Thesis for Degree of Master of Fisheries Science

Development of feed additives by using fisheries by-products in juvenile Nile tilapia, *Oreochromis niloticus* and olive flounder, *Paralichthys olivaceus*

by

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Advisors: Prof. Sungchul C. Bai

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A dissertation

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요 약

치어기 틸라피아와 넙치에 있어서 수산가공부산물에 이노신산(IMP)첨가와 가공 과정의 pH 조절을 통한 양어사료용 첨가제 개발가능성을 확인하기 위해 수행되었다. 실험 1 에서는, 4.9 ± 0.07g (mean ± SD) 인 치어기 틸라피아를 8 주간 사육실험하였다. 총 6 개의 사료구는 대조구 (CON)와 수산가공부산물 각각 2%의 100% 새우가공부산물 (SSE), 98% SSE + 2% IMP ($SSEP_2$), 96% SSE + 4% IMP ($SSEP_4$), 100% 오징어가공부산물 (SQSE) 그리고 100% 틸라피아가공부산물 (TSE)를 대조구사료 내 대두박과 다른 사료원 2%를 대체하여 제작하였다. 실험 2 에서는, 13.4 ± 0.13g (mean ± SD) 인 치어기 넙치를 9 주간 사육실험하였다. 총 5 개의 사료구는 대조구 (CON)와 수산가공부산물 각각 2%의 100% SSE at pH 2 (SSEL), 100% SSE at pH 4 (SSEH), 95% SSE at pH 2 + 5% IMP (SSELP) 그리고 95% SSE at pH 4 + 5% IMP (SSEHP)를 대조구사료 내 Lysine 과 다른 사료원 2%를 대체하여 2x2 factorial design 으로 제작하였다. 사육실험 종료 후, 실험 1 에서는 IMP 가 첨가되지 않은 SSE 와 4% IMP 가 첨가된 $SSEP_4$ 의 수산가공부산물 첨가가 치어기 틸라피아에 있어 성장과 면역반응에 효과를 보이는 것으로 판단되었다. 실험 2 에서는 가공 과정의 pH 조절에 상관없이 IMP 가 첨가되지 않은 SSEL 및 SSEH 와 가공 과정의 pH 가 2 와 4 로 조절 되고 5% IMP 가 첨가된 SSELP 및 SSEHP 의 수산가공부산물 첨가가 치어기 넙치에 있어 SSEL 및 SSEH 실험구는 성장에 효과를 보였으며, SSELP 및 SSEHP 실험구는 면역반응에 효과를 보이는 것으로 판단되었다.

실험 1 : 치어기 틸라피아에 있어서 사료내 3 가지 수산가공부산물과 이노신산(IMP)이 첨가된 양어사료용 첨가제 개발 가능성 평가

총 8 주간의 본 실험은 치어기 틸라피아에 있어서 사료 내 3 가지 수산가공부산물과 그 중 이노신산(IMP)이 첨가된 양어사료용 첨가제 개발 가능성을 평가하기 위해 수행되었다. 실험어는 4.9 ± 0.07g (mean ± SD) 인 치어기 틸라피아를 대상으로 3 반복으로 한 탱크당 20 미씩 사육하였다. 총 6 개의 사료구는 대조구(CON)와 수산가공부산물 각각 2%의 100% 새우가공부산물 (SSE), 98% SSE + 2% IMP ($SSEP_2$), 96% SSE + 4% IMP (SSEP4), 100% 오징어가공부산물 (SQSE) 그리고 100% 틸라피아가공부산물 (TSE)를 대조구사료 내 대두박과 다른 사료원 2%를 대체하여 제작하였다. 8 주간의 사육 실험 종료 후, 증체율 일간성장률에 있어서 SSE, $SSEP_2$ 그리고 SSEP 실험구는 CON 실험구와 SQSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 하지만, SSE, $SSEP_2$ 그리고 $SSEP_4$ 실험구는 TSE 실험구와 유의적인 차이를 보이지 않았다 ($P > 0.05$). Myeloperoxidase (MPO)활성에 있어서, SSE 그리고 $SSEP_4$ 실험구는 CON, $SSEP_2$ 그리고 SQSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 하지만, SSE 그리고 $SSEP_4$ 실험구는 TSE 실험구와는 유의적인 차이를 보이지 않았다 ($P > 0.05$). Superoxide dismutase (SOD)활성에 있어서도, 결과 SSE 그리고 $SSEP_4$ 실험구는 CON, $SSEP_2$, SQSE 그리고 TSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 또한, SOD 활성에서 $SSEP_2$ 그리고 TSE 실험구는 CON 그리고 SQSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 공격실험으로 *Aremonas hydrophila* ($1x10^7$cfu/ml)를 복강주사한 후 10 일 동안 누적생존율을 조사해본 결과, SSE 실험구가 CON, SQSE 그리고 TSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 또한, $SSEP_2$ 실험구는 SQSE 그리고 TSE 실험구보다 유의적으로 높은 값을 나타내었다 ($P < 0.05$). 하지만, CON, $SSEP_4$, SQSE 그리고 TSE 실험구에서는 유의적인 차이를 보이지 않았다 ($P > 0.05$). 따라서, IMP 가 첨가되지 않은 SSE 와 4% IMP 가 첨가된 $SSEP_4$의 수산가공부산물 첨가가 치어기 틸라피아에 있어 성장과 면역반응에 효과를 보이는 것으로 판단되었다.

실험 2 : 치어기 넙치에 있어서 새우가공부산물에 이노신산(IMP)첨가와 가공 과정의 pH 조절을 통한 양어사료용 첨가제 개발가능성 평가

총 9 주간의 본 실험은 치어기 넙치에 있어서 수산가공부산물에 이노신산(IMP) 첨가와 가공 과정의 pH 조절을 통한 양어사료용 첨가제 개발가능성을 확인하기 위해 수행되었다. 실험어는 13.4 ± 0.13g (mean ± SD) 인 치어기 넙치를 대상으로 3 반복으로 한 탱크당 15 미씩 사육하였다. 총 5 개의 사료구는 대조구 (CON)와 수산가공부산물 각각 2%의 100% SSE at pH 2 (SSEL), 100% SSE at pH 4 (SSEH), 95% SSE at pH 2 + 5% IMP (SSELP) 그리고 95% SSE at pH 4 + 5% IMP (SSEHP)를 대조구사료 내 Lysine 과 다른 사료원 2%를 대체하여 2x2 factorial design 으로 제작하였다. 9 주간의 사육 실험 종료 후, 증체율 일간성장률에 있어서 SSEL 그리고 SSEH 실험구가 CON, SSELP 그리고 SSEHP 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). 사료효율과 단백질 전환효율에 있어서 SSEL, SSEH, SSELP 그리고 SSEHP 실험구는 CON 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). 하지만, SSEL, SSEH, SSELP 그리고 SSEHP 실험구는 서로 유의적인 차이를 보이지 않았다 (P > 0.05). Myeloperoxidase (MPO)활성에 있어서, SSEHP 실험구는 CON, SSEL 그리고 SSEH 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). Superoxide dismutase (SOD)활성에 있어서도, SSEHP 실험구는 CON, SSEL 그리고 SSEH 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). 하지만, SSELP 와 SSEHP 실험구는 유의적인 차이를 보이지 않았다 (P > 0.05). Lysozyme 활성에 있어서, SSELP 그리고 SSEHP 실험구가 CON, SSEL 그리고 SSEH 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). 공격실험으로 *Edwardsiella tarda* ($1x10^7$cfu/ml)를 복강주사한 후 9 일 동안 누적생존율을 조사해본 결과, SSEHP 실험구는 SSEH 그리고 SSELP 실험구보다 유의적으로 높은 값을 나타내었다 (P < 0.05). 하지만, CON 와 SSEL 실험구는 유의적인 차이를 보이지 않았다 (P > 0.05). 따라서, 가공 과정의 pH 조절에 상관없이 IMP 가 첨가되지 않은 SSEL 및 SSEH 와 가공 과정의 pH 가 2 와 4 로 조절 되고 5% IMP 가 첨가된 SSELP 및 SSEHP 의 수산가공부산물 첨가가 치어기 넙치에 있어 SSEL 및 SSEH 실험구는 성장에 효과를 보였으며, SSELP 및 SSEHP 실험구는 면역반응에 효과를 보이는 것으로 판단되었다.

Development of feed additives by using fisheries by-products in juvenile Nile tilapia, *Oreochromis niloticus* and olive flounder, *Paralichthys olivaceus*

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Abstract

Two experiments were conducted to investigate the effects of dietary supplementation of different fisheries by-product extracts with and without inosine momophosphate (IMP) on growth performance and non-specific immune responses in juvenile Nile tilapia and juvenile olive flounder. In the first experiment, juvenile Nile tilapia averaging 4.9 ± 0.07g (mean ± SD) were fed one of the six experimental diets; A basal diet without feed additives was used as control (CON), the other five experimental diets were formulated to include 2% each of 100% shrimp soluble extract (SSE), 98% shrimp soluble extract + 2% IMP ($SSEP_2$), 96% shrimp soluble extract + 4% IMP ($SSEP_4$), 100% squid soluble extract (SQSE) and 100% tilapia soluble extract (TSE) replacing 2% of soybean meal and other ingredients from CON diet. In the second experiment, juvenile olive flounder averaging 13.4 ± 0.13g (mean ± SD) were fed one of the five experimental diets. A basal diet without feed additives was used as control (CON), the other four diets were formulated to include 2% each of 100% SSE processed at low pH (SSEL), 100% SSE processed at high pH (SSEH), 95% SSE + 5% IMP processed in low pH (SSELP) and 95% SSE + 5% IMP processed in high pH (SSEHP) replacing 2% of wheat flour and other ingredients from

CON diet. The first experiment's results that supplementation shrimp soluble extract without (SSE) and with 4% IMP ($SSEP_4$) as feed additives could have beneficial effects on growth performance and immune responses in juvenile Nile tilapia. The second experiment's results indicated that supplementation of shrimp soluble extract produced in low or high pH without (SSEL and SSEH) and with IMP (SSELP and SSEHP) could have beneficial effects on growth performance by SSEL and SSEH and on immune responses by SSELP and SSEHP in juvenile olive flounder.

Experiment 1.

Effects of three fisheries by-product extracts on growth performance and non-specific immune responses in juvenile Nile tilapia, *Oreochromis niloticus*

An 8-week feeding trial was conducted to investigate the effects of dietary supplementation of three different fisheries by-product extracts as the feed additives in juvenile Nile tilapia, *Oreochromis niloticus*. Twenty fish averaging 4.9 ± 0.07 g (mean ± SD) were randomly distributed into 18 rectangular 30-L volume tanks and fed one of the six experimental diets. A basal diet without feed additives was used as control (CON), other five diets were formulated to include 2% each of 100% shrimp soluble extract (SSE), 98% shrimp soluble extract + 2% inosine monophosphate ($SSEP_2$), 96% shrimp soluble extract + 4% inosine monophosphate ($SSEP_4$), 100% squid soluble extract (SQSE) and 100% tilapia soluble extract (TSE) replacing 2% of soybean meal and other ingredients from CON diet. After the feeding trial, weight gain and specific growth rate of fish fed SSE, $SSEP_2$ and $SSEP_4$ diets were significantly higher than those of fish fed CON and SQSE diets (P<0.05), and however, there were no significant differences among fish fed SSE, $SSEP_2$, $SSEP_4$ and TSE diets (P>0.05). Myeloperoxidase activities of fish fed SSE and $SSEP_4$ diets were significantly higher than those of fish fed CON, $SSEP_2$ and SQSE diets (P<0.05), and however, there were no significant differences in among fish fed SSE, $SSEP_4$ and TSE diets (P>0.05). Superoxide dismutase activities (SOD) of fish fed SSE and $SSEP_4$ diets were significantly higher than those of fish fed CON, $SSEP_2$, SQSE and TSE diets (P<0.05). Also, SOD of fish fed $SSEP_2$ and TSE diets were significantly higher than those of fish fed CON and SQSE diets (P<0.05). In challenge test with *Aeromonas hydrophila* for 10 days, cumulative survival rate (CSR) of fish fed SSE diet were significantly higher than those of fish fed CON, SQSE and TSE diets (P<0.05). Also, CSR of fish fed $SSEP_2$ diet were significantly higher than those of fish fed SQSE and TSE diets (P<0.05), and however, there were no significant differences in CSR among fish fed CON, $SSEP_4$, SQSE and TSE diets (P>0.05). Therefore, these results indicated that supplementation of shrimp soluble extract without (SSE) or with inosine monophosphate ($SSEP_4$) as feed additives could have beneficial effects on growth performance and non-specific immune responses of juvenile Nile tilapia.

Experiment 2.

Effects of dietary shrimp soluble extract produced by high/low pH with and without additional inosine monophosphate in juvenile olive flounder, *Paralichthys olivaceus*

A nine-week feeding trial was conducted to investigate the effects of dietary supplementation of shrimp soluble extract (SSE) produced by high/low pH with and without inosine monophosphate (IMP) as feed additives in juvenile olive flounder, *Paralichthys olivaceus*. Fifteen fish averaging 13.4 ± 0.13g (mean ± SD) were randomly distributed into 18 rectangular 45-L volume tanks and fed one of the five experimental diets. A basal diet without feed additives was used as control (CON), and 2x2 factorial designed with the other four diets were formulated to include 2% each of 100% SSE processed at low pH (SSEL), 100% SSE processed at high pH (SSEH), 95% SSE + 5% IMP processed in low pH (SSELP) and 95% SSE + 5% IMP processed in high pH (SSEHP) replacing 2% of wheat flour and other ingredients from CON diet. After the feeding trial, weight gain and specific growth rate of fish fed SSEL and SSEH diets were significantly higher than those of fish fed CON, SSELP and SSEHP diets ($P < 0.05$). Feed efficiency and protein efficiency ratio of fish fed SSEL, SSEH, SSELP and SSEHP diets were significantly higher than fish fed CON diet ($P<0.05$), however, there were no significant differences among fish fed SSEL, SSEH, SSELP and SSEHP diets ($P>0.05$). Myeloperoxidase activities of fish fed SSEHP diet were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P<0.05$). Superoxide dismutase activities of fish fed SSEHP diet were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P < 0.05$), however, there were no significant differences among fish fed SSELP and SSEHP diets ($P>0.05$). Lysozyme activities of fish fed SSELP and SSEHP diets were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P<0.05$). In challenge test with *Edwardsiella tarda* for 9 days, cumulative survival rate (CSR) of fish fed SSEHP diet was significantly higher than those of fish fed SSEH and SSELP diets ($P<0.05$), however, there were no significant differences in CSR among fish fed CON and SSEL diets ($P>0.05$). Therefore, these results indicated that supplementation of feed by shrimp soluble extract produced in low or high pH (SSEL and SSEH) without IMP could have beneficial effects on growth performance without positive immune responses. However, supplementation of IMP (SSELP and SSEHP) could have beneficial effects on immune responses without positive growth performance in juvenile olive flounder.

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I. Introduction

Aquaculture is one of the fastest growing animal-producing sectors now supplying nearly 50% of the world's food fish. The use of dietary additives in fish feeds is one of the methods commonly used to improve weight gain, feed efficiency, and disease resistance in cultured fish. Marine by-products additives of the contain valuable nutrients that can be utilized as functional ingredients in feed industry. Studies have reported the beneficial effects of 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). With the aim of using these by-products, enzymatic hydrolysis has been employed as one of the promising ways for conversion of fish by-products into acceptable forms with improved quality and functional characteristics (Shahidi., 1994; Gildberg., 1993; Chalamaiah et al., 2012). Also, based on their low molecular weight compounds and well-balanced amino acid profile, hydrolysates have been used as chemo-attractant and fish meal replacer in aquafeeds (Aksnes et al., 2006a, 2006b; Cahu et al., 2001; Kolkovski et al., 2000; Refstie et al., 2004). And 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 (Hevroy et al., 2005; Kolkovski et al., 2000; Refstie et al., 2004). In this point of view, marine by-product like species can also be considered as potential ingredients for fish feed additives.

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). Shrimp head and shell generally contain good percentage of protein with balanced amino acid profile and minerals like Ca, P, Na and Zn (Ibrahim et al., 1999). Shrimp waste hydrolysates produced under controlled conditions yield desirable functional properties, high nutritive value and reduced bitterness (Kristinsson et al., 2000). And,

the shrimp soluble extract (SSE) has high levels of amino acids and active peptides that are highly digestible and absorbable for animals (Gildberg et al., 2001; Aksnes et al., 2006). Also, use of shrimp soluble extract could reduce environmental problems that are caused by the improper dumping of the inedible parts of shrimp, such as head, shell, and tail (Heu et al., 2003). Recovery of the shrimp waste by enzymatic hydrolysis results in the formation of biologically active peptides with pharmaceutical and growth-stimulating properties (Gildberg et al., 2001).

Tilapia processing industries generate a huge quantity of by-product having potentially high nutritional and functional values. Tilapia protein hydrolysate is a desirable source of essential amino acids and minerals suggesting it as a potential ingredient in aquafeeds (Foh et al., 2011). 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).

In the same way, the level of protein in squid-processing by product is high enough for proteolytic hydrolysis for the generation of peptides and free amino acids. It also possesses most of the amino acids essential to the growth and survival of fish (Jobling M., 1998). At this high-protein level, the most viable approach to the full utilization of squid by product would be a bioconversion into hydrolysate as an aquaculture nutrient additive (Lee et al., 2008). And use of squid hydrolysate has feed attractant and stimulant properties and improved growth and survival (Lian et al., 2003a, 2003b).

Nucleotides are the base units for DNA and RNA synthesis during cell construction, provide energy for normal cellular process and are therefore essential to growth and development (Vanburen et al., 1994). Over the last 35 years, the roles of nucleotides and their related products in fish diets have been sparingly studied as functional nutrients (Li et al., 2006; Hossain et al., 2016a, b). However, dietary supplementations of nucleotides or nucleosides have been shown to benefit many mammalian physiological and nutritional functions (Carver., 1994; Hasko et al., 2000; Quan., 1992; Uauy., 1989). Inosine monophosphate (IMP), a nucleoside monophosphate, is the first compound formed during the synthesis of purine. IMP may be involved in cell signaling pathways as well as serve as nutrients for biosynthesis. Numerous studies on different

aquatic species already reported that dietary supplementation of inosine or IMP, either alone or in combination with certain free amino acids, significantly improves the growth rate of fish (Lin et al., 2009; Hossain et al., 2016a, 2016b) and it can also improve disease resistance and immune responses such as lysozyme activity, myloperoxidase activity and nitro-blue-tetrazolium activity of Japanese flounder, (*Paralichthys olivaceus*) (Song et al., 2012). Such biological activities could find application in new development of fish feed for higher performances.

Nile tilapia, *Oreochromis niloticus* has become iconic fresh water cultured fish species around the world by contributing a global production of 3.9million MT in 2015 (FAO., 2017). The tilapia is an omnivorous species that has a digestive system that differs both from that of carnivorous and many herbivorous fish (Albino et al., 2009). It uses a wide spectrum of foods (Sklan et al., 2004a), efficiently uses dietary carbohydrates (Boscolo et al., 2002) and has a great ability to digest plant protein (Olvera-Novoa et al., 2002; Shelton et al., 2006; Gatlin et al., 2007). Also, Nile tilapia is one of the most economically important species in aquaculture to culture because of its rapid growth, good survival in high density culture and disease tolerance (El-Sayed., 2006).

Olive flounder, *Paralichthys olivaceus* is a carnivorous fish and is one of the most important marine finfish for aquaculture in East Asian countries such as Korea, Japan and China. The aquaculture of olive flounder started from the late 1980s in Korea (Kim et al., 2002). In 2016, the production of olive flounder in Korea is approximately 41,620 metric tons and maximum aquaculture production is nearly 55,000 tons in 2009 (KOSTAT., 2017). Accordingly, many feeding trials have been performed to determine dietary nutrient requirements (Lee et al. 2000a, 2002), the optimum feeding frequency (Lee et al. 2000b), alternative protein sources for fish meal (Kikuchi et al., 1994, 1997; Kikuchi., 1999), and the best feeding strategy (Cho et al., 2005; Cho et al. 2006c).

In this study, the effects of dietary fisheries by-product with and without additional inosine monophosphate at process pH in juvenile Nile tilapia and olive flounder were determined for growth performance and non-specific immune responses.

II. Effects of dietary soluble extracts additive on growth performance and non-specific immune responses in juvenile Nile tilapia, *Oreochromis niloticus*

Materials and Methods

Experimental diets

Fisheries by-products tested in this study were provided by VNF Company (Vietanm Food Joint Stock Company), Ca Mau, Vietnam. Fisheries by-products production process in divided into the following key stages. Heads collected from seafood processors are processed to eliminate extraneous matter, then cut and pressed to obtain liquid extract. The shell leftovers are used to produce chitin and glucosamine. This liquid extract will go through centrifugal processing to acquire its purest form. The extract is then sent to chemical processing area where the protein will be broken into peptides and digestible single protein (amino acid) such as: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, cystein, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine. Many among these amino acids are widely used as growth stimulants for livestock and aquaculture (aspartic acid, threonine, methionine, lysine). Chemically processed extract is densified to create products that will meet various levels of quality standards according to customer demands. Densified soluble is mixed and added with flavor-preservation additives, to maintain the product's unique scent. Amino acid profile of the fisheries by-product have been provided in Tables 1 respectively.

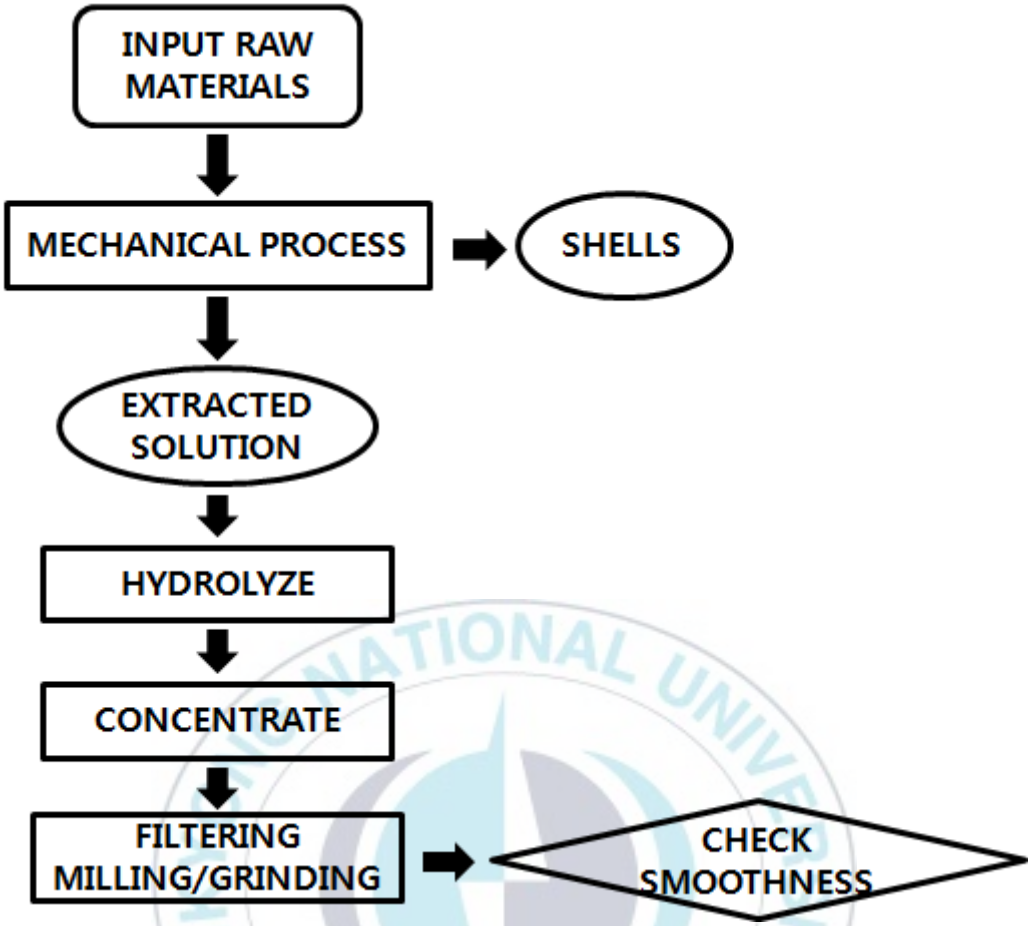

Fig 1. Fisheries by-products production process

Ingredients and proximate composition of the six experimental diets are shown in Table 2. Six experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (33%) and gross energy (kcal energy/kg). The amino acid compositions of all experimental diets are presented in Table 3. A basal diet without feed additives was used as control (CON), other five diets were formulated to include 2% each of 100% shrimp soluble extract (SSE), 98% shrimp soluble extract + 2% inosine monophosphate ($SSEP_2$), 96% shrimp soluble extract + 4% inosine monophosphate ($SSEP_4$), 100% squid soluble extract (SQSE) and 100% tilapia soluble extract (TSE) replacing 2% of soybean meal and other ingredients from CON diet. Fish meal, soybean meal, rapeseed meal, mate and bone meal, poultry offal meal and squid liver powder were used as the protein sources, soybean oil and fish oil as the lipid source, and wheat flour as the carbohydrate

source in the experimental diets. Pellets were air-dried for 48-96 h, broken and sieved to achieve the desired particle size and stored at -20℃ until use.

Table 1. Free amino acid contents of the fisheries by-products (% of wet matter basis)

Amino acids	Fisheries by-products				
	100% SSE	98% SSE + 2% IMP	96% SSE + 4% IMP	100% SQSE	100% TSE
Alanine	1.33	1.25	1.27	0.62	1.43
Arginine	0.61	0.52	0.62	0.60	1.24
Aspartic acid	1.42	1.26	1.28	0.90	1.81
Glutamic acid	1.97	1.78	1.89	1.06	2.71
Glycine	0.94	1.02	1.49	0.64	1.95
Histidine	1.03	0.68	0.95	0.68	0.99
Isoleucine	0.78	0.70	0.74	0.49	0.76
Valine	0.97	0.87	0.91	0.51	0.91
Leucine	1.12	1.02	1.07	0.70	1.30
Lysine	0.93	0.86	0.88	0.56	1.42
Phenylalanine	0.71	0.62	0.67	0.37	0.72
Proline	0.63	0.60	0.64	0.53	1.15
Serine	0.46	0.42	0.46	0.38	0.77
Threonine	0.53	0.48	0.51	0.42	0.79
Tyrosine	0.24	0.26	0.24	0.08	0.21

Table 2. Composition and proximate analysis of the basal diet for juvenile Nile tilapia (% of dry matter basis)

Ingredients	%
Fish meal (Tuna)[1]	8.00
Soybean meal[1]	34.6
Wheat flour[1]	34.99
Rapeseed meal[1]	10.0
Meat and bone meal[1]	2.00
Poultry offal meal[1]	2.00
Squid liver powder[1]	2.00
Soybean oil[1]	1.30
Fish oil[1]	2.00
Others[2]	3.11
Proximate analysis (% of DM basis)	
Moisture	9.42
Crude protein	33.3
Crude lipid	5.20
Crude ash	8.02

[1] CJ CheilJedang Co. Seoul, Korea.

[2] Protide (nucleotide by product), C-IMP (inosine monophosphate), phos mono, fish mineral, protase, vitamin C, koking tocopherol and koking choline

Table 3. Amino acid contents of the juvenile Nile tilapia experimental diets (% of dry matter basis)

	Diets					
	CON	SSE	$SSEP_2$	$SSEP_4$	SQSE	TSE
Alanine	1.60	1.65	1.64	1.67	1.59	1.65
Arginine	2.09	2.18	2.11	2.15	2.16	2.11
Aspartic acid	3.20	3.30	3.13	3.31	3.10	3.22
Glutamic acid	6.54	6.74	6.52	6.74	6.49	6.62
Glycine	1.79	1.85	1.80	1.85	1.76	1.65
Histidine	1.01	1.06	1.03	1.09	1.12	1.02
Isoleucine	1.48	1.52	1.49	1.54	1.49	1.55
Valine	1.68	1.69	1.67	1.73	1.64	1.74
Leucine	2.43	2.50	2.45	2.52	2.44	2.48
Lysine	1.82	1.88	1.90	1.88	1.85	1.90
Phenylalanine	1.54	1.59	1.52	1.59	1.56	1.56
Proline	2.07	1.98	1.65	2.09	2.16	2.11
Serine	1.44	1.55	1.50	1.53	1.49	1.39
Threonine	1.25	1.30	1.27	1.31	1.27	1.25
Tyrosine	0.89	0.93	0.92	0.94	0.94	0.89
Total	30.84	31.72	30.60	31.93	31.04	31.31

Experimental fish and feeding trial

This experiment was conducted at the Institute of Fisheries Sciences, Pukyong National University, Busan, Korea, and juvenile Nile tilapia were obtained from a private hatchery (Docheon Aquafarm, Changnyeong, Republic of Korea) and fed a commercial diet for two weeks to be acclimated to the experimental conditions and facilities. Three hundred sixty fish averaging 4.9 ± 0.07 g (mean ± SD) were weighed and randomly distributed into 18 indoor tanks (20 fish/tank) with a 30-L volume receiving a constant flow (0.8~1.0 L/min) of fresh water. Each tank was then assigned randomly to one of the three replicates of the six dietary treatments. Fish were fed twice daily (09:00 and 18:00 h) for 8 wk to apparent satiation. Throughout the experimental period, the water temperature and pH were maintained at 27 ± 0.5℃ and 7.5 ± 0.3, respectively. And supplemental aeration was provided to maintain the dissolved oxygen near saturation.

Sample collection and analysis

At the end of the feeding trial, fish were starved for 24h, and the total number and weight of fish in each tank were determined for calculation of initial body weight, final body weight, weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival. Three fish per tank were randomly sampled, individually weighed, and then dissected to obtain liver and viscera samples for determination of hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF), respectively (Yoo et al. 2007; Kim et al. 2014).

Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

Specific growth rate (SGR, %/day) = (loge final wt. - loge initial wt.) × 100 / days

Feed Efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

Survival rate (%) = (total fish - dead fish) × 100 / total fish

Protein efficiency ratio (PER) = (wet weight gain / protein intake)

Daily feed efficiency (DFI, %) = specific growth rate × 100 / feed efficiency

Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt

Visceralsomatic index (VSI, %) = viscera wt. × 100 / body wt

Condition factor = (wet weight / total length^3) × 100

Three additional fish per tank were randomly captured and anesthetized with ethylene glycol phenyl ether (200mg/L) and blood samples were obtained from the caudal vein using 1 mL disposable syringe without anticoagulant. The blood sample was separated by centrifugation (5000 x g) for 10 min. Then, the serum was stored at -70℃ for later analysis of plasma glucose, total cholesterol and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan). Another set of blood samples of the same fish were allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 x g for 10 min and stored at -70℃ for the analysis of non-specific immune responses including lysozyme, superoxide dismutase (SOD) and myeloperoxidase (MPO) activities.

Analyses of moisture, crude protein, lipid, and ash of whole-body samples and experimental diets were performed using standard methods (AOAC 1995). Samples of diets and fish were dried to constant weights at 105℃ to determine their moisture content. Ash was determined by incineration at 550℃, crude lipid was determined by Soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and crude protein content was determined by the Kheldahl method (N x 6.25) after acid digestion. The plasma glucose, total cholesterol and activities of AST and ALT were measured by a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

Myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997). Briefly, 20 μL of serum was diluted with HBSS (Hanks balanced salt solution) without Ca^{2+} or Mg^{2+} (Sigma-Aldrich) in 96-well plates. Then, 35 μL o f 3.3'5.5'- tetramethlybenzidine hydrochloride (TMB, 20 mM) (Sigma-Aldrich) and H_2O_2 (5 mM) was 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 micro-plate reader.

Superoxide dismutase (SOD) activity was measured by the superoxide radical based on reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) in accordance with the procedure of products. Each endpoint assay was observed by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 effect with superoxide) and after 20 minutes of reaction time at 37°C. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg.

A turbidimetric assay was used for determination of serum lysozyme level by the method described by Hultmark et al. (1980) with slight modification. Briefly, *Micrococcus lysodeikticus* (0.75 mg/mL) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200μL 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 570nm after incubation at room temperature for 0 and 30 min in a microplate reader (UNM340, Biochrom, Cambridge, UK).

Challenge test

Challenge test a bacterial pathogen, *Aeromonas hydrophila*, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Fish (n = 5 per tank) were distributed according to their dietary treatment groups into 50 L aquarium for the challenge test with

no water exchange. Fish were injected intraperitoneally with 0.1 mL of culture suspension of pathogenic *A. hydrophila* containing 1×10^8 CFU/mL. Fish mortality was recorded daily for 10 days.

Statistical Analysis

After confirming normality and homogeneity of variance, data were analyzed by one-way ANOVA using SAS version 9.1 (SAS Institute, Cary, NC, USA). LSD's multiple range test LSD was used to compare significant differences among the treatments diets at $P < 0.05$ significance.

Results

Table 4 and Figures 2-6 shows the growth performance and survival rate of juvenile Nile tilapia fed different experimental diets for 8 weeks. weight gain (WG) and specific growth rate (SGR) of fish fed SSE, $SSEP_2$ and $SSEP_4$ diets were significantly higher than those of fish fed CON and SQSE diets ($P < 0.05$), however, there were no significant differences among fish fed SSE, $SSEP_2$, $SSEP_4$ and TSE diets ($P > 0.05$). Feed efficiency (FE) and Protein efficiency ratio (PER) were no significant differences in of fish fed all experimental diets ($P > 0.05$). Daily feed intake (DFI) of fish fed SSE diet was significantly higher than those of fish fed CON diet ($P < 0.05$), however, there was no significant differences among fish fed SSE, $SSEP_2$, $SSEP_4$, SQSE and TSE diets ($P > 0.05$). There were no significant differences in Hematosomatic index, Viscerosomatic index and Condition factor among the treatments. Also, there were no significant differences in survival rate of fish fed all experimental diets ($P > 0.05$). There were no significant ($P > 0.05$) differences in whole-body proximate composition of fish in all experimental groups (Table 5). Table 6 shows hematological parameters of juvenile Nile tilapia fed different experimental diets. There were no significant differences in Aspartate aminotransferase, Alanine aminotransferase, glucose and total cholesterol contents of fish fed experimental diets. Table 7 and Figures 7-9 shows the Myeloperoxidase (MPO), superoxide dismutase (SOD) and lysozyme activities of fish fed different experimental diets for 8 weeks. MPO activities of fish fed SSE and $SSEP_4$ diets were significantly higher than those of fish fed CON, $SSEP_2$ and SQSE diets ($P < 0.05$), however, there were no significant differences in among fish fed SSE, $SSEP_4$ and TSE diets ($P > 0.05$). SOD of fish fed SSE and $SSEP_4$ diets were significantly higher than those of fish fed CON, $SSEP_2$, SQSE and TSE diets ($P < 0.05$). Also, SOD of fish fed $SSEP_2$ and TSE diets were significantly higher than those of fish fed CON and SQSE diets ($P < 0.05$). Lysozyme of fish fed $SSEP_4$ and SQSE diets were significantly higher than those of fish fed SSE and $SSEP_2$ diets ($P < 0.05$), however, there were no significant differences in among fish fed CON, SSEP4, SQSE and TSE diets ($P > 0.05$).

Challenge test

Mortality was initially observed in all fish groups at 1 day after *A. hydrophila* infection (Fig. 10). At the end of day 1, the cumulative survival rate (CSR) of fish fed the CON, SSE, $SSEP_2$ and $SSEP_4$ diets were significantly higher than TSE diet ($P < 0.05$). However, there was no significant differences in CSR among fish fed CON, SSE, $SSEP_2$, $SSEP_4$ and SQSE diets ($P > 0.05$). At day 10, CSR of fish fed SSE diet were significantly higher than those of fish fed CON, SQSE and TSE diets ($P < 0.05$). Also, CSR of fish fed $SSEP_2$ diet was significantly higher than those of fish fed SQSE and TSE diets ($P < 0.05$). However, there was no significant differences in CSR among fish fed CON, $SSEP_4$, SQSE and TSE diets ($P > 0.05$).

Table 4. Growth performance, feed efficiency and organosomatic indices of juvenile Nile tilapia fed the experimental diets for 8 weeks[1]

	Diets						Pooled SEM[12]
	CON	SSE	$SSEP_2$	$SSEP_4$	SQSE	TSE	
IBW[2]	4.9	4.8	4.9	4.9	4.9	4.9	0.02
FBW[3]	17.2	17.6	17.6	17.8	17.4	17.5	0.09
WG (%)[4]	254^{b}	264^{a}	265^{a}	265^{a}	253^{b}	257^{ab}	1.56
SGR (%/day)[5]	2.63^{b}	2.69^{a}	2.70^{a}	2.70^{a}	2.63^{b}	2.65^{ab}	0.01
FE (%)[6]	76.9	71.4	75.0	74.2	72.9	72.6	0.77
PER[7]	2.31	2.14	2.28	2.23	2.25	2.25	0.02
DFI (%)[8]	3.43^{b}	3.77^{a}	3.60^{ab}	3.64^{ab}	3.61^{ab}	3.66^{ab}	0.04
HSI (%)[9]	0.69	0.74	0.72	0.73	0.71	0.71	0.04
VSI (%)[10]	6.68	6.77	6.64	6.71	6.66	6.75	0.21
CF[11]	1.53	1.56	1.55	1.53	1.55	1.55	0.04

[1] Values are means from triplicate groups of shrimp where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] Initial body weight (g).

[3] Final body weight (g).

[4] Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

[5] Feed efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

[6] Specific growth rate (SGR, %) = ($\log_e$ final wt. - $\log_e$ initial wt.) × 100 / days

[7] Protein efficiency ratio (PER) = (wet weight gain / protein intake)

[8] Daily feed intake (DFI, %) = specific growth rate × 100 / feed efficiency

[9] Hematosonatic index (HSI, %) = liver wt. × 100 / body wt.

[10] Visceralsomatic index (VSI, %) = viscera wt. x 100 / body wt.

[11] Condition factor = (wet weight / total length^3) × 100

[12] Pooled standard error of mean : SD/√n

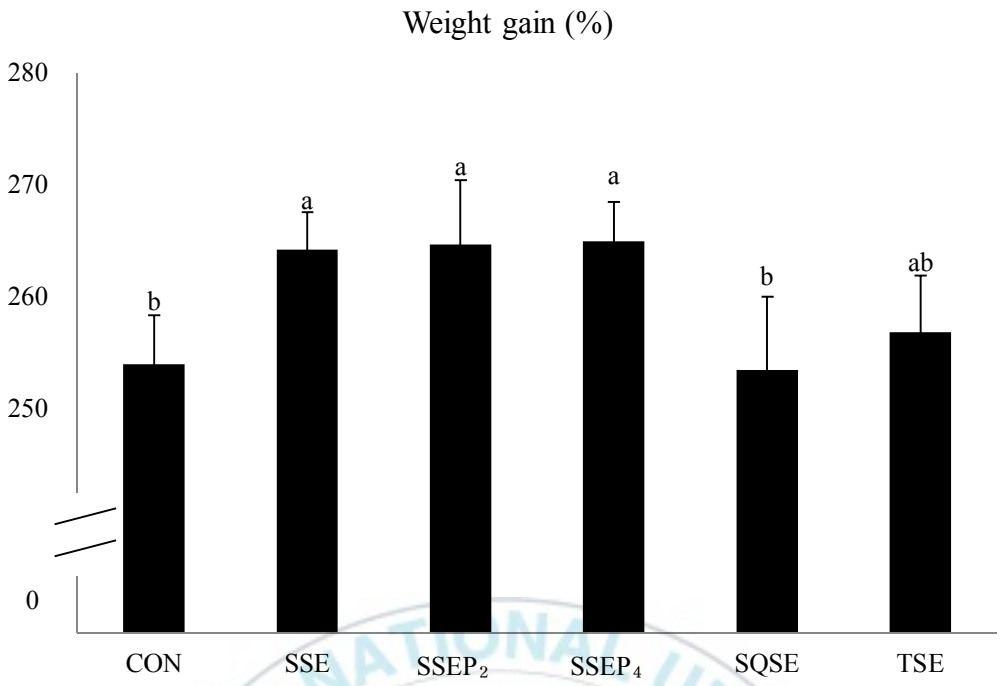

Fig.2. Weight gain of juvenile Nile tilapia fed the experimental diets for 8 weeks.

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

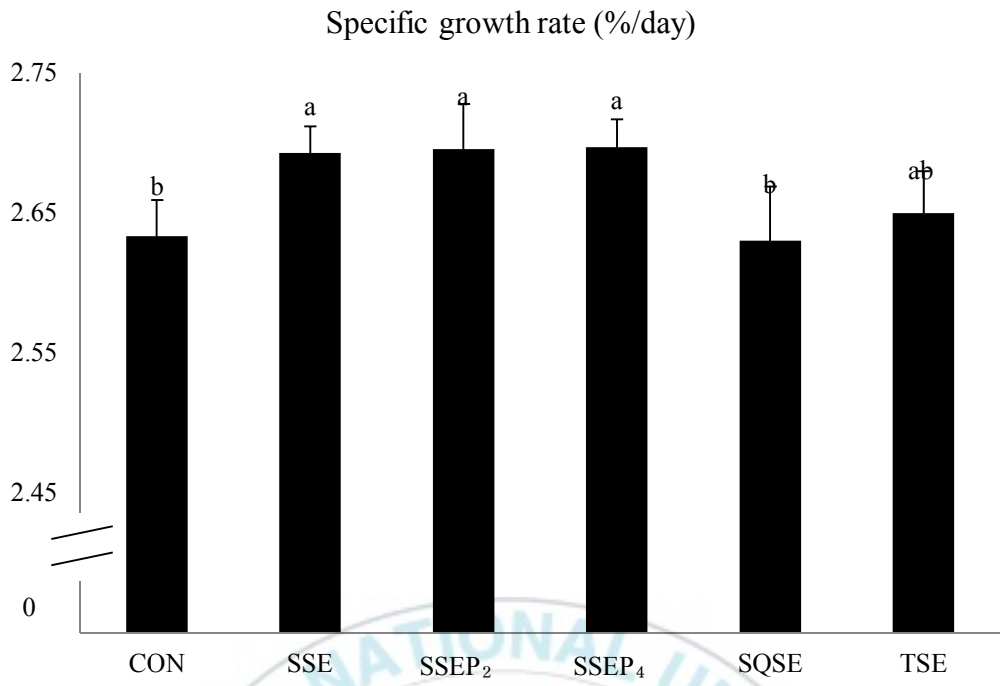

Fig.3. Specific growth rate of juvenile Nile tilapia fed the experimental diets for 8 weeks.

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

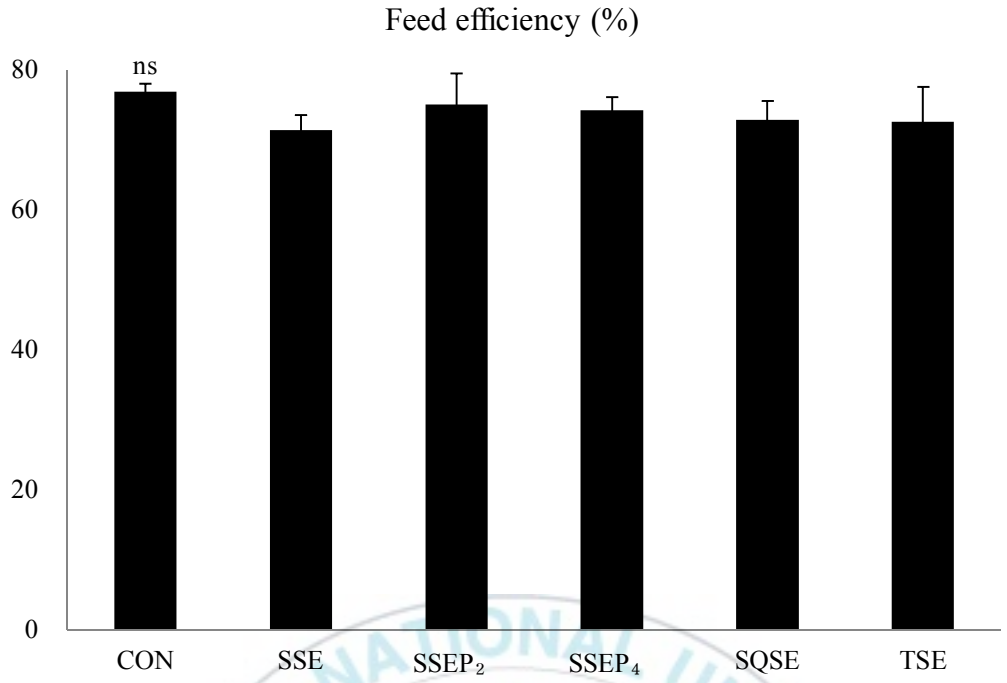

Fig.4. Feed efficiency of juvenile Nile tilapia fed the experimental diets for 8 weeks.

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

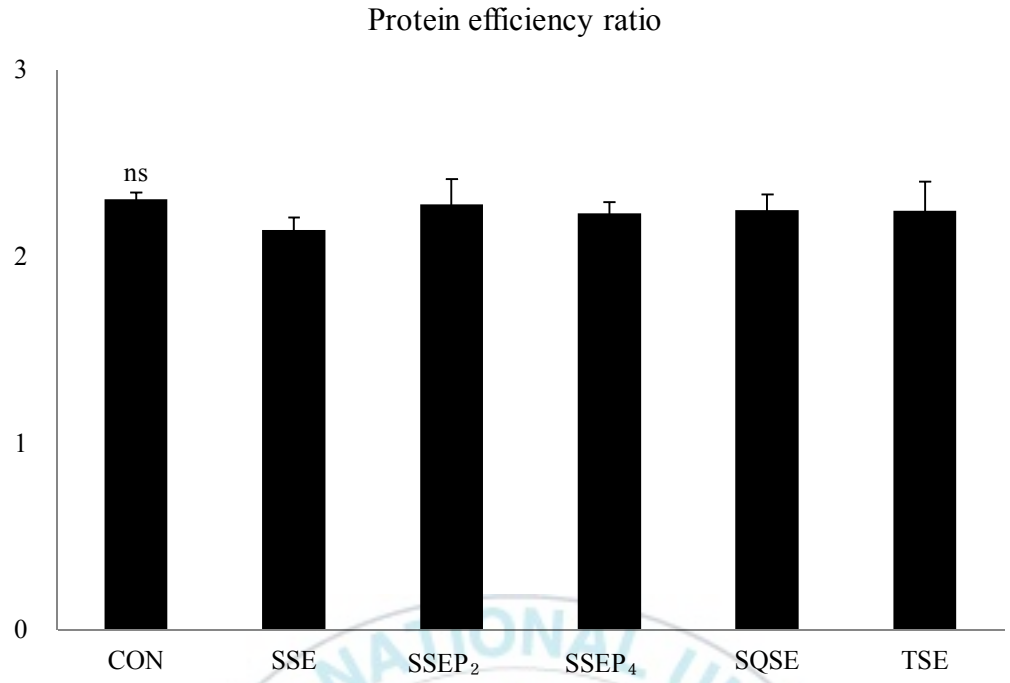

Fig.5. Protein efficiency ratio of juvenile Nile tilapia fed the experimental diets for 8 weeks.

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

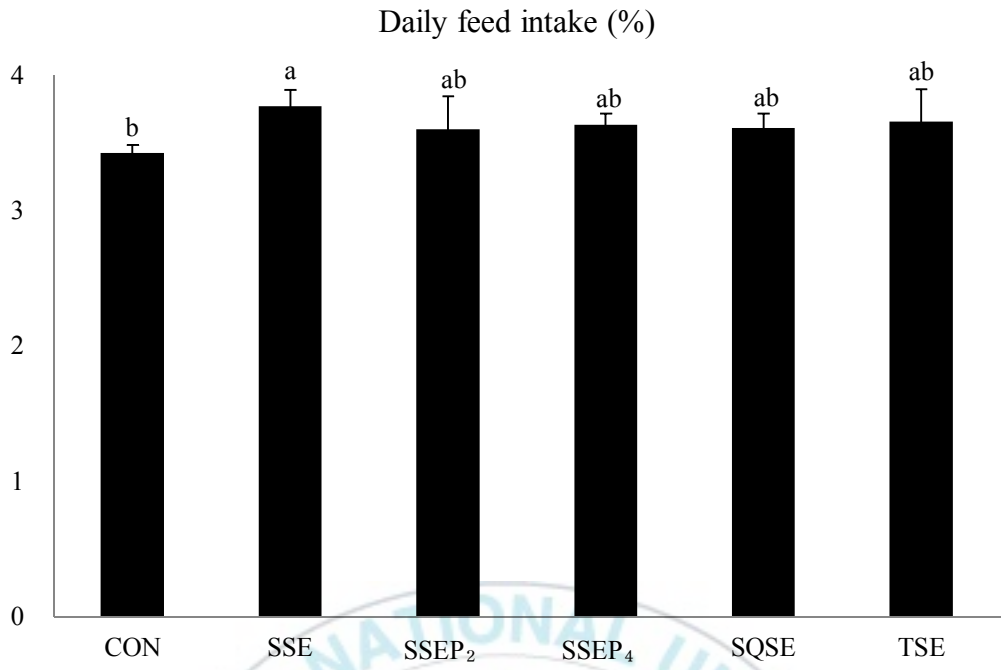

Fig.6. Daily feed intake of juvenile Nile tilapia fed the experimental diets for 8 weeks.

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

Table 5. Whole-body proximate composition of juvenile Nile tilapia fed the experimental diets for 8 weeks (%, dry matter basis)[1]

	Diets						Pooled SEM[2]
	CON	SSE	$SSEP_2$	$SSEP_4$	SQSE	TSE	
Moisture (%)	6.86[ns]	5.87	4.90	7.00	6.44	6.61	0.38
Crude protein (%)	57.7[ns]	57.7	58.4	57.2	58.7	58.0	0.44
Crude lipid (%)	24.1[ns]	24.4	23.7	25.4	25.2	23.3	0.67
Crude ash (%)	16.7[ns]	16.8	17.9	17.2	17.4	16.6	0.36

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] Pooled standard error of mean : SD/√n

Table 6. Hematological parameters of juvenile Nile tilapia fed the experimental diets for 8 weeks[1]

	Diets						Pooled SEM[4]
	CON	SSE	$SSEP_2$	$SSEP_4$	SQSE	TSE	
AST (U/L)[2]	76.0	77.0	75.0	75.0	75.0	74.0	0.6
ALT (U/L)[3]	3.0	2.0	3.0	2.7	2.7	2.7	0.2
Glucose (mg/dl)	44.3	43.0	45.3	44.0	43.0	43.0	0.3
T-cholesterol (mg/dl)	224	224	225	224	224	225	0.3

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] AST (U/L) : Aspartate aminotransferase.

[3] ALT (U/L) : Alanine animotransferase.

[4] Pooled standard error of mean : SD/√n

Table 7. Non-specific immune responses of juvenile Nile tilapia fed the experimental diets for 8 weeks[1]

	Diets						Pooled SEM[4]
	CON	SSE	$SSEP_2$	$SSEP_4$	SQSE	TSE	
MPO[2]	0.98^{c}	1.09^{a}	0.96^{c}	1.09^{a}	1.04^{b}	1.06^{ab}	0.01
SOD[3]	66.5^{d}	88.3^{a}	77.6^{b}	89.2^{a}	70.5^{c}	76.2^{b}	2.08
Lysozyme (U/ml)	0.82^{ab}	0.78^{b}	0.61^{c}	0.90^{a}	0.88^{a}	0.86^{ab}	0.03

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] MPO (absorbance) : Myeloperoxidase

[3] SOD (% inhibition): Superoxide dismutase

[4] Pooled standard error of mean : SD/√n

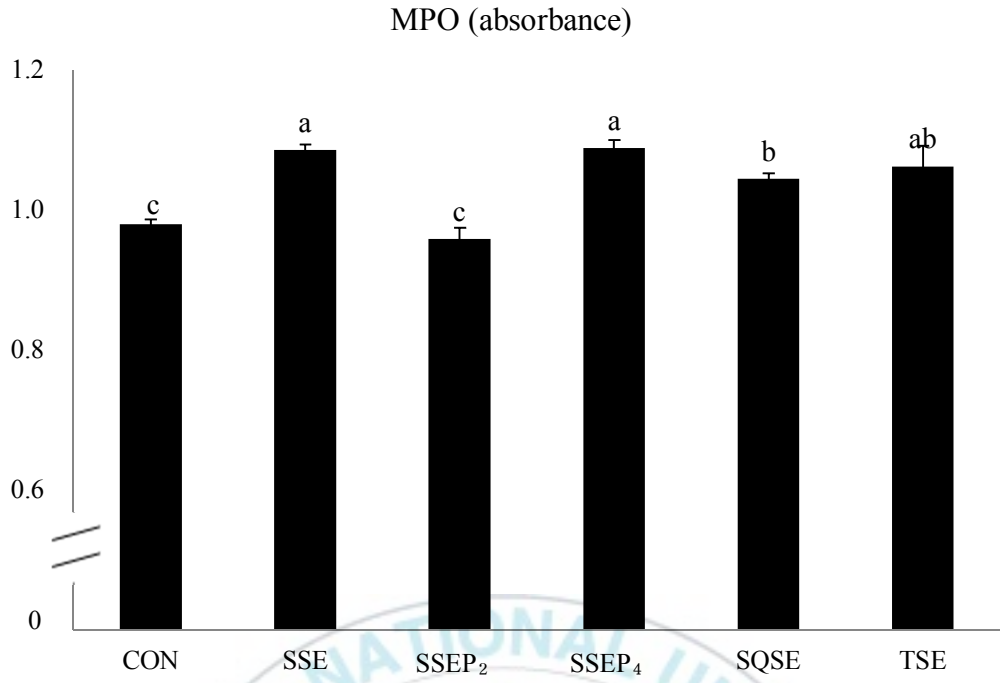

Fig.7. Myeloperoxidase (MPO) in juvenile Nile tilapia fed the experimental diets for 8 weeks

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

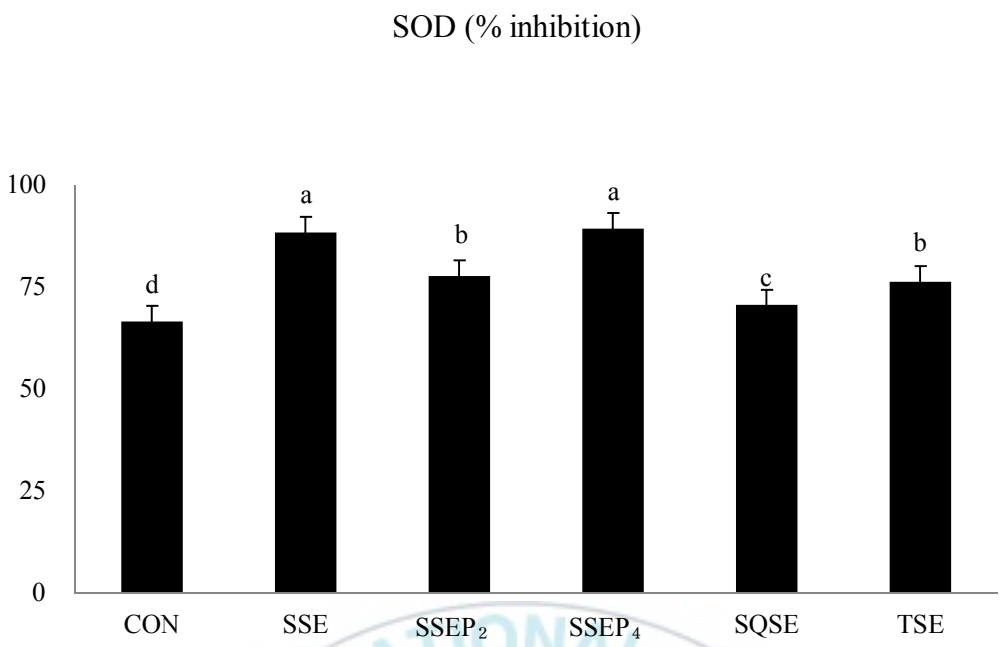

Fig.8. Superoxide dismutase (SOD) in juvenile Nile tilapia fed the experimental diets for 8 weeks

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

Lysozyme (U/ml)

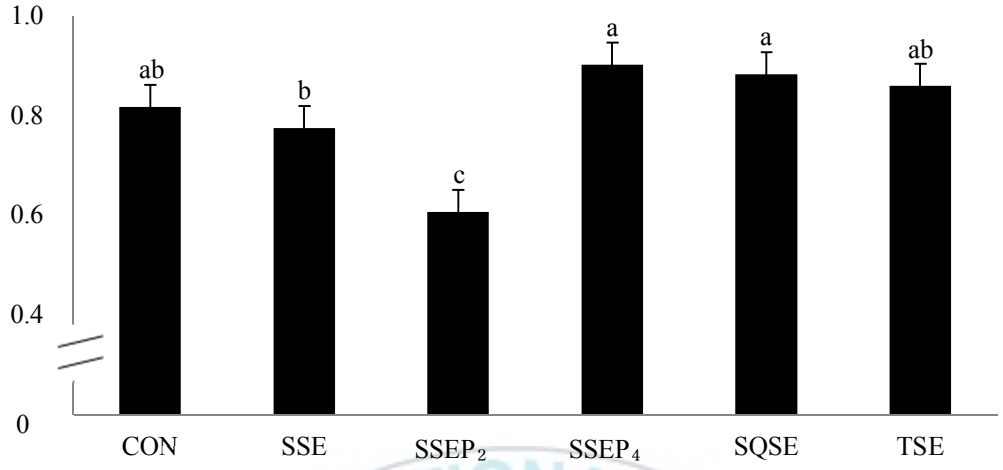

Fig.9. Lysozyme (U/ml) in juvenile Nile tilapia fed the experimental diets for 8 weeks

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

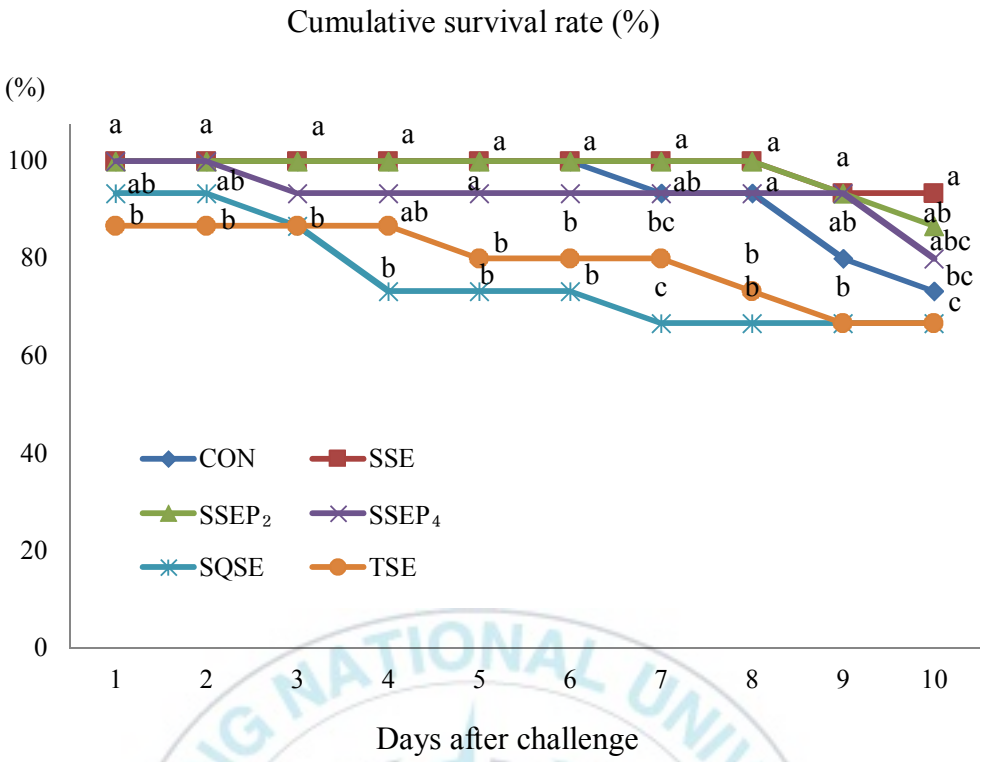

Fig.10. Cumulative survival after intraperitoneal injection with *A. hydrophila* in six experimental groups of juvenile Nile tilapia fed diets. Each value represents mean ± SE (n=3). Different letters are significantly ($P < 0.05$) different by LSD test

CON - Basal diet (0 % of soluble extract)

SSE – 2% of 100% shrimp soluble extract

$SSEP_2$ – 2% of 98% shrimp soluble extract + 2% inosine monophospate

$SSEP_4$ – 2% of 96% shrimp soluble extract + 4% inosine monophospate

SQSE – 2% of 100% squid soluble extract

TSE – 2% of 100% tilapia soluble extract

Discussion

Result of this study showed that fish fed the SSE, $SSEP_2$ and $SSEP_4$ diets showed a significantly higher growth performance. Consistent with our observations, some studies reported the use of shrimp soluble extract that showed better growth performances in Nile tilapia (Albino., 2009). Shrimp soluble extract is replaced with alternative protein sources, adverse effects related to deficiencies of certain essential amino acids. Fish nutritionists have supplemented the diet with amino acids to improve growth performance (Hardy., 2010). This study use of top-coated protein sources with supplementation of soluble extract. (Plascencia-Jatomea et al., 2002) concluded that shrimp protein silage could be included in tilapia diets at concentrations as high as 15%, improving fish growth rate. The experimental amino acid composition of all experimental diets appeared to meet the requirement levels that were reported for growing Nile tilapia (NRC., 2011). The essential amino acid requirements for juvenile Nile tilapia have been known, for lysine 1.10 to 1.90 % of the diet, for methionine 0.15 % to 1.35 % of the diet (in the presence of 0.02% of cystein) (NRC., 2011). (Santiago et al., 1988) experiment showed weight gains analyzed by the broken line regression method indicated the following requirements as a percentage of the dietary protein: lysine, 5.12; arginine, 4.20; histidine, 1.72; valine, 2.80; leucine, 3.39; isoleucine, 3.11; threonine, 3.75; tryptophan, 1.00; methionine with cystine (0.54% of the protein), 3.21; and phenylalanine with tyrosine (1.79% of the protein), 5.54. Consistent with our observations, some studies reported the supplement of protein hydrolysates that showed better growth performances in fish and abalone. (Bautista-Teruel et al., 2003; Zhu et al., 2011; Burr et al., 2012; Kader et al., 2012; Jo et al., 2016). In other studies, it has been demonstrated that supplementation of blended protein sources and SSE had a beneficial effect on growth performance in Malabar grouper, *Epinephelus malabaricus* (Li et al., 2009), and red sea bream, *Pagrus major* (Khosravi et al., 2015), respectively. Hematological parameters are useful indicators for evaluating the physiological and health status (Maita., 2007). In the biochemical parameters, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose and total cholesterol were found to be not much affected by dietary supplements of fisheries by-products. And

blood enzymes such as ALT and AST are known to be health markers of the animal's physiological condition (Ozgur et al., 2010). It is also known that these markers are sensitive indicators for tissue damage (De la Tore et al., 2000). Other researchers have reported that the blood parameters of fish are not affected by the dietary substitution of alternative protein sources for fishmeal (Cho et al., 2005; Jeon et al., 2014; Lee et al., 2012). The non-specific defense mechanism of fish include neutrophil activation, the production of peroxidase, oxidative radicals, and initiation of other inflammatory factors (Ainsworth et al. 1991). And they have an important role in the immune function of teleost fish (Irianto et al., 2002). In this study, SOD and MPO activities of fish fed the SSE and $SSEP_4$ diets were significantly higher than those of fish fed other diets. Enhancement of SOD provides further evidence for earlier in vitro studies that found production of superoxide anion was stimulated by peptides from fish protein hydrolysate in Atlantic salmon leucocytes (Gildberg et al., 1996). The MPO is an important enzyme having microbicidal activity, utilize one of the oxidative radicals (H_2O_2) to produce hypochlorous acid. The enzymes such as SOD and MPO activities play significant roles *in vivo* due to their antioxidant function, and their elevated expression and activities are indication of oxidative stress (Yonar., 2012). A similar tendency was observed in our study, indicating the enhancement of the fish immune system by inclusion of SSE (Jo et al., 2016). These effects of SSE are mainly attributed to their bioactive peptide contents that have antioxidative, antimicrobical, and immunomoculatory acivities (He et al., 2013). (Khosravi et al., 2015) showed that inclusion of 3% shrimp hydrolysate enhanced the lysozyme activity in diets of the sea bream. These effects of SSE are mainly attributed to their bioactive peptide contents that have antioxidative, antimicrobial, and immunomodulatory activities (He et al., 2013). Dietary nucleotides have most recently received considerable attention as immunomodulating compounds for various fish species; however, these compounds also may actually influence diet intake of fish. Inosine and inosine 5'-monophosphate have been effective in improving diet consumption by different fish species (Gatlin et al., 2007). Numerous studies on humans and animals have reported that dietary supplementation of nucleotides has positive influence on growth performance, immune responses and disease resistance (Carver., 1995; 1990; Devresse., 2000). Dietary supplementation of other nucleotides also showed an

improvement in growth performance of different fish species such as grouper (*Epinephelus malabaricus)*, rainbow trout, (*Oncorhynchus mykiss)*, Atlantic salmon, (*Salmo salar)* and red sea bream, (*Pagrus major)* (Burrells et al., 2001; Lin et al., 2009; Tahmasebi-Kohyani et al., 2011; Hossain et al., 2016b). In consistent with our findings, recent studies also reported that dietary supplementation of inosine monophosphate (IMP) improved growth of red sea bream (*Pagrus major*) (Hossain et al., 2016a) and olive flounder (*Paralichthys olivaceus*) (Song et al., 2012). In conclusion, this study indicated that supplementation of shrimp soluble extract without (SSE) or with inosine monophosphate ($SSEP_4$) as feed additives could have beneficial effects on growth performance and non-specific immune responses of juvenile Nile tilapia.

III. Effects of dietary shrimp soluble extract produced by high/low pH with and without additional inosine monophosphate in juvenile olive flounder, *Paralichthys olivaceus*

Materials and Methods

Experimental diets

Shrimp soluble extract source tested in this study were provided by VNF Company (Vietanm Food Joint Stock Company), Ca Mau, Vietnam. Shrimp soluble extract production process in divided into the following key stages. Shrimp heads collected from seafood processors are processed to eliminate extraneous matter, then cut and pressed to obtain liquid extract. The shell leftovers are used to produce chitin and glucosamine. This liquid extract will go through centrifugal processing to acquire its purest form. The extract is then sent to chemical processing area where the protein will be broken into peptides and digestible single protein (amino acid) such as: aspartic acid, threonine, serine, glutamic acid, proline, glycine, alanine, valine, cystein, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine, histidine, arginine. Many among these amino acids are widely used as growth stimulants for livestock and aquaculture (aspartic acid, threonine, methionine, lysine). Chemically processed extract is densified to create products that will meet various levels of quality standards according to customer demands. Densified soluble is mixed and added with flavor-preservation additives, to maintain the product's unique scent. Amino acid profile of the shrimp soluble extract have been provided in Tables 8 respectively.

Ingredients and proximate composition of the six experimental diets are shown in Table 9. Six experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (53%) and gross energy (kcal energy/kg). The amino acid compositions of all experimental diets are presented in Table 10. A basal diet without feed additives was used as control (CON), and 2x2 factorial designed with the other four diets were formulated to include 2% each of 100% SSE processed at low pH (SSEL), 100% SSE processed at high pH (SSEH), 95% SSE + 5% IMP processed in low pH (SSELP) and 95% SSE + 5% IMP processed in high pH (SSEHP) replacing 2% of wheat flour and other ingredients from CON diet. Fish meal, meat and bone meal, poultry by-product meal, blood meal and squid liver powder were used as the protein sources, fish oil as the lipid source, and wheat flour and starch as the carbohydrate source in the experimental diets. Pellets were air-dried for 48-96 h, broken and sieved to achieve the desired particle size and stored at -20℃ until use.

Table 8. Free amino acid contents of the fisheries by-products (% of wet matter basis)

Amino acids	Fisheries by-products			
	100% SSEL	100% SSEH	95% SSEL + 5% IMP	95% SSEH + 5% IMP
Alanine	1.35	1.44	1.26	1.49
Arginine	0.61	1.18	0.56	1.22
Aspartic acid	1.41	2.13	1.32	2.17
Glutamic acid	1.99	2.79	1.83	2.89
Glycine	0.96	1.28	1.64	2.11
Histidine	0.71	0.96	0.93	1.14
Isoleucine	0.78	0.92	0.72	0.97
Valine	0.97	1.10	0.91	1.15
Leucine	1.11	1.42	1.04	1.49
Lysine	0.94	1.36	0.87	1.42
Phenylalanine	0.69	0.93	0.64	0.98
Proline	0.66	0.97	0.61	1.02
Serine	0.45	0.73	0.43	0.73
Threonine	0.54	0.81	0.50	0.82
Tyrosine	0.20	0.45	0.22	0.40

Table 9. Composition and proximate analysis of the basal diet for juvenile olive flounder (% of dry matter basis)

Ingredients	%
Fish meal (Sardin)[1]	50.0
Fish meal (Tuna) [1]	10.0
Meat and bone meal[1]	5.00
Polutry by-product meal[1]	5.00
Blood meal[1]	2.00
Squid liver powder[1]	2.00
Starch(Tapioca) [1]	4.00
Wheat flour[1]	12.5
Fish oil[1`]	2.00
Others[2]	7.48
Proximate analysis (% of DM basis)	
Moisture	12.8
Crude protein	52.7
Crude lipid	11.3
Crude ash	9.33

[1] CJ CheilJedang Co. Seoul, Korea.

[2] Protide (nucelotide by product), fish mineral, koking tocopherol, vitamin C and premier vitamin A, Lysine, Methionine

Table 10. Amino acid contents of the juvenile olive flounder experimental diets (% of dry matter basis)

	Diets					
	CONT	SSEL	SSEH	SSELP	SSEHP	SQSE
Alanine	3.47	3.87	3.71	3.72	3.77	3.75
Arginine	3.07	3.46	3.27	3.35	3.31	3.28
Aspartic acid	5.27	5.85	5.59	5.43	5.68	5.58
Glutamic acid	7.44	8.33	7.90	8.02	8.13	8.01
Glycine	3.61	3.79	3.68	3.69	3.76	3.79
Histidine	1.86	2.13	2.01	1.98	2.02	1.99
Isoleucine	2.24	2.60	2.47	2.54	2.56	2.49
Valine	2.77	3.24	3.07	3.09	3.15	3.11
Leucine	3.95	4.50	4.36	4.33	4.40	4.35
Lysine	4.31	4.80	4.63	4.67	4.75	4.70
Phenylalanine	2.17	2.47	2.40	2.41	2.42	2.40
Proline	2.89	2.30	3.26	2.81	3.05	3.17
Serine	2.08	2.29	2.17	2.19	2.11	2.08
Threonine	2.21	2.46	2.36	2.37	2.36	2.32
Tyrocine	1.32	1.49	1.50	1.50	1.40	1.43
Total	48.6	53.59	52.38	52.1	52.8	52.4

Experimental fish and feeding trial

This experiment was conducted at the Institute of Fisheries Sciences, Pukyong National Universilty, Busasn, Korea, and juvenile olive flounder were obtained from a private hatchery (Hwang-geum Aquafarm, Goheung, Republic of Korea) and fed a commercial diet for two weeks to be acclimated to the experimental conditions and facilities. Two hundred seventy fish averaging 13.4 ± 0.13 g (mean ± SD) were weighed and randomly distributed into 18 indoor tanks (15 fish/tank) with a 45-L volume receiving a constant flow (0.8~1.0 L/min) of sea water. Each tank was then assigned randomly to one of the three replicates of the six dietary treatments. Fish were fed twice daily (09:00 and 16:00 h) for 9 wk to apparent satiation. Throughout the experimental period, the water temperature and pH were maintained at 18 ± 1℃ and 7.5 ± 0.3, respectively. And supplemental aeration was provided to maintain the dissolved oxygen near saturation.

Sample collection and analysis

At the end of the feeding trial, fish were starved for 24 h, and the total number and weight of fish in each tank were determined for calculation of initial body weight, final body weight, weight gain (WG), specific growth rate (SGR), feed efficiency (FE), protein efficiency ratio (PER), and survival. Three fish per tank were randomly sampled, individually weighed, and then dissected to obtain liver and viscera samples for determination of hepatosomatic index (HSI), viscerosomatic index (VSI) and condition factor (CF), respectively (Yoo et al., 2007; Kim et al., 2014).

Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

Specific growth rate (SGR, %/day) = (loge final wt. - loge initial wt.) × 100 / days

Feed Efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

Survival rate (%) = (total fish - dead fish) × 100 / total fish

Protein efficiency ratio (PER) = (wet weight gain / protein intake)

Daily feed intake (DFI, %) = specific growth rate × 100 / feed efficiency

Hepatosomatic index (HSI, %) = liver wt. × 100 / body wt

Visceralsomatic index (VSI, %) = viscera wt. x 100 / body wt

Condition factor = (wet weight / total length^3) × 100

Three additional fish per tank were randomly captured and anesthetized with ethylene glycol phenyl ether (200mg/L) and blood samples were obtained from the caudal vein using 1 mL disposable syringe without anticoagulant. The blood sample was separated by centrifugation (5000 x g) for 10 min. Then, the serum was stored at -70℃ for later analysis of plasma glucose, total cholesterol and activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured by a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan). Another set of blood samples of the same fish were allowed to clot at room temperature for 30 min. Then, the serum was separated by centrifugation at 5000 x g for 10 min and stored at -70℃ for the analysis of non-specific immune responses including lysozyme, superoxide dismutase (SOD) and myeloperoxidase (MPO) activities.

Analyses of moisture, crude protein, lipid, and ash of whole-body samples and experimental diets were performed using standard methods (AOAC 1995). Samples of diets and fish were dried to constant weights at 105℃ to determine their moisture content. Ash was determined by incineration at 550℃, crude lipid was determined by Soxhlet extraction using the Soxtec system 1046 (Tecator AB, Hoganas, Sweden), and crude protein content was determined by the Kheldahl method (N x 6.25) after acid digestion. The plasma glucose, total cholesterol and activities of AST and ALT were measured by a chemical analyzer (Fuji DRI-CHEM3500i, Fuji Photo Film, Ltd., Tokyo, Japan).

Myeloperoxidase (MPO) activity was measured according to Quade and Roth (1997). Briefly, 20 µL of serum was diluted with HBSS (Hanks balanced salt solution) without Ca^{2+} or Mg^{2+} (Sigma-Aldrich) in 96-well plates. Then, 35 µL o f 3.3'5.5'- tetramethlybenzidine hydrochloride (TMB, 20 mM) (Sigma-Aldrich) and H_2O_2 (5 mM) was 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 micro-plate reader.

Superoxide dismutase (SOD) activity was measured by the superoxide radical based on reaction inhibition rate of enzyme with WST-1 (Water Soluble Tetrazolium dye) substrate and xanthine oxidase using the SOD Assay Kit (Sigma-Aldrich, 19160) in accordance with the procedure of products. Each endpoint assay was observed by absorbance at 450 nm (the absorbance wavelength for the colored product of WST-1 effect with superoxide) and after 20 minutes of reaction time at 37°C. The percent inhibition was normalized by mg protein and expressed as SOD unit/mg.

A turbidimetric assay was used for determination of serum lysozyme level by the method described by Hultmark et al. (1980) with slight modification. Briefly, *Micrococcus lysodeikticus* (0.75 mg/mL) was suspended in sodium phosphate buffer (0.1 M, pH 6.4), 200µL 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 570nm after incubation at room temperature for 0 and 30 min in a microplate reader (UNM340, Biochrom, Cambridge, UK).

Challenge test

Challenge test a bacterial pathogen, *Edwardsiella tarda*, was obtained from the Department of Biotechnology, Pukyong National University, Busan, Republic of Korea. Fish (n = 5 per tank) were distributed according to their dietary treatment groups into 50 L aquarium for the challenge test with

no water exchange. Fish were injected intraperitoneally with 0.1 mL of culture suspension of pathogenic *E. tarda* containing 1×10^8 CFU/mL. Fish mortality was recorded daily for 9 days.

Statistical Analysis

All data were analyzed by two-way ANOVA to test for the effects of the dietary treatments. When significant differences were found, a least significant difference (LSD) test used to identify differences among experimental groups. Treatment effects were considered with the significance level at $P < 0.05$. All statistical analyses were carried out by SAS version 9.1 (SAS Institute, Cary, NC, USA).

Results

Table 11 and Figures 11-15 shows the growth performance and survival rate of juvenile olive flounder fed different experimental diets for 9 weeks. weight gain (WG) and specific growth rate (SGR) of fish fed SSEL and SSEH diets were significantly higher than those of fish fed CON, SSELP and SSEHP diets ($P < 0.05$). Feed efficiency (FE) and protein efficiency ratio (PER) of fish fed SSEL, SSEH, SSELP and SSEHP diets were significantly higher than fish fed CON diet ($P < 0.05$), however, there were no significant differences among fish fed SSEL, SSEH, SSELP and SSEHP diets ($P > 0.05$). Daily feed intake (DFI) of fish fed CON diet was significantly higher than those of fish fed SSEHP diet ($P < 0.05$), however, there were no significant differences among fish fed CON, SSEL, SSEH and SSELP diets ($P > 0.05$). There were no significant differences in Hematosomatic index, Viscerosomatic index and Condition factor among the treatments. Also, there were no significant differences in survival rate of fish fed all experimental diets ($P > 0.05$). There were no significant ($P > 0.05$) differences in whole-body proximate composition of fish in all experimental groups (Table 12). Table 13 shows hematological parameters of juvenile Nile tilapia fed different experimental diets. There were no significant differences in Aspartate aminotransferase, Alanine aminotransferase, glucose and total cholesterol contents of fish fed experimental diets. Table 14 and Figures 16-18 shows the Myeloperoxidase (MPO), superoxide dismutase (SOD) and lysozyme activities of fish fed different experimental diets for 9 weeks. MPO activities of fish fed SSEHP diet were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P < 0.05$). SOD activities of fish fed SSEHP diet were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P < 0.05$), however, there were no significant differences among fish fed SSELP and SSEHP diets ($P > 0.05$). Lysozyme activities of fish fed SSELP and SSEHP diets were significantly higher than those of fish fed CON, SSEL and SSEH diets ($P < 0.05$).

Challenge test

Mortality was initially observed in all fish groups at day 2 after *E. tarda* infection (Fig. 19). At day 3, the cumulative survival rate (CSR) of fish fed the SSELP and SSEHP diets were significantly higher than SSEL, SSEH, and SQSE diets ($P < 0.05$), however, there was no significant differences in CSR among fish fed CON, SSELP and SSEHP diets ($P > 0.05$). At day 9, CSR of fish fed SSEHP diet was significantly higher than those of fish fed SSEH and SSELP diets ($P < 0.05$), however, there were no significant differences in CSR among fish fed CON and SSEL diets ($P > 0.05$).

Table 11. Growth performance, feed efficiency and organosomatic indices of juvenile olive flounder fed the experimental diets for 9 weeks[1]

	Diets					Pooled SEM[12]
	CON	SSEL	SSEH	SSELP	SSEHP	
IBW[2]	13.4	13.4	13.4	13.2	13.3	0.03
FBW[3]	41.4	51.7	49.2	47.9	49.0	1.24
WG (%)[4]	217^{c}	312^{a}	314^{a}	270^{b}	268^{b}	9.10
SGR (%/day)[5]	2.51^{c}	3.08^{a}	3.09^{a}	2.85^{b}	2.83^{b}	0.05
FE (%)[6]	116^{b}	152^{a}	149^{a}	139^{a}	147^{a}	3.52
PER[7]	2.25^{b}	2.67^{a}	2.72^{a}	2.53^{a}	2.67^{a}	0.05
DFI (%)[8]	2.15^{a}	2.03^{ab}	2.08^{ab}	2.04^{ab}	1.92^{b}	0.03
HSI (%)[9]	1.08	1.03	1.05	0.99	1.06	0.03
VSI (%)[10]	3.18	3.03	3.18	3.07	3.09	0.10
CF[11]	0.83	0.86	0.86	0.86	0.83	0.01

[1] Values are means from triplicate groups of shrimp where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] Initial body weight (g).

[3] Final body weight (g).

[4] Weight gain (WG, %) = (final wt. - initial wt.) × 100 / initial wt

[5] Feed efficiency (FE, %) = (wet weight gain / dry feed intake) × 100

[6] Specific growth rate (SGR, %) = ($\log_e$ final wt. - $\log_e$ initial wt.) × 100 / days

[7] Protein efficiency ratio (PER) = (wet weight gain / protein intake)

[8] Daily feed intake (DFI, %) = specific growth rate × 100 / feed efficiency

[9] Hematosonatic index (HSI, %) = liver wt. × 100 / body wt.

[10] Visceralsomatic index (VSI, %) = viscera wt. × 100 / body wt.

[11] Condition factor = (wet weight / total length^3) × 100

[12] Pooled standard error of mean : SD/√n

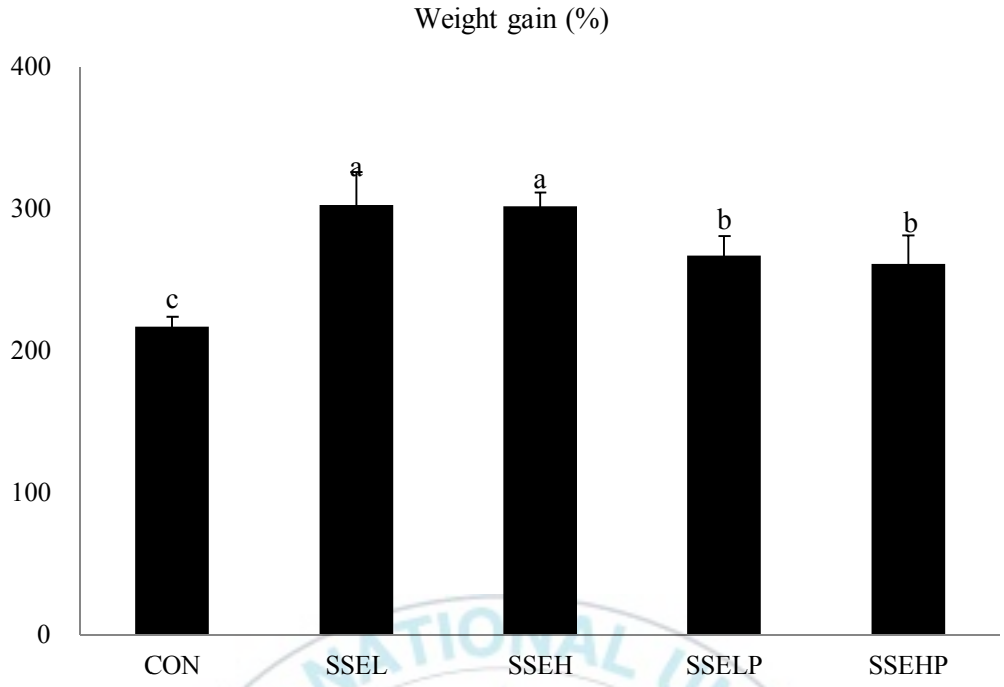

Fig.11. Weight gain of juvenile olive flounder fed the experimental diets for 9 weeks.

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

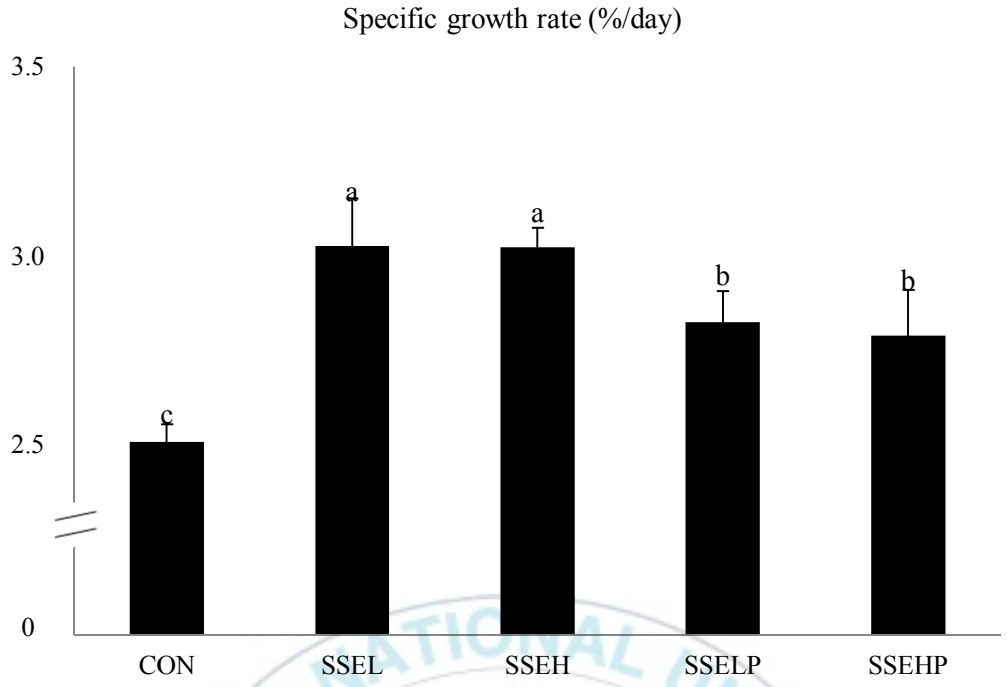

Fig.12. Specific growth rate of juvenile olive flounder fed the experimental diets for 9 weeks.

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

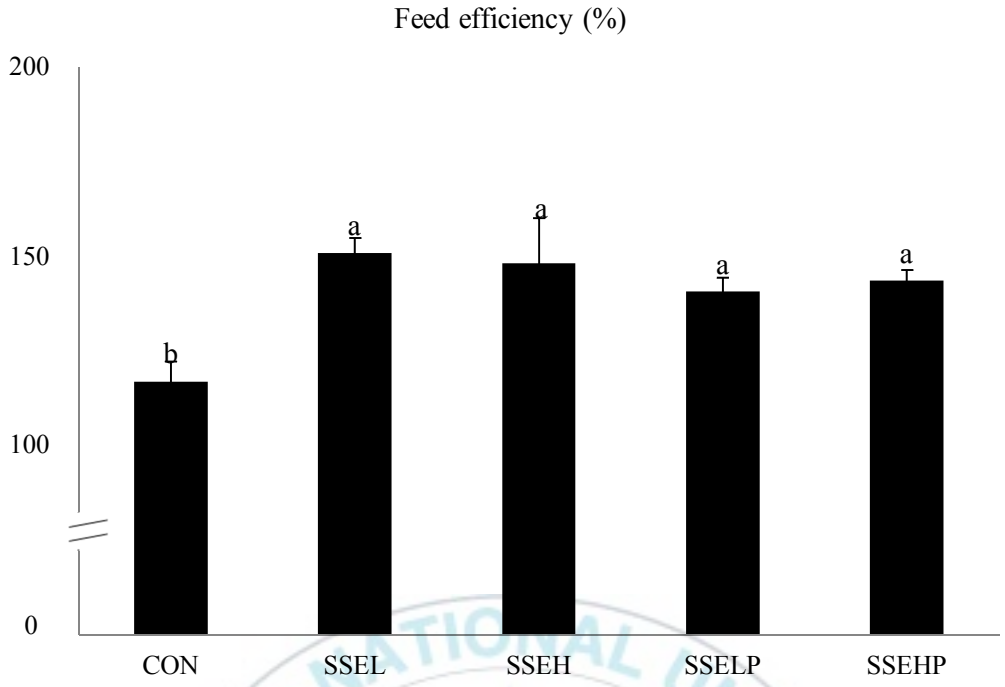

Fig.13. Feed efficiency of juvenile olive flounder fed the experimental diets for 9 weeks.

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

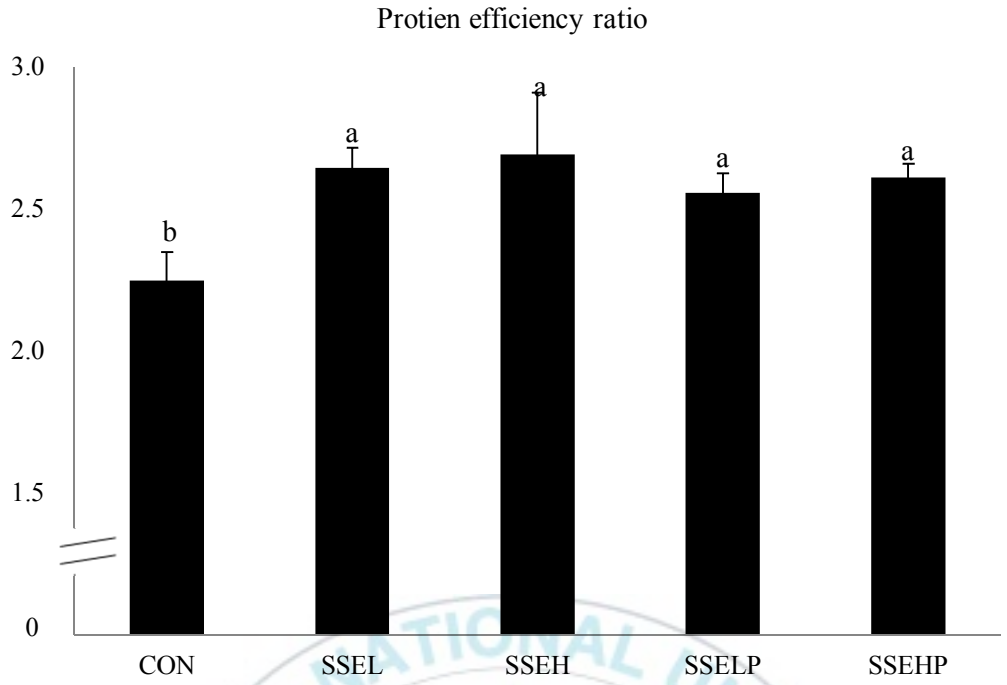

Fig.14. Protein efficiency ratio of juvenile olive flounder fed the experimental diets for 9 weeks.

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

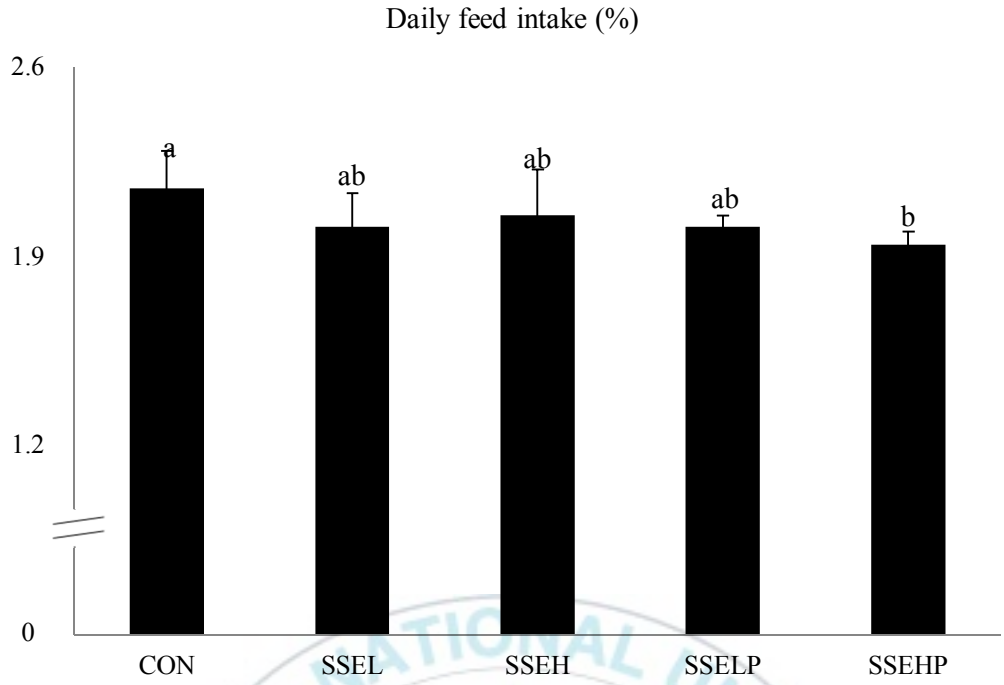

Fig.15. Daily feed intake of juvenile olive flounder fed the experimental diets for 9 weeks.

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

Table 12. Whole-body proximate composition of juvenile olive flounder fed the experimental diets for 9 weeks (%, dry matter basis)[1]

	Diets					Pooled SEM[2]
	CON	SSEL	SSEH	SSELP	SSEHP	
Moisture (%)	2.39	2.42	2.14	2.55	2.57	0.10
Crude protein (%)	76.0	75.6	76.3	76.8	76.2	0.29
Crude lipid (%)	10.0	10.1	10.4	9.66	10.6	0.19
Crude ash (%)	14.8	14.8	14.5	14.4	15.8	0.20

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] Pooled standard error of mean : SD/√n

Table 13. Hematological parameters of juvenile olive flounder fed the experimental diets for 9 weeks[1]

	Diets					Pooled SEM[4]
	CON	SSEL	SSEH	SSELP	SSEHP	
AST (U/L)[2]	11.0	11.3	10.7	11.3	11.0	0.1
ALT (U/L)[3]	6.3	6.3	6.0	6.3	6.3	0.1
Glucose (mg/dl)	21.7	21.3	21.7	21.7	21.3	0.3
T-cholesterol (mg/dl)	207	209	234	215	231	4.9

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] AST (U/L) : Aspartate aminotransferase.

[3] ALT (U/L) : Alanine animotransferase.

[4] Pooled standard error of mean : SD/√n

Table 14. Non-specific immune responses of juvenile olive flounder fed the experimental diets for 9 weeks[1]

	Diets					Pooled SEM[4]
	CON	SSEL	SSEH	SSELP	SSEHP	
MPO[2]	1.64^{e}	1.78^{cd}	1.80^{bc}	1.84^{ab}	1.87^{a}	0.02
SOD[3]	61.6^{c}	68.9^{ab}	70.0^{ab}	72.3^{a}	73.2^{a}	1.04
Lysozyme (U/ml)	0.42^{cd}	0.47^{bc}	0.52^{b}	0.62^{a}	0.63^{a}	0.03

[1]Values are means from triplicate groups of fish where the values in each row with different superscripts are significantly different ($P < 0.05$)

[2] MPO (absorbance) : Myeloperoxidase

[3] SOD (% inhibition): Superoxide dismutase

[4] Pooled standard error of mean : SD/√n

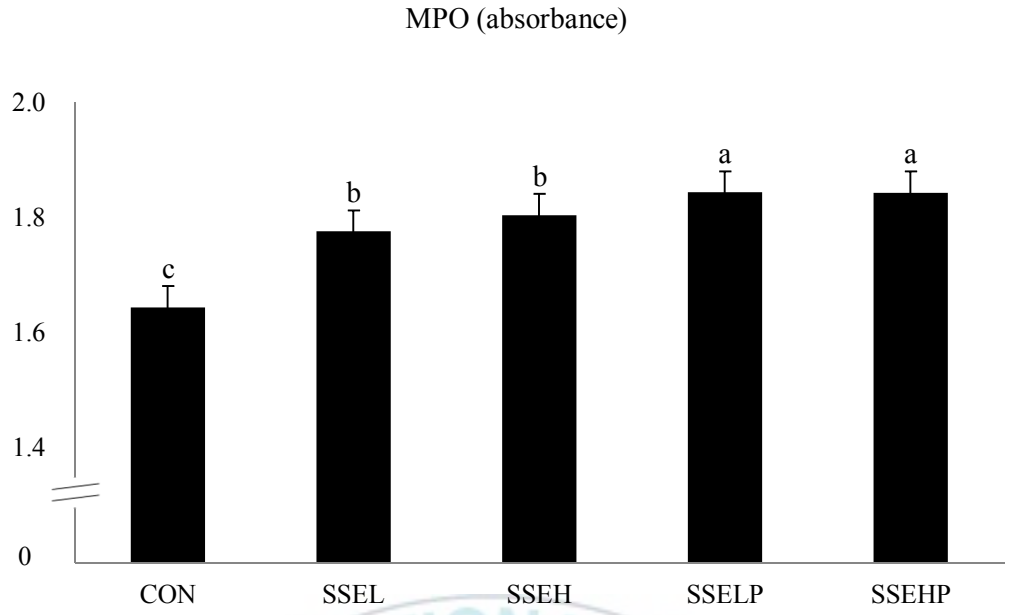

Fig.16. Myeloperoxidase (MPO) in juvenile olive flounder fed the experimental diets for 9 weeks

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

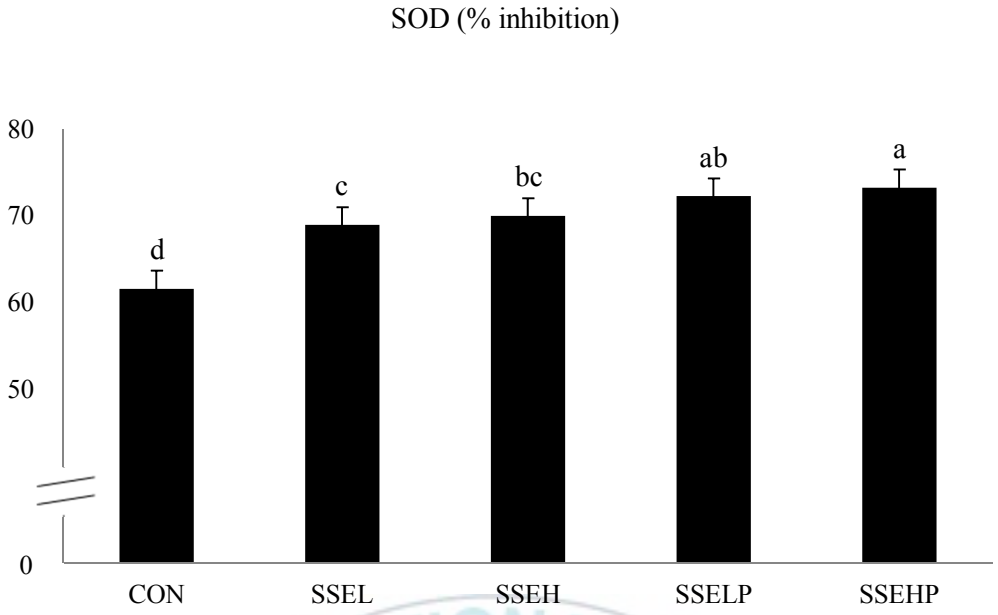

Fig.17. Superoxide dismutase (SOD) in juvenile olive flounder fed the experimental diets for 9 weeks

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

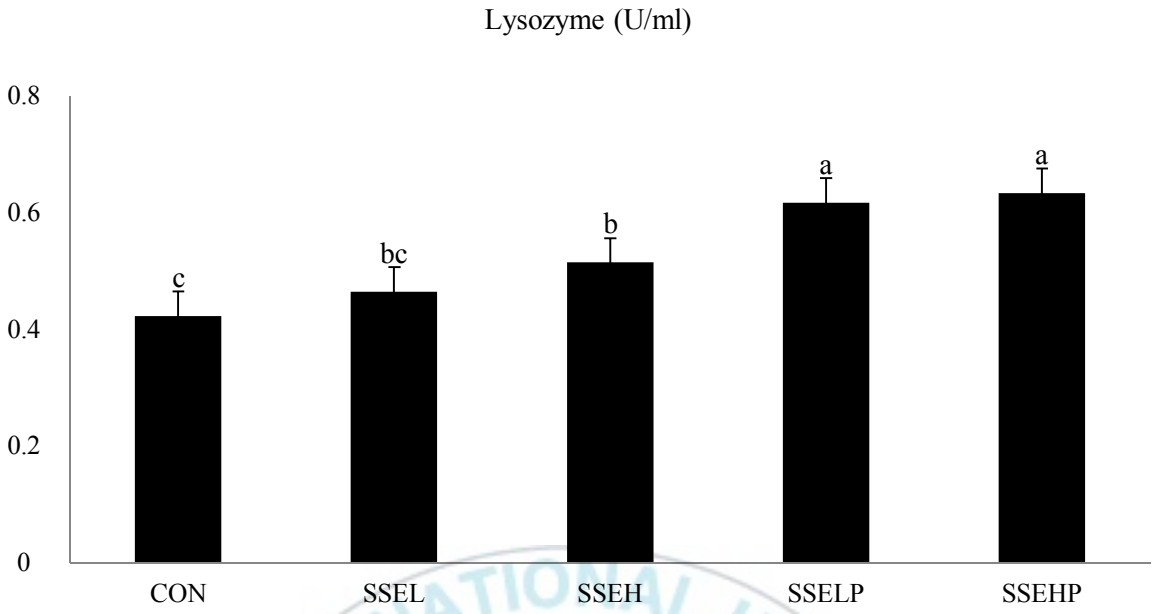

Fig.18. Lysozyme activity in juvenile olive flounder fed the experimental diets for 9 weeks

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

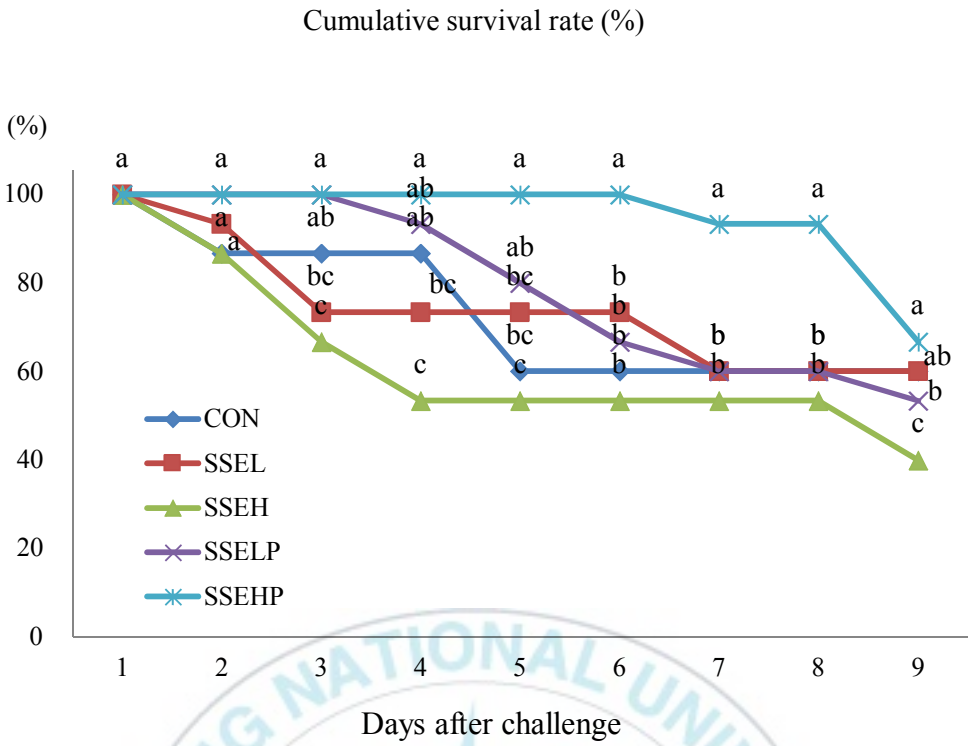

Fig.19. Cumulative survival after intraperitoneal injection with *Edwardsiella tarda* in six experimental groups of juvenile olive flounder fed diets. Each value represents mean ± SE (n=3). Different letters are significantly ($P < 0.05$) different by LSD test

CON - Basal diet (0 % of soluble extract)

SSEL – 2% of 100% shrimp soluble extract (pH2)

SSEH – 2% of 100% shrimp soluble extract (pH4)

SSELP – 2% of 95% shrimp soluble extract (pH2) + 5% inosine monophospate

SSEHP – 2% of 95% shrimp soluble extract (pH4) +5% inosine monophospate

Discussion

Result of this study showed that fish fed the SSEL and SSEH diets showed a significantly higher growth performance. (Kolkovski et al., 2000) reported the growth promoting effect of krill hydrolysate when it was supplemented in diets for larval and juvenile fish. Increased growth performance of fish following dietary krill hydrolysate application has been reported to be enhanced diet ingestion rate as kirll hydrolysate is a rich source of low molecular weight compounds acting as chemo-attractant in fish diets (Kolkovski et al., 2000). Shrimp soluble extract is replaced with alternative protein sources, adverse effects related to deficiencies of certain essential amino acids. Fish nutritionists have supplemented the diet with amino acids to improve growth performance (Hardy., 2010). This study use of top-coated protein sources with supplementation of SSE. The experimental amino acid composition of all experimental diets appeared to meet the requirement levels that were reported for growing olive flounder (NRC., 2011). The essential amino acid requirements for olive flounder have been known, for lysine 1.5 to 2.1 % of the diet, for methionine 1.4 % to 1.5% of the diet (in the presence of 0.06% of cystein) (NRC., 2011). Consistent with our observations, some studies reported the supplement of protein hydrolysates that showed better growth performances in fish and abalone. (Bautista-Teruel et al., 2003; Zhu et al., 2011; Burr et al., 2012; Kader et al., 2012; Jo et al., 2016). (Kader et al., 2012) reported that some additives such as fish soluble extract, krill meal, and 2015 fish feeds. In other studies, it has been demonstrated that supplementation of blended protein sources and SSE had a beneficial effect on growth performance in Malabar grouper, *Epinephelus malabaricus* (Li et al. 2009), and red sea bream, *Pagrus major* (Khosravi et al. 2015), respectively. Hematological parameters are useful indicators for evaluating the physiological and health status (Maita., 2007). And blood enzymes such as AST and ALT are known to be health markers of the animal's physiological condition (Ozgur et al., 2010). It is also known that these markers are sensitive indicators for tissue damage (De la Tore et al., 2000). In the biochemical parameters, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose and total cholesterol were found to be not much affected by dietary supplements of shrimp soluble extract.

Other researches have reported that the blood parameters of fish are not affected by the dietary substitution of alternative protein sources for fishmeal (Cho et al., 2005; Jeon et al., 2014; Lee et al., 2012). The non-specific defense mechanism of fish include neutrophil activation, the production of peroxidase, oxidative radicals, and initiation of other inflammatory factors (Ainsworth et al., 1991). And they have an important role in the immune function of teleost fish (Irianto et al., 2002). In this study, MPO activities of fish fed SSEHP diets were significantly higher than those of fish fed CON, SSEL, and SSEH diets. The MPO is an important enzyme having microbicidal activity, utilize one of the oxidative radicals (H_2O_2) to produce hypochlorous acid. In this study, SOD activities of fish fed the SSEHP diet were significantly higher than those of fish fed CON, SSEL and SSEH diets. Enhancement of SOD provides further evidence for earlier in vitro studies that found production of superoxide anion was stimulated by peptides from fish protein hydrolysate in Atlantic salmon leucocytes (Gildberg et al., 1996). The enzymes such as MPO and SOD activities play significant roles *in vivo* due to their antioxidant function, and their elevated expression and activities are indication of oxidative stress (Yonar., 2012). A similar tendency was observed in our study, indicating the enhancement of the fish immune system by inclusion of SSE (Jo et al., 2016). These effects of SSE are mainly attributed to their bioactive peptide contents that have antioxidative, antimicrobical, and immunomoculatory acivities (He et al., 2013). (Khosravi et al., 2015) showed that inclusion of 3% shrimp hydrolysate enhanced the lysozyme activity in diets of the sea bream. These effects of SSE are mainly attributed to their bioactive peptide contents that have antioxidative, antimicrobial, and immunomodulatory activities (He et al., 2013). Dietary nucleotides have most recently received considerable attention as immunomodulating compounds for various fish species; however, these compounds also may actually influence diet intake of fish. Inosine and inosine 5'-monophosphate have been effective in improving diet consumption by different fish species (Gatlin et al., 2007). Numerous studies on humans and animals have reported that dietary supplementation of nucleotides has positive influence on growth performance, immune responses and disease resistance (Carver., 1995; 1990; Devresse., 2000). Dietary supplementation of other nucleotides also showed an improvement in growth performance of different fish species such as grouper (*Epinephelus*

malabaricus), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and red sea bream (Burrells et al., 2001; Lin et al., 2009; Tahmasebi-Kohyani et al., 2011; Hossain et al., 2016b). In consistent with our findings, recent studies also reported that dietary supplementation of inosine monophosphate (IMP) improved growth of red sea bream (*Pagrus major*) (Hossain et al., 2016a) and olive flounder (*Paralichthys olivaceus*) (Song et al., 2012).

In conclusion, this study indicated that supplementation of feed by shrimp soluble extract produced in low or high pH (SSEL and SSEH) without IMP could have beneficial effects on growth performance without positive immune responses. However, supplementation of IMP (SSELP and SSEHP) could have beneficial effects on immune responses without positive growth performance in juvenile olive flounder.

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무사히 석사를 졸업할 수 있도록 석사 과정 동안 아낌없는 가르침을 주신 배승철 교수님께 감사의 인사를 드립니다. 또한, 매번 손수 맛있는 음식을 만들어주시며 댁에 초대해주시고 저희의 식단과 건강을 챙겨주신 사모님께도 감사의 말을 전하고 싶습니다. 졸업논문의 심사를 해주신 김창훈 교수님과 최윤희 교수님께도 진심으로 감사의 인사를 드립니다. 지금까지 많은 가르침을 주신 김종명 교수님, 김동수 교수님, 남윤권 교수님, 공승표 교수님, 박정환 교수님께도 깊은 감사를 드립니다.

실험을 진행하는 동안에도 많은 분들의 도움이 있었습니다. 항상 적극적으로 아낌없이 저에게 도움을 준 실험실 분들의 도움이 없었으면 실험이 무사히 진행되지 못했을 것입니다. 주영이오빠, 진혁이오빠, 승한이오빠, 성훈이오빠, 영진이오빠, 진호오빠, 민지언니한테 많은 것을 배웠으며 도움을 받았습니다. 또한 원석이오빠, 하함, 형우, 민혜에게도 많은 도움을 받아 실험이 무사히 진행이 되어 고맙다는 말을 하고 싶습니다.

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마지막으로 공부를 계속할 수 있도록 아낌없는 지원을 해주시고 항상 제 편이 되어주시면서 용기를 주시고 타지생활을 하면서 저에게 큰 힘과 원동력이 되었던 사랑하는 부모님 너무 감사드립니다. 또한 저에게 큰 힘이 되어주고 고민상담도 언제든지 해주던 하나뿐인 언니와 동생에게도 고맙다는 말을 전하고 싶습니다. 오랜만에 만나도 웃으면서 반겨주고 항상 제 편이 되어준 친구들에게도 고맙다는 말을 전하고 싶습니다.

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모두들 진심으로 고맙습니다. 사랑합니다. 다들 좋은 일과 행복한 일들만 가득하길 바랍니다.

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Appendix

Exp. 1

	Rep	WG (%)	SGR (%/day)	FE (%)	PER	DFI (%)	Survival (%)
CON	1	344.0	2.63	78.1	2.35	3.36	100
	2	348.8	2.66	76.5	2.30	3.48	100
	3	340.2	2.61	75.9	2.28	3.44	100
SSE	1	348.1	2.69	68.9	2.07	3.91	100
	2	350.7	2.67	72.6	2.17	3.69	100
	3	357.5	2.71	72.9	2.19	3.72	100
$SSEP_2$	1	353.5	2.73	71.7	2.18	3.81	100
	2	3.52.3	2.67	80.1	2.44	3.33	100
	3	355.5	2.68	73.2	2.23	3.67	100
$SSEP_4$	1	360.0	2.68	74.5	2.24	3.60	100
	2	357.1	2.72	75.9	2.28	3.58	100
	3	353.6	2.69	72.1	2.17	3.73	100
SQSE	1	355.7	2.66	76.0	2.35	3.50	100
	2	332.9	2.59	71.5	2.21	3.62	100
	3	338.4	2.65	71.2	2.20	3.72	94.7
TSE	1	347.9	2.68	69.8	2.16	3.83	100
	2	341.1	2.66	78.3	2.42	3.39	100
	3	347.1	2.62	69.6	2.16	3.76	94.7

Exp. 2

	Rep	WG (%)	SGR (%/day)	FE (%)	PER	DFI (%)	Survival (%)
CON	1	213.5	2.48	118	2.28	2.09	100
	2	213.0	2.48	120	2.33	2.05	100
	3	22.51	2.56	110	2.13	2.31	92.9
SSEL	1	306.4	3.05	147	2.58	2.07	92.9
	2	278.1	2.89	154	2.72	1.87	100
	3	324.2	3.14	150	2.64	2.09	92.9
SSEH	1	312.6	3.08	153	2.79	2.01	92.9
	2	294.3	2.98	156	2.84	1.91	100
	3	298.3	3.00	134	2.44	2.24	92.9
SSELP	1	251.7	2.73	136	2.49	2.00	100
	2	271.7	2.85	144	2.63	1.98	100
	3	277.7	2.89	140	2.56	2.06	92.9
SSEHP	1	276.4	2.88	145	2.65	1.98	100
	2	239.0	2.65	140	2.56	1.98	100
	3	268.9	2.84	144	2.63	1.97	100