantic salmon head kidney leucocytes (Bogwald et al., 1996; Gildberg et al., 1996). Furthermore, it has also been reported that positively-charged low molecular weight soy peptides are effective in stimulating immunomodulating activity against lymphocyte proliferation and macrophage phagocytosis (Yoshikawa and Takahashi, 1993; Kong et al., 2008). Accordingly, this may partly explain the higher serum lysozyme activity in the hydrolysate treated fish, as monocytes/macrophages and neutrophils are considered to be the main sources of lysozyme (Torsteinsdottir et al., 1999; Fischer et al., 2006). Lysozyme is a bacteriolytic enzyme killing bacteria by hydrolyzation of the peptidoglycan of bacterial cell walls. Moreover, besides an antibacterial function, it promotes phagocytosis by directly activating polymorphonuclear leucocytes and macrophages or indirectly by an opsoninic effect (Saurabh and Sahoo, 2008). Song et al. (2014) have shown that partial replacement of fish meal protein ($\leq$ 85%) by soy protein hydrolysate in diet for juvenile starry flounder significantly elevated serum lysozyme activity. This was also evident in our present study where supplementation of KH and TH in FM-based diets significantly enhanced the serum lysozyme activity of juvenile olive flounder compared to those fed the control diet. However, this trend was not supported by the findings on juvenile coho salmon (Murray et al., 2003) and turbot (*Scophthalmus maximus* L.) (Zheng et al., 2013).

There is evidence that the production of immunoglobulins also has a critical role in forming complexes with pathogens causing opsonization or cellular lysis (Alexander and Ingram, 1992). In this study, dietary inclusion of the SH and KH in SPC-based diets resulted in significant increase in plasma Ig content that were similar to those observed in our

previous experiment (Khosravi et al., 2015). Tang et al. (2008) also showed a significant increase in serum IgM level when large yellow croaker (*Pseudosciaena crocea* R.) were fed a diet supplemented with 10 or 15% fish PH compared with fish fed basal diet.

Superoxide dismutase (SOD), catalase and glutathione system are considered as principal components of antioxidant enzymatic defense. SOD is an oxidoreductase that accelerates the conversion of O^{-}_{2} to H_2O_2 and O_2 and thereby negates the direct toxic effects of the reactive oxygen species (Fridovich, 1989). In the present study, serum SOD activity in the hydrolysate-fed groups was higher than those of the HFM and LFM groups, and significant difference was observed when the fish were offered SH diet. These observations agreed with our previous findings in respect to the beneficial properties of fish PH for promoting SOD activity in juvenile olive flounder fed the FM-based diets (Khosravi et al., 2015). In the same way, Song et al. (2014) found that diets containing soy protein hydrolysates significantly elevated serum SOD activity in fish. Several studies have demonstrated that bioactive peptides derived from different PHs act as potential antioxidants by upregulating antioxidant enzyme activities (Young et al., 2010), sequestering metal-ion catalysts (Wang and Xiong, 2005), or scavenging free radicals and reactive oxygen species (Kim and Wijesekara, 2010). It can therefore be suggested that dietary inclusion of the tested PHs, in the current study, may play a role in the antioxidant defense status in fish sera by enhancing the SOD activity to maintain the superoxide anion level which is expected to increase bactericidal capacity of phagocytes. This is further supported by the fact that fish fed the SH diet showed significantly higher SOD activity, as it contains much higher level of low molecular weight peptides (< 500) than two other PHs. Moure et al. (2006) found that peptide fractions with low molecular weight presented the highest antioxidant activity compared to the fractions with higher molecular weight.

It is well documented that nutritional manipulation is an effective way to modulate innate immune response, accompanied with improved disease resistance (Magnadottir, 2010; Oliva-Teles, 2012). Thus, the increments observed in the non-specific immune response in the current study may correlated with the enhance resistance to *E. tarda* infection. However, even though significantly higher survival rate was recorded in fish fed the KH diet than those of the HFM and LFM groups; there were no significant differences among all the other experimental groups. This finding is in line with our previous results with the same species showing no significant effect on fish disease resistance whenever offered the similar PHs compared to those fed the control diet. Furthermore, Liang et al. (2006) also indicated that although dietary supplementation of fish PH in practical diets for Japanese sea bass could enhance the innate immunity indices in the fish, no significant differences were found on fish disease resistance following challenge with *Vibrio anguillarum*. It has been suggested that PHs origin as well as the molecular weight of their peptide profiles play a key role in their impact on fish performance (Liang et al., 2006) with lower molecular weight fractions possessing more efficient activities than larger peptides and proteins (Carvalho et al., 2004; Kim, 2013).

In summary, these findings may offer convincing evidence that 50% of FM protein could be substituted by SPC and PHs blend without any adverse impact on juvenile olive flounder growth performance and health/welfare condition, when compared to the fish fed the

high FM diet. Dietary inclusion of the tested PHs, in exchange for FM, also improved the innate immune responses of fish which is most probably responsible for the enhanced disease resistance of fish against bacterial infection caused by *E. tarda*, which was depressed in fish fed the LFM diet. In addition, these results suggest that KH, apparently, is the most effective dietary supplement for this species than two other tested PHs. However, further studies are needed to clarity the highest possible replacement level of FM by SPC and PHs blend in juvenile olive flounder diet, as well as its consequences on fish health status fed the SPC-based diets in long-term feeding trials.

Table 3.1. Proximate compositions of marine protein hydrolysates (from product technical data sheets).

	KH	SH	TH
Dry matter (%)	56.1	96.0	95.0
Protein (% DM)	44.1	64.9	71.2
Lipid (% DM)	2.3	10.8	15.4
Ash (% DM)	10.5	10.3	4.8
Soluble Nitrogen (% N)	96.3	91.1	91.5
Essential Amino Acids (% products)			
Arg	2.94	4.15	4.06
His	0.50	1.55	1.36
Ile	1.39	2.71	2.45
Leu	2.32	4.28	4.38
Lys	2.85	4.13	4.23
Met	0.87	1.26	1.58
Phe	1.25	2.95	2.42
Thr	1.24	2.62	2.77
Val	1.57	3.34	2.94

KH, Krill Hydrolysate; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate

Table 3.2. The molecular weight of krill hydrolysate (KH), shrimp hydrolysate (SH) and tilapia hydrolysate (TH) (%).

Molecular weight (Da)	KH	SH	TH
>30000	<0.1	<0.1	<0.1
20000-30000	<0.1	<0.1	<0.1
10000-20000	<0.1	<0.1	1.0
5000-10000	2.0	<0.5	2.0
1000-5000	18	4	27
500-1000	16	5	18
<500	64	90	52

KH, Krill Hydrolysate; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate

Table 3.3. Formulation and proximate composition of the experimental diets for red sea bream (g kg^{-1}).

Ingredients	Experimental diets				
	HFM	LFM	SH	TH	KH
White fishmeal	550	275	242	241	241
SH	0	0	33.4	0	0
TH	0	0	0	28.8	0
KH	0	0	0	0	31.2
Soy protein concentrate	50	220	220	220	220
Corn gluten meal	50	100	100	100	100
Wheat flour	235	238	237	242	240
Squid liver oil	40	60	60	60	60
Soybean oil	40	40	40	40	40
Mineral Mix[a]	10	10	10	10	10
Vitamin Mix[b]	10	10	10	10	10
CMC[c]	10	10	10	10	10
Choline chloride	5	5	5	5	5
L-lysine	0	10	10	10	10
L-methionine	0	3	3	3	3
Taurine	0	5	5	5	5
Di-calcium phosphate	0	15	15	15	15
Chromic oxide	10	10	10	10	10
Proximate composition (g kg^{-1} in dry matter)					
Dry matter	910	899	896	899	913
Crude protein	510	508	510	507	510
Crude lipid	142	138	132	135	139
Ash	97	71	70	67	72

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

[a] Mineral premix (g kg^{-1} of 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, 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

[b] Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α 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

[c] CMC, Carboxy Methyl Cellulose

Table 3.4. Growth performance, feed utilization and somatic parameters of olive flounder fed the five experimental diets for 11 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
FBW[1] (g)	121±0.6[a]	99±6.8[b]	117±6.1[a]	120±3.4[a]	127±8.1[a]
WG[2] (%)	699±7.3[a]	552±43.3[b]	673±43.4[a]	693±21.8[a]	742±50.6[a]
SGR[3] (%)	2.89±0.01[a]	2.60±0.09[b]	2.84±0.08[a]	2.88±0.04[a]	2.96±0.08[a]
FI[4] (g fish^{-1})	113±1.2[a]	96±3.6[c]	107±1.0[b]	107±1.8[b]	111±1.2[ab]
FCR[5]	1.18±0.03[ab]	1.27±0.06[a]	1.14±0.05[ab]	1.11±0.05[b]	1.07±0.06[b]
PER[6]	1.66±0.04[ab]	1.56±0.08[b]	1.73±0.08[ab]	1.78±0.08[ab]	1.83±0.10[a]
Survival (%)	83.9±3.2	89.2±1.9	91.4±6.7	92.5±4.9	91.4±4.9

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

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] Final Mean Body Weight (g)

[2] Weight Gain (%) = [(final body weight − initial body weight) / initial body weight × 100]

[3] SGR (%) = [(ln final body weight − ln initial body weight) / days] × 100

[4] Feed Intake (g fish^{-1}) = dry feed consumed / fish number

[5] Feed Conversion Ratio = dry feed fed / wet weight gain

[6] Protein Efficiency ratio = wet weight gain / total protein given

Table 3.5. Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for olive flounder.

	Experimental diets				
	HFM	LFM	SH	TH	KH
ADCd (%)[1]	81.2±0.8[a]	71.8±3.6[b]	77.5±5.6[ab]	81.9±2.7[a]	82.7±0.8[a]
ADCp (%)[2]	94.4±0.5[a]	88.0±1.5[b]	93.2±1.5[a]	94.7±0.8[a]	94.2±0.4[a]

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

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

Table 3.6. Hematological parameters of olive flounder fed the five experimental diets for 11 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
Hematocrit (%)	32.2±3.3	30.8±1.7	29.3±2.08	29.6±2.3	30.4±2.2
Hemoglobin (g dL^{-1})	5.59±0.13	5.36±0.09	5.07±0.37	5.26±0.45	5.67±0.61
Glucose (mg dL^{-1})	28.9±3.3	29.3±5.1	26.8±3.4	26.4±2.7	28.3±2.4
Triglyceride (mg dL^{-1})	378±21	399±44	346±59	379±53	381±48
Total protein (g dL^{-1})	4.02±0.44	4.02±0.12	4.04±0.12	4.29±0.67	4.30±0.47
Total cholesterol (mg dL^{-1})	307±55	302±35	262±48	296±60	284±19
AST (U L^{-1})[1]	32.0±1.7	35.2±6.7	30.9±7.1	33.2±5.1	28.8±5.9
ALT (U L^{-1})[2]	6.24±0.38^{ab}	7.00±0.38^{a}	6.63±0.20^{ab}	6.36±0.39^{ab}	5.87±0.26^{b}

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

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] Aspartate aminotransferase activity

[2] Alanine aminotransferase activity

Table 3.7. Whole-body proximate composition of olive flounder fed the five experimental diets for 11 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
Moisture	70.5±0.4	71.1±0.2	71.1±0.3	70.7±0.7	71.2±0.4
Protein	18.8±0.2	18.7±0.1	18.8±0.4	18.6±0.2	18.7±0.1
Lipid	6.43±0.20	5.58±0.26	6.36±0.29	6.39±0.40	6.06±0.13
Ash	3.45±0.00	3.41±0.07	3.34±0.19	3.28±0.21	3.28±0.05

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

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

Table 3.8. Morphometric parameters of olive flounder intestine fed the five experimental diets for 11 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
GC[1]	1324±176[a]	935±127[b]	1235±115[a]	1223±186[a]	1392±111[a]
Vh[2]	505±45[a]	398±33[b]	508±28[a]	502±40[a]	502±12[a]
Eh[3]	39.1±1.8[a]	30.4±2.5[b]	36.4±0.8[a]	35.8±0.4[a]	36.3±0.6[a]

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate

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

[2]Villi height (μm)

[3]Enterocyte height (μm)

Table 3.9. Non-specific immune parameters of olive flounder fed the five experimental diets for 11 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
NBT[1]	0.71±0.05^{ab}	0.69±0.02^{b}	0.75±0.02^{ab}	0.80±0.05^{a}	0.80±0.03^{a}
Ig[2]	21.7±1.1^{ab}	18.5±0.8^{b}	23.2±0.5^{a}	21.9±1.6^{ab}	24.6±2.6^{a}
Lysozyme[3]	24.5±2.2^{ab}	21.2±1.6^{b}	28.1±1.2^{a}	26.4±1.3^{a}	27.3±1.1^{a}
MPO[4]	1.95±0.30	1.98±0.11	2.08±0.07	2.08±0.22	2.26±0.21
Antiprotease[5]	32.0±2.4	32.4±2.5	33.4±3.6	33.3±1.9	35.8±0.3
SOD[6]	67.5±3.3^{b}	67.7±1.7^{b}	78.7±3.7^{a}	75.2±2.7^{ab}	72.1±3.6^{ab}
GPx[7]	16.7±0.4	14.6±0.6	15.4±0.6	16.8±0.8	15.8±1.6

HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate powder; TH, Tilapia Hydrolysate powder; KH, Krill Hydrolysate powder

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

[3]Lysozyme activity (μg mL^{-1})

[4]Myeloperoxidase level

[5]Antiprotease (% inhibition)

[6]Superoxide dismutase (% inhibition)

[7]Glutathione peroxidase activity (mU mL^{-1})

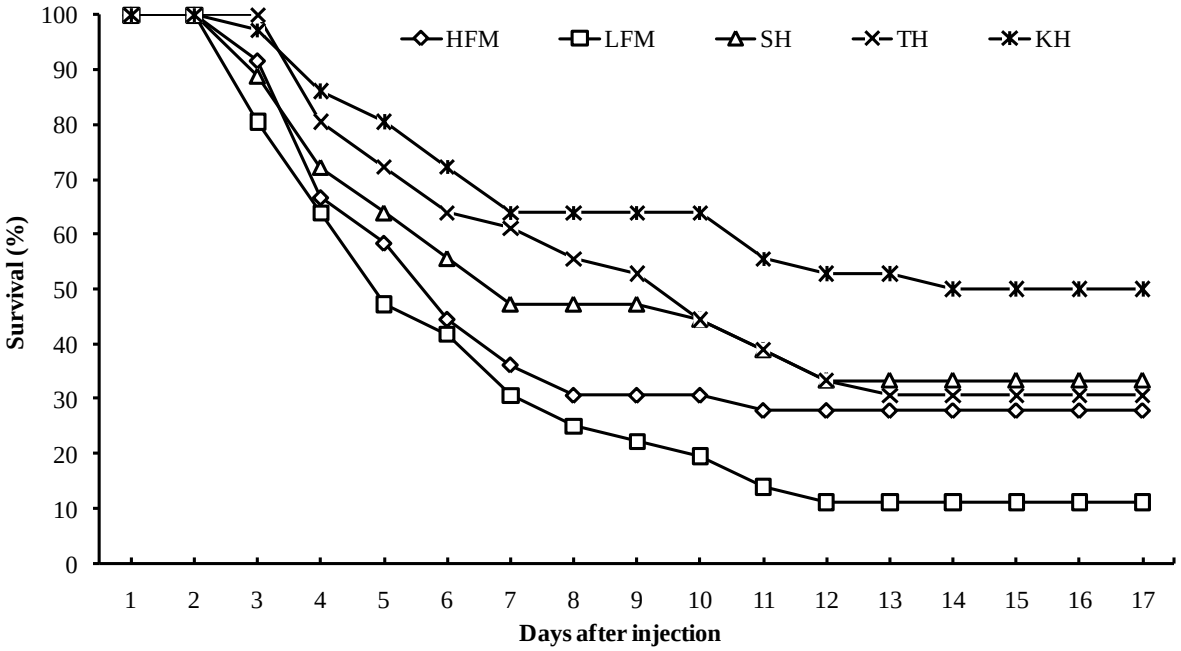


Figure 1. Survival rate of olive flounder after challenge with *E. tarda*. HFM, High Fish Meal; LFM, Low Fish Meal; SH, Shrimp Hydrolysate; TH, Tilapia Hydrolysate; KH, Krill Hydrolysate.

CHAPTER 4

Effects of Protein Hydrolysate Supplementation in Low Fish Meal Diets on Growth Performance, Innate Immunity and Disease Resistance of Red Sea Bream *Pagrus major*

Effects of Protein Hydrolysate Supplementation in Low Fish Meal Diets on Growth Performance, Innate Immunity and Disease Resistance of Red Sea Bream *Pagrus major*

Abstract

This study was conducted to evaluate the supplemental effects of three different types of protein hydrolysates in a low fish meal (FM) diet on growth performance, feed utilization, intestinal morphology, innate immunity and disease resistance of juvenile red sea bream. A FM-based diet was used as a high fish meal diet (HFM) and a low fish meal (LFM) diet was prepared by replacing 50% of FM by soy protein concentrate. Three other diets were prepared by supplementing shrimp, tilapia or krill hydrolysate to the LFM diet (designated as SH, TH and KH, respectively). Triplicate groups of fish (4.9 ± 0.1 g) were fed one of the test diets to apparent satiation twice daily for 13 weeks and then challenged by *Edwarsellia tarda*. At the end of the feeding trial, significantly ($P < 0.05$) higher growth performance was obtained in fish fed HFM and hydrolysate treated groups compared to those fed the LFM diet. Significant improvements in feed conversion and protein efficiency ratios were obtained in fish fed the hydrolysates compared to those fed the LFM diet. Significant enhancement in digestibility of protein was found in fish fed SH and KH diets and dry matter digestibility was increased in the group fed SH diet in comparison to LFM group. Fish fed the LFM diet showed significantly higher glucose level than all the other treatments. Whole-body and dorsal muscle compositions were not significantly influenced by dietary treatments. Histological analysis revealed significant reductions in goblet cell numbers and enterocyte length in the proximal intestine of fish fed the LFM diet. Superoxide dismutase activity and total immunoglobulin level were significantly increased in fish fed the diets containing protein hydrolysates compared to the LFM group. Also, significantly higher lysozyme and antiprotease activities were found in fish fed the hydrolysates and HFM diets compared to those offered LFM diet. Fish fed the LFM diet exhibited the lowest disease resistance against *E. tarda* and dietary inclusion of the hydrolysates resulted in significant enhancement of survival rate. The results of the current study indicated that the inclusion of the tested protein hydrolysates, particularly SH, in a LFM diet can improve growth performance, feed utilization, digestibility, innate immunity and disease resistance of juvenile red sea bream.

Introduction

Red sea bream is one of the main marine finfish species cultured in Korea and Japan. The demand for red sea bream has grown tremendously for the last decade since it is a high-quality sashimi grade fish with high market value. According to the ministry of maritime affairs and fisheries of the Korea, total amount of 2,755 tons red sea bream was produced in 2013. However, red sea bream is a strictly carnivorous fish which is generally fed with practical or commercially formulated feeds with high FM content (Huang et al., 2007; Kader et al., 2012a). This may, itself, place increasing demands on global supply of fish meal and the associated rise in its price. Therefore, it is not surprising that the issues pertaining to economical alternative sources of protein are always a hot topic in fish nutrition field (Hardy, 2004; Hardy, 2010) for promoting a sustainable aquaculture industry.

Several cost-effective alternative protein sources have been investigated as substitutes for FM in aquafeeds (Gatlin et al., 2007; Tacon and Metian, 2008). Despite some promising results, utilization of conventional plant protein sources in carnivorous fish feeds face a number of challenge/limitations due to their undesirable characteristics such as imbalanced amino acid profile and antinutritional factors (ANFs) that can affect growth performance and health status of fish (Francis et al., 2001; Gatlin et al., 2007). Among these plant proteins, soy products are of major interest as protein source in fish feed primarily for their low cost, stable supply and generally good nutritional and economical value to FM (Storebakken et al., 2000; Drew et al., 2007). In fact, there are several ways to overcome some of the major limitations with regard to the use of soy products in aquafeeds. Soy protein concentrate (SPC) is one of the diverse products obtained during the processing of mature soybeans which is believed to reduce its ANFs and improve its functional and nutritional properties (Drew et al., 2007). However, it has been found that the substitution of FM with SPC in diets for marine carnivorous finfish such as red sea bream, particularly at high inclusion level, most likely require essential amino acid supplementation as well as other essential nutrients in to the diet in order to support normal growth performance and physiological condition in fish (Takagi et al., 2001; Takagi et al., 2006; Takagi et al., 2011). Meanwhile, research efforts are underway to identify an appropriate blend of plant products and other alternative feed ingredients that prevent nutritional deficiencies and ensure a proper supply of essential nutrients. This may further increase the replacement level of FM without detrimental effect on fish performance by restoring a proper balance of amino acid and increasing the palatability of the diet (Kader et al., 2012a,b).

By development of the modern food processing technologies, interest has been directed towards the potentially marketable raw materials derived from fisheries by catch and seafood processing leftovers which have long been discarded as waste or processed into low market value products (Hsu, 2010). Furthermore, conversion and dietary utilization of these nutrient-rich feed stuffs in aquafeed can prevent environmental pollution and excessive expenses for their disposal. In view of utilizing these nutrient rich by-products, enzymatic hydrolysis biotechniques have been developed to improve the functional and nutritional properties of the byproducts through the conversion of native proteins into smaller biologically active peptides (Chalamaiah et al., 2012).

Crustacean protein hydrolysates have long been used in aquafeeds as potential protein source (Plascencia-Jatomea et al., 2002) or as dietary supplements in small amounts for improvement of diet palatability (Kolkovski et al., 2000). Processing of shrimp for human consumption has led to the production of a huge amount of by-products accounting for 50–70% of the processed materials. These waste materials contain valuable components such as protein, chitin and astaxanthin (Bataille and Bataille, 1983; Shahidi and Synowiecki, 1991; Tacon et al., 2006) making them as potential ingredients for aquafeeds. Recovery of the shrimp wastes by enzymatic hydrolysis results in the formation of biologically active peptides with pharmaceutical and growth-stimulating properties (Gildberg and Stenberg, 2001). Krill is regarded as one of the most promising marine protein sources and has been examined as a potential dietary ingredient in aquafeeds (Storebakken, 1988; Gildberg and Stenberg, 2001; Tacon et al., 2006). It is also a rich source of high-quality protein compared with the other

animal protein sources due to its low-fat content and being rich in omega-3 fatty acids (Nicol et al., 2000; Tou et al., 2007). In the same way, tilapia processing industry generates a huge quantity of by-products with high nutritional and functional value. Strong antioxidant activity has been reported for tilapia hydrolysate (Fan et al., 2012; Zhang et al., 2012). Such biological activities could find application in the development of fish feed designed for higher performances.

It has previously been demonstrated that a combination of soybean protein and marine by-products can be used to successfully replace FM in diets for juvenile red sea bream (Kader et al., 2010; 2011; 2012). Therefore, the aim of the present study was to evaluate the supplemental effects of three different types of protein hydrolysates including krill, shrimp and tilapia hydrolysates in low FM diets, and to compare their efficiency with that of a high FM diet for juvenile red sea bream.

Material and methods

Experimental diets and design

Three protein hydrolysate sources tested in this study were provided by AQUATIV Company (Aquaculture Division of DIANA, Member of SYMRISE Group), Elven, France. Briefly, whole Antarctic krill (*Euphausia superba*) was enzymatically hydrolyzed and defatted to produce krill hydrolysate concentrate. Farmed white shrimp (*Litopenaeus vannamei*) head co-products were enzymatically hydrolyzed and spray-dried to produce shrimp hydrolysate powder. Farmed tilapia co-products containing heads, bones, trims and viscera were enzymatically processed, defatted and spray-dried to produce tilapia hydrolysate powder. Proximate composition and peptide profile of the protein hydrolysates have been provided in Tables 1 and 2, respectively. Peptide profiles of the hydrolysates were determined using size exclusion chromatography method (Guerard et al., 2001).

Five experimental diets were formulated to be isonitrogenous (500 g kg^{-1} crude protein) and isocaloric (18 MJ kg^{-1}) (Table 3). A basal FM-based diet was regarded as a high fish meal diet (HFM) and a diet containing soy protein concentrate, as a substitute for FM, at replacement level of 50% was considered as low FM diet (LFM). Three other experimental diets were prepared by partial replacement of FM with shrimp, tilapia or krill hydrolysate in low FM diet (designated as SH, TH and KH, respectively). Doses of the three hydrolysates were fixed to supplement equivalent levels of crude protein on a dry matter basis. All dry ingredients were thoroughly mixed and, after addition of squid liver oil, soybean oil and double-distilled water, were pelleted through a meat chopper machine (SMC-12; Kuposlice, Busan, Korea) in 3 mm diameter. Then the diets were freeze-dried at -40 °C for 24 h, crushed into desirable particle sizes, and stored at -20 °C until used.

Fish and feeding trial

The feeding trial was conducted at the Marine and Environmental Research Institute of the Jeju National University (Jeju, South Korea). Juvenile red sea breams were transported from a private hatchery (Jeju, South Korea). The health status of the fish was checked upon arrival, and the fish were immediately treated with 100 mg L^{-1} formalin for 20 min. Fish were fed a commercial diet for 2 weeks to be acclimated to the experimental conditions and facilities. At the end of the acclimation period, the fish (initial mean body weight, 4.92 ± 0.1

g) were randomly distributed into fifteen polyvinyl circular tanks of 150 L capacity at a density of 32 fish per tank and 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 hand-fed with one of the test diets to apparent satiation (twice a day, 09:00 and 17:00 h) for 13 weeks. The average water temperature during the feeding trial was 23.4 ± 4.1 °C. The photoperiod was maintained on a 12:12 h (light/dark) schedule. The uneaten feed was collected and weighed to determine the feed intake level. The insides of the tanks were routinely cleaned by a sponge to prevent the growth of microflora. Growth of fish was measured with 3-week intervals. Feeding was stopped 24 h prior to weighing or blood sampling to minimize handling stress on fish.

Sample collection and analyses

At the beginning of the experiment, 20 fish were sampled and stored at -20 °C for analysis of initial whole-body and muscle proximate composition. At the end of the feeding trial, all fish in each tank were counted and individually weighed for calculation of growth parameters and survival rate. Body length of fish from each tank was measured to the nearest 0.1 mm. Three intact fish per tank were randomly selected and kept at -20 °C for whole-body proximate composition analyses. Epaxial sample of white muscle were taken from three other fish per tank and kept at -70 °C for proximate analyses. Viscera and liver were separately dissected out from these fish and weighted for the determination of viscerosomatic index (VSI) and hepatosomatic index (HSI), respectively. Three fish per tank (nine fish per dietary treatment) were randomly captured, anaesthetized with 2-phenoxyethanol (200 mg L^{-1}), and blood samples were collected from the caudal vein with heparinized syringes for the determination of hematocrit and hemoglobin levels, and respiratory burst activity. After the above-mentioned measurements with whole blood, plasma was separated by centrifugation at 5000 × *g* for 10 min using a high-speed refrigerated microcentrifuge (Micro 17 TR; HanilBioMed Inc., Gwangju, Korea) and stored at -70 °C for the determination of total immunoglobulin (Ig) level and blood biochemical parameters including glucose, total protein, total cholesterol, triglyceride, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). Another set of blood samples (three fish per tank, nine fish per dietary treatment) was taken 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 non-specific immune responses and antioxidant enzyme activities including lysozyme, myeloperoxidase (MPO), antiprotease, superoxide dismutase (SOD), and glutathione peroxidase (GPx) activities.

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

Proximate composition of diet, whole-body and muscle samples was analyzed according to standard methods (AOAC, 1995). Crude protein was measured by using an automatic Kjeltec Analyzer Unit 2300 (Foss Tecator, Höganäs, Sweden). Crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6,

Korea). Moisture was determined by oven drying at 105 °C for 6 h and ash content was determined after combustion at 600 °C for 4 h in a muffle furnace.

Non-specific immune parameters and antioxidant enzyme activities

Oxidative radical production by phagocytes during respiratory burst was measured through the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995) with some modifications. Briefly, blood and 0.2% NBT (Sigma, St. Louis, MO, 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. One milliliter of dimethylformamide (Sigma) was added and centrifuged at 2000 × *g* for 5 min. Finally, the optical density of supernatant was measured at 540 nm using a spectrophotometer (Genesys 10UV, Rochester, NY, USA). Dimethylformamide was used as the blank.

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1994). Briefly, plasma total protein content was measured using a microprotein determination method (C-690; Sigma), prior to and after precipitating down the Ig molecules, using a 12% solution of polyethylene glycol (Sigma). The difference in protein content represents the Ig content.

A turbidimetric assay was used for the determination of serum lysozyme level by the method described by Hultmark et al. (1980) with slight modifications. Briefly, *Micrococcus lysodeikticus* (0.75 mg mL^{-1}) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), and then, 200 µL of suspension was placed in each well of 96-well plates, and 20 µL serum was added subsequently. The reduction in absorbance of the samples was recorded at 570 nm after incubation at room temperature for 0 and 30 min in a microplate reader (UVM 340; Biochrom, Cambridge, UK). Hen egg white lysozyme (Sigma) was used for the standard curve. Values are expressed as µg mL^{-1}.

MPO activity was measured according to Quade and Roth (1997). Briefly, serum (20 µL) was diluted with HBSS (Hanks Balanced Salt Solution) without Ca^{2+} or Mg^{2+} (Sigma) in 96-well plates. Then, 35 µL of 20 mM 3,3′,5,5′-tetramethylbenzidine hydrochloride (TMB; Sigma) and 5 mM H_2O_2 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 the microplate reader.

Antiprotease activity was measured according to the method described by Ellis (1990), with some modifications (Magnadottir et al., 1999). Briefly, 20 µL of serum was incubated with 20 µL of standard trypsin solution (Type Π-S, from porcine pancreas, 5 mg mL^{-1}, Sigma) for 10 min at 22 °C. Then, 200 µL of phosphate buffer (0.1 M, pH 7.0) and 250 µL azocasein (2%; Sigma) were added and incubated for 1 h at 22 °C. Five-hundred microlitres of 10% trichloro acetic acid (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 1 N NaOH. Optical density was read at 430 nm. For a 100% positive control, buffer replaced the serum, while for the negative control, buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

Trypsin inhibition % = $(A_1 – A_2 / A_1) \times 100$

where A_1= control trypsin activity (without serum); A_2= trypsin activity remained after adding serum.

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 colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent inhibition was normalized by mg protein and presented as SOD activity units.

GPx activity was measured using a GPx Assay Kit (Biovision, Inc., Milpitas, CA, USA) according to the manufacturer's instructions. In this assay, cumene hydroperoxide was used as a peroxide substrate (ROOH), and glutathione reductase (GSSG-R) and NADPH (b-nicotinamide adenine denucleotide phosphate, reduced) were included in the reaction mixture. GPx catalyses the oxidation of glutathione by cumene hydroperoxide. In the presence of glutathione reductase and NADPH, the oxidized form of glutathione is immediately converted to the reduced form with the concomitant oxidation of NADPH to $NADP^+$. The change in 340 nm due to NADPH oxidation was monitored for GPX activity. Briefly, 50 µL of serum was added to the reaction mixture and incubated for 15 min and then 10 µL of cumene hydroperoxide was added. The optical density of NADPH was measured at 340 nm and 25 °C, and the rate of the reaction was estimated from the absorbance readings in the first 5 min after adding cumene hydroperoxide. Specific activity was expressed as GPx mU mL^{-1}. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25 °C.

Digestibility test

For estimation of apparent digestibility coefficient of the experimental diets, chromic oxide (Cr_2O_3) (Sigma) was included in the diets as an inert indicator at a concentration of 10 g kg^{-1}diet. The digestibility trial was conducted in a fecal collection system equipped with five fiberglass tanks of 300 L capacity, designed according to Cho et al. (1982). New sets of red sea bream with mean body weight of 70 g were stocked into each tank at a density of 40 fish per tank, and each group of fish was fed one of the test diets. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. The digestibility trial consisted of three periods of 10 days. In each 10-day period, the fish were allowed to become acclimatized to the feed for the first 3 days, and feces were collected over the next 7 days. Then, diets were randomly changed between tanks, and the procedure repeated for two more times, giving a total of three fecal samples for each diet. All feces collected from each tank in each period were pooled and frozen at -20 °C until analysis. After feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed waste and fecal residues. Chromium oxide content of diet and feces samples were analyzed by the method described by Divakaran et al. (2002). The apparent digestibility coefficients of dry matter, protein and lipid of the experimental diets were calculated through the following formula:

ADC = 1 – ((Cr_2O_3 in feed / Cr_2O_3 in feces) × (nutrient content of feces / nutrient content of feed)).

Challenge trial

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly selected and subjected to a bacterial challenge. *Edwardsiellia tarda* was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The bacterium, originally isolated from diseased olive flounder, was cultured in 10 mL BHI broth (Difco, Detroit, MI, USA) with 1.5% NaCl and incubated with shaking for 24 h at 37 °C. Bacterial growth was measured at an optical density of 700 nm followed by plate counting in BHI-NaCl. The isolated bacteria were identified using the API 20E commercial identification kit (bioMérieux, Marcy l'Etoile, France). The fish were injected intraperitoneally with *E. tarda* suspension containing 1×10^4 CFU mL^{-1}. The pathogenic dose of bacterium had previously been determined in a preliminary test using fish of a similar size. After injection, the fish were distributed into fifteen 65 L plastic tanks, and their mortality was monitored and recorded for 23 days.

Intestinal histology

Two individual fish from each tank (6 per treatment) were euthanized with an overdose of 2-phenoxyethanol. Subsequently, the body cavity was opened and the entire gastrointestinal tract was dissected for histological examination of the anterior intestine for light microscopical analysis (Hur et al., 2013). The fragments of anterior intestine were fixed in Bouin's solution, dehydrated in graded ethanol concentrations and embedded in paraffin according to routine procedures. For each specimen, multiple sets of cross-sections (5 µm thick) were stained with Alcian blue (AB) and periodic acid-Schiff (PAS).

The intestinal diameter, villus height, enterocyte height and the number of goblet cells in the epithelium (defined as the region between the lamina propria and the microvilli brush border), across the whole cross-sectional epithelial area, were monitored and, then, calculated by averaging the values from all specimens.

These measures were made using the Image J 1.32j image analysis software (National Institutes of Health, USA). Images were obtained with Olympus CKX41 (Tokyo, Japan) microscopy equipped with a JUJAK 5.5 digital camera (DIXI Optics, Daejeon, South Korea) and eXcope V 5.0.1 software. Results were expressed as mean values with their standard deviation.

Statistical analysis

All dietary treatments were assigned by a completely randomized design. Data were subjected to one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the difference in means was made with Tukey's HSD multiple range test. Statistical significance was determined at $P < 0.05$. Data are presented as mean ± SD. Percentage data were arcsine transformed before analysis.

Results

All the experimental diets were readily accepted by the juvenile red sea bream at the start of the feeding trial and they fed aggressively during the 13 weeks of the feeding trial. The results of growth performance, feed utilization and organosomatic indices are provided in Table 4. The lowest growth performance was obtained in fish fed LFM diet which significantly ($P < 0.05$) differed from that of the other experimental groups. Dietary inclusion

of the hydrolysate sources in LFM diets resulted in significant improvement of growth performance and made it comparable to that of HFM group, even higher growth rate was found in fish fed SH containing diet. Significantly higher protein efficiency ratio and lower feed conversion ratio were obtained in fish fed the hydrolysate supplemented diets compared with those of fish fed the LFM diet. However, fish survival rate and organosomatic indices including condition factor, HSI and VSI did not significantly differ among dietary treatments ($P > 0.05$).

Fish fed the SH diet showed significantly higher dry matter digestibility than the LFM group ($P < 0.05$) (Table 5). Dietary inclusion of SH and KH significantly improved apparent digestibility coefficient of protein and comparable values to that of HFM were obtained. Significantly higher lipid digestibility was found in fish fed HFM diet than that of fish fed the LFM diet and dietary supplementation of the protein hydrolysates improved dietary lipid digestibility, however, the differences were not significant among treatments.

Plasma glucose level was significantly increased in fish fed LFM diet compared to the other experimental groups (Table 6). In addition, replacement of FM by SPC led to significant reduction of plasma total cholesterol concentration ($P < 0.05$). The rest of the hematological parameters were not significantly influenced by dietary treatments ($P > 0.05$).

Proximate compositions of whole-body and muscle are shown in Table 7. The results revealed no significant differences among dietary treatments.

Dietary treatments had no significant effects on morphological structure of anterior intestine in terms of villus height (Vh) and intestinal diameter (Id) (Table 8). However, significantly higher number of goblet cells was found in fish fed the hydrolysate and HFM diets compared to that of fish fed LFM diet. Also, fish fed the HFM diet showed significantly higher enterocyte height than those fed LFM or TH diet.

Non-specific immune responses and antioxidant enzyme activities were positively affected by dietary protein hydrolysate supplementation (Table 9). Significant enhancement in SOD activity was observed in all hydrolysates fed groups in comparison to LFM group. The groups fed HFM, TH and KH diets exhibited significantly higher Ig level than fish fed the LFM diet. Also, lysozyme and antiprotease activities were significantly increased in fish fed HFM and hydrolysates containing diets compared to fish fed the LFM diet. Although, numerically higher NBT, MPO and GPx activities were detected in the hydrolysate groups than LFM group, there were no significant differences among treatments.

During the *E. tarda* challenge, the first dramatic mortality was observed on the eighteenth day after injection in fish fed the LFM diet compared to all the other experimental groups (Fig. 1). At the end of 23 days of the challenge test, the LFM group had the lowest survival rate (44.4%) and significantly differed from that of the other groups. Also, hydrolysate groups exhibited numerically higher disease resistance than HFM group.

Discussion

It is generally expected that the use of plant proteins in exchange of marine protein sources in diets for carnivorous fish species adversely affects the fish growth performance and feed utilization. Therefore, it is difficult to sustain growth and feed efficiency in carnivorous fish with high levels of plant feed ingredients as they are different from marine protein sources in nutrient content which may not meet the dietary requirements (Kader et al., 2012a). Thus, this fact may explain the observed inferior results in terms of growth performance and feed utilization in LFM group compared to all the other experimental groups in the current study. Yet, it doesn't seem to be in accordance with the improved performance of fish fed LFM diets containing protein hydrolysates, in the present study, as these experimental diets were formulated with comparatively higher replacement levels of FM protein. It has been demonstrated that supplementation of essential nutrients or blending of individual plant protein with other ingredients from plant and/or animal sources, specially marine by-products, in red sea bream diet had a beneficial effect in dealing with a number of nutritional deficiencies (Kader et al., 2010; 2012a). Interestingly, the results of the current study indicated that the growth performance and feed utilization of juvenile red sea bream can be remarkably improved by dietary inclusion of the tested protein hydrolysates in LFM diets and even better performance can be achieved by SH supplementation compared to the use of FM-based diet. Similarly, Kader et al. (2010) showed that supplementing feed additives including krill meal, fish soluble and squid meal in high SPC based diets can recover the depleted performance of juvenile red sea bream. Several authors have reported that dietary supplementation of protein hydrolysates at low inclusion levels has positive impact on growth performance and health condition of fish (Aksnes et al., 2006a,b; Liang et al., 2006; Kotzamanis et al., 2007; Zheng et al., 2012). These observations are also in agreement with previous studies in Atlantic cod (*Gadus morhua*) (Aksnes et al., 2006a) and rainbow trout (*Oncorhynchus mykiss*) (Aksnes et al., 2006b) fed high plant protein diets supplemented with different level of hydrolysate. Enhanced growth performance by the inclusion of the tested protein hydrolysates in the current study might be explained by the presence of certain bioactive peptides derived from hydrolysis processes which most likely are essential for promoting the biological performance of fish (Aksnes et al., 2006a,b). In addition, a tendency to enhanced feed intake was found in the SH and KH groups, made it comparable to the HFM group. Thereby, it appears that the LFM diets in the current study were able to provide sufficient nutrient and energy to meet the requirements of fish for normal growth and health in the presence of protein hydrolysates. This is also supported by the fact that somatic indices of red sea bream did not show any detrimental effect and neither was any sign of green live syndrome among dietary treatments.

Our results clearly indicated that dietary supplementation of the protein hydrolysates can improve dry matter and protein digestibility in high plant protein diets. Similar results were observed in rainbow trout (Aksnes et al., 2006b). Such enhancement in ADC of nutrients by the hydrolysate supplementation can be due to their better absorption (Aksnes et al., 2006b; Hevroy et al., 2005), as the enzymatic hydrolysis improves the functional properties of the native proteins (Chalamaiah et al., 2012). Nevertheless, inclusion of the tested protein hydrolysates, in the current study, has been unable to overcome the adverse effects of soy protein on lipid digestibility. It has been suggested that the alcohol-soluble

components in soy products are responsible for their detrimental effects, disturbing the normal digestion/absorption process of dietary ingested fat that results in poor lipid digestibility (Refstie et al., 2001; Romarheim et al., 2006; Deng et al., 2010).

Hematological parameters are useful indicators for evaluating the physiological and health status of fish (Maita et al., 2007). The results of the present study showed increased glucose level in fish fed the LFM diet while supplementation of the protein hydrolysates reduced its concentration. This finding is supported by Kader and Koshio (2012) who reported higher plasma glucose level in red sea bream fed a low or non FM diet. However, previous studies found no significant changes in the plasma glucose level of red sea bream fed high SPC based diets (Takagi et al., 2001; Kader et al., 2010). This discrepancy can be attributed to various factors including fish size, experimental conditions and handling methods, as they can strongly affect fish physiological condition (Maita et al., 2007; Chatzifotis et al., 2010). Moreover, the significant reduction of plasma cholesterol concentration in the present study may provide further evidence for impaired lipid assimilation (malabsorption) caused by dietary inclusion of SPC. This is in agreement with previous studies on red sea bream where significantly lower cholesterol levels were found following administration of SPC based diets (Takagi et al., 2001; Kader et al., 2010). The hypocholesterolemic effect of soy products has been attributed to increased faecal excretion of bile acids, increased apolipoprotein B/E receptor activity and decreased hepatic lipoprotein secretion which is suggested to be due to the imbalanced amino acid profile or presence of certain non-starch polysaccharides (Dias et al., 2005; Romarheim et al., 2006). The other measured hematological parameters in this study were not affected by the dietary treatments, and the values were within the reported ranges for juvenile red sea bream (Takagi et al., 2001; Kader et al., 2010; 2011; 2012a).

It has been demonstrated that fish proximate composition is affected by several endogenous (e.g., fish size and life cycle stage) and exogenous (e.g., nutritional and environmental condition) factors (Shearer, 1994; Cook et al., 2000; Chatzifotis et al., 2010). There are several reports indicating that replacing FM with soy protein significantly affects red sea bream whole-body and filet composition (Takagi et al., 2001; Kader et al., 2010; 2011; 2012a). In fact, significant effects were found in the body composition of juvenile red sea bream fed SPC based diets while no such differences were observed in larger yearling red sea bream fed with the same diets (Takagi et al., 2006). Therefore, it is likely that unaltered chemical composition observed in the current study could be a result of the relatively larger size of red sea bream compared to those of the earlier studies (Takagi et al., 2001; Kader et al., 2010; 2011; 2012a).

The histological results of the current study showed that dietary inclusion of SPC affects the structural integrity of the anterior intestine in juvenile red sea bream. Histological aspects of the gastrointestinal (GI) tract are considered to be of great importance for fish nutrition studies, as it is the major site for nutrient absorption. Morphological alterations in the GI tract have been detected by several studies in response to dietary inclusion of soy bean meal (Refstie et al., 2000; Krogdahl et al., 2003; Bonaldo et al., 2006; 2008). It has also been demonstrated that the severity of the adverse effects strongly depends on fish size and species, nutritional value of the processed plant protein and experimental condition

(Storebakken et al., 2000; Refstie et al., 2001; Bonaldo et al., 2006; Overland et al., 2009). The possible negative impacts on structural and functional properties of fish GI tract caused by plant protein based diets have been attributed to the presence of antinutritional factors [7], affecting the efficiency of feed utilization through the induction of structural abnormalities and/or interference with the activity of mucosal enzymes (Krogdahl et al., 2003; Martinez-Llorens et al., 2012). It is believed that the factors responsible for soy-induced enteritis (Uran et al., 2009) are absent in the final products of soy processing industry, e.g. SPC, as SPC-fed fish did not develop the pathological signs (Refstie et al., 2001; Escaffre et al., 2007). This notion was further supported by the results of this study where no remarkable pathological changes were detected in the anterior intestine of red sea bream except for the decreased Eh in LFM and TH groups. This also accords with an earlier report in rainbow trout indicating that total replacement of FM by SPC may not provoke gut inflammatory reaction at the macromorphological level (Escaffre et al., 2007). These authors suggested that the significant reduction in Eh of SPC fed fish may result from altered physiological and metabolic status in response to dietary SPC.

Goblet cells synthesize and secrete bioactive molecules, generally known as mucus, and are assumed to be involved in protection or digestion processes in the alimentary tract, according to the type of their mucin content (Marchetti et al., 2006; Cerezuela et al., 2013). In the present study, significant reduction in the number of GC were found in LFM fed fish and hydrolysates supplementation led to enhancement of GC number. This observation is in agreement with earlier studies where dietary inclusion of plant proteins resulted in reduced number of GC (Nogales-Merida et al., 2010; Martinez-Llorens et al., 2012).

The results of the present study indicated that dietary supplementation of the protein hydrolysates in low FM diets caused an improvement in the non-specific immune response and antioxidant enzyme activities of red sea bream. It has been demonstrated that dietary inclusion of the protein hydrolysates in FM-based diets can stimulates the innate immunity of fish (Bogwald et al., 1996; Gildberg et al., 1996; Liang et al., 2006; Tang et al., 2008; Kotzamanis et al., 2007; Bui et al., 2014; Khosravi et al., 2015). Significant enhancement of SOD activity by protein hydrolysates in the present study provides further evidence for earlier in vitro studies where superoxide anion production in Atlantic salmon leucocytes was stimulated by medium-size peptides from fish protein hydrolysate (Bogwald et al., 1996; Gildberg et al., 1996). Lysozyme is a mucolytic enzyme of leucocytic origin, and is considered as an important marker of the humoral innate immune response (Saurabh and Sahoo, 2008). Liang et al. (2006) showed that inclusion of 15% fish protein hydrolysate in diets for sea bass enhances the lysozyme activity. A similar tendency was observed in the current study and dietary inclusion of the protein hydrolysate in low FM diets augmented the lysozyme activity. Total Ig is considered as a reliable immunological marker of the non-specific immune system. In accordance with our previous study on red sea bream (Bui et al., 2014), dietary protein hydrolysates enhanced plasma total Ig level. Protease inhibitors are involved in defense mechanisms by providing protection against invading pathogens that secrete proteolytic enzymes for facilitated nutrient availability and intrusion into the host body cells (Magnadottir et al., 2006). Our results indicated that dietary inclusion of the protein hydrolysates can promote the serum antiprotease activity. However, these findings are

in contrast with the results of previous studies which did not show any positive effect of hydrolysate supplementation on innate immune functions of juvenile coho salmon (*Oncorhynchus kisutch*) (Murray et al.,. 2003) and juvenile turbot (*Scophthalmus maximus* L.) (Zheng et al., 2012). Moreover, it is assumed that the level of inclusion in the diet can be of great importance for promoting an immunostimulatory effect in fish (Murray et al., 2003) . Beneficial effects of hydrolysates are mainly attributed to their bio-active peptide content that exert anti-oxidative, antimicrobial and immunomodulatory activities (Korhonen et al., 2006; He et al., 2013). Indeed, Robert et al. (2014a,b) reported the existence of a number of bio-active peptides with antibacterial properties isolated from shrimp and tilapia hydrolysates used in the current study. However, to date, the physiological relevance of distinct bio-active peptides and their contribution to promoting fish immune response is not well understood. Therefore, further studies are required to ascertain the contributing factors responsible for the elevated humoral innate immune response following hydrolysate administration.

It is well-documented that the nutritional status of fish influences its innate immune system which is considered as an essential component in combating infections thereby modulating the resistance to disease (Kiron, 2012). There are substantial evidences that inclusion of plant proteins in diets for carnivorous fish may cause or trigger immune dysfunction leading, subsequently, to higher susceptibility to bacterial infections (Maita et al., 1998; 2006; 2007). Results of the present study agree with those of previous studies where lower disease resistance was observed in fish fed SPC-based diets compared to the use of FM-based diets (Maita et al., 1998; 2006). It has been suggested that the incidence of hypocholesterolemia (Maita et al., 2006) and/or anemia (Maita et al., 1998), caused by taurine deficiency, are the most important pathophysiological changes that may correspond with reduced disease resistance in fish fed SPC-based diets (Goto et al., 2001). The results of this study provide more evidence for this notion as LFM fed fish exhibited the lowest hematocrit value and plasma total cholesterol concentration than other experimental groups. Significant improvements in fish disease resistance were observed by the supplementation of hydrolysate sources to the LFM diet, and even higher survival rates were obtained compared to the HFM fed fish. Similarly, the results of an earlier research conducted in our laboratory (Bui et al., 2014) showed the improvement in red sea bream resistance against *E. tarda* when they were fed FM-based diets supplemented with protein hydrolysates. Also, Kotzamanis et al. (2007) reported the significant enhancement in resistance of European sea bass larvae (*Dicentrarchus labrax*) to *Vibrio anguillarum* challenge following dietary administration of fish protein hydrolysates. However, supplementation of tuna viscera protein hydrolysate in diets for Persian sturgeon (*Acipenser persicus* L.) failed to enhance disease resistance against *Aeromona hydrophila* (Ovissipour et al., 2014). The increased disease resistance of red sea bream by the hydrolysates supplementation in this study can be related to the significant enhancement of innate immune responses including lysozyme and antiprotease activities and total Ig level. The differences in the effect of hydrolysates on fish disease resistance can be attributed to the variation of bioactive peptide profile of hydrolyzed protein depending on raw material, enzyme specifications, hydrolysis conditions, and dietary inclusion level of hydrolysates, experimental period and species-specific differences (Klompong et al., 2009).

However, more investigations on this topic are needed to determine the causal link between the protein hydrolysate utilization and fish disease resistance.

In conclusion, the findings in this study showed that inclusion of the tested protein hydrolysates in low FM diets for red sea bream improves the feed nutritional value resulting in enhanced growth performance, non-specific immune response and disease resistance. Also, it is suggested that SPC could replace up to 50% of the FM by the supplementation of the tested protein hydrolysates, particularly SH. However, further studies are required for determination of optimal inclusion level of the examined hydrolysate sources.

Table 4.1. Proximate compositions of krill, shrimp and tilapia hydrolysates (from product technical data sheets).

	Krill hydrolysate	Shrimp hydrolysate	Tilapia hydrolysate
Dry matter (%)	56.1	96.0	95.0
Protein (% DM)	44.1	64.9	71.2
Lipid (% DM)	2.3	10.8	15.4
Ash (% DM)	10.5	10.3	4.8
Soluble Nitrogen (% N)	96.3	91.1	91.5
Essential Amino Acids (% products)			
Arg	2.94	4.15	4.06
His	0.50	1.55	1.36
Ile	1.39	2.71	2.45
Leu	2.32	4.28	4.38
Lys	2.85	4.13	4.23
Met	0.87	1.26	1.58
Phe	1.25	2.95	2.42
Thr	1.24	2.62	2.77
Val	1.57	3.34	2.94

Table 4.2. The molecular weight of krill, shrimp and tilapia hydrolysates (%).

Molecular weight (Da)	Krill hydrolysate	Shrimp hydrolysate	Tilapia hydrolysate
>30000	<0.1	<0.1	<0.1
20000-30000	<0.1	<0.1	<0.1
10000-20000	<0.1	<0.1	1.0
5000-10000	2.0	<0.5	2.0
1000-5000	18	4	27
500-1000	16	5	18
<500	64	90	52

Table 4.3. Formulation and proximate composition of the experimental diets for red sea bream (g kg^{-1}).

Ingredients	Experimental diets				
	HFM	LFM	SH	TH	KH
White fishmeal[a]	500	250	216	216	215
Shrimp hydrolysate	0	0	33.4	0	0
Tilapia hidrolysate	0	0	0	28.8	0
Krill hidrolysate	0	0	0	0	31.2
Soy protein concentrate[b]	70	240	240	235	233
Corn gluten meal	60	100	100	100	100
Wheat flour	255	244	244	253	254
Squid liver oil	40	60	60	60	60
Soybean oil	40	40	40	40	40
Mineral Mix	10	10	10	10	10
Vitamin Mix	10	10	10	10	10
CMC[e]	10	10	10	10	10
Choline chloride	5	5	5	5	5
L-lysine	0	8	9	9	9
L-methionine	0	3	3	3	3
Taurine	0	5	5	5	5
Di-calcium phosphate	0	15	15	15	15
Chromic oxide	10	10	10	10	10
Proximate composition (g kg^{-1} in dry matter)					
Dry matter	905	908	901	889	881
Crude protein	509	511	506	509	502
Crude lipid	137	134	139	135	137
Ash	90	68	67	64	64

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

[a] Suhyup Feed Co. Ltd., Uiryeong, Korea (crude protein: 65%, crude lipid: 8.6%)

[b] Soy protein concentrate, Soycomil R., Koog aan de Zaan, Netherlands (crude protein: 80%, crude lipid: 3.4%)

[c] Mineral premix (g kg^{-1} of 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, 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

[d] Vitamin premix (g kg^{-1} of mixture): L-ascorbic acid, 121.2; DL-α 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

[e] CMC, Carboxy methyl cellulose

Table 4.4. Growth performance, feed utilization and somatic parameters of red sea bream fed the five experimental diets for 13 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
FBW[1] (g)	66.7±4.4[b]	59.1±1.3[a]	68.0±1.0[b]	65.3±1.9[ab]	66.7±2.3[b]
WG[2] (%)	1259±56[b]	1104±24[a]	1290±40[b]	1234±45[b]	1239±20[b]
SGR[3] (% /day)	3.03±0.05[b]	2.89±0.02[a]	3.06±0.03[b]	3.01±0.04[b]	3.02±0.02[b]
FI[4] (g fish^{-1})	79.5±2.7	75.0±1.2	76.3±4.7	74.6±4.6	75.9±2.8
FCR[5]	1.27±0.06[ab]	1.36±0.01[b]	1.19±0.05[a]	1.23±0.04[a]	1.20±0.05[a]
PER[6]	1.71±0.08[ab]	1.59±0.01[a]	1.84±0.07[bcd]	1.80±0.06[bcd]	1.88±0.08[cd]
CF[7] (%)	2.12±0.10	2.30±0.10	2.26±0.16	2.13±0.10	2.07±0.17
HSI[8] (%)	0.97±0.12	1.03±0.33	1.10±0.17	0.98±0.23	0.96±0.16
VSI[9] (%)	6.44±0.31	5.84±0.20	5.79±0.38	5.38±1.08	5.65±0.56
Survival (%)	95.8±1.8	93.8±3.1	94.8±4.8	97.9±3.6	92.7±3.6

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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] Final mean body weight (g)

[2] Weight gain (%) = [(final body weight − initial body weight) / initial body weight × 100]

[3] SGR (% /day) = [(ln final body weight − ln initial body weight) / days] × 100

[4] Feed intake (g fish^{-1}) = dry feed consumed / fish number

[5] Feed conversion ratio = dry feed fed / wet weight gain

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

[7] Condition factor (%) = weight of fish / (length of fish)^3 × 100

[8] Hepatosomatic index (%) = weight of liver / weight of fish × 100

[9] Viscerasomatic index (%) = weight of viscera / weight of fish × 100

Table 4.5. Apparent digestibility coefficients (%, ADC) for dry matter, protein and lipid of the experimental diets for red sea bream.

	Experimental diets				
	HFM	LFM	SH	TH	KH
ADCd (%)[1]	82.7±1.2^{ab}	78.6±3.6^{a}	85.1±2.1^{b}	81.1±0.4^{ab}	80.5±3.0^{ab}
ADCp (%)[2]	91.8±0.6^{c}	84.4±2.6^{a}	91.8±1.1^{c}	87.6±0.2^{ab}	88.5±1.8^{bc}
ADCl (%)[3]	87.6±0.1^{b}	82.6±1.5^{a}	86.5±2.1^{ab}	86.0±0.3^{ab}	86.0±2.3^{ab}

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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

[3] Apparent digestibility coefficient of lipid

Table 4.6. Hematological parameters of red sea bream fed the five experimental diets for 13 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
Hematocrit (%)	45.0±6.5	39.3±1.0	43.8±5.3	42.5±1.1	42.3±2.4
Hemoglobin (g dL^{-1})	6.95±0.56	6.56±0.13	7.32±0.53	6.78±0.45	6.89±0.30
Glucose (mg dL^{-1})	$38.3±0.8^{d}$	$42.2±0.7^{e}$	$34.6±0.8^{a}$	$36.3±0.3^{bc}$	$37.2±0.2^{cd}$
Triglyceride (mg dL^{-1})	291±50	359±57	323±36	330±61	307±58
Total protein (g dL^{-1})	4.15±0.44	3.89±0.15	4.22±0.36	4.29±0.46	4.14±0.55
Total cholesterol (mg dL^{-1})	$232±19^{b}$	$170±28^{a}$	$199±26^{ab}$	$201±24^{ab}$	$191±16^{ab}$
AST (U L^{-1})[1]	51.6±12.6	51.4±2.5	38.5±9.6	52.3±9.4	42.2±4.9
ALT (U L^{-1})[2]	15.9±1.1	26.4±7.7	19.1±5.3	19.2±4.4	14.9±3.3

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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] Aspartate aminotransferase activity

[2] Alanine aminotransferase activity

Table 4.7. Whole-body and muscle proximate composition of red sea bream fed the five experimental diets for 13 weeks.

	Experimental diets					
	Initial	HFM	LFM	SH	TH	KH
Whole-body (%)						
Moisture	72.9±0.4	65.3±0.6	66.2±0.9	64.7±0.7	65.7±0.7	65.9±0.7
Protein	16.3±0.7	18.4±0.1	18.4±0.7	18.8±0.5	18.7±0.6	17.9±0.3
Lipid	6.13±0.33	12.3±0.3	11.8±0.3	12.2±0.5	11.9±0.7	11.2±0.4
Ash	4.55±0.23	4.49±0.26	4.52±0.27	4.93±0.38	4.69±0.11	4.73±0.64
Muscle (%)						
Moisture	74.6±0.1	74.9±0.1	73.9±0.2	73.8±0.7	73.6±0.8	74.4±0.6
Protein	19.5±1.3	21.9±1.0	22.1±0.7	22.2±0.3	22.5±0.8	22.3±0.7
Lipid	2.85±0.25	1.86±0.41	1.83±0.29	2.07±0.27	2.30±0.55	1.49±0.14
Ash	1.55±0.08	1.60±0.01	1.50±0.16	1.48±0.06	1.54±0.05	1.56±0.03

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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

Table 4.8. Morphometric parameters of red sea bream intestine fed the five experimental diets for 13 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
GC[1]	938±46[b]	558±41[a]	918±78[b]	820±79[b]	879±49[b]
Id[2]	2020±75	1865±178	1990±105	1975±120	1981±58
Vh[3]	695±26	590±51	659±54	653±48	666±75
Eh[4]	47.1±4.1[b]	36.1±2.1[a]	41.6±2.5[ab]	38.2±3.8[a]	40.0±4.1[ab]

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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

[2]Intestinal diameter (µm)

[3]Villi height (µm)

[4]Enterocyte height (µm)

Table 4.9. Non-specific immune parameters and antioxidant enzymes activity of red seabream fed the five experimental diets for 13 weeks.

	Experimental diets				
	HFM	LFM	SH	TH	KH
NBT[1]	0.763±0.08	0.741±0.05	0.840±0.05	0.880±0.07	0.828±0.11
MPO[2]	1.47±0.31	1.44±0.28	1.53±0.20	1.70±0.09	1.56±0.23
SOD[3]	42.3 ± 3.5^{ab}	39.3 ± 1.0^{a}	48.2 ± 1.5^{bc}	53.6 ± 2.8^{c}	47.7 ± 2.9^{bc}
Ig[4]	31.1 ± 0.4^{bc}	28.4 ± 0.8^{a}	29.9 ± 0.9^{ab}	32.8 ± 0.7^{c}	30.7 ± 0.3^{b}
Lysozyme[5]	18.7 ± 0.5^{b}	14.2 ± 0.5^{a}	18.6 ± 1.2^{b}	21.8 ± 0.3^{c}	17.8 ± 0.6^{b}
Antiprotease[6]	70.9 ± 0.8^{b}	68.1 ± 0.4^{a}	70.6 ± 1.5^{b}	70.6 ± 0.4^{b}	70.9 ± 1.2^{b}
GPx[7]	174±17	165±9	181±16	169±2	176±10

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate

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 (% inhibition)

[7]Glutathione peroxidase activity (mU mL^{-1})

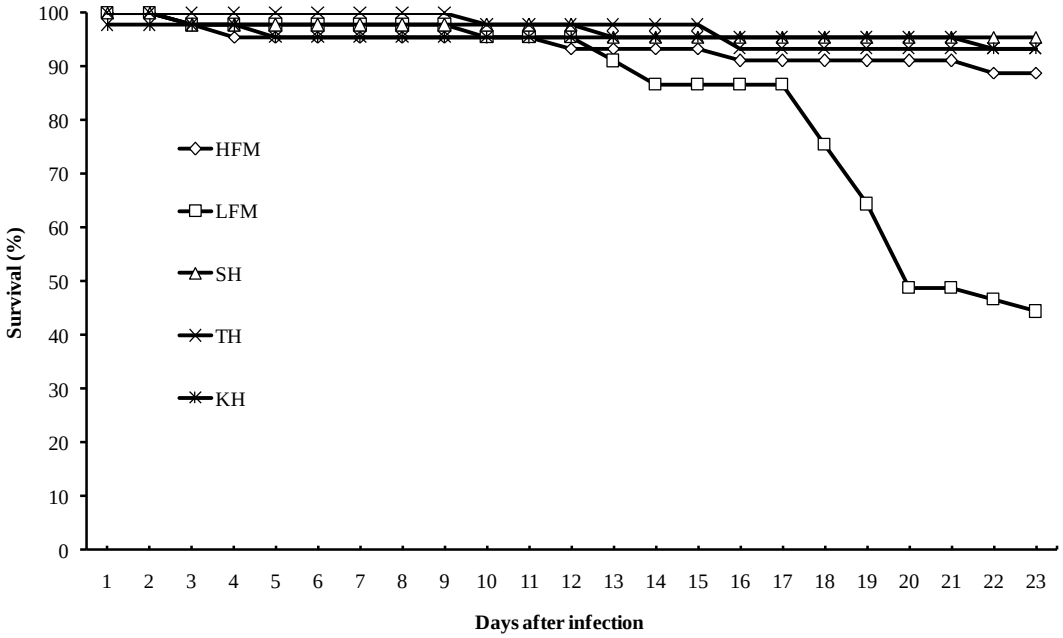


Figure 4.1. Survival rate of red sea bream after challenge with *E. tarda*. HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate; KH, Krill hydrolysate.

CHAPTER 5

Effects of Different Dietary Levels of Protein Hydrolysates on Growth, Feed Utilization, Intestinal Morphology, Innate Immunity and Resistance to *Edwardsiella tarda* in Juvenile Olive Flounder (*Paralichthys olivaceus*)

Effects of Different Dietary Levels of Protein Hydrolysates on Growth, Feed Utilization, Intestinal Morphology, Innate Immunity and Resistance to *Edwardsiella tarda* in Juvenile Olive Flounder (*Paralichthys olivaceus*)

Abstract

A feeding trial was conducted to evaluate the effect of protein hydrolysate at different inclusion levels of 1.5, 3.0 and 4.5%, on growth performance, feed utilization, diet digestibility, the expression levels of liver insulin-like growth factor I (IGF-I), intestinal morphology, innate immunity and disease resistance of juvenile olive flounder (*Paralichthys olivaceus*) using diets low in dietary FM inclusion. A fish meal based diet was regarded as a high fish meal diet (HFM) and a diet contained soy protein concentrate, as a substitute for FM, at replacement level of 50% was considered as low fish meal diet (LFM). Six other experimental diets were prepared by dietary supplementation of shrimp hydrolysate (SH) and tilapia hydrolysate (TH) in low FM diet at different inclusion levels of 1.5, 3.0 and 4.5%, in exchange for FM (designated as HFM, LFM, SH-1.5, SH-3.0, SH-4.5, TH-1.5, TH-3.0 and TH-4.5, respectively). Triplicate groups of fish (initial body weight, 22.4 ± 0.2 g) were fed one of the experimental diets to apparent satiation twice daily for ten weeks. At the end of the feeding trial, significantly ($P < 0.05$) higher growth performance was obtained in fish fed HFM, SH-3.0, SH-4.5, TH-1.5 and TH-3.0 diets. Feed utilization of the fish groups fed the diet supplemented with the highest level of SH was significantly improved compared to those of fish fed the LFM diet. Significantly higher digestibility of dietary dry matter was found in fish fed SH-3.0, SH-4.5, TH-1.5 and TH-3.0 while higher protein digestibility was observed at all the inclusion levels of TH. Dietary inclusion of the protein hydrolysates had no significant effect on fish biochemical indices whereas fish fed the HFM diet showed significantly higher plasma total protein and cholesterol levels compare to those of the LFM group. Significantly higher respiratory burst activity was found in fish fed hydrolysate-supplemented diets than the LFM group. Fish fed the TH-3.0 diet showed significantly higher total immunoglobulin level compared to that of the LFM group. Dietary inclusion of the SH at the levels of 3.0 and 4.5% resulted in significant enhancement of lysozyme activity in comparison to LFM group while supplementing the low FM diets with TH showed the same trend at all the inclusion levels. Liver IGF-I mRNA expression was significantly higher in fish fed with SH-3.0, SH-4.5, TH-1.5, TH-3.0 and TH-4.5 diets than fish fed with LFM diet. Dietary inclusion of the protein hydrolysates significantly increased the disease resistance of fish against *Edwardsiella tarda*, at the highest inclusion level (4.5%). Quantitative changes in the structural characteristics of the gastrointestinal tract of olive flounder fed the various diets were evaluated using histological methods. Enterocyte height in proximal intestine was not significantly affected by dietary treatment. Intestinal diameter was significantly increased by dietary inclusion of the SH at 1.5 and 3% and TH at the level of 1.5%. Feeding the TH-1.5 diet resulted in a significant increase in villus length (VL) in comparison to the fish fed LFM diet. In addition, the number of the goblet cells was significantly increased in fish fed the SH supplemented diet at all the inclusion levels as well as TH at the level of 1.5% compared to that of the LFM group. In conclusion, the results indicate that dietary supplementation of the

hydrolysate products can improve growth performance, feed utilization, intestinal morphology, innate immunity and disease resistance of juvenile olive flounder fed low FM diets. Based on the results of the growth performance in the current study, shrimp hydrolysate (SH) seems to be the most effective dietary supplement for juvenile olive flounder whereas tilapia hydrolysate (TH), is apparently the best choice for induction of the innate immune response causing enhanced disease resistance.

Introduction

Aquaculture industry is one of the fastest growing animal protein producing sectors, currently accounting for roughly 46% of the whole fish food 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).

Fish meal (FM) has been the most important protein source in aqua-feeds for several decades especially for carnivorous fish species because it is an excellent source of high quality protein, essential nutrients, attractants and potentially unidentified growth factors. However, world FM production is not expected to increase yet increasing demand for aqua-feed protein, unstable supplies and high prices seen with the dramatic expansion of aquaculture make it prudent to search for alternative protein sources or proper combination of those (Hardy and Tacon, 2002; Gatlin et al., 2007). For this reason, replacement of FM by alternative protein sources has long been of interest and will increasingly be important for the development of low-cost highly efficient aquafeeds (Lee et al., 2002; Gatlin et al., 2007; Pham et al., 2008).

During the past decades, many studies have been conducted aiming at finding appropriate protein sources including both plant and animal origin for aqua-feed industry. In this regard, there has been a great interest in using plant proteins as potential protein source in fish feed. However, their inclusion level in fish diets is limited by their deficiencies in essential amino acids and minerals, and high contents of anti-nutritional factors and complex carbohydrates (NRC, 1993).

One of the most promising ways of meeting the future demands for protein sources is the efficient utilization of marine by-products including fisheries by-catch and seafood processing leftovers (Hardy et al., 2001). Furthermore, utilization of these products in feed industry would prevent the environmental pollution from their disposal. Fisheries processing by-products are generally regarded as low-value resources with negligible market value (Choudhury and Bublitz, 1996). Hydrolysis process is regarded as one of the promising ways for conversion of byproducts into acceptable forms by improvement of their quality and functional characteristics (Gildberg, 1993; Shahidi, 1994). There have been increasing interests in protein hydrolysates, because these compounds contribute towards improving feed palatability. In addition, protein hydrolysates are used as FM replacer and feed attractants, as well as to stimulate the non-specific immune defense

system. In general, protein hydrolysates provide more digestible diets and improve growth and survival rates (Silk et al., 1985; Rust, 1995; Roennestad et al., 2001; Savoie et al., 2006).

Considerable amounts of shrimp processing by-products (SBP), mainly composed of head and body carapace, are discarded each year, while the SBP are important sources of bioactive molecules. The major components (on dry weight basis) of shrimp waste are protein (35–50%), chitin (15–25%), minerals (10–15%), and carotenoids (Tacon et al. 2006). Among cultured fish species in the world, tilapia has the third production rate after carps and salmonids (El-Sayed, 2006). Tilapia protein hydrolysate is a desirable source of essential amino acids and minerals for use in food industry (Foh et al., 2011). Krill is a rich source of high-quality protein with the advantage over other animal proteins of being low in fat and a rich source of omega-3 fatty acids. Krill hydrolysates have been developed as additives for aquaculture feed (Nicol et al., 2000). It is currently used as a nutrient-rich additive in many commercial aquaculture diets. It contains high levels of carotenoid pigments and attractants. Freeze-dried krill hydrolysate, in contrast to ordinary krill meal, has a higher content of free amino acids that theoretically increase the digestibility and attractant qualities of krill.

Therefore, in view of finding an appropriate inclusion level for the hydrolysate products that showed the best results in terms of growth performance, the current study was conducted to verify the effects of different dietary levels of shrimp and tilapia hydrolysates in low FM diet on growth performance, feed utilization, intestine histology, digestibility, innate immunity and disease resistance against pathogens for olive flounder.

Material and methods

Experimental diets

Eight experimental diets were formulated to be isonitrogenous (50% crude protein) and isocaloric (18 kJ g^{-1}) (Table 1). A FM-based diet was regarded as a high FM diet and a diet contained soy protein concentrate (SPC), as a substitute for FM, at replacement level of 50% was considered as low-FM diet. Six other experimental diets were prepared by dietary inclusion of shrimp hydrolysate powder (SH) or tilapia hydrolysate powder (TH) in low FM based diet at different inclusion levels of 1.5, 3 and 4.5% in exchange for FM (designated as HFM, LFM, SH-1.5, SH-3.0, SH-4.5, TH-1.5, TH-3.0 and TH-4.5, respectively). All dry ingredients were thoroughly mixed and, after addition of squid liver oil, soybean oil and double-distilled water, the dough was extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) in ideal size, freeze-dried at −40 °C for 24 h and stored at −20 °C until use.

Fish and Feeding trial

Juvenile olive flounder (*Paralichthys olivaceus*) were transported from a private hatchery (Chang-Hae Fisheries, Jeju Island, Korea) 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, 22.4 ± 0.2 g) were randomly distributed into twenty four tanks of 150 L capacity polyvinyl circular tanks at a density of 33 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 (twice a day, 08:00 and 18:00 h) for thirteen weeks. Uneaten feed was collected 30 min after feeding and reweighed to determine feed intake.

Sample collection and analyses

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.

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).

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 Soxhlet Extraction System C-SH6 (Korea).

Immunological assays

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 dimethylformamide (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. Dimethylformamide 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 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 colored product of WST-1 reaction with superoxide) after 20 min of reaction time at 37 °C. The percent 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 µlazocasein (2%) (Sigma-Aldrich) were added and incubated for 1 h at 22 °C. Five hundred microliter of trichloro 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 thenegativecontrol buffer replaced both serum and trypsin. The trypsin inhibition percentage was calculated using the following equation:

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.

Estimation of apparent digestibility coefficients

For estimation of apparent digestibility coefficient 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 1.0%. All dry ingredients were thoroughly mixed and extruded through a pelletizer machine (SP-50, Gum Gang Engineering, Daegu, Korea) in ideal size after addition of squid liver oil and 10% double distilled water. The pellets were dried with electric fans at room temperature and maintained in a freezer at -20 °C until used.

New sets of flounder (initial body weight, 50 g) were distributed into 300 L capacity Guelph system (fecal collection system) tanks at a density of 40 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 18:00h. One hour after feeding, the rearing tanks were brushed 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 stored at -40 °C. Then the fecal samples were freeze-dried for 24 h and stored at -20 °C until analyses.

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 milliliter 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 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.

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

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)

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.

Slides will be 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 measurements of villus length (VL), intestine diameter (ID) and enterocyte height (EH) were made, using the ImageJ 1.44 analysis software.

Expression levels of liver IGF-I mRNA

Liver total RNA was isolated using E.Z.N.A.^{TM} 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 ×·PrimeScript Buffer (for Real Time), 1 µL of PrimeScript RT Enzyme Mix I, 1 µL of RT Primer Mix and 4 µL of RNase-free dH_2O 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.

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. 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 (FM treatment) and expressed in comparison with the reference gene (18S rRNA), according to Pfaffl's mathematical model (Pfaffl, 2001).

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

Challenge test

At the end of the feeding trial, twelve flounder from each tank (36 fish per treatment) were intraperitoneally injected with *Edwardsiella tarda* (ATCC 15947, Korea Collection for Type Cultures) suspension (1×10^4 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 four 60 L plastic tanks and their behavior and mortality were monitored and recorded for 14 days.

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 19.0 (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.

Results

All the experimental diets were readily accepted by the juvenile olive flounder at the start of the feeding trial and they fed aggressively during the 10 weeks of the feeding trial. The growth and survival data after 10 weeks of the feeding trial is provided in Table 2. The lowest growth was found in fish fed the LFM diet and significant improvements in growth performance and feed utilization were obtained by hydrolysates supplementation. In fact, a significantly higher weight gain value was observed at the highest level of supplementation with HP1 while a similar pattern was found for the dietary inclusion of HP2 up to 3.0%, compared to that of the LFM group. Significantly lower feed conversion ratio (FCR) was found in the groups of fish fed diets supplemented with HP1 at the level of 4.5% compared to that of the LFM group, which was comparable to the HFM value. In the same way, the highest protein efficiency ratio (PER) was observed in the groups of fish fed HFM and HP1-4.5 diets compared to that of the LFM group. However, feeding rate of fish was not affected by dietary treatments (P > 0.05). Also, at the end of the trial, no significant differences were found between survival rates for the different dietary groups.

Fish fed the HP1 diet at the inclusion levels of 3.0 and 4.5% showed remarkably higher digestibility of dry matter compared to that of the LFM group while significantly higher dry matter digestibility was observed with 1.5 and 3.0% of HP2. The protein digestibility followed the same trend as the dry matter; whereas the fish offered the HP2 diets indicated significantly higher values at all the inclusion levels compared to the fish fed the LFM diet (Table 3).

The hemoglobin and total protein contents of the fish plasma samples were significantly higher in fish fed the HFM diet compared to the LFM values. Other fish biochemical indices were not significantly affected by dietary treatment (Table 4).

Analysis of the innate immune parameters revealed significant increase in NBT activity in fish fed the hydrolysate-supplemented diets compared to those fed the LFM diet (Table 5). Dietary inclusion of the HP2 at the level of 3.0% resulted in a significant increment in the immunoglobulin level in comparison to LFM group. Significantly higher lysozyme activity was found in fish fed 3.0 and 4.5% HP1 while fish fed the HP2-supplemented diets showed significantly higher values at all the inclusion levels compared to those fed the LFM diet. Although, numerically higher MPO, SOD and anti-protease activities were detected in fish fed diets containing the hydrolysates, the differences were not significant (Table 5).

The results of the fish whole-body composition revealed no significant differences among dietary treatments (Table 6).

As shown in Fig. 1, liver IGF-I mRNA level was significantly influenced by dietary inclusion of the tested protein hydrolysates ($P < 0.05$). Fish fed with HP1-4.5 and HP2-1.5 diets had significantly higher IGF-I mRNA level than all the other hydrolysate treated groups ($P < 0.05$).

The survival of fish after the challenge with *E. tarda* is shown in Fig 2. During the *E. tarda* challenge test, the first dramatic mortality was observed on the 8th day after injection where the fish fed the LFM diet showed lower disease resistance compared to those fed the other experimental diets (Fig. 2). At the end of the challenge test the LFM group had the lowest survival rate (27.7%) which was significantly differed from those of fish fed HFM, HP1-4.5 and HP2-4.5 diets. Dietary inclusion of the protein hydrolysates significantly enhanced the disease resistance of fish against *E. tarda*, at the highest inclusion level (Fig. 2).

The number of the goblet cells was significantly enhanced by dietary supplementation of the HP2 at 1.5% and HP1 at all the inclusion levels (Table 7; Fig. 3). Intestinal diameter was significantly enhanced by dietary inclusion of the HP1 at 1.5 and 3% and HP2 at the level of 1.5%. Also, fish fed the HP2-1.5 diet had significantly longer villi than those fed the LFM diet. Although, numerically higher values for enterocyte height were detected in fish fed the diets containing the hydrolysates, the differences were not significant (Table 7).

Discussion

In the present study replacing 50% of FM with SPC resulted in significant decrease of growth performance. This is in agreement with results of previous studies showing inferior growth performance of mutton snapper (*Lutjanus analis*) (Freitas et al., 2011), golden pompano (*Trachinotus ovatus*) (Wu et al., 2015) and red sea bream (Kader et al., 2010; Khosravi et al., 2015) fed high SPC based diets. Plant protein sources are significantly different in the contents

of small nitrogen compounds as the content of free amino acids, taurine, anserine and free nucleotides as compared to marine protein sources (Aksnes, 2005). Exchanging FM with plant protein sources will therefore also affect the dietary level of various non-protein nitrogen compounds. Some of these are shown to have effect on growth performance and health of fish (Stapleton et al., 1997; Abe, 2000; Burrells et al., 2001). This difference in composition between protein sources of marine and plant origin and thus dietary differences in potential growth promoters, may be one of the explanations why it is difficult to sustain growth and feed efficiency in carnivorous fish with high levels of plant feed ingredients. Our results showed that supplementing an appropriate dose of SH and TH to the LFM diet can recover the depleted growth of olive flounder. Similarly, in our previous study (Khosravi et al., 2015b) we found a significant improvement in growth performance of red sea bream when krill, shrimp or tilapia hydrolysate were supplemented to a SPC-based diet. In the same way, Kader et al. (2010) showed that supplementing feed additives including krill meal, fish soluble and squid meal in high SPC based diets can recover the performance of juvenile red sea bream. In the current study, supplementation of 1.5 and 3.0% SH to the LFM diet slightly improved growth performance, however, still the values were not significantly different from that of the LFM group. But fish fed the diet containing 4.5% SH showed significantly higher growth than those fed LFM diet and the data was comparable to that of fish fed HFM diet indicating that the optimal dose of SH supplementation is around 4.5%. Arredondo-Figueroa et al. (2012) examined the effects of supplementing different levels of shrimp hydrolysate (2.5, 5.0, 10 and 15%) to a diet in which 30% of FM was replaced by soy bean meal. Their results showed that red claw crayfish hatchlings (*Cherax quadricarinatus*) grew best when they were fed a diet containing 5.0% shrimp hydrolysate while significantly lower growth performances were achieved at lower and higher inclusion levels of shrimp hydrolysate. In the present study, an opposed trend was observed when TH was used as protein hydrolysate source; fish fed the diet containing 1.5% TH exhibited similar growth performance to those fed the HFM diet but further increase in its inclusion level led to a reduced growth. It has been reported that differences in molecular weight distribution of peptides and degree of solubility affects the efficiency of protein hydrolysates (Kotzamanis et al., 2007). The observed different trends for the two hydrolysate sources in this study could be ascribed to the differences in the molecular weight distribution of their peptides. Baker (1984) stated that increased growth performance in animals by dietary supplements can be due to increased feed intake and/or metabolic efficiency. In the present study, we did not find any significant difference in the feed intake among the groups fed with the test diets. Enhancement of growth performance in this study by dietary supplementation of hydrolysates was mainly due to the improved feed utilization because we observed improved FCR and PER values compared to those fed LFM diet and this was more evident when SH was used. It has earlier been demonstrated that the inclusion of a moderate level of protein hydrolysate influence fish digestive enzyme activity (Cahu et al., 2004; Santos et al., 2013; Zambonino Infante and Cahu, 2007). Although in this study the effects of hydrolysates were not evaluated on activity of

digestive enzymes, the significant enhancements in dry matter and protein digestibility may signify the facilitated enzymatic activity by hydrolysates supplementation.

In this study fish whole-body composition was not significantly influence by dietary treatments. Similarly, the results of our previous study (Khosravi et al., 2015a) showed no significant effect of TH supplementation to FM based diet for red sea bream and olive flounder. Also, Khosravi et al. (2015b) reported no significant changes in whole-body and dorsal muscle composition of red sea bream fed high SPC diets supplemented with SH and KH.

Blood parameters are important tools for the indication of the physiological stress response as well as the general health condition of fish. Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to those of the previous findings (Kader et al., 2010; 2011; Uyan et al., 2007). In the current study, hemoglobin and total protein levels were significantly decreased in fish fed with LFM diet. Total protein levels can be used as a diagnostic tool and a valuable test for evaluating the general physiological state in fish (Pedro et al., 2005). In general, blood parameters suggest that physiological condition or welfare of fish was not greatly influenced by the LFM diet, based on protein hydrolysates and SPC compared to the high quality FM based diet.

Insulin-like growth factors I (IGF-I) is an important hormone with multiple biological functions for regulating cell metabolism, accelerating cell growth, division and differentiation, advancing embryonic development and growth regulation, and for adjusting osmosis pressure. The expression of IGF-I in mammals, fish and birds is regulated by nutrition (Clemmons and Underwood, 1991). In the present study, liver IGF-I mRNA level was significantly influenced by dietary inclusion of the tested protein hydrolysates while fish fed the LFM diet showed the lowest level. Fish fed SH-4.5 and TH-1.5 diets had significantly higher IGF-I mRNA level than all the other experimental groups. Similarly, Gomez-Requeni et al., (2004) reported that replacement of FM by plant protein sources leads to decrease in liver IGF-I mRNA transcripts of gilthead sea bream (*Sparus aurata*). It has also been found that liver IGF-I expression level was significantly influenced by dietary protein level and showed a significant correlation with SGR in mud carp (*Cirrhinus molitorella*) (Jiang et al., 2010). In agreement with the results of the current study, Zheng et al., 2012 showed significant increase in liver IGF-I mRNA expression by dietary utilization of the protein hydrolysate in high plant protein diet in juvenile flounder.

The results of our present study showed that the assayed diets improve villus height which was significant in fish fed the TH-1.5 diet compared to the fish fed the LFM diet. In aquatic animals, intestinal villus height is regarded as a sign of absorption ability. It has been proposed that the increase in the length of the villi implied an increase of surface area for the greater absorption of available nutrients (Caspary, 1992). The main functions of intestinal mucous cells are producing and secreting mucous gel which helps in lubricating, immobilizing enzyme, protecting the mucosal surface and trapping pathogenic bacteria and parasites (Dharmani et al., 2008). The results of the current study showed that the number of the goblet cells was significantly enhanced by dietary supplementation of the TH at 1.5% and SH at all the inclusion levels.

Currently, fish farmers tend to use immunostimulants on a daily basis. Even though many natural and synthetic substances have been reported to potentiate the fish immune system to increase disease resistance, the search for new immunostimulants continues in an attempt to improve intensive fish farming. It has been suggested that the immune system may also be affected by fish protein hydrolsyate supply. There have been reports of biologically active peptides with immuno-stimulating and antibacterial properties being produced during the hydrolysing procedure (Bøgwald et al., 1996; Gildberg et al., 1996; Daoud et al., 2005). The general health and immunocompetence of fish from the protein hydrolysate treated groups in this study were evaluated using humoral and cellular measures of certain nonspecific immune functions, and later using *E. tarda* challenge to compare their ability to produce a protective immune response. The results of the current study revealed significant increase in respiratory burst activity activity in fish fed the hydrolysate-supplemented diets compared to those fed the LFM diet. In the same way, Liang et al. (2006) found that phagocytic index of Japanese sea bass, *Lateolabrax japonicas*, significantly increased by partial replacement of dietary FM protein with fish protein hydrolysate. In contrast, Murray et al. (2003) reported that rearing coho salmon, *Oncorhynchus kisutch*, on practical diets that contained either fish meal (control), fish meal supplemented with cooked fish by-products or fish meal supplemented with hydrolysed fish protein alone or with hydrolysed fish protein and processed fish bones for 6 weeks had no effect on NBT% activity. Based on the results of the current study, significantly higher lysozyme activity was found in fish fed diets contained 1.5% ≤ SH while fish fed the TH-supplemented diets showed significantly higher values at all the inclusion levels compared to those fed the LFM diet. In agreement to this, it has been indicated that 15% fish protein hydrolysate in the diet could enhance the lysozyme activity of sea bass after feeding for 30 or 60 days (Liang et al. 2006). However, lysozyme activity didn't show any significant differences by dietary treatments in groups of coho salmon which were reared on practical feeds that supplemented with hydrolyzed fish protein alone, or with hydrolyzed fish protein and processed fish bones (Murray et al., 2003). These increases may have been biologically significant under the conditions of the current study, as the better survival of fish from all groups fed the hydrolysates following challenge with *E. tarda* suggested that the feed supplements provide a beneficial effect on the innate immune functions.

The results of this study showed positive effects of the supplementation of hydrolysate products in LFM diets for Olive flounder. The improvements in growth performance, feed utilization, apparent digestibility, liver IGF-I mRNA expression, intestinal morphology, innate immunity and disease resistance by these products seems to be due to the formation of biologically active compounds during the hydrolysis process. Also, the results of these experiments clearly indicated that dietary inclusion of a moderate amount of the tested protein hydrolysates in high soy protein based diets could effectively facilitate higher FM replacement in this species diet. Based on these findings, the highest growth performance and feed utilization efficiency were observed in fish fed 4.5% dietary shrimp hydrolysate (SH) which were comparable to those of the HFM group. Moreover, the significant improvement of liver IGF-I

mRNA expression by increment of dietary SH level in this study further confirmed that supplementation of appropriate level of SH in diets for olive flounder would enhance IGF-I production, which is fundamentally involved in growth regulation. In contrast, fish fed the highest tilapia hydrolysate (TH) level exhibited lower growth performance and feed utilization efficiency than other inclusion levels, even though the difference was not significant. Therefore, based on the fish growth performance, shrimp hydrolysate seemed to be the most effective dietary supplement for juvenile olive flounder whereas tilapia hydrolysate, apparently, might be the best choice for induction of the innate immune response causing enhanced disease resistance.

Table 5.1. Formulation and proximate composition of the eight experimental diets for olive flounder (%, dry matter basis).

Ingredients	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
Brown fish meal	46.0	23.0	21.6	20.2	18.8	21.4	19.8	18.2
Soy protein concentrate	5.0	21.0	21.0	21.0	21.0	21.0	21.0	21.0
SH	0.0	0.0	**1.5**	**3.0**	**4.5**	0.0	0.0	0.0
TH	0.0	0.0	0.0	0.0	0.0	**1.5**	**3.0**	**4.5**
Corn gluten meal	6.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Wheat flour	31.5	30.3	30.2	30.1	30.0	30.4	30.5	30.6
Squid liver oil	4.0	5.5	5.5	5.5	5.5	5.5	5.5	5.5
Soybean oil	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Mineral Mix[1]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin Mix[2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CMC	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Choline chloride	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Lysine	0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
L-Methionine	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Taurine	0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Di-calcium phosphate	0.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Proximate composition								
Dry matter	92.8	91.8	92.4	92.4	93.0	93.2	92.7	92.4
Crude protein	48.7	49.3	49.1	49.1	48.7	48.6	48.2	48.5
Crude lipid	14.8	14.6	14.0	14.1	14.1	14.1	14.6	14.1
Ash	8.57	7.11	6.97	7.03	6.87	7.15	7.10	7.03

HFM, High fish meal; LFM, Low fish meal; SH, Shrimp hydrolysate; TH, Tilapia hydrolysate

[1] Mineral premix (g kg^{-1} of 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, 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^{-1} of mixture): L-ascorbic acid, 121.2; DL-α 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.

Table 5.2. Growth performance and feed utilization of olive flounder fed the eight experimental diets for 10 weeks.

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
Final mean body weight (g)	133±3.40^{c}	113±6.55^{a}	117±1.74^{ab}	122±2.37^{b}	125±3.11^{bc}	124±1.67^{bc}	123±2.49^{b}	119±1.57^{ab}
Weight gain (%)	494±18.0^{c}	403±30.6^{a}	423±11.5^{ab}	444±4.94^{ab}	459±16.5^{bc}	454±8.10^{bc}	454±13.7^{bc}	434±14.6^{ab}
Feed intake (g fish^{-1})	124±4.60	119±9.08	121±6.32	119±4.79	118±9.96	115±7.46	116±4.03	113±8.12
Feed conversion ratio	1.11±0.06^{a}	1.29±0.02^{b}	1.26±0.02^{ab}	1.18±0.05^{ab}	1.12±0.09^{a}	1.13±0.09^{ab}	1.14±0.03^{ab}	1.16±0.07^{ab}
Protein efficiency ratio	1.85±0.10^{b}	1.58±0.03^{a}	1.62±0.03^{ab}	1.72±0.07^{ab}	1.85±0.15^{b}	1.82±0.14^{ab}	1.83±0.05^{ab}	1.78±0.10^{ab}
Survival rate (%)	95.9±3.50	93.9±6.06	98.0±3.50	99.0±1.75	92.9±7.63	100.0±0.00	94.9±4.63	99.0±1.75

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.

Table 5.3. Apparent digestibility coefficients (%, ADC) for dry matter and protein of the experimental diets for olive flounder.

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
ADCd (%)[1]	72.7±0.66^{d}	63.6±1.35^{a}	64.3±0.14^{ab}	67.9±2.11^{bc}	71.7±0.98^{cd}	69.0±0.18^{cd}	69.0±2.28^{cd}	64.6±2.13^{ab}
ADCp (%)[2]	92.3±0.19^{e}	81.6±0.68^{a}	82.1±0.07^{a}	87.5±0.83^{c}	89.4±0.37^{d}	88.3±0.07^{cd}	88.0±0.88^{cd}	84.4±0.94^{b}

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

Table 5.4. Hematological parameters of olive flounder fed the eight experimental diets for 10 weeks.

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
Ht[1]	30.0±2.88	26.0±1.52	31.2±2.57	30.8±2.14	30.0±3.38	29.1±0.52	28.0±1.09	27.2±2.90
Hb[2]	4.82±0.32^{b}	3.83±0.19^{a}	4.40±0.09^{ab}	4.08±0.07^{ab}	4.16±0.22^{ab}	4.41±0.44^{ab}	4.33±0.20^{ab}	4.15±0.48^{ab}
Glucose[3]	71.7±4.96	69.2±4.97	67.6±0.90	66.8±3.32	68.3±3.81	68.6±1.86	67.2±4.94	67.7±5.85
Total protein[4]	4.06±0.59^{b}	2.95±0.16^{a}	3.77±0.39^{ab}	3.33±0.32^{ab}	3.23±0.25^{ab}	3.10±0.48^{ab}	3.18±0.25^{ab}	3.17±0.26^{ab}
Total cholesterol[5]	239±23.4	231±7.82	234±10.9	236±37.3	234±22.3	240±39.0	243±18.0	225±29.2
Triglyceride[6]	253±47.8	335±17.8	328±46.5	299±38.5	286±28.6	302±12.0	276±34.5	270±18.3

[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]Triglyceride (mg dL^{-1})

Table 5.5. Non-specific immune response of olive flounder fed the eight experimental diets for 10 weeks.

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
NBT[1]	0.53±0.05^{b}	0.27±0.04^{a}	0.48±0.06^{b}	0.50±0.01^{b}	0.45±0.07^{b}	0.46±0.05^{b}	0.47±0.06^{b}	0.47±0.03^{b}
MPO[2]	1.94±0.26	1.54±0.09	1.85±0.06	1.94±0.29	1.87±0.50	1.66±0.33	1.89±0.38	1.71±0.05
SOD[3]	46.5±3.30	42.9±5.03	57.5±2.74	52.6±11.6	54.3±5.65	49.5±3.27	50.5±8.25	54.1±8.98
Ig[4]	19.7±1.90^{ab}	16.0±3.14^{a}	20.0±1.37^{ab}	18.0±0.39^{ab}	18.6±1.97^{ab}	20.3±1.51^{ab}	22.3±1.29^{b}	20.7±1.46^{ab}
Lysozyme[5]	19.5±1.28^{cd}	14.0±1.19^{a}	14.5±1.08^{ab}	18.6±2.49^{c}	18.4±0.63^{bc}	18.7±1.58^{c}	21.3±1.02^{cd}	23.4±1.40^{d}
Antiprotease[6]	22.2±5.01	18.8±0.15	20.3±5.52	21.1±3.15	21.5±5.01	20.2±2.51	19.6±2.90	21.9±4.93

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 (% inhibition)

Table 5.6. Whole-body composition of olive flounder fed the eight experimental diets for 10 weeks (% DM).

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
Dry matter	28.5±0.7	27.6±0.5	26.9±0.9	27.8±0.9	27.5±0.0	27.5±0.5	27.8±1.4	27.3±0.4
Protein	65.1±2.7	65.5±3.2	65.8±0.9	64.0±2.4	64.0±0.6	66.6±0.2	64.1±2.7	64.9±1.5
Lipid	21.4±0.4	20.7±1.2	20.4±1.8	20.6±1.0	21.5±0.6	20.8±1.0	21.2±1.3	21.7±1.0
Ash	11.2±3.0	10.1±2.6	10.5±0.4	12.1±1.7	11.3±1.7	9.9±2.1	10.9±0.7	10.4±2.8

Values are mean of triplicate groups and presented as mean ± SD.

Table 5.7. Morphometric parameters of olive flounder intestine fed the eight experimental diets for 10 weeks.

	Experimental diets							
	HFM	**LFM**	**SH-1.5**	**SH-3.0**	**SH-4.5**	**TH-1.5**	**TH-3.0**	**TH-4.5**
GC[1]	1050±101^{bc}	695±67^{a}	977±59^{bc}	963±86^{bc}	1091±59^{c}	990±75^{bc}	899±76^{abc}	833±116^{ab}
ID[2]	2543±136^{ab}	2373±154^{a}	2714±75^{b}	2719±99^{b}	2655±48^{ab}	2755±103^{b}	2588±118^{ab}	2512±151^{ab}
VL[3]	777±44^{ab}	661±105^{a}	808±64^{ab}	833±50^{ab}	836±53^{ab}	862±45^{b}	817±68^{ab}	737±63^{ab}
EH[4]	41.7±0.7	38.7±1.6	41.7±1.7	42.1±1.3	41.5±2.8	40.6±2.7	42.2±1.9	38.9±1.0

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

[2]Intestinal Diameter (μm)

[3]Villi Length (μm)

[4]Enterocyte Height (μm)

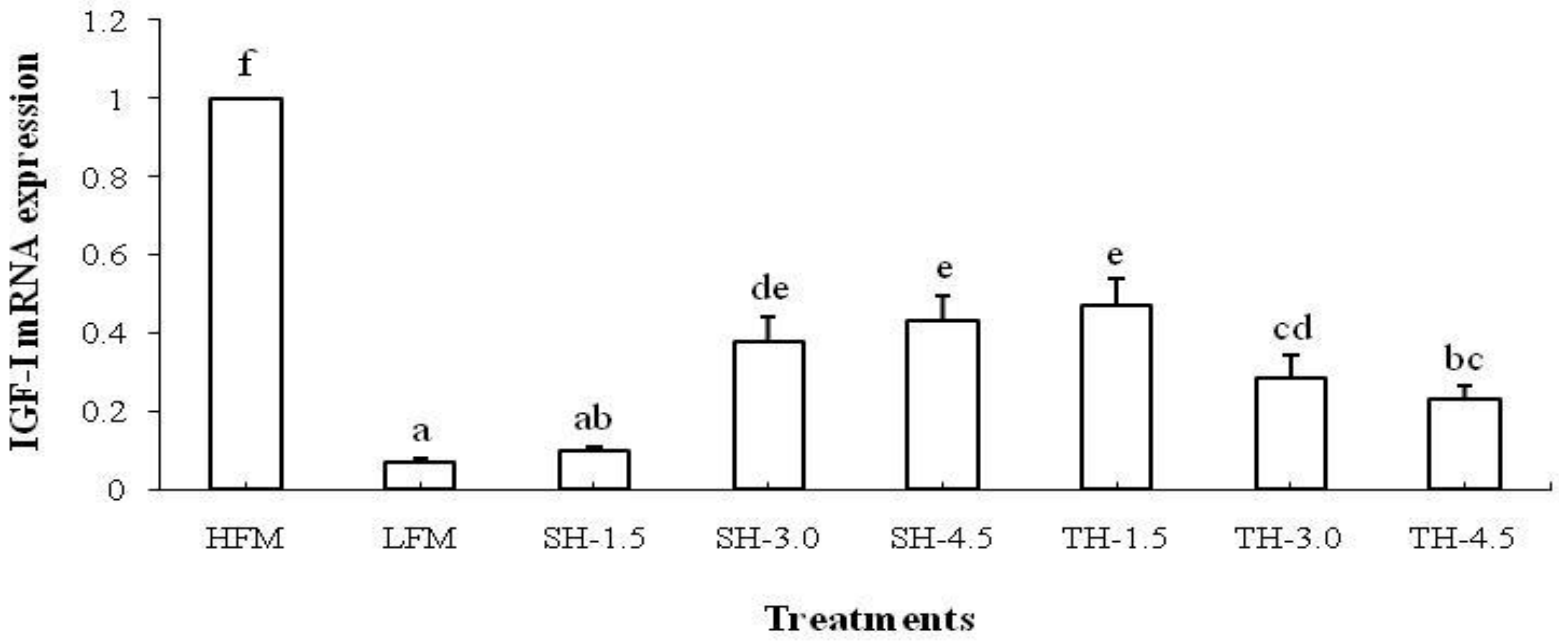


Figure 5.1. Liver insulin-like growth factors I mRNA expression for each diet group expressed as a ratio to HFM diet values. Data are presented as mean ± SD from three replicate tanks. Different letters above the bars denote significant differences between diet groups at the $P < 0.05$ level.

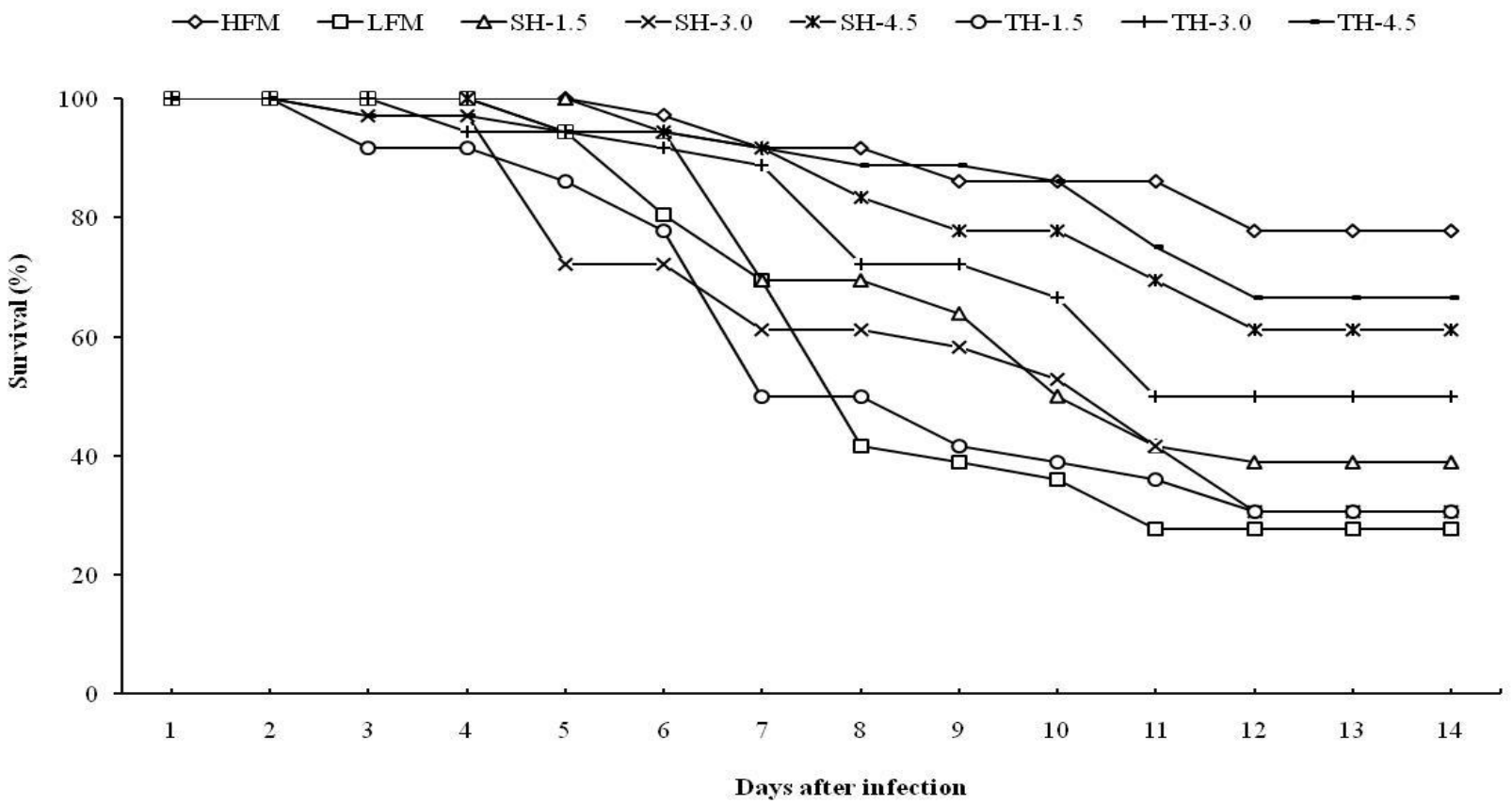


Figure 5.2. Survival rate of olive flounder after challenge with *E. tarda*. .

CHAPTER 6

Dietary Supplementation of Marine Protein Hydrolysates in Fish-Meal Based Diets for Red Sea Bream (*Pagrus major*) and Olive Flounder (*Paralichthys olivaceus*)

Dietary Supplementation of Marine Protein Hydrolysates in Fish-Meal Based Diets for Red Sea Bream (*Pagrus major*) and Olive Flounder (*Paralichthys olivaceus*)

Abstract

Two feeding trials were conducted to investigate the effects of dietary supplementation of krill hydrolysate (KH) and tuna hydrolysate (TH) on growth performance, feed utilization, nutrient digestibility, innate immunity and disease resistance of juvenile red sea bream (Exp I) and olive flounder (Exp II). In both experiments a basal fish meal-based diet was regarded as a control and two other diets were prepared by top-coating 2% of KH or TH (designated as Con, KH and TH, respectively). Triplicate groups of red sea bream (29.0 ± 0.1 g) and olive flounder (24.5 ± 0.3 g) were fed one of the test diets for 12 and 9 weeks, respectively. In both experiments growth performance and feed utilization of the fish were significantly improved by dietary supplementation of KH compared to those of fish fed the control diet. Significantly higher digestibility of dietary protein was found in both species fed KH and TH diets, while higher dry matter digestibility was only found inflounder fed the KH and TH diets. No significant changes were observed in fish hematological parameters and whole-body composition. Red sea bream had significantly higher NBT activity by TH and higher lysozyme activity by both TH and KH diets compared to Con diet. In olive flounder, the KH diet led to significantly higher NBT and superoxide dismutase activities, and the TH diet resulted in a significantly higher lysozyme activity than Con diet. At the end of the challenge test, red sea bream fed the TH diet showed higher disease resistance against *Edwardsiella tarda*. Results of this study indicated that supplementation of 2% KH in diets for red sea bream and olive flounder can enhance growth performances and feed efficiency, and that non-specific immune response can be positively affected by both KH and TH.

Introduction

Every year a considerable amount of marine by-products including fisheries by-catch and seafood processing leftovers is discarded while these by-products contain valuable nutrients that can be utilized as functional ingredients in feed industry. With the aim of using these by-products, enzymatic hydrolysis has been employed as one of the promising ways for conversion offish by-products into acceptable forms with improved quality and functional characteristics (Chalamaiah et al., 2012; Gildberg, 1993; Shahidi, 1994). Protein hydrolysates derived from marine processing by-products have been considered as a great ingredient in aquafeed due to their good nutritional value and functional properties (Chalamaiah et al., 2012). Hence, based on their low molecular weight compounds and well balanced amino acid profile, hydrolysates have been used as chemoattractant and fish meal replacer in aquafeeds (Aksnes et al., 2006a, 2006b; Cahu and Zambonino Infante, 2001; Kolkovski et al., 2000; Refstie et al., 2004). The beneficial effects on growth performance and feed utilization followed by dietary inclusion of protein hydrolysates at moderate levels are often attributed to the improvement in palatability of the feed related to their contents of free amino acids and peptides with short chain length (Hevrøy et al., 2005; Kolkovski and Tandler, 2000; Refstie et al., 2004). Moreover, bio-active peptides derived from enzymatic hydrolysis of marine by-products have been reported to possess physiological

and biological functions including immunomodulatory, antimicrobial or antioxidant activities (Chalamaiah et al., 2012; Harnedy and Fitzgerald, 2012) depending on their molecular weight and amino acid sequences (Kim and Wijesekara, 2010).

In this point of view, wastes of processed tuna (*Thunus* spp.) and tuna-like species can also be considered as potential ingredients for fish feed industry (Herpandi et al., 2012) where approximately 50–70% of the initial raw materials are discarded and/or traditionally processed into other low market-value products, such as fish meal for animal feeds and fertilizers (Benjakul and Morrissey, 1997; Guérard et al., 2002; Tekinay et al., 2009). Tuna hydrolysate (TH) showed potential to be used as a high value feed additive for fish (Ovissipour et al., 2012) and shrimp (Nguyen et al., 2012). In the same way, krill is also regarded as one of the most promising marine protein sources which has been used as a nutrient-rich dietary supplement in aquafeeds for a long time (Storebakken, 1988; Tacon et al., 2006). Many positive results demonstrated that krill hydrolysate (KH) could be regarded as a high quality protein source to replace fish meal and/or as feed attractant resulting in enhanced feed intake and stimulation of fish digestive system (Kolkovski et al., 2000; Storebakken, 1988).

Olive flounder and red sea bream are very important cultured marine fish species in Asian countries, especially Korea, Japan and China (Kang et al., 2008; Watanabe and Vassallo-Agius, 2003). However, there is limited information on the use of protein hydrolysates in diets for both species. In this study, therefore, two feeding trials were carried out for the two species to investigate the effects of dietary supplementation of KH and TH on growth performance, feed utilization, digestibility, innate immunity and disease resistance against *Edwardsiella tarda*.

Material and methods

Experimental diets

Marine protein hydrolysates were provided by AQUATIV Company. Briefly, fresh tuna co-products and whole Antarctic krill (*Euphausia superba*) were enzyme hydrolyzed to produce TH liquid (Actipal OL4, AQUATIV Thailand) and KH concentrate (Actipal HC6 AQUATIV France). Proximate composition and peptide profiles of the protein hydrolysates are presented in Table 1. Peptide profiles of the hydrolysates were determined using size exclusion chromatography method (Guérard et al., 2001). Two sets of experimental diets were formulated in similar way. Two fish meal-based diets were formulated to meet the nutrient requirements of the target species (Table 2). A basal fish meal based diet was regarded as a control, and two other experimental diets were prepared by supplementation of either 2% (by weight) KH or TH (designated as Con, KH and TH, respectively). Protein hydrolysates were applied on basal diet by top-coating pellets using an air-pressure spray gun during constant mixing of the diet. All the diets were freeze-dried and kept at −20 °C until used.

Experimentalfish and feeding trial

The two feeding trials were conducted at the Marine Science Institute, Jeju National University, Jeju, South Korea. The fish in genetically homogenous stock were obtained from a private hatchery, Jeju Island, and fed a commercial diet for two weeks to be acclimated to the experimental conditions and facilities. The health status of the fish was checked upon arrival and

the fish were immediately treated with 100 mg L^{-1} formalin for 20 min. Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

In the first experiment (Exp I), red sea bream (initial mean body weight, 29.0 ± 0.1 g) were randomly distributed into nine polyvinyl circular tanks of 150 L capacity at a density of 33 fish per tank and in the second experiment (Exp II) olive flounder (initial mean body weight, 24.5 ± 0.3 g) were randomly distributed into a similar rearing systemat a density of 35 fish per tank. Triplicate groups of fish were fed one of the experimental diets to apparent satiation (twice a day, 08:00 and 18:00 h) for 12 and 9 weeks, in Exp I and Exp II, respectively. The tanks were supplied with sand filtered seawater at a flow-rate of 3 L min^{-1} and aeration to maintain enough dissolved oxygen. The photoperiod was maintained on a 12:12 h (light/dark) schedule. The average water temperature during the trial was 22.0 ± 1.8 °C. Uneaten feed was collected 20 min after feeding and reweighed to determine feed intake. Growth of fish was measured every three weeks. Feeding was stopped 24 h prior to weighing or blood sampling to minimize stress on fish.

Sample collection and analytical methods

At the end of the feeding trial, all the fish in each tank were bulk-weighed and counted for the calculation of growth parameters and survival. Blood samples were collected from individualfish, and each sample was analyzed separately. Threefish per tank (nine fish per dietary treatment) were randomly captured, and anesthetized with 2-phenoxyethanol solution (200 mg L^{-1}) and blood samples were taken from the caudal vein with heparinized syringes for determination of hematocrit and hemoglobin concentration, and respiratory burst activity. After the above-mentioned measurements with whole blood, plasma was separated by centrifugation at 5000 × g for 10 min and stored at −70 °C for the determination of total immunoglobulin (Ig), glucose, total protein, total cholesterol and triglyceride levels. Another set of blood samples was taken (three fish per tank, nine fish per dietary treatment) without heparin 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 response including lysozyme, superoxide dismutase (SOD), myeloperoxidase (MPO) and antiprotease activities. Hematocrit was determined by a microhematocrit technique (Brown, 1980). Hemoglobin and plasma levels of glucose, total protein, total colesterol and triglyceride were determined by using an automated blood analyzer (SLIM; SEAC Inc., Florence, Italy).

Analysis of moisture and ash contents of diets and whole-body samples was performed by the standard procedures (AOAC, 1995). Crude protein was measured by using automatic Kjeltec Analyzer Unit 2300 (FossTecator, Höganäs, Sweden) and crude lipid was determined using the Soxhlet method with extraction in diethyl ether (Soxhlet Extraction System C-SH6, Korea).

Monitoring of non-specificimmuneresponse

Plasma Ig level was determined according to the method described by Siwicki and Anderson (1993). The oxidative radical production by phagocytes during respiratory burst was measured using the NBT (nitro-blue-tetrazolium) assay described by Anderson and Siwicki (1995). A turbidimetric assay was used to determine serum lysozyme levels using the method

described by Hultmark et al. (1980) with slight modifications. Serum MPO activity was measured according to Quade and Roth (1997). 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. Serum antiprotease activity was measured according to the method described by Ellis et al. (1990) with slight modifications (Magnadóttir et al., 1999).

Digestibility test

For the estimation of apparent digestibility coefficient (ADC) of the experimental diets, chromic oxide (Cr2O3) (Sigma-Aldrich, St. Louis, USA) was included in the diets as an inert indicator at a concentration of 1.0%. The digestibility trial was conducted in fiberglass fecal collection tanks of 300 L capacity designed according to Cho et al. (1982). New sets of red sea bream and olive flounder with mean body weight of ~50 g were stocked into each tank at a density of 40 fish per tank and each group of fish was fed one of the test diets. The tanks were supplied with cartridge-filtered seawater at a flow rate of 1 L min^{-1} and aeration to maintain enough dissolved oxygen. The digestibility trial consisted of three periods of 10 days. In each 10-day period, the fish were allowed to become acclimatized to the feed for the first three days and feces were collected over the next 7 days. Then, diets were randomly changed between tanks and the procedure was repeated for two more times giving a total of three fecal samples for each diet. All feces collected from each tank in each period were pooled and frozen at −20 °C for analysis. After each feeding, the tanks and the settling columns were thoroughly cleaned to eliminate all feed wastes and fecal residues. Chromium oxide content of diet and feces samples was analyzed by the method described by Divakaran et al. (2002). The apparent digestibilit coefficient of the experimental diets was calculated through the following formula:

ADC = 1- ((Cr_2O_3 in feed = Cr_2O_3 in feces) × (Nutrient content of feces / Nutrient content of feed))

Bacterial challenge

At the end of the feeding trial, 15 fish from each tank (45 fish per treatment) were randomly captured and subjected to a bacterial challenge. *E. tarda* (ATCC 15947, Korea Collection for Type Cultures) was used as the pathogenic agent (provided by the Marine Microbiology Laboratory of Jeju National University). The bacterium was cultured in 10 mL BHI broth (Difco, Detroit, MI, USA) with 1.5% NaCl and incubated with shaking for 24 h at 37 °C. Bacterial growth was measured at an optical density of 700 nm followed by plate counting in BHI-NaCl. The isolated bacteria were identified using API 20E commercial identification kit (97.4% accuracy) (bioMérieux, Marcy l'Etoile, France). The bacterium concentration was determined by plate counting on BHI agar. Fish were injected intraperitoneally with *E. tarda* suspension containing 1×10^5 and 1×10^3 CFU mL^{-1} for Exp I and Exp II, respectively. The pathogenic dose of bacterium had previously been determined in a preliminary test using fish of a similar size. After injection, the respective fish groups (15 fish) were distributed into plastic tanks of 65 L capacity in triplicates and their mortality was monitored and recorded for 21 (Exp I) and 10 (Exp II) days. Relative percent survival was calculated using the following formula:

Relative % survival = [(Mortality (%) Control – Mortality (%) Treatment) / Mortality (%) Control] × 100.

Statistical analysis

All diets were assigned by a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 12.0 (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.

Results

Experiment I (red sea bream)

All the experimental diets were readily accepted by both fish species at the start of the experiment and they fed aggressively during the feeding trial. Growth performance and feed utilization of red sea bream were significantly improved by dietary supplementation of KH compared to those of fish fed the Con diet (Table 3). Although the fish fed the KH diet showed numerically higher feed intake than the other groups, no significant difference was found among dietary treatments. Survival rate was not affected by the dietary treatments. The results of the digestibility trial showed a significant increase in apparent digestibility coefficient of protein by dietary supplementation of both tested hydrolysates (Table 3). No significant differences were found in whole-body proximate composition as well as hematological parameters among dietary treatments (Tables 4 and 5).

Dietary supplementation of TH resulted in significant increase of NBT activity compared to the control group (Table 6). Also, significantly higher lysozyme activity was found infish fed the diets supplemented with both protein hydrolysates. Although, numerically higher Ig, MPO, SOD and antiprotease values were detected infish fed diets containing the hydrolysates, the differences were not significant.

During the *E. tarda* challenge test, the first dramatic mortality was observed on the third day after injection, and the fish fed the Con and KH diets showed lower disease resistance compared to those fed the TH diet (Fig. 1). Although fish fed the TH diet exhibited numerically higher disease resistance than the two other experimental groups, the differences were not significant at the end of the challenge test.

Experiment II (oliveflounder)

The fish fed the KH diet showed significantly ($P < 0.05$) higher growth performance than those fed the Con diet. Significantly lower feed conversion ratio was obtained in fish fed the KH diet compared to those fed the Con diet. Fish survival varied from 82 to 87% without any significant differences among dietary treatments. Digestibility of dry matter and protein was significantly higher infish fed hydrolysate containing diets (Table 3).

The results of whole-body composition analysis (Table 4) and hematological indices (Table 5) revealed no significant differences among treatments.

Dietary supplementation of the KH significantly increased NBT and SOD activities. Also, significantly higher lysozyme activity was recorded infish fed TH diet. Total Ig level and antiprotease activity were not significantly influenced by the dietary treatments (Table 6).

All the fish in the control group were dead within six days following the bacterial infection, while the other two groups exhibited higher disease resistance even 10 days post infection (Fig. 1). However, the differences were not significant among treatments.

Discussion

In the present study, significant improvements in growth performance of red sea bream and olive flounder were achieved by dietary supplementation of KH. Similarly, Kolkovski et al. (2000) reported the growth promoting effect of KH when it was supplemented in diets for larval and juvenile fish. Increased growth performance of fish following dietary KH application has been reported to be due to enhanced diet ingestion rate as KH is a rich source of low molecular weight compounds acting as chemo-attractant in fish diets (Kolkovski et al., 2000). In the current study, slight improvement in feed intake was observed in groups offered KH coated diets and this was more evident in olive flounder, although the differences were not significant. Therefore, the potential attractive properties of KH may not fully explain the considerable improvement of fish performance in the present study. The increased growth of fish fed KH coated diets was mainly due to improved feed utilization in both species, as significantly lower FCR values were found by KH supplementation. Also, numerically higher protein efficiency ratio was obtained in KH fedfish. It has been reported that the inclusion of a moderate proportion of protein hydrolysate might affect fish digestive enzyme activity (Cahu et al., 2004; Santos et al., 2013; Zambonino Infante and Cahu, 2007). In the present study, the changes in digestive enzymes were not taken into account but the results of digestibility trials highlighted the significant enhancement of protein digestibility by KH and TH supplementation in bothfish species. In accordance with the present results, previous studies have demonstrated significant improvement in apparent nutrient digestibility by the dietary hydrolysate supplementation in juvenile olive flounder (Khosravi et al., in press; Zheng et al., 2012) and red sea bream diets (Bui et al., 2014).

It has been demonstrated that fish immune system can be triggered by dietary supplementation of protein hydrolysates (Kotzamanis et al., 2007; Zheng et al., 2013). In this regard, there are several reports on improvement of the fish immune system by dietary supplementation of protein hydrolysates (Bui et al., 2014; Liang et al., 2006; Khosravi et al., in press; Tang et al., 2008). The present results also indicated that coating the diets with KH or TH can enhance non-specific immune responses in fish. Nevertheless, contradictory results have been reported for the effect of protein hydrolysate onfish immune response. While dietary fish protein hydrolysates did not show significant impact on the certain indices of defense mechanism of fish (Murray et al., 2003; Zheng et al., 2013), several in vitro and in vivo studies indicated significant enhancement in fish immune response by various protein hydrolysates (Bøgwald et al., 1996; Bui et al., 2014; Khosravi et al., in press; Liang et al., 2006; Tang et al., 2008). The enzymatic hydrolysis of proteins may result in the formation of biologically active peptides (Kim and Wijesekara, 2010; Korhonen and Pihlanto, 2006) that have been found to exhibit numerous

physicochemical properties and bioactivities such as immunostimulating and antibacterial properties depending on their molecular weight and amino acid sequence (Kim and Mendis, 2006). Bøgwald et al. (1996) found that peptide fractions obtained from fish protein hydrolysate with the molecular weight ranging from 500 to 3000 Da were able to stimulate the activity of fish macrophages. Therefore, the contradictory results obtained in previous studies may be due to the variations in peptide profiles of hydrolysates that could be influenced by the raw material, the enzyme specifications and the hydrolysis conditions (Klompong et al., 2009).

The improvement of immune function, either cellular or humoral, accompanied with enhanced disease resistance has already been demonstrated in various fish species (Kotzamanis et al., 2007; Liang et al., 2006; Murray et al., 2003). However, there is no strong evidence to support such a claim even though the immunostimulatory effects of the protein hydrolysates may get them as potential dietary ingredients for promoting disease resistance of fish. In the current study juvenile red sea bream fed the TH coated diet showed higher disease resistance than the other experimental groups.

In conclusion, the findings in the present study indicate that coating fish meal-based diets with KH can improve growth performance of both red sea bream and olive flounder juveniles. Moreover, KH and TH can be used as potential stimulator of innate immunity in both fish species.

Table 6.1. Chemical compositions and peptide profiles of the tested protein hydrolysates.

	KH[a]	TH[b]
Dry matter (%)	58.2	35.7
Protein (% DM)	44.8	22.0
Lipid (% DM)	1.6	2.6
Ash (% DM)	11.6	7.1
Taurine (%)	1.4	6.1
Total amino acids (% protein)	73.1	72.1
Soluble nitrogen (% N)	96.3	85.2
Molecular weight repartition (% of peptides)		
Peptides >30000 Da	<0.1	<0.1
Peptides 20000-30000 Da	<0.1	0.9
Peptides 10000-20000 Da	0.3	8.2
Peptides 5000-10000 Da	2.0	9.6
Peptides 1000-5000 Da	17.7	10.5
Peptides 500-1000 Da	16.0	4.3
Peptides <500 Da	64.0	66.5

[a]Krill hydrolysate concentrate

[b]Tuna hydrolysate liquid

Table 6.2. Dietary formulation and proximate composition of the basal diets for red sea bream and olive flounder (% dry matter).

Ingredients	Fish meal basal diets	
	Red sea bream	Olive flounder
White fishmeal	52.00	50.00
Soybean meal	6.00	7.00
Corn gluten meal	6.00	7.00
Wheat flour	22.50	24.50
Squid liver oil	5.00	4.00
Soybean oil	5.00	4.00
Mineral Mix[1]	1.00	1.00
Vitamin Mix[2]	1.00	1.00
CMC[3]	1.00	1.00
Choline chloride	0.50	0.50
Proximate composition (% dry matter)		
Dry matter	90.9	91.2
Protein	45.7	44.4
Lipid	14.8	13.0
Ash	13.8	18.3
Gross energy (MJ kg^{-1})[4]	21.1	19.9

[1] Mineral premix (g kg^{-1} of 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, 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^{-1} of mixture):L-ascorbic acid, 121.2; DL-αtocopheryl 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.

[3]Carboxymethyl cellulose.

[4]Gross energy of experimental diets was calculated according to gross energy values of 23.60 MJ kg^{-1} protein, 17.20 MJ kg^{-1}carbohydrate, and 39.50 MJ kg^{-1} fat, respectively (NRC, 1993).

Table 6.3. Growth performance and feed utilization of red sea bream (initial body weight, 29.0 ± 0.1 g) and olive flounder (initial body weight, 24.5 ± 0.3 g) fed the experimental diets for 12 and 9 weeks, respectively.

Diets	FBW[1]	SGR[2]	FCR[3]	PER[4]	FI[5]	Survival	ADCd	ADCp
Red sea bream								
Con	103±2^{a}	1.49±0.03	1.80±0.05^{b}	1.34±0.03	133±1	92.2±5.1	70.9±1.1	88.0±0.2^{a}
TH	108±1^{ab}	1.54±0.02	1.68±0.03^{ab}	1.43±0.03	133±3	91.1±1.9	71.7±1.1	91.0±0.4^{b}
KH	113±3^{b}	1.57±0.04	1.63±0.05^{a}	1.44±0.05	136±3	93.3±3.3	72.4±2.3	91.9±1.1^{b}
Olive flounder								
Con	74.8±4.1^{a}	1.89±0.07	1.52±0.03^{b}	1.48±0.03	81.4±2.4	87.6±6.6	75.2±1.5^{a}	93.6±0.7^{a}
TH	82.6±5.5^{ab}	2.00±0.14	1.36±0.12^{ab}	1.60±0.13	83.6±4.9	81.9±11.5	81.1±1.1^{b}	95.1±0.3^{b}
KH	86.6±5.2^{b}	2.11±0.08	1.31±0.08^{a}	1.68±0.09	85.9±5.8	86.7±5.9	80.3±0.8^{b}	95.5±0.2^{b}

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

[1]Final body weight (g)

[2]Specific growth rate (%) = [(ln final body weight - ln initial body weight) / days] × 100

[3]Feed conversion ratio = dry feed fed / wet weight gain

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

[5]Feed intake = dry feed consumed (g) / fish

[6]Apparent digestibility coefficient of dry matter (%)

Table 6.4. Whole-body composition of red sea bream and olive flounder fed the experimental diets for 12 and 9 weeks, respectively.

	Dry matter (%)	Protein (% DM)	Lipid (% DM)	Ash (% DM)
Red sea bream				
Con	35.3±0.6	51.4±1.3	33.8±0.4	14.3±1.2
TH	35.9±0.9	49.9±2.1	35.6±1.4	13.9±0.8
KH	36.3±0.8	49.9±1.8	34.3±1.1	12.8±1.2
Olive flounder				
Con	26.2±0.8	70.3±1.6	15.3±1.2	13.6±0.5
TH	26.5±0.4	70.3±1.4	15.0±1.4	13.2±0.5
KH	26.6±0.2	69.7±0.6	16.8±0.2	13.0±0.6

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

Table 6.5. Plasma biochemical indices of red sea bream and olive flounder fed the experimental diets for 12 and 9 weeks, respectively.

	Ht[1]	Hg[2]	Glu[3]	TP[4]	Cho[5]	TG[6]
Red sea bream						
Con	39.1±1.9	7.67±1.20	55.1±9.3	4.33±0.50	370±48	386±89
TH	42.9±2.5	7.90±0.54	55.7±3.8	4.60±0.34	377±34	380±37
KH	42.1±1.5	7.43±0.54	53.7±3.3	4.61±0.53	372±53	395±27
Olive flounder						
Con	25.9±3.8	4.21±0.69	42.4±6.5	3.41±0.19	271±61	412±53
TH	27.5±2.9	3.83±0.35	41.8±6.0	3.90±0.15	229±22	307±68
KH	29.1±4.7	4.35±0.48	39.6±5.3	3.87±0.18	226±35	372±51

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

[1]Haematocrit

[2]Haemoglobin

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

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

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

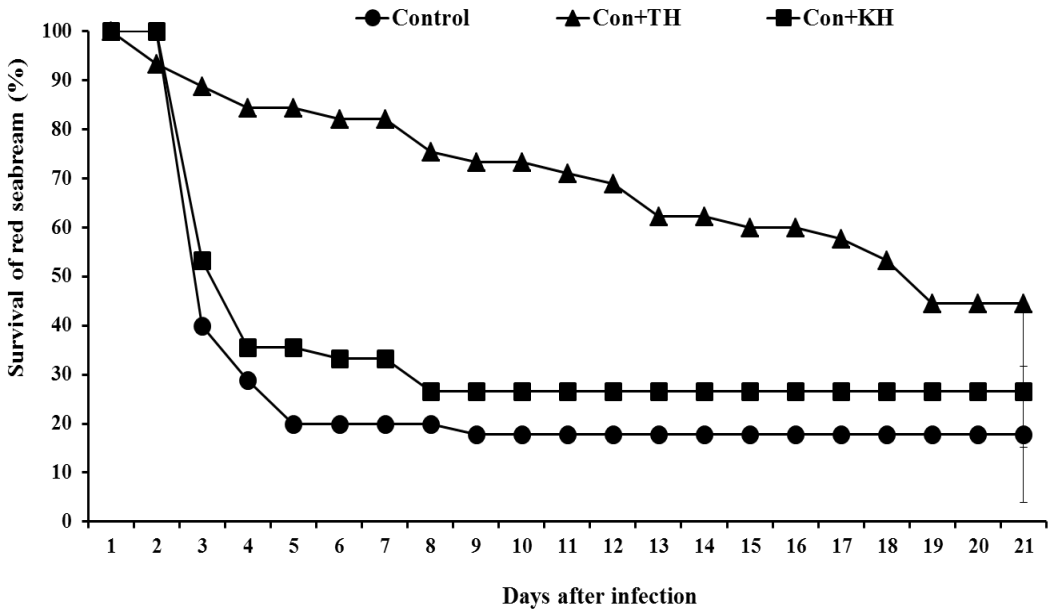
[6]Triglyceride (mg dL^{-1})

Table 6.6. Non-specific immune response of red sea bream and olive flounder fed the experimental diets for 12 and 9 weeks, respectively.

	NBT[1]	Ig[2]	Lysozyme[3]	MPO[4]	SOD[5]	Antiprotease[6]
Red seabream						
Con	1.12±0.03[a]	15.4±1.4	10.9±0.7[a]	1.26±0.15	30.6±4.3	28.7±1.9
TH	1.31±0.08[b]	17.3±1.1	12.6±0.6[b]	1.43±0.10	31.7±1.8	33.9±2.8
KH	1.23±0.06[ab]	17.6±0.9	12.9±0.7[b]	1.50±0.10	32.5±2.6	30.9±2.1
Olive flounder						
Con	0.47±0.01[a]	12.5±0.5	18.4±2.0[a]	1.71±0.17	42.5±4.4[a]	16.1±2.7
TH	0.55±0.04[ab]	14.9±0.5	26.9±3.1[b]	2.12±0.29	49.4±4.4[ab]	22.7±4.3
KH	0.60±0.03[b]	15.4±2.0	25.7±4.7[ab]	2.23±0.10	53.7±3.6[b]	22.5±2.1

Values are mean of triplicate groups and presented as mean ± SD. Values with different superscripts in the same column are significantly different ($P < 0.05$). The lack of the superscript letter indicates no significant differences among treatments.
[1]Nitro blue tetrazolium activity
[2]Immunoglobulin (mg ml^{-1})
[3]Lysozyme activity (μg ml^{-1})
[4]Myeloperoxidase level
[5]Superoxide dismutase (% inhibition)
[6]Antiprotease (% inhibition)

a)



b)

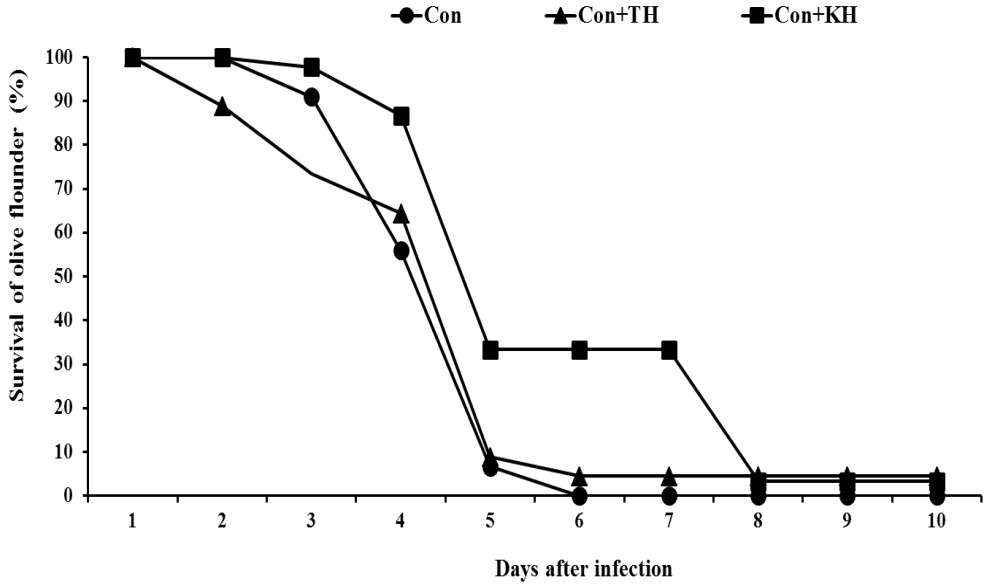


Figure 6.1. Survival rate of red sea bream (a) and olive flounder (b) fed the experimental diets after challenge with *Edwardsiella tarda*.

CONCLUSION

CONCLUSION

Chapter 2:

- The tested protein hydrolysates can be used as a valuable protein source by at least 10% of fish meal protein repralcement in juvenile flounder diet.
- Dietary inclusion of the krill hydrolysate (KH) can positively affect the fish growth performance and feed utilization efficiency as well as diet digestibility.
- Immune function of the fish was significantly improved in goups offered the KH diet.
- Dietary treatment has no significant effect on fish disease resistance against bacterial infection caused by *Edwardsiella tarda*.

Chapter 3:

- These findings may offer convincing evidence that 50% of FM protein could be substituted by SPC and PHs blend without any adverse impact on juvenile olive flounder growth performance and health/welfare condition, when compared to the fish fed the high FM diet.
- Dietary inclusion of the tested PHs, in exchange for FM, also improved the innate immune responses of fish which is most probably responsible for the enhanced disease resistance of fish against bacterial infection caused by *E. tarda*, which was depressed in fish fed the LFM diet.
- These results, also, suggest that KH, apparently, is the most effective dietary supplement for this species than two other tested PHs.

Chapter 4:

- The study showed that dietary inclusion of the tested protein hydrolysates in low FM diets for red sea bream improves the feed nutritional value resulting in enhanced growth performance, non-specific immune response and disease resistance.
- SPC could replace up to 50% of the FM by the supplementation of the tested protein hydrolysates, particularly SH.

Chapter 5

- The highest growth performance and feed utilization efficiency were observed in flounder fed 4.5% dietary shrimp hydrolysate which were comparable to those of the HFM group.
- The significant improvement of liver IGF-I mRNA expression by increment of dietary SH level in this study further confirmed that supplementation of appropriate level of SH in diets for olive flounder would enhance IGF-I production, which is fundamentally involved in growth regulation.
- In contrast, fish fed the highest tilapia hydrolysate level exhibited lower growth performance and feed utilization efficiency than other inclusion levels, even though the difference was not significant.
- Based on the fish growth performance, shrimp hydrolysate seemed to be the most effective dietary supplement for juvenile olive flounder whereas tilapia hydrolysate,

apparently, might be the best choice for induction of the innate immune response causing enhanced disease resistance.

Chapter 6:

- This chapter indicates that coating the fish meal-based diets with KH can improve growth performance of both red sea bream and olive flounder juveniles.
- KH and TH can be used as potential stimulator of innate immunity in both fish species.

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