Thesis for the Degree of Doctor of Philosophy

Evaluation of the bioprocessed protein concentrates as the dietary fish meal replacer in rainbow trout, *Oncorhynchus mykiss* and whiteleg shrimp, *Litopenaeus vannamei*

by

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무지개송어와 흰다리새우에 있어서 어분대체원으로서
응용생물학적 처리를 통한 단백질 농축물의 평가

Advisor: Sungchul C. Bai

by

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A dissertation

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Abstract

The present study evaluated the potential use of bioprocessed protein concentrates (BPCs) as an alternative of fish meal (AF) in the diets of juvenile rainbow trout, *Oncorhynchus mykiss* and juvenile whiteleg shrimp, *Litopenaeus vannamei* reared in semi-circulating aquaria. In the first experiment, results demonstrated that dietary fish meal (FM) level could be reduced up to 30% by BPC in rainbow trout diet. The second experiment showed that BPC pre-treated with acid hydrolyses and/or added with shrimp soluble extract (SSE) showed better digestibility over other plant protein based ingredients such as soybean meal or protease enzyme treated fermented soybean meal, corn gluten meal (CGM), and commercially produced fermented protein concentrate (Soytide) meal in rainbow trout. The third experiment similarly dealt with FM replacement in whiteleg shrimp, the results revealed that FM could be substituted by BPC with acid hydrolyses and/or SSE supplements at a 30% replacement level without compromising the health status of shrimp. In the fourth and final experiment, results postulated that BPC with acid hydrolyses and/or added SSE had better digestibility than those of SM, CGM, or enzyme treated BPC diets. These research works have potential implications in high quality plant protein based feed ingredient development as an alternative of fish meal for sustainable production of two commercially important aquaculture species such as rainbow trout and whiteleg shrimp.

First experiment:

Effect of bioprocessed protein concentrates in partial replacement of fish meal on growth, blood chemistry, innate immunity and gut histology in juvenile rainbow trout, *Oncorhynchus mykiss*

An 8-week feeding trial evaluated the potential use of bioprocessed protein concentrates (BPCs) as a fish meal replacer in diets for juvenile rainbow trout, *Oncorhynchus mykiss*. Ten isonitrogenous and isoenergetic diets were formulated to contain high quality low temperature fish meal (LT-FM) as positive control, low quality Vietnam fish meal (VT-FM) as negative control and four different kinds of bioprocessed protein concentrates as an alternatives of fish meal (AF-A, B, C, and D) at levels of 30% and 50% FM replacement. The protein concentrates (PC) were prepared by different processing techniques. The diet AF-A was added with a protein concentrate fermented by *Bacillus* spp.; diet AF-B was pre-treated with acid hydrolyzed AF-A, whereas diets C and D were designed by AF-A+shrimp soluble extract (SSE) and AF-B+SSE, respectively. Fifteen fish with an average weight of 15.4±0.03 (mean±SD) were randomly distributed into 30 aquaria and fed the experimental diets in triplicate at satiation twice daily on dry matter basis. At the end of the feeding trial, fish fed AF-B, AF-C, and AF-D diets showed no significant differences in weight gain (WG) and feed efficiency (FE) than fish fed rest of the diets at 30% of fish meal replacement level in juvenile rainbow trout. Non-specific immune response, hematology, and gut histology followed the same trend of growth performance. The results show that AF-B, AF-C, and AF-D diets could replace up to 30% of fish meal in rainbow trout.

Second experiment:

Evaluation of bioprocessed protein concentrates by the determination of apparent digestibility of protein and digestive enzyme activities in rainbow trout, *Oncorynchus mykiss*

Apparent digestibility coefficient (ADC) of five types of bioprocessed protein concentrates (BPCs) in terms of AF-A (only fermented protein concentrate, BPC), AF-B (BPC pre-treated with acid hydrolyses), AF-C (BPC + shrimp soluble extract), AF-D (BPC pre-treated with acid hydrolyses + shrimp soluble extract), AF-E (BPC + protease enzyme), soybean meal (SM), corn gluten meal (CGM), and a commercial fermented soybean meal (SOY-T) were assessed indirectly in rainbow trout by applying chromic oxide (Cr_2O_3) marker based stripping method. In the study, one diet with low temperature fish meal (LT-FM) or reference diet and another diet with Vietnam local fish meal (VT-FM) were used as controls. Each of ten experimental diets consisted of 70% reference diet and 30% test ingredient. Fifteen fish with an average initial weight of 40.26±1.36 g (mean±SD) were randomly distributed in each of 30 semi-circulated tanks in triplicate. Fish were fed the experimental diets to apparent satiation. The feces collection was carried out twice a day by stripping process for 30 days. Results revealed that apparent digestibility of diets (ADDs) and apparent digestibility of ingredients (ADIs) for crude protein (CP) were significantly higher in fish fed AF-B, C, and D diets compared to other plant protein based diets. There were no significant differences among fish fed AF-B, AF-D, and LT-FM diets. The digestive enzymes such as protease, lipase, and amylase followed the same trend of protein digestibility. The results suggested that AF-B, C, and D are good protein sources for rainbow trout.

Third experiment:

Effects of bioprocessed protein concentrates in partial replacement of fish meal on growth, hematology and non-specific immune responses in juvenile whiteleg shrimp, *Litopenaeus vannamei*

An 8-week feeding trial evaluated the potential use of bioprocessed protein concentrates (BPCs) to partially replace fish meal in juvenile whiteleg shrimp**,** *Litopaeneus vannamei*. Ten experimental diets were high quality low temperature fish meal based diet (LT-FM) as positive control, low quality Vietnamese fish meal based diet (VT-FM) as negative control and rest of the eight diets constituted with four kinds of BPCs as alternatives of fish meal (AF-A, B, C, and D) at 30% and 50% replacement levels of high quality fish meal or LT-FM. The BPCs were prepared by different processing techniques using protein concentrates (PC). The diet AF-A was added with a protein concentrate fermented by *Bacillus* spp. or BPC diet. AF-B was pre-treated with acid hydrolyzed BPC, whereas diet C and D were supplemented with AF-A+shrimp soluble extract (SSE) and AF-B+SSE, respectively. Fifteen juvenile whiteleg shrimp averaging 3.88±0.05 g (mean±SD) were randomly distributed in each treatment tank and each treatment was prepared in triplicate. Shrimp were fed one of the ten experimental diets containing 40% crude protein to apparent satiation for 8 weeks. At the end of the feeding trial, shrimp fed AF-B, AF-C, and AF-D diets showed no significant differences in growth and non-specific immune responses at a 30% replacement level of fish meal in juvenile whiteleg shrimp. In conclusion, AF-B, AF-C, and AF-D ingredients at 30% FM replacement levels could be recommended in the diets of whiteleg shrimp.

Fourth experiment:

Evaluation of different bioprocessed protein concentrates on nutrient digestibility and digestive enzyme activities in juvenile whiteleg shrimp*, Litopenaeus vannamei*

A feeding trial to assessed five types of bioprocessed a protein concentrates (BPCs) such as AF-A (only fermented protein concentrate), AF-B (pre-treated with acid hydrolyzed AF-A), AF-C (AF-A + 2% shrimp soluble extract), AF-D (AF-B + 2% shrimp soluble extract) and AF-E (AF-A + protease enzyme), soybean meal (SM), corn gluten meal (CGM), and a commercial fermented protein concentrate meal (SOY-T) were examined indirectly in terms of apparent digestibility of diets (ADDs) and apparent digestibility of ingredients (ADIs) for protein in whiteleg shrimp by conventional method. In the study, one diet with low temperature fish meal (LT-FM) or reference diet and another diet with Vietnam local fish meal (VT-FM) were used as controls. The experimental diets consisted of 70% reference diet (LT-FM) and 30% test ingredient. Fifteen whiteleg shrimp with an average initial weight of 6.83±0.32 g (mean±SD) were randomly distributed in each of 30 semi-circulated tanks in triplicate. Shrimp were fed the experimental diets to apparent satiation. The feces collection was carried out four times a day for 30 days by sieving process. Results demonstrated that apparent digestibility of diets (ADDs) and ingredients (ADIs) for crude protein (CP) were significantly higher in shrimp fed AF-B, C, and D diets compared to other plant protein based diets. However, there were no significant differences between shrimp fed AF-D and LT-FM diets. The digestive enzymes such as protease, lipase and amylase followed the same trend of the protein digestibility. In conclusion, shrimp fed AF-B and AF-D diets could be recommended as a vegetable protein based ingredient in the diets of whiteleg shrimp based on digestibility of the plant-based ingredients.

무지개송어와 흰다리새우에 있어서 어분대체원으로서 응용생물학적 처리를 통한 단백질 농축물의 평가

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요약

본 연구는 반순환여과식 시스템에서 사육실험이 진행된 치어기 무지개송어(*Oncorhynchus mykiss*)와 치하기 흰다리새우(*Litopenaeus vannamei*)의 어분대체재로 생물학적 공정처리를 통한 농축단백질(Bioprocessed protein concentrate, BPC)의 잠재적인 이용가능성을 평가하기 위해 실시되었다. 첫번째 실험을 통해 무지개송어 사료 내 BPC 를 이용하여 어분(FM)을 30%까지 줄일 수 있었다. 두번째 실험결과는 산 가수분해 처리 그리고/또는 새우가수분해물(SSE)를 처리를 한 BPC 가 무지개송어 사료에 있어서 대두박, 단백질 가수분해 효소 처리된 발효대두박, 콘글루텐밀, 상업적으로 생산되고 있는 발효 농축단백질(Soytide)과 같은 식물성단백질보다 더 높은 소화율을 보여주었다. 세번째 흰다리새우 실험에서는 무지개송어의 어분 대체 실험과 유사하게 진행되었으며, 새우 사료 내 산 가수분해 처리 그리고/혹은 새우가수분해물(SSE)를 처리한 BPC 를 어분(FM)의 30%까지 대체하여도 새우의 건강상태에는 손상이 없었다. 네번째 실험에서는 산 가수분해 처리 그리고/혹은 새우가수분해물(SSE) 처리한 BPC 가 대두박, 콘글루텐밀, 효소 처리된 BPC 보다 더 나은 소화율을 보여주었다. 이러한 연구결과를 통해 고품질 식물성 단백질이 어분대체재로써 충분히 잠재력이 있다고 볼 수 있으며, 더 나아가 이러한 사료원 개발을 통해 상업적으로 중요한 양식종인 무지개송어와 흰다리새우의 지속가능한 생산에도 기여할 수 있을 것이라고 생각된다.

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My mother, Monowara Begum, the founding teacher in my life

and

My wife, Farjana Akther, who continuously supported during my PhD study

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CHAPTER 1

General introduction

General introduction

As global consumption of cultured fish has been increasing every year, feed which accounts for 50-60% of total cost in aquaculture is being recognized as a factor affecting the economic development of the sector in many countries. Fish meal which is the most expensive component in aquatic feed is an important source of highly digestible protein, long chain omega-3 fatty acids (EPA and DHA), and essential vitamins and minerals (IFOMA 2000). However, the intensification of aquaculture around the world is increasing the demand for fish meal even though the supply has limited. According to recent report, the global production of fish meal is decreasing by about 2.3 million tons compared with production in 2000 (IFFO 2016). Consequently, there have been a number of studies to develop cost effective fish meal replacers such as soybean, poultry by-product, microalgae meal, etc. Finding and evaluating fish meal replacer is important to the aquatic feed industry (Kiron et al. 2012).

In the trends of the increase in the price of fish meal and oil, plant protein sources are increasingly used to replace fish meal, since they represent good substitutes for partial or total replacement of fish meal in fish diets (Hardy 2010). Among the plant protein sources, the most frequently used ones are legumes such as soybean, pea, lupin (Kaushik et al. 1995; Kaushik et al. 2004; Dias et al. 2005; Pereira et al. 2002; Glencross et al. 2004), and corn gluten meal (Pereira and Oliva-Teles 2003) that have already been tested for European sea bass, turbot, Atlantic salmon, and carp. The aim of the studies on evaluating the possibility of plant protein is not only testing the nutritional value in aspect of fish quality, but also taking into consideration their eventual effects on fish health. However, high dietary levels of plant proteins usually result in reduced growth performance due to reduced feed consumption, essential amino acid deficiency, and the presence of anti-nutritional factors (ANFs) and indigestible components. ANFs are mainly

alkaloids that play a limiting effect on fish growth and may cause pathomorphological changes in the intestinal epithelium of soybean meal-fed Atlantic salmon (Krogdahl et al. 2003) and rainbow trout (Ostaszewska et al. 2005).

Soybeans have two groups of protease inhibitors; the Kunitz soybean trypsin inhibitor and the Bowman-Birk protease inhibitor. For this reason, moist heat treatment (autoclaving for 15-30 min) is recommended to reduce the concentration of trypsin inhibitor. Fermentation is also one promising approach for reducing antinutritional factors and improving nutritional values of soybean meal with increased availability of certain vitamins including riboflavin, cyanocobalamin, thiamine, niacin, B6, B12 and folic acid (Kiers et al. 2000; Shiu et al. 2015). In recent years, there have been a number of studies reported on the use of fermented soybean meal in livestock (Hirabayashi et al. 1998; Kiers et al. 2003; Feng et al. 2007) and in aqua feeds (Yamamoto et al. 2010; Zhou et al. 2011; Barnes et al. 2012). Furthermore, bacterial fermentation of soybean meal plays an important role as a growth enhancement and immunostimulants in aquaculture. Barnes et al (2012) reported that dietary *Aspergillus* and *Bacillus* fermented soybean meal could replace at least 60% of fish meal in rainbow trout, *Oncorhynchus mykiss* with improved growth and no evidence of gross gut inflammation.

Crustacean protein hydrolysates have long been used in aqua feeds as potential protein source (Plascencia et al. 2002) or as dietary supplements in small amounts for improvement of diet palatability (Kolkovski et al. 2000). Among those hydrolysates, shrimp soluble extract (SSE) has high levels of amino acids and active peptides that are highly digestible and absorbable for animals (Gildberg and Stenberg 2001; Aksnes et al. 2006). Dietary shrimp soluble extract produced through the extraction and chemical and enzymatic processing of raw shrimp heads could increase the level of fish meal replacement without negative

effects on growth performance and hematology in tilapia, *Oreochromis niloticus* L. (Hung et al. 2014) and growing rainbow trout (Jo et al. 2016). Also, SSE may serve as a useful source of protein and flavorants in feed formulations (Heu et al. 2003).

Rainbow trout, *Oncorhynchus mykiss* is a widely cultured freshwater fish species around the world. The global aquaculture production of rainbow trout has been increasing rapidly and reached 814,068 metric tons (mt) in 2013 (FAO 2015). A massive expansion along with the intensification has led to sudden increase in the annual production of rainbow trout in the Republic of Korea and reached 3,304 ton in 2014 (KOSTAT 2015). A great attention has been paid to increase its productivity per unit area in Korea recently.

Whiteleg shrimp, *Litopenaeus vannamei*, is the most important cultivated shrimp species and has presented the highest value of all traded crustacean products. The global production of whiteleg shrimp increased from 146,362 mt in 2000 to 3,178,721 metric ton in 2012 (FAO 2014) and domestic production from 661 mt in 2006 to 4,488 mt in 2014 (MOF 2015). This species is more suitable for aquaculture than other penaeid species has several characteristics such as rapid growth, good survival in high-density culture and disease tolerance (Cuzon et al. 2004), high adaptability to wide ranges of salinity and temperature (Moss et al. 2007; Lightner et al. 2009; Rocha et al. 2010).

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CHAPTER 2

Effects of bioprocessed protein concentrates in partial replacement of fish meal on growth, blood chemistry, innate immunity and gut histology in juvenile rainbow trout, *Oncorhynchus mykiss*

1. Introduction

Fish meal (FM) is a good source of high-quality protein and highly digestible essential amino acids (Cho and Kim 2011; Anderson et al. 2016). However, due to its high pricing and the imbalance in demand and supply of FM as well as its sustainability issue in fisheries sector have led to many studies on alternative of FM (AF) protein in aquafeeds (Tacon et al. 2011; Alam et al. 2012; Kader et al. 2012). In this case, replacement of FM with plant protein source has utmost importance for sustainability of the world aquaculture (Gatlin et al. 2007). However, not all plant proteins are suitable as aquafeed ingredients in their unprocessed forms, as many of them contain antinutrients, which are detrimental in terms of fish nutrition (Francis et al. 2001). For example, among the terrestrial plant ingredients used for fish diet formulation, soy proteins have been recognized as the most promising plant protein source due to its sufficient supply, low price, balanced amino acids and highly digestible protein (Hardy 1999; Gatlin et al. 2007). However, due to the presence of anti-nutritional factors (ANFs) in soy proteins, such as protease inhibitors, lectins, phytates, glucosinolates, saponins, and tannins has limited its inclusion in fish diets (Francis et al. 2001). In addition, soy protein is limiting in sulfur-amino acids (Amerio et al. 1998). Some studies have reported that replacement of FM with soy proteins at high levels resulted in decline of growth and alterations of intestinal morphology in fish (Wang et al. 2006; Kikuchi and Furuta 2009; Krogdahl et al. 2003).

Plant-based protein concentrate has a high quality crude protein (65-67%) among the terrestrial plant protein sources (USSEC-ASA 2008; Kokou et al. 2015). However, plant protein when produced by extraction with water alone may contain higher levels of saponins than soybean meal (Ireland et al. 1986). It has been reported that saponins in high plant protein diets may decrease the growth performance of fish (Francis et al. 2002; Chikwati et al. 2012); whereas some

researchers claimed that saponins in diet do not affect the growth performance in European sea bass (Couto et al. 2015). Moreover, studies have reported that FM replacement with soy protein have adverse effects on carnivorous marine fish species in terms of growth performance (Lim et al. 2011; Song et al. 2014), feed efficiency (Silva-Carrilo et al. 2012), and health condition (Ye et al. 2011). Gatlin et al. (2007) reported that technical removal of ANFs could increase the FM replacement level by soy protein in fish diets. There have been a numerous processing techniques proposed to remove or inactivate soy ANFs such as heating, soaking, cooking, gamma-irradiation, alcohol extraction or bioprocess technology (Refstie et al. 2005; Drew et al. 2007; Yamamoto et al. 2010; Kokou et al. 2012; Zhang et al. 2014).

Bioprocess of soy protein is a good choice in replacing fish meal in the diet of fish (Kokou et al. 2012). It has been reported that bioprocessing of soy protein can act as functional food in terms of increasing the nutritive value and decreasing ANFs of soy protein (Refstie et al. 2005; Tibaldi et al. 2006). Kokou et al. (2012) and Zhang et al. (2014) reported that bioprocessed or fermented soybean meal (SBM) can contain about 56% protein; whereas solvent extracted SBM can contain only 44% protein (NRC 2011). Fermentation is a useful bioprocess technique for drying wet products with minimal nutrient loss (Yamamoto et al. 2004). It allows the utilization of beneficial bacteria such as *Bacillus subtilis* to breakdown complex compounds to yield a unique tasting and aromatic foods (Kader et al. 2012; Azarm et al. 2012; Zhang et al. 2014). The process can be done by sub-merged or liquid and solid-state condition. It has been reported that solid-state fermentation (SSF) has more beneficial effects over liquid or sub-merged fermentation in terms of increasing value addition of feeds (Lio and Wang 2012). Lio and Wang (2012) reported that SSF can be done by using microorganisms on solid substrate without the presence of free liquid. Kokou et al. (2012) reported that bioprocessed SBM can replace up to 40% of

FM in gilthead sea bream. In another report, the same researchers found that the FM replacement level could be more than 40% in case of SPC with same fish species (Kokou et al. 2012). However, Zhang et al. (2014) reported that gamma-irradiated SBM can replace about 16% of FM in the diets of Japanese sea bass. Yaghoubi et al. (2016) postulated the FM replaced by soy products (mixture of SBM and isolated soy protein) ranged between 16.5 and 27.3% after the broken-line model analysis in juvenile silvery-black porgy. It has been reported that supplementation of crystalline amino acids in soy protein diets could also improve the FM replacement level in carnivorous fish species without affecting growth and feed efficiency (Kader et al. 2012; Silva-Carrilo et al. 2012; Zhang et al. 2014). Some studies have reported that attractant substance such as shrimp soluble extract (SSE) has high levels of amino acids and active peptides that are highly digestible and absorbable for animals (Gilberg and Stenberg 2001; Aksnes et al. 2006). Hung (2014) and Jo et al. (2016) found that supplementation of 2% SSE in the diets of tilapia and growing rainbow trout, respectively, could effectively increase the FM replacement level.

Rainbow trout, *Oncorhynchus mykiss*, is a widely cultured freshwater fish species around the world. The global aquaculture production of rainbow trout has been increasing rapidly and reached 814,068 metric tons (mt) in 2013 (FAO 2015). A massive expansion along with the intensification has led to sudden increase in the annual production of rainbow trout in the Republic of Korea and reached 3,304 mt in 2014 (FAO 2015). A great attention has been paid to increase its productivity per unit area in Korea recently. Numerous studies have been conducted on the FM replacement in rainbow trout (Hauptman et al. 2014). However, so far our knowledge, no study is found on the use of bioprocessed protein concentrate (BPC) for replacing FM in juvenile rainbow trout. Therefore, the present study evaluates the different types of BPCs at 30% and 50% FM

replacement levels in terms of growth performance, hematology, non-specific immune responses and distal intestinal morphology in juvenile rainbow trout.

2. Materials and methods

2.1. Experimental diets

Ten isonitrogenous (42% CP) and isoenergetic (16.7 kJ/g energy) diets were formulated to contain high quality low temperature fish meal (LT-FM) as positive control, low quality Vietnam fish meal (VT-FM) as negative control and four different kinds of bioprocessed protein concentrates (BPCs) as alternatives of fish meal (AF-A, B, C, and D) at the levels of 30% and 50% replacement. The plant-based protein ingredients were prepared by different processing techniques using protein concentrate (PC). The diet AF-A or mixture of soybean meal (SBM) and corn gluten meal (CGM) (1:1) was fermented by *Bacillus* spp. at solid state fermentation process (SSF), diet AF-B was pre-treated acid hydrolyzed AF-A; whereas AF- C and -D diets were supplemented with AF-A+shrimp soluble extract (SSE) and AF-B+SSE, respectively. Additionally, methionine (0.06%) and lysine (0.61%) were added in the bio-processed diets (AF-A and AF-B) to balance the amino acids content. The experimental diets were prepared followed by Bai and Kim (1997). In brief, ingredients of the experimental diets were thoroughly mixed with a mixer and then fish oil, and soybean oil, and other micronutrients with 30% water was added and further mixed. The mixture of feeds was finally passed through a laboratory pelleting machine to get 2-mm diameter pellets and dried for 72 hrs. The dried pellets were then stored at -20 ^{0}C in small bags in air-tight condition before use every time.

2.2. Experimental fish and feeding trial

Juvenile rainbow trout, *Oncorhynchus mykiss* were transported from a local fish farm, Sangju, Korea to Feeds and Foods Nutrition Research Center (FFNRC), Pukyong National University, Busan, Korea. Before the start of the experiment,

all fish were reared in a circular plastic tank with 5000 L well fresh water and were fed a commercial diet for 2 weeks. For experimental purposes, 30 flow-through aquaria were used for rearing fish. After 2 weeks conditioning period, fish averaging 15.4±0.03 g (mean±SD) were randomly distributed into 30 aquaria as groups of 15 fish and fed the experimental diets in triplicate at satiation twice daily (09:00 and 18:00) on dry matter basis.

Total fish weight in each tank was determined every 2 weeks after anaesthesia with 100 ppm of MS 222 (tricaine methanesulfonate) and the amount of feeds were adjusted accordingly. During the experimental period water flow rate was maintained at 3 L/min and water temperature maintained at 16 ± 1℃. Aeration was practiced to maintain dissolved oxygen levels near saturation.

2.3. Sample collection, analyses and calculations

At the end of the feeding trial, all fish were weighed and counted to calculate growth parameters such as percent weight gain (WG), feed efficiency (FE), and specific growth rate (SGR) as well as biometrics such as HSI, VSI and CF following the formula:

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

Specific growth rate (SGR, %/day) = [($\log_e$ final wt. - $\log_e$ 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)

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

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

Condition factor = (wet weight / total length^3) × 10

Blood parameters such as hematocrit, hemoglobin, ALT, AST, T-protein, and glucose, and non-specific immune parameters such as superoxide dismutase (SOD) and lysozyme were determined. After the final weighing, five fish were

randomly collected from each aquarium and blood samples were obtained using syringes from the caudal vein of fish and pooled in the vials according to the number of diets. The blood serum was collected by centrifuging blood samples at 3,000 × *g* for 20 min and stored at −20°C. Then the non-specific immune parameters were analyzed by spectrophotometer (Infinite 200 PRO NannoQuant, Tecan, Mannedorf, Switzerland) using the manufacturer protocols (Sigma Aldrich, MO, USA) and serum parameters were analyzed by blood analyzer (DRI-CHEM 4000i- Fuji Dri-Chem Slide- 3150, Minato-ku,Tokyo, Japan). Fish liver and whole viscera were collected by dissected for measuring HSI and VSI, respectively. Crude protein, lipid, moisture and ash of whole-body samples were determined by the AOAC methods (1995). In brief, samples of diets and fish were dried to a constant weight at 135°C for 2 h to determine moisture content. Ash was determined by incineration using muffle furnace at 550°C for 3 hr. Crude lipid was determined by soxhlet extraction unit using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein content analyzed by Kjeldahl method (N × 6.25) after acid digestion.

Hematocrit (packed cell volume, PCV) was determined using the microhematocrit technique. Hemoglobin concentration was measured according to the cyanmethemoglobin method (Taati et al. 2011). The plasma levels of ALT, AST, total protein, and glucose were measured using a chemical analyzer (Fuji DRI-CHEM 3500i; Fuji Photo Film, Tokyo, Japan).

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), 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 (UVM340, Biochrom, Cambridge, UK). A

reduction in absorbance of 0.001/min was regarded as one unit of lysozyme activity.

Superoxide dismutase activity was measured by the percentage reaction inhibition rate of the enzyme with a WST-1 (water soluble tetrazolium dye) substrate and xanthine oxidase using an SOD Assay Kit (Sigma-Aldrich 19160, St. Louis, MO, USA) 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 ^{0}C. The percent inhibition was normalized by milligram protein and presented as SOD activity units.

Myeloperoxidase (MPO) activity was measured according to the method described by 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 of 3, 3´, 5, 5´- tetramethylbenzidine hydrochloride (TMB, 20 mM; Sigma-Aldrich) and H_2O_2 (5 mM) were added. The color 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 a microplate reader.

The SBM, CGM and FPC-A (AT65-A, AQUATIDE65^{TM}) protein analysis was performed with the Agilent 2100 Bio-analyzer system (Agilent, Waldbronn, Germany) using Protein 80 Kit.

Histological analysis of distal intestine was done using the standard histological procedure. Briefly, after dissection of whole intestine of fish hind gut or distal intestine of fish were separated and placed in the small cassettes. The cassettes were then placed in the 10% buffered formalin according to the diet numbers. The sections of the distal intestine were stained in hematoxylin and eosin (H & E stain). The intestinal muscle thickness, goblet cell numbers and villi length were observed under light microscope (AX70 Olympus, Tokyo, Japan). Data were expressed as mean values with their standard deviation. Here can be

mention, to determine the *villus* length, 10 *villi* of a distal intestinal section of each fish were measured from the muscularis mucosa to the basal lamina of the epithelial cell (Pirarat et al. 2011). The *villi* were chosen based on their integrity and higher length. The fused *villi* were considered as one. Goblet cells with clear and rounded cytoplasm were counted on the measured villi. The thickness of the muscular layer of intestinal section was measured from serosa to submucosa of three representative samples (Batista et al. 2015).

2.4. Statistical analysis

All the data were analyzed by one-way ANOVA using SAS version 9.1 software (SAS Institute, Cary, NC, USA) to test the effects of dietary protein. When a significant effect of the treatments was observed, a least significant difference (LSD) *post-hoc* test was used to compare means. Treatment effects were considered significant at $P<0.05$.

3. Results and discussion

At the end of the feeding trial, fish fed LT-FM, AF-B, AF-C, and AF-D at 30% FM replacement level showed significantly higher WG and SGR than those of fish fed AF-A at 30% FM and AF-A, AF-B, AF-C, and AF-D at 50 % FM replacement levels. However, fish fed LT-FM, AF-B, AF-C, and AF-D diets at 30% FM replacement showed no significant differences in terms of WG and SGR in juvenile rainbow trout. Feed efficiency and PER followed the same trend as WG and SGR of fish. There were no significant differences in HSI, VSI, and CF among fish fed the diets. Azarm and Lee (2014) reported that about 40% FM in the diets of juvenile black sea bream could be replaced by fermented soybean meal which is similar to our findings. However, Zhang et al. (2014) found that only 16% FM could be substituted by gamma-irradiated soybean meal in the diet of Japanese sea bass.

There were no specific trends in hematology of fish fed the experimental diets. Non-specific immune responses such SOD activity of fish fed AF-D diet at 30% FM replacement was significantly higher than those of fish fed AF-A and AF-B diets at 50% FM replacement. Lysozyme activity of fish fed AF-A at 50% FM replace diet was significantly lower than those of fish fed LTFM, VTFM, AF-B, AF-C, and AF-D diets at 30% FM replacement. Alanine aminotransferase of fish fed AF-C diet at 30% FM replacement was significantly higher than those of fish fed AF-A, AF-B, and AF-D diets at 50% replacement. Whole body proximate composition of fish was not affected by dietary treatments.

In this study, we checked the presence of *Bacillus* spp. in the bioprocessed protein concentrates. The results revealed that near about $3\sim5\times10^{8}$ CFU/g *Bacillus* spp. was indentified in the ingredients after the microbiological analyses. The histological photomicrographs of the present study showed that dietary higher inclusion (50% FM replacement) of BPC diets affect the distal intestinal morphology of juvenile rainbow trout. However, fish fed the LT-FM and 30% inclusion of BPC diets showed no changes in the intestinal morphology of fish. In consistent of our results, Barnes et al. (2015) reported no significant changes in intestine at 35% FM replacement with fermented SBM in Shasta and McConaughy strains of rainbow trout. Moreover, Yamamoto et al. (2010) found that fermentation techniques of SBM influenced the morphological changes in distal intestine of rainbow trout. However, in this study, morphological changes in distal intestine at 50% FM replacement with BPCs may be due to the presence of higher saponins in the diets which is associated to the soybean-induced enteritis in salmonids (Knudsen et al. 2008; Krogdahl et al. 2010).

In the present study, dietary inclusion of BPC did not significantly affect the mucosal *villi* length in distal intestine of juvenile rainbow trout fed the 30% FM replaced diet compared to LT-FM diet. The results suggest that these groups of fish might have higher nutrient absorption capacity due to the wide surface area

of *villi* as well as low saponin or other ANFs content in the diets in relation to the 50%-BPC based diets which is in agreement with Yamamoto et al. (2010).

Goblet cells (GC) are known as the source of mucus which helped in protection and digestion in the gastro-intestinal tract (Marchetti et al. 2006; Cerezuela et al. 2013; Khosravi et al. 2015). In this study, GCs did not significantly change among fish fed the experimental diets and this in agreement with Ramos et al. (2016) who reporting no effects of dietary probiotics (*Bacillus* spp.) on the GC in distal intestine of rainbow trout fed a SBM based diet. Van et al. (1991) and Cerezuela et al. (2013) reported that dietary SBM and additives like probiotics and prebiotics can change the GC numbers in fish intestine through the microbial modulation (Bakke-McKellep et al. 2007). In contrast to our study, Khosravi et al. (2015) reported that this GC number can be reduced in SPC based diet compared with protein hydrolysates supplemented diets.

In the present study, gut muscular layers in distal intestine of rainbow trout was more thickened in fish fed the LT-FM and 30% FM replaced diets with BPCs compared with VT-FM and 50% FM replaced diets with different BPCs. The results may suggest that the higher inclusion level of BPC could be detrimental for intestinal integrity of fish which is in agreement with Yamamoto et al. (2010) and Barnes et al. (2015). Bakke-McKellep et al. (2007) reported that gut muscular thickness can be increased by supplementation of additives in the diet of Atlantic salmon.

The novel finding of the present study is the successful replacement of high fish meal protein with different bioprocessed protein concentrates (BPCs) at a 30% replacement. Interestingly, we found that fish fed the AF-B, AF-C, and AF-D diets at 30% FM replacement level performed better than those of fish fed AF-A diet at same replacement. This suggests that bioprocessing of PC with supplementation of lysine and methionine is not enough to replace high FM in the diet of juvenile rainbow trout. The results also suggest that acid hydrolyzed BPC

with supplementation of lysine and methionine in the diet or acid hydrolyzed BPC with addition of 2% SSE and BPC with addition of 2% SSE in absence of lysine and methionine in both of the diets could replace up to 30% of high fish meal protein in the diet of rainbow trout without compromising the growth and health status of fish. However, further research should be conducted on low inclusion of BPC to replace the high fish meal protein in the diet of rainbow trout.

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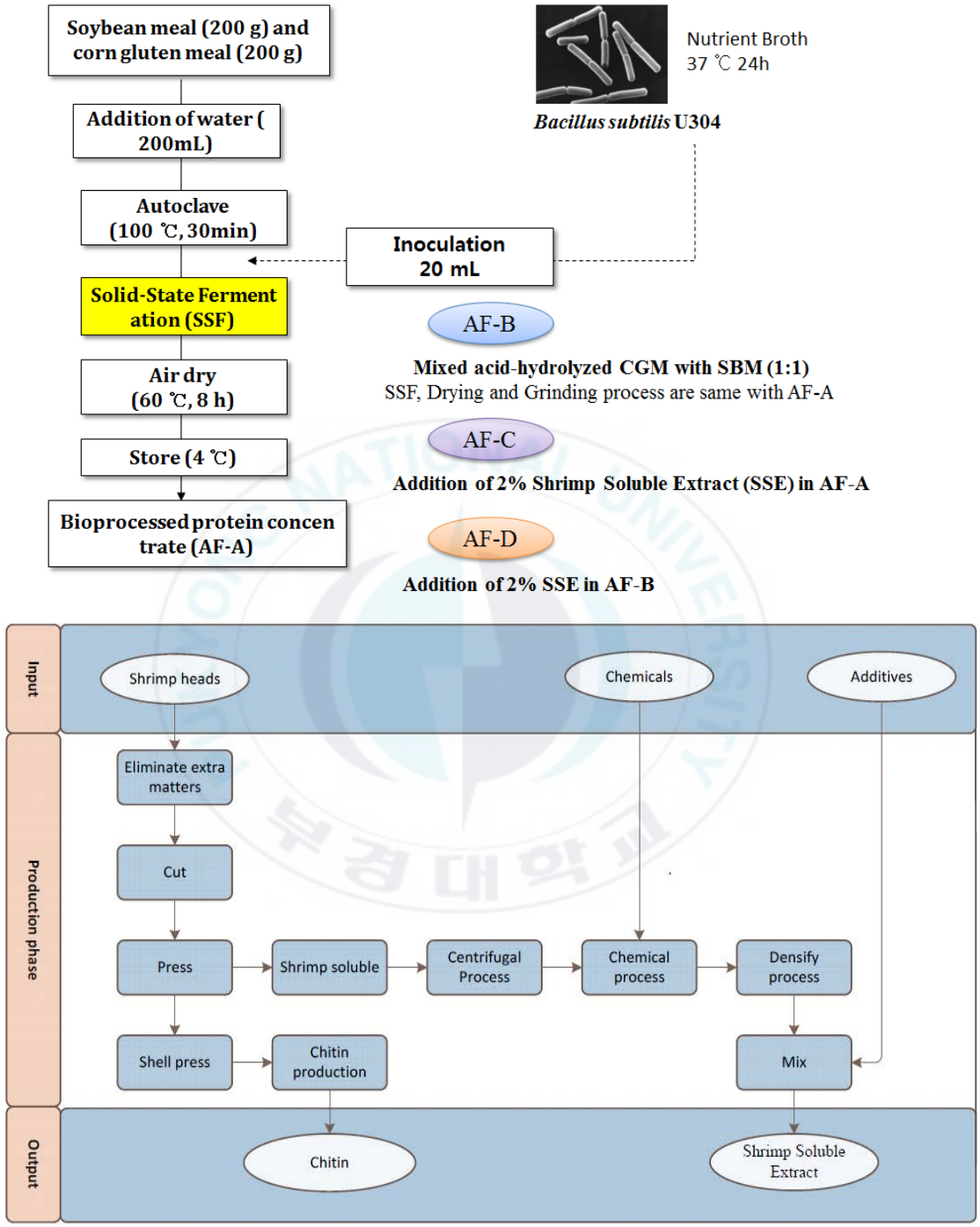


Figure 1. Production process of bioprocessed protein concentrates (BPCs) and shrimp soluble extract (SSE)

Table 1. Proximate composition (% dry matter basis) and amino acid profile (% of sample) of bioprocessed protein concentrates (BPC) such as only fermented soy protein concentrate (AF-A), acid hydrolyzed AF-A (AF-B), AF-A+shrimp soluble extract (AF-C), and AF-B+shrimp soluble extract (AF-D)

	AF-A	AF-B	AF-C	AF-D
Proximate composition				
Moisture	5.6	4.9	4.6	4.8
Crude protein	65.6	65.9	66.3	66.7
Crude lipid	3.4	3.4	3.3	3.7
Ash	4.4	6.6	5.3	6.2
Amino acids profile				
Asp	5.7	5.8	5.7	5.9
Thr	2.4	2.4	2.3	2.4
Ser	3.2	3.3	3.3	3.2
Glu	12.9	13.3	13.3	13.4
Pro	5.5	5.6	5.8	5.9
Gly	2.3	2.3	2.2	2.5
Ala	4.6	4.4	4.2	4.6
Val	3.2	3.2	3.2	3.2
Ile	3.2	2.9	2.9	3.1
Leu	8.5	8.6	9.2	7.9
Tyr	2.5	2.5	2.6	2.4
Phe	4.0	4.1	3.9	4.1
His	2.2	2.3	2.2	2.4
Lys	2.7	2.5	2.6	2.6
Arg	2.9	2.9	2.9	3.1

Table 2. Composition of the experimental diets in rainbow trout, *Oncorhynchus mykiss* (% of DM basis)

Ingredients [a]	LTFM	VTFM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)
Fish meal, Denmark-LT [b]	40.0	0.00	28.0	28.0	28.0	28.0	20.0	20.0	20.0	20.0
Fish meal, Vietnam	0.00	43.0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AF-A	0.00	0.00	12.9	0.00	0.00	0.00	21.5	0.00	0.00	0.00
AF-B [c]	0.00	0.00	0.00	12.9	0.00	0.00	0.00	21.5	0.00	0.00
AF-C	0.00	0.00	0.00	0.00	13.2	0.00	0.00	0.00	22.0	0.00
AF-D	0.00	0.00	0.00	0.00	0.00	13.2	0.00	0.00	0.00	22.0
SBM 44%,South America	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Wheat hard red,Small	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
Squid liver powder	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Wheat flour	14.7	10.6	12.4	12.4	12.8	12.8	11.2	11.2	11.5	11.5
Fish oil	4.80	6.00	5.50	5.50	5.50	5.50	6.00	6.00	6.00	6.00
Soybean oil	4.80	4.80	4.80	4.80	4.80	4.80	4.80	4.80	4.80	4.80
Lysine	0.00	0.00	0.61	0.61	0.00	0.00	0.66	0.66	0.00	0.00
Methionine	0.00	0.00	0.06	0.06	0.00	0.00	0.10	0.10	0.00	0.00
Others [d]	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80	2.80
Total	100	100	100	100	100	100	100	100	100	100

Proximate composition (% of dry matter basis)										
Moisture %	8.6	7.8	9.0	9.2	9.5	8.4	9.0	8.7	8.6	9.1
Crude protein %	42.2	41.5	42.6	42.1	42.6	42.3	42.4	41.7	41.7	41.9
Crude lipid %	15.8	16.6	16.1	16.3	16.2	16.1	16.3	16.6	16.6	16.7
Ash %	10.7	10.6	9.6	9.8	9.4	9.7	9.4	9.4	9.7	9.1

[a] Feed stuff not mentioned here are the same feed stuffs as the domestic aquaculture feed companies are using currently.

[b] Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.

[c] AQUATIDE65 (AT-65) provided by CJ CheilJedang Corporation, Seoul, Korea

[d] Others (as g/100 g): Phos-mono 24%, 0.10; Mineral premix, 1.0; Koking Toco-50, 0.30; Choline 50%, 0.20; vitamin C-100, 0.15; vitamin A, 1.0; in all the experimental diets.

Table 3. Growth performance and biological indices of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks[1]

Items	LTFM	VTFM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	*P*-values
WG (%)	243±3.2^{ab}	232±0.7^{bcd}	220±3.9^{de}	237±2.1^{abc}	244±3.7^{ab}	246±6.1^{a}	206±12.2^{f}	217±6.4^{ef}	227±9.1^{cde}	223±12.3^{de}	0.0001
SGR (%/day)	3.00±0.1^{ab}	2.93±0.1^{abc}	2.84±0.1^{cd}	2.91±0.1^{bcd}	3.01±0.1^{a}	3.03±0.1^{a}	2.72±0.1^{e}	2.81±0.1^{de}	2.86±0.1^{cd}	2.82±0.1^{cd}	0.0001
FE (%)	92.3±0.7^{a}	89.0±4.1^{ab}	84.0±0.8^{c}	87.4±0.1^{ab}	91.2±2.3^{a}	92.2^{a}±1.3^{a}	83.1±1.3^{c}	83.8±2.1^{c}	86.3±3.9^{bc}	86.6±2.2^{bc}	0.0009
PER	2.18±0.1^{ab}	2.15±0.1^{ab}	1.98±0.1^{c}	2.02±0.1^{c}	2.14±0.1^{ab}	2.18±0.1^{a}	1.96±0.1^{c}	1.97±0.1^{c}	2.08±0.1^{abc}	2.07±0.1^{bc}	0.0014
Survival (%)	100	100	100	97.8±3.8	100	100	100	100	100	100	0.0000
HSI (%)	0.78±0.1^{a}	0.82±0.2^{a}	0.77±0.1^{a}	0.71±0.1^{ab}	0.73±0.1^{ab}	0.69±0.1^{ab}	0.75±0.1^{ab}	0.70±0.1^{ab}	0.63±0.1^{b}	0.71±0.1^{ab}	0.2497
VSI (%)	7.64±0.5^{ab}	7.55±0.3^{ab}	7.20±1.4^{ab}	7.49±0.9^{ab}	7.60±0.6^{ab}	7.49±0.5^{ab}	7.74±1.6^{ab}	8.46±1.8^{a}	7.26±0.9^{ab}	6.52±0.7^{b}	0.7553
CF	1.09±0.1^{ab}	1.02±0.1^{ab}	0.99±0.1^{b}	0.99±0.1^{b}	1.05±0.1^{ab}	1.04±0.1^{ab}	1.11±0.1^{ab}	1.14±0.1^{a}	1.09±0.1^{ab}	0.99±0.1^{b}	0.2138

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

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

Specific growth rate (SGR, %/day) = [($\log_e$ final wt. - $\log_e$ 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).

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

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

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

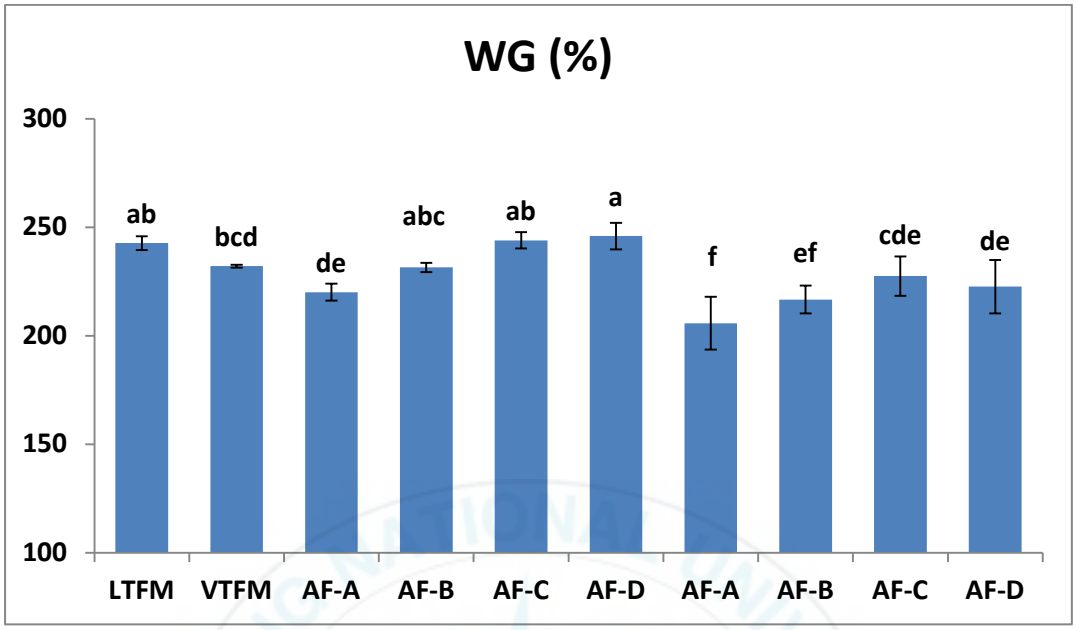


Figure 2. Weight gain (%) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0001$).

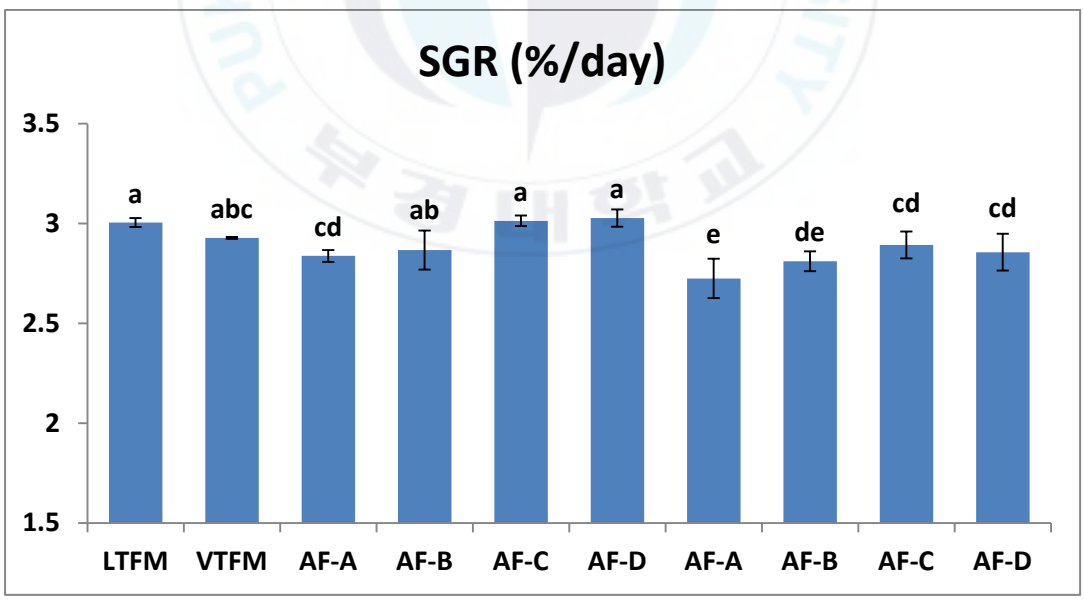


Figure 3. Specific growth rate (%/day) of juvenile rainbow, *Oncorhynchus mykiss* trout fed experimental diets for 8 weeks ($P < 0.0001$).

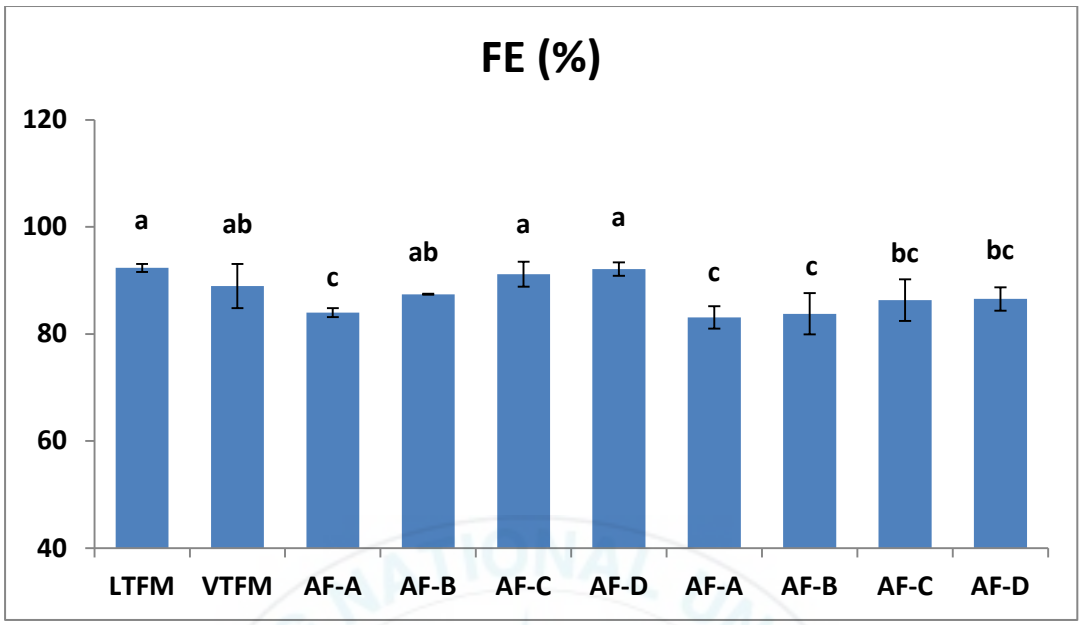


Figure 4. Feed efficiency (%) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0009$).

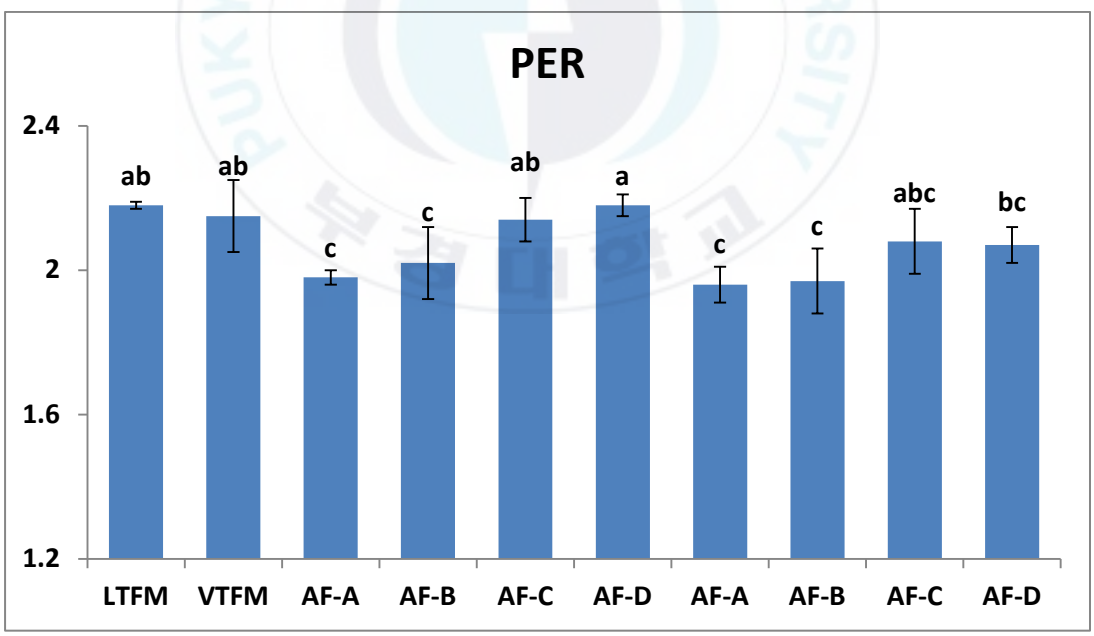


Figure 5. Protein efficiency ratio (%) of juvenile rainbow, *Oncorhynchus mykiss* trout fed experimental diets for 8 weeks ($P < 0.0014$).

Table 4. Whole body proximate composition of juvenile rainbow trout, *Oncorhynchus mykiss* fed the experimental diets for 8 weeks[1]

	Experimental diets										*P*-values
	LT-FM	VT-FM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	
Moisture %	71.5±0.1	71.5±0.1	71.6±0.1	71.5±0.2	71.5±0.1	71.6±0.2	71.5±0.1	71.4±0.2	71.5 ±0.2	71.4 ±0.2	0.8299
Crude protein %	56.2±0.5	55.6±0.4	55.6±0.3	55.4±0.3	55.4±0.4	55.2±0.3	55.6±0.1	55.8±0.9	55.9±0.2	55.5±0.2	0.4996
Crude lipid %	33.8±0.1	33.6±0.1	32.5±0.4	32.6±0.5	33.7±0.1	33.8±0.1	33.2±0.1	32.7±0.1	32.6±0.1	31.2±0.1	0.2186
Ash %	8.6±0.1	8.5±0.1	8.8±0.1	8.5±0.1	8.6±0.1	8.7±0.1	8.5±0.1	8.7±0.1	8.5±0.1	8.6±0.1	0.1463

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

Table 5. Hematological analysis of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks[1]

Para-meters	Experimental diets										*P*-values
	LT-FM	VT-FM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	
PCV (%)	36.2±0.3^{a}	32.5±0.8^{c}	34.7±0.5^{b}	29.6±1.5^{d}	29.5±0.9^{d}	32.6±1.0^{c}	32.1±0.6^{c}	29.3±0.6^{d}	31.4 ±0.9^{c}	32.3 ±0.6^{c}	0.01
Hb (g/dl)	7.33±0.2^{a}	6.93±0.1^{b}	6.73±0.1^{bc}	6.37±0.2^{de}	6.07±0.2^{ef}	6.97±0.2^{b}	6.73±0.1^{bc}	5.97±0.2^{f}	5.97±0.2^{f}	6.50±0.2^{cd}	0.01
ALT (U/L)	2.67±0.6^{d}	4.67±2.1^{abc}	3.33±0.6^{cd}	5.33±1.5^{ab}	5.67±2.1^{a}	3.67±0.6^{bcd}	2.67±0.6^{d}	2.67±0.5^{d}	4.33±0.5^{abcd}	3.33±0.6^{cd}	0.01
AST (U/L)	185±2.6^{ab}	181±3.0^{bc}	178±2.0^{c}	183±2.6^{ab}	185±1.5^{ab}	186±2.1^{a}	187±1.5^{a}	186±2.1^{a}	187±4.9^{a}	184±2.1^{ab}	0.01
T-Protein (g/dl)	3.43±0.1^{cd}	3.73±0.1^{a}	3.40±0.1^{cd}	3.67±0.1^{ab}	3.43±0.1^{cd}	3.23±0.1^{d}	3.70±0.1^{a}	3.47±0.1^{bc}	3.77±0.1^{a}	3.47±0.2^{bc}	0.01
Glucose (mg/dl)	89.0±1.0^{de}	90.7±1.5^{cde}	88.7±0.6^{e}	93.7±2.1^{ab}	92.3±1.5^{abc}	93.0±1.0^{abc}	91.3±1.5^{bcd}	94.7±1.5^{a}	92.7±1.5^{abc}	91.3±1.5^{bcd}	0.01

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

PCV (%): Packed cell volume or hematocrit

Hb (g/dL): Hemoglobin

AST (U/L): Aspartate transaminase

ALT (U/U): Alanine transaminase

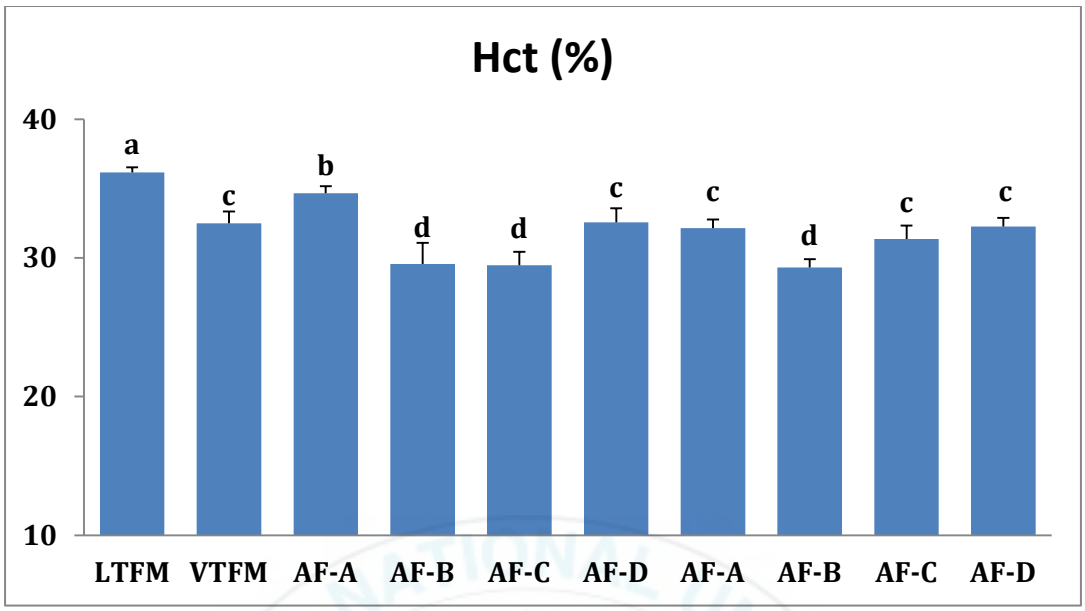


Figure 6. Hct (%) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0001$).

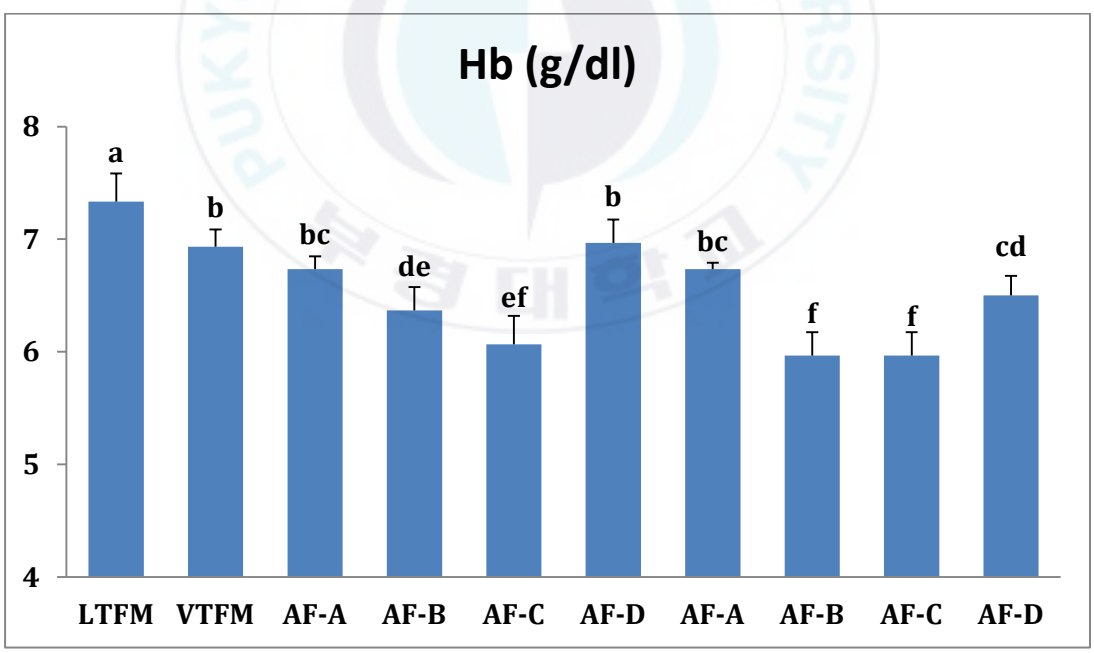


Figure 7. Hb (g/dl) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0001$).

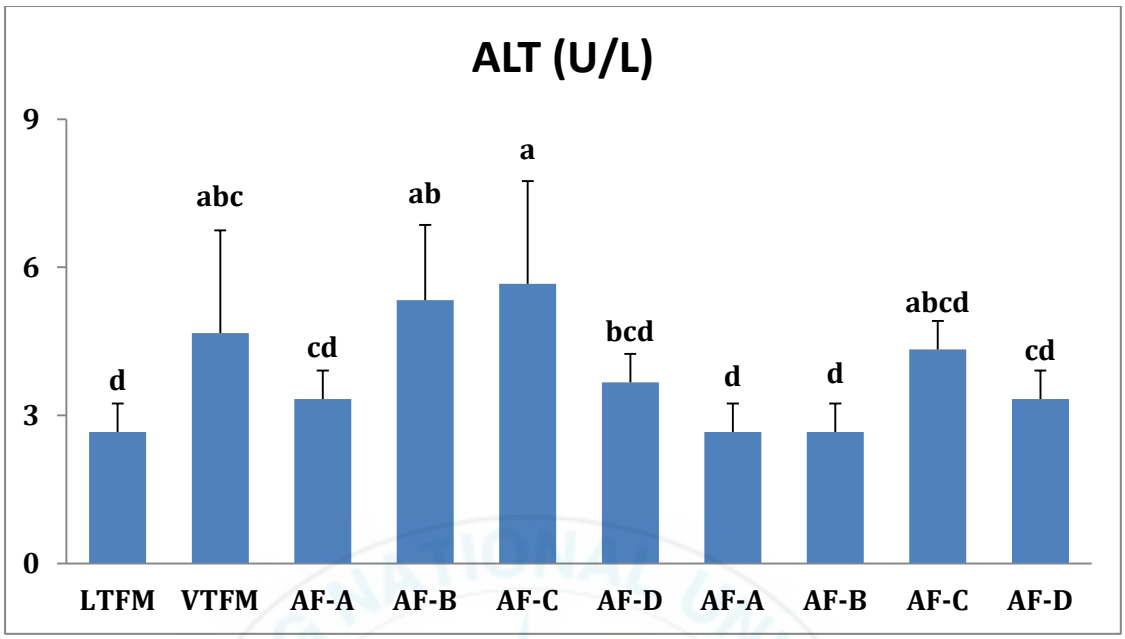


Figure 8. ALT (U/L) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0268$).

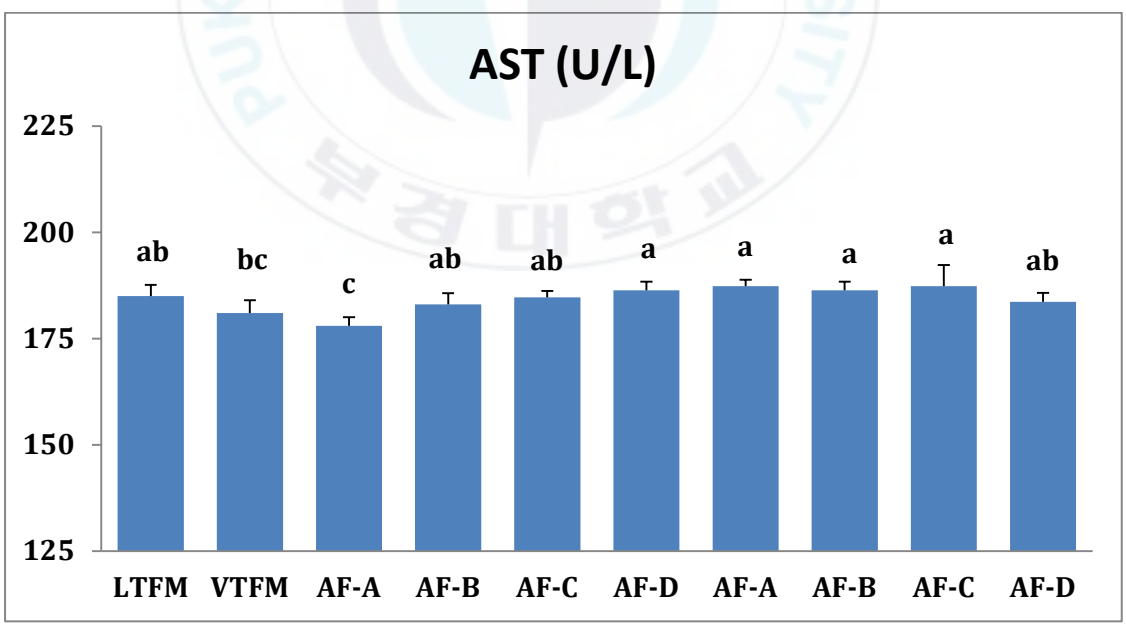


Figure 9. AST (U/L) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0057$).

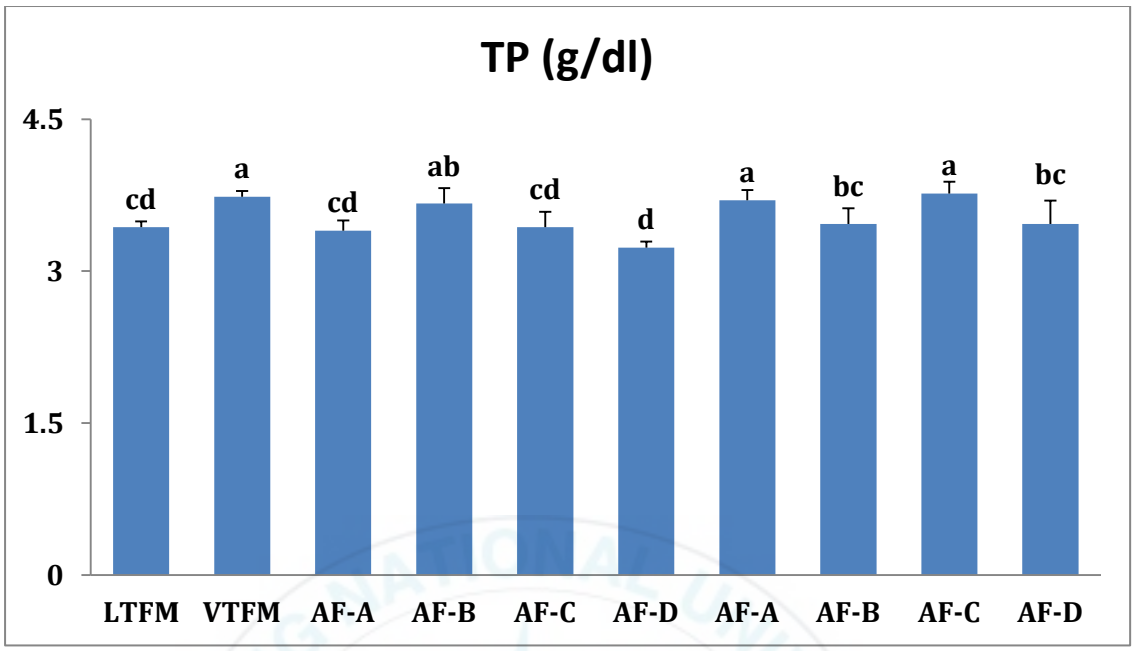


Figure 10. TP (g/dl) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0007$).

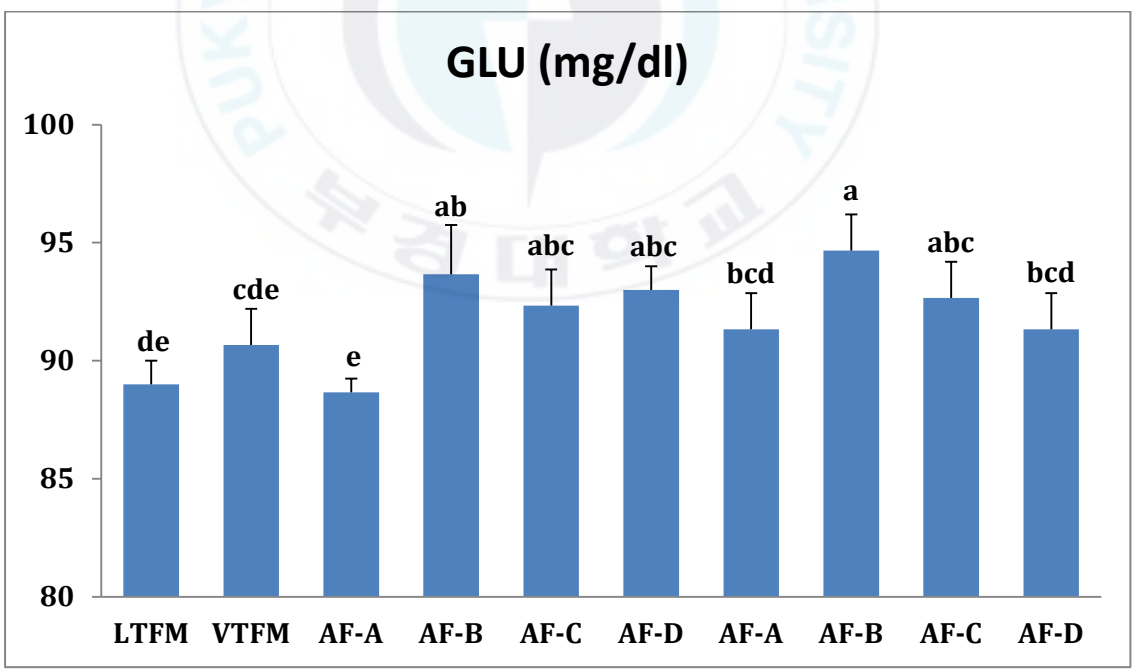


Figure 11. Glucose (mg/dl) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0008$).

Table 6. Non-specific immune responses of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks[1]

Para-meters	LT-FM	VT-FM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	*P*-values
SOD	57.4±1.1^{ab}	52.2±1.6^{abc}	55.1±3.2^{ab}	57.6±2.6^{ab}	54.3±2.9^{ab}	59.9±5.62^{a}	42.0±13.1^{bc}	38.1±9.1^{c}	44.4±8.7^{abc}	43.0±11.3^{bc}	0.1023
LYZ	7.8±0.9^{a}	6.8±0.4^{ab}	5.7±0.1^{abc}	5.6±2.1^{abc}	6.3±1.0^{ab}	6.83±1.9^{ab}	3.48±0.3^{c}	6.0±0.2^{abc}	5.3±1.4^{abc}	4.54±0.64^{bc}	0.1031
MPO	2.43±0.1^{a}	2.41±0.1^{a}	2.32±0.1^{ab}	2.32±0.1^{ab}	2.27±0.1^{ab}	2.29±0.1^{ab}	2.15±0.1^{b}	2.19±0.2^{b}	2.21±0.4^{b}	2.17±0.6^{b}	0.0856

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

SOD (% inhibition): Super oxide dismutase activity.
LYZ (U/ml): Lysozyme activity.
MPO (absorbance): Myeloperoxidase activity .

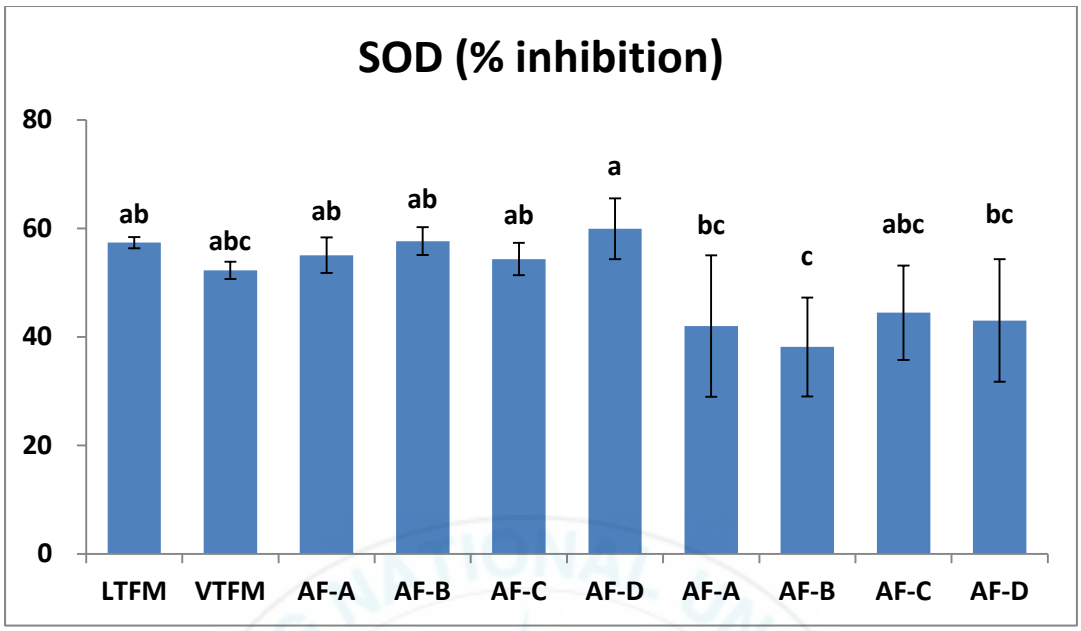


Figure 12. Superoxide dismutase activity (% inhibition) of rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.1023$).

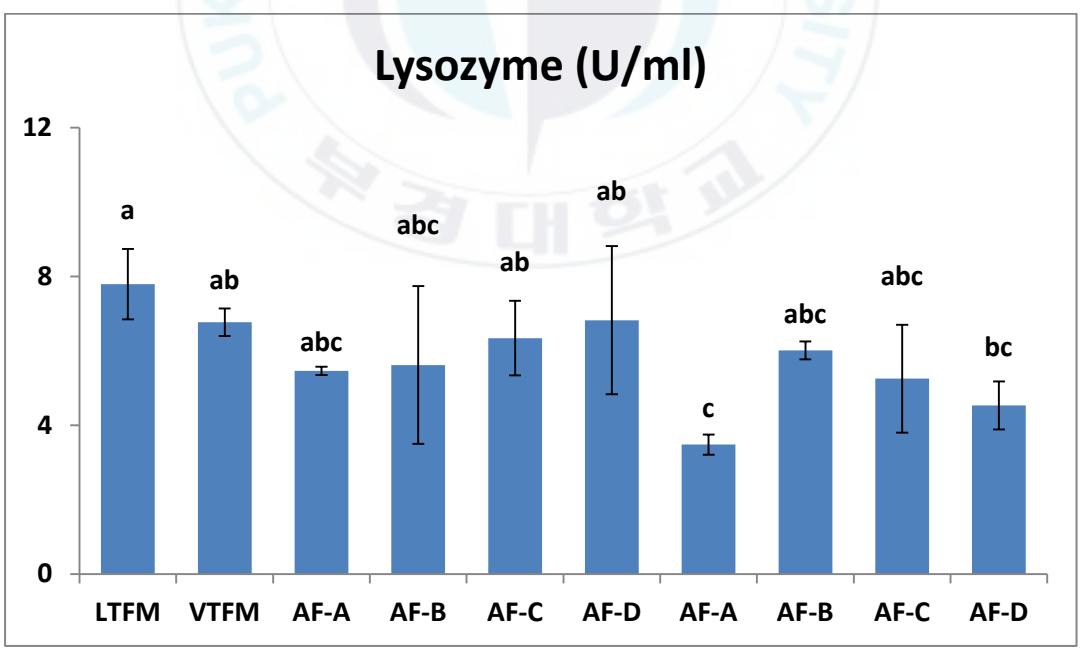


Figure 13. Lysozyme activity (U/ml) of rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.1031$).

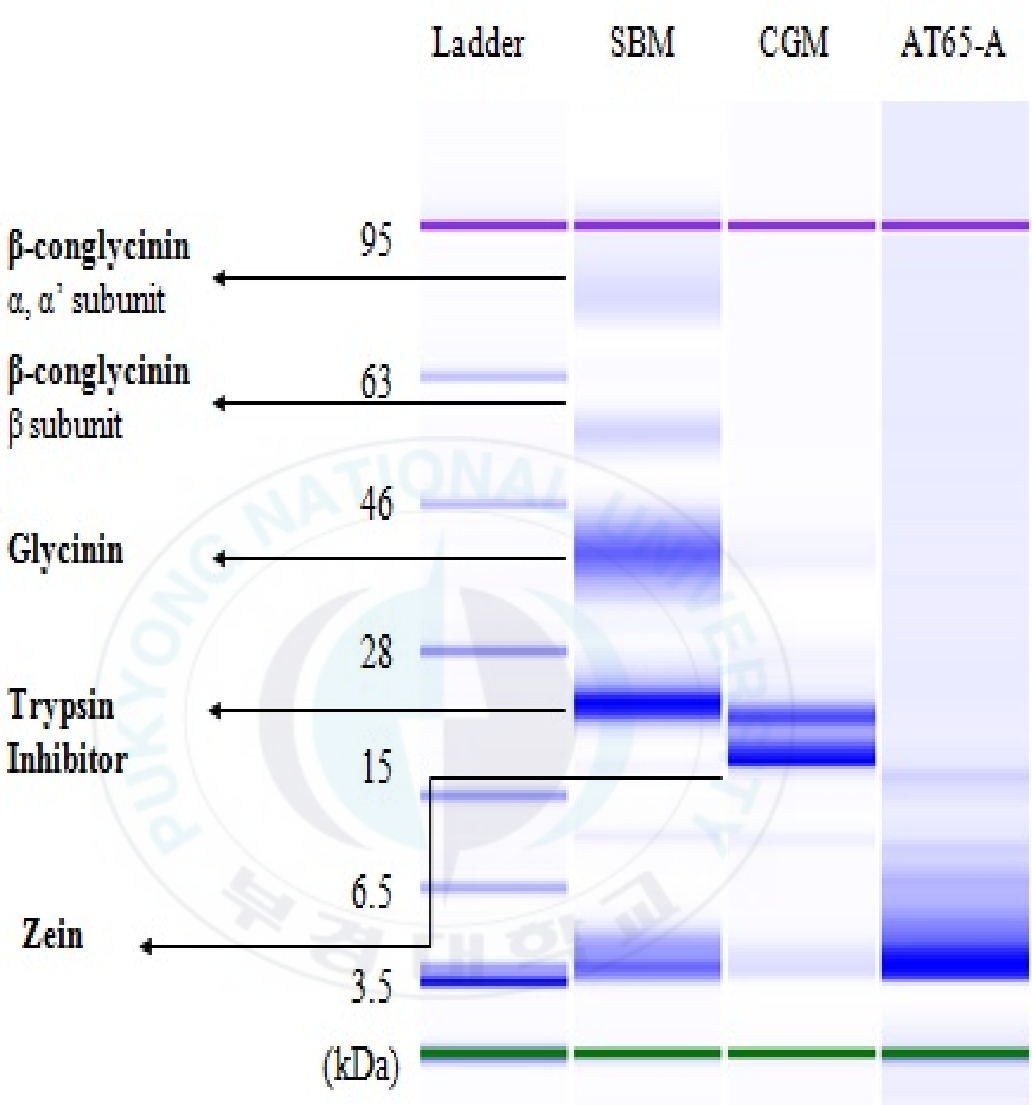


Figure 14. Protein profile of the SBM, CGM and AT65-A (or, AF-A) run with Protein 80 kit.

Table 7. Distal intestinal morphology of juvenile rainbow trout, *Oncorhynchus mykiss* fed the ten experimental diets for 8 weeks[1]

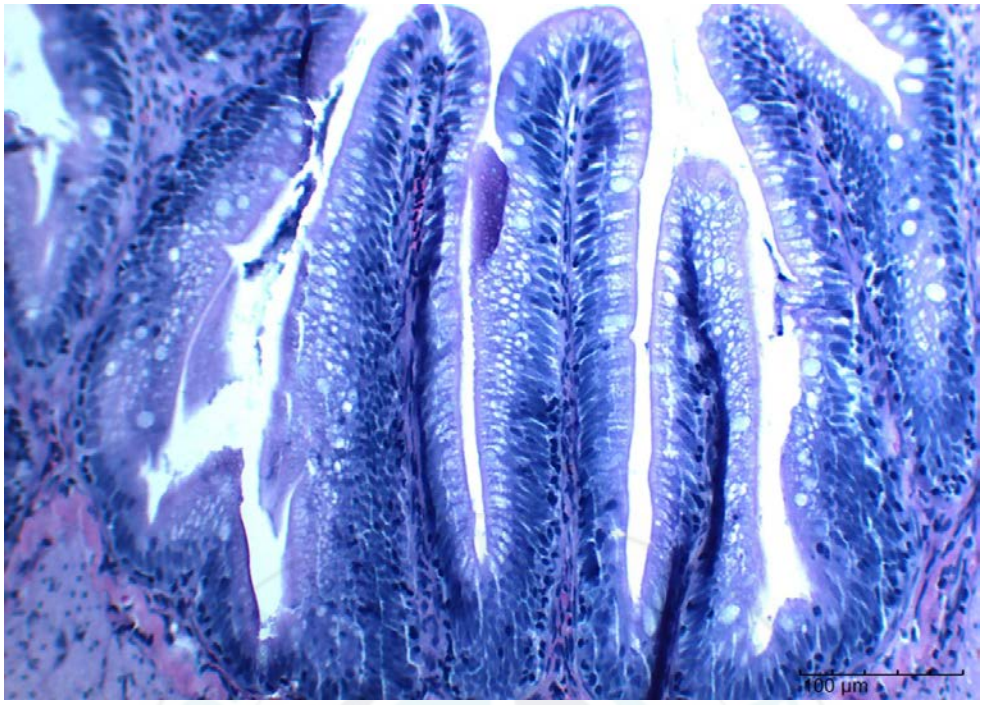
	Experimental diets										*P*-values
	LT-FM	VT-FM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	
GC	29.3±6.1^{ab}	24.7±3.5^{ab}	27.3±4.2^{ab}	28.3±5.5^{ab}	30.0±2.0^{a}	27.7±1.5^{ab}	23.7±6.4^{ab}	22.0±4.0^{b}	22.7±5.0^{ab}	22.0±1.7^{b}	0.2045
VL	366±8.5^{a}	347 ± 10.1^{c}	358 ± 5.5^{abc}	366 ±7.1^{a}	363±5.0^{ab}	365±8.5^{a}	348 ± 4.0^{c}	347±5.0^{c}	351±4.6^{c}	354±5.1^{bc}	0.0027
MT	68.3±3.5^{ab}	61.0±4.0^{c}	65.7±2.5^{ab}	65.0±2.0^{ab}	67.7±3.1^{ab}	68.7±3.1^{a}	60.3±3.1^{c}	62.3±4.5^{bc}	63.3±3.1^{abc}	62.0±4.0^{c}	0.0792

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

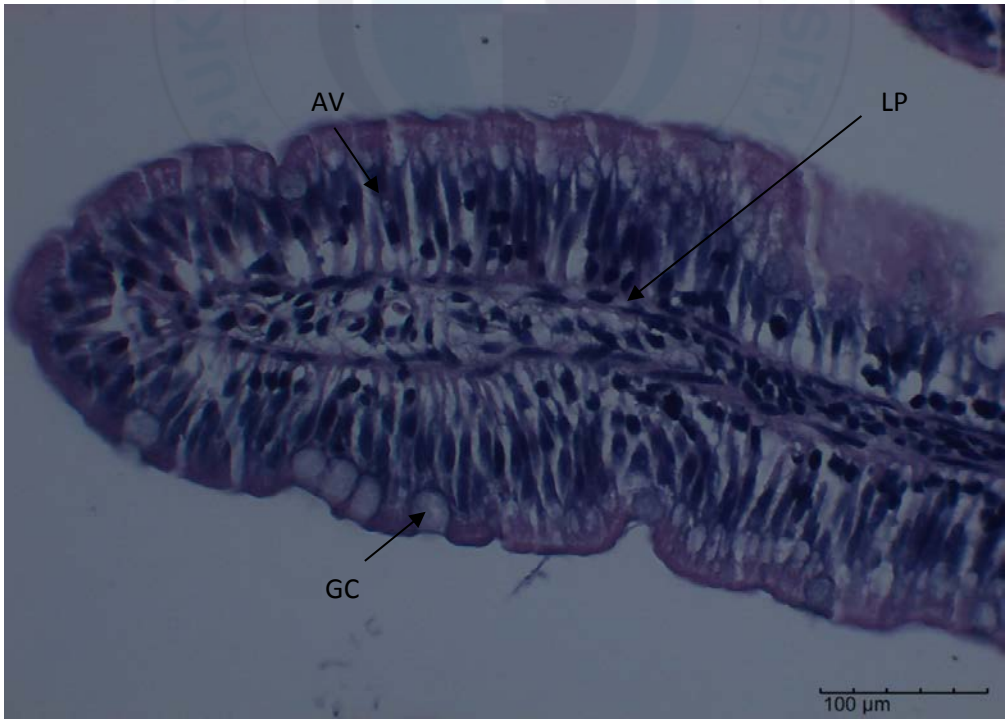
GC: Goblet cells per *villus*

VL: *villi* length (μm)

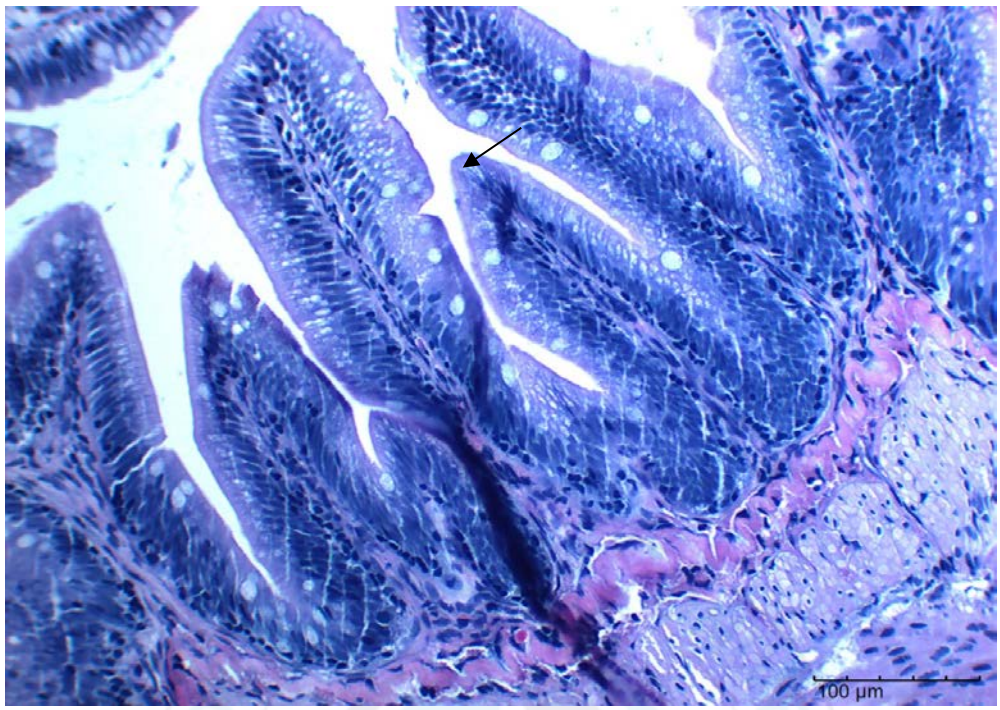
MT: muscular thickness (μm)



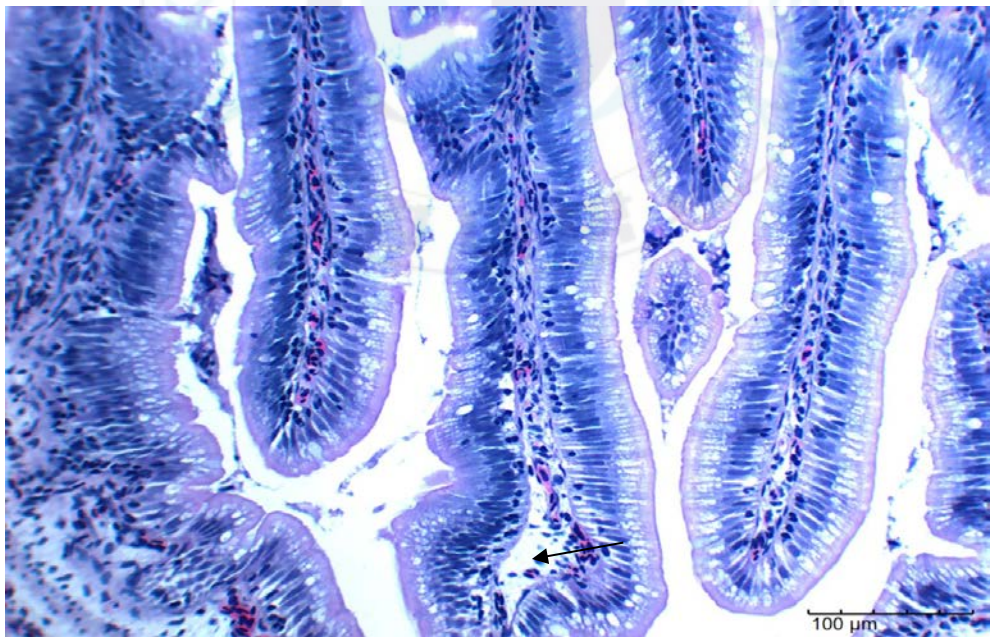
(a) LT-FM



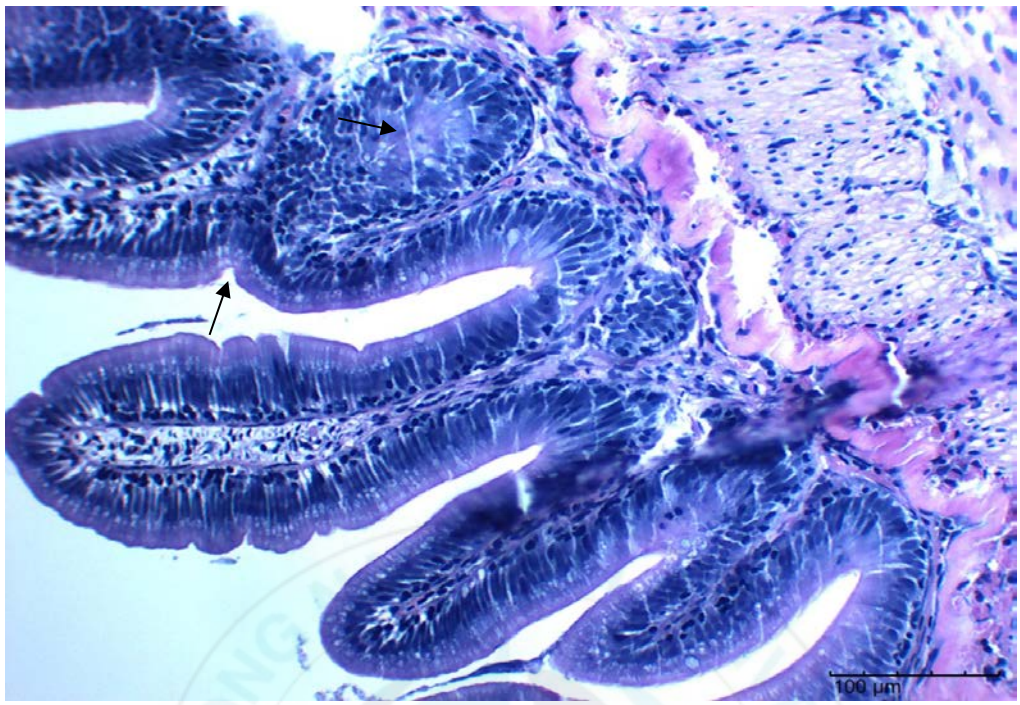
(b) LT-FM



(c) VT-FM



(d) BPC at 30% FM replacement level



(e) BPC at 50% FM replacement level

Figure 15. Representative photomicrographs from the distal intestine of juvenile rainbow trout, *Oncorhynchus mykiss* fed the experimental diets for 8 weeks. LT-FM (A) depicting the normal and elongated *villi* (B) showing wide surface of *villus* with goblet cells (GC), *lamina propria* and absorptive vacuoles (AV) in arrows; VT-FM (C) depicting normal *villi* with some short *villi* (irregular); BPC at 30% FM replacement level (D) showing normal *villi* with some dilation (in arrows); BPC at 50% FM replacement level (e) depicting short and fused *villi* with huge dilation.

CHAPTER 3

Evaluation of bioprocessed protein concentrates by the determination of apparent digestibility of protein and digestive enzyme activities in rainbow trout, *Oncorhynchus mykiss*

1. Introduction

Dietary fish meal (FM) is considered as the main protein sources for carnivorous fish species (Naylor et al. 2009; FAO 2010; Hardy 2010). However, global demand of fish meal increases day-by-day which is forcing the aquaculture researcher to search for alternative protein sources such as high quality plant based-protein which have high digestibility as well (Booth et al. 2001; Gatlin et al. 2007; Hardy 2010). However, the uses of plant protein sources in aquaculture have some limitations because of having anti-nutritional factors (ANFs) such as trypsin inhibitors, saponins, phytic acids, conglycinin, glycinin, zein and limiting amino acids such as methionine, lysine, and cystine (Francis et al. 2001; Krogdahl et al. 2010).

It has been reported that processing of plant protein ingredients could reduce the anti-nutritional factors and improve the digestibility of the diets in fish (Kokou et al. 2012). This can be done by fermentation, irradiation, heat treatment, or chemical extraction (Kokou et al. 2015; Rumsay 1994). Soybean meal (SBM) and corn gluten meal (CGM) are widely used plant protein ingredients in aqua feeds. Soybean meal has high protein content (48%) and has high amino acid profile, except methionine (El-Sayed 2000). On the other hand, CGM has also high protein content (63% CP), low fiber, and low ANFs and has good amino acids profile except lysine and arginine (Pereira and Oliva-Teles 2003). It has been reported that combination of SBM and CGM could increase the FM replacement in gilthead seabream (Kisssil and Lupatsch 2004).

The nutritional values of aqua feed ingredients are dependent on their digestibility and bioavailability to the species being fed (Chi et al. 2016). The digestibility coefficient values of diets and ingredients are very important to maximize the fish growth by delivering appropriate amount of nutrients. There have been a numerous methods are proposed to determine the nutrient digestibility in terms of collection of feces in fish. The methods are categorized

into passive and active method. For most of the fish species passive methods such as automatic or manual filtration, or sedimentation by settling chambers according to Guelph system (Cho et al. 1982; Choubert et al. 1982; Heinitz et al. 2016). However, these methods have some drawbacks such as leaching of soluble nutrients which may overestimate the digestibility and inaccurate reading of marker in the feces if feces are very soft which increases loss of nutrients in the feces (NRC 2011; Bureau et al. 2002; Glencross et al. 2007). In active method, recently stripping of fish for feces collection is commonly using in some carnivorous fish species such as rainbow trout, Atlantic salmon, *Salmo salar* and cobia, *Rachycentron canadum* (Gaylord et al. 2010; Burr et al. 2011; Hansen et al. 2011; Zhang et al. 2012; Chi et al. 2016) as well as in herbivorous fish species like common carp (Heinitz et al. 2016). The benefit of the stripping method is leaching of nutrient in water contact can be avoid which can ultimately give more accurate result than the other passive methods.

The determinations of intestinal enzyme activities such as protease, lipase, and amylase enzymes are also useful tools to understand the ingredient digestibility in fish feeds. It has been reported that most of the digestive enzymes are located in the brush border of intestine (Fountoulaki et al. 2005). Moreover, Ray et al. (2012) reported that amylase and protease may be triggered by the indigenous intestinal microbiota. However, due to the presence of ANFs in plant proteins these digestive enzymes could be disrupted in fish which may ultimately affect the growth and nutrient digestibility in fish (Sørensen et al. 2011; Chickwati et al. 2012; Kokou et al. 2015).

Rainbow trout is a widely cultured species around the world. There are very limited information available on the fermented protein concentrates digestibility and the digestive enzyme activities in juvenile rainbow trout. So, the aim of this experiment is to evaluate the apparent digestibility of protein and digestive

enzyme activities in juvenile rainbow trout fed diets supplemented with fermented protein concentrates in comparison to other plant proteins.

2. Materials and methods

2.1. Experimental diets

To determine apparent digestibility coefficient (ADC) of protein, ten experimental diets including five types of bio-processed protein concentrates (BPC) in terms of AF-A (only fermented protein concentrate), AF-B (BPC pre-treated with acid hydrolyses), AF-C (BPC + shrimp soluble extract), AF-D (BPC pre-treated with acid hydrolyses + shrimp soluble extract), AF-E (BPC + protease enzyme), soybean meal (SM), corn gluten meal (CGM), and a commercial fermented soybean meal (Soy-T) were assessed indirectly in rainbow trout by applying 0.5% chromic oxide (Cr_2O_3) in the diets as marker based stripping method. In the study, one diet with low temperature fish meal (LT-FM) or reference diet and another diet with Vietnam local fish meal (VT-FM) were used as controls. Each of ten experimental diets was consisted with 70% reference diet and 30% test ingredient.

The apparent protein digestibility in fish was calculated by:

ADC of protein (%) = (1-(Cr in diet × Protein in feces)/ (Cr in feces × Protein in diet)) ×100

The apparent ingredient digestibility in fish was calculated by:

ADC Ingr (%) = ADC Feed+[(ADC Feed-ADC ref.diet) × (0.7× Protein in Ref/ 0.3 × Protein in Ingr)]

2.2. Experimental fish and feeding

Fifteen fish with an average initial weight of 40.26±1.36 g (mean±SD) were randomly distributed in each of 30 semi-circulated tanks in triplicate. Fish were fed the experimental diets twice a day (09:00 and 18:00) to apparent satiation. The feces collection was carried out twice a day by stripping process for 30 days.

Crude protein content in feeds and feces were determined by the AOAC methods (1995). In brief, samples of diets and feces were dried to a constant weight at 135°C for 2 h to determine moisture content. Ash was determined by incineration using muffle furnace at 550°C for 3 hr. Crude lipid was determined by soxhlet extraction unit using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein content analyzed by Kjeldahl method (N × 6.25) after acid digestion. Chromium (Cr) content in feeds and feces were determined by Inductively Coupled Plasma Mass Spectromy (ICP-MS, Perkin-Elmer, Waltham, USA). Argon was used as a carrier gas.

For the analysis of digestive enzymes from fish intestine, five fish were randomly collected from each aquarium and ventral part of fish dissected to collect intestine and pooled in the vials for homogenization. After homogenization, supernatant of intestinal samples were further processed for digestive enzyme analyses such as lipase, amylase and protease following the manufacturers protocols (Biovision, USA).

2.3. Statistical analysis

All the data were analyzed by one-way ANOVA using SAS version 9.1 software (SAS Institute, Cary, NC, USA) to test the effects of dietary protein. When a significant effect of the treatments was observed, a least significant difference (LSD) test was used to compare means. Treatment effects were considered significant at $P<0.05$.

3. Results and discussion

The results revealed that apparent digestibility of diets (ADDs) and apparent digestibility of ingredients (ADIs) for crude protein (CP) were significantly higher in fish fed AF-B, C, and D diets compared to other plant protein based diets. Results support the findings of the first experiment. There were no significant differences among fish fed AF- B, D, and LT-FM diets. The results

suggested that AF-B, C, and D diets could be good sources of protein in the diets of rainbow trout in terms of digestibility of protein in feeds as well as in ingredients.

Dietary protein quality can affect the fish performance and protein digestibility in fish (Ye et al. 2011). In the present study, the ADC of protein of above mentioned diets must be highly digestible by rainbow trout. This indicates that each of these fish meals can be utilized efficiently as protein sources for rainbow trout. The ADC of protein for the AF-B, C and D diets is supported previously reported for rainbow trout (Gaylord et al. 2008). In this study, the ADC of protein for the diets are lower than that reported for rainbow trout (Burrells et al. 1999), silvery-black porgy (Yaghoubi et al. 2016), Japanese sea bass (Zhang et al. 2014) and Azarm and Lee (2012). The reason might be because of using stripping method for feces collection of fish in this study. The results of our study is in agreement with Heinitz et al. (2016) who also found the same ranges of digestibility of plant based protein ingredients in common carp. In the present study, the protein ADCs of the AF-B, C, and D diets were significantly higher than those of the other plant based ingredients tested. The differences in ADC of protein among plant based meals can be attributed to their different nutrient compositions, raw materials, species, locations, seasons of catch, and processing conditions used to produce the meal (Luo et al. 2008; Lemos et al. 2009; Terrazas-Fierro et al. 2010).

The evaluation of the digestive enzymes activity in fish species for aquaculture may be helpful in the selection of feed ingredients (Yaghoubi et al. 2016). In the present study, digestive enzyme such as protease followed the same trends as ADD and ADI of protein in juvenile rainbow trout. Kokou et al. (2012) reported that bioprocessing can increase the nutrient digestibility and decrease the ANFs in SBM. Yamamoto et al. (2010) postulated that nutritional benefit of fermented SBM is dependent on fermentation conditions, and fish meal in diets

for rainbow trout can be completely replaced by well-fermented SBM. In this experiment, we used solid-state fermented BPCs which might increase the digestibility of feeds in terms of increasing protease enzyme activity. However, compared to BPC diets other plant based diets such as SBM and CGM showed significantly lower protease activity probably due to the presence of high ANFs like trypsin inhibitors (Krogdahl et al. 2010). However, Egounlety and Aworh (2003) reported that SBM fermentation can increase trypsin inhibitors slightly. Moreover, lactic acid fermentation such as *Bacillus* spp. could eliminate the ANFs in SBM with increasing digestibility of protein, lipid, and carbohydrate for rainbow trout (Refstie et al. 2005). In this study, in line with protease enzyme, amylase enzyme also have shown higher activities in fish fed the BPC diets compared to other plant based protein diets. However, there were no significant differences in lipase activity among fish fed the experimental diets.

In this experiment, the most interesting finding is the digestibility of diets or ingredients in terms of protein are lower than the studies reported previously. The reason might be due to the use of different feces collection methodologies. In our study we used stripping method whereas, in all other studies, digestibilities were measured by sedimentation method except Kim et al. (1998) and recently Heinitz et al. (2016). It has been reported that use of passive collection or sedimentation methods have leaching problem of soluble nutrients which ultimately overestimate the digestible data (NRC 2011). Overestimation may occur due to high solubility of feces leading to loss of soluble nutrients (Glencross et al. 2007). To avoid this problem it is necessary to separate the feces from water as quick as possible (Heinitz et al. 2016). In case of existed feces collection methodologies such as settling column modified from the Guelph system (Chu et al. 1991), centrifuging of settled feces from water (Watanabe et al. 1996), and collection feces from sedimentation column (Ogino and Chen 1973), all having problem with contact of feces highly where results might be also highly overestimated.

However, stripping method could prohibit feces contact with water which may give more accurate digestibility data (Heinitz et al. 2016).

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Table 8. Composition of the digestibility evaluation feed in rainbow trout, *Oncorhynchus mykiss* (% of DM basis)

Ingredients	LTFM	FM-VIE	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T
FISH MEAL	590	413	413	413	413	413	413	413	413	413
WHEAT FLOUR	180	126	126	126	126	126	126	126	126	126
SOYBEAN MEAL	55.0	38.5	38.5	38.5	38.5	38.5	38.5	38.5	38.5	38.5
SOYBEAN LECITHIN	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
FISH OIL	160	112	112	112	112	112	112	112	112	112
VITA-MINE PREMIX	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
FM-VIE		300								
AF-A			300							
AF-B				300						
AF-C					300					
AF-D						300				
AF-E							300			
SOYBEAN MEAL								300		
CORN GLUTEN									300	
SOYTIDE										300
CHORMIC OXIDE	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000

Proximate composition (% of dry matter basis)										
Moisture %	9.4	9.1	9.4	9.7	9.2	9.4	8.9	9.1	9.4	9.8
Crude protein %	49.1	47.3	47.9	46.9	46.2	47.2	49.3	42.3	43.5	42.6
Crude lipid %	22.9	23.2	23.1	22.5	22.9	22.4	21.5	21.9	21.4	20.6
Ash %	9.3	7.7	7.3	7.2	7.6	7.4	7.1	7.3	5.4	7.2

Table 9. Amino acids profile of the experimental feeds in rainbow trout, *Oncorhynchus mykiss* (% of sample)

Amino acids	LTFM	FM-VIE	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T
Asp.	4.90	5.02	4.38	4.40	4.31	4.21	4.54	4.43	2.58	4.44
Thr.	2.18	2.26	1.87	1.92	1.85	1.81	1.91	1.78	1.08	1.77
Ser.	2.18	2.27	2.14	2.25	2.11	2.07	2.17	1.98	1.11	1.95
Glu.	7.55	7.98	8.24	8.48	8.25	7.87	8.42	7.28	4.12	7.12
Pro.	2.14	2.50	2.61	3.02	2.66	2.57	2.77	2.10	1.41	2.14
Gly.	3.05	3.33	2.32	2.31	2.29	2.18	2.36	2.21	1.52	2.31
Ala.	3.22	2.20	3.13	3.10	3.11	2.94	3.19	2.48	1.68	2.49
Val.	2.80	1.96	2.61	2.56	2.59	2.49	2.71	2.35	1.61	2.38
Ile.	2.20	4.46	2.06	2.06	2.03	1.98	2.15	1.89	1.15	1.93
Leu.	4.25	0.97	4.86	4.91	4.79	4.55	4.96	3.65	2.38	3.63
Tyr.	1.68	2.49	1.32	1.50	1.23	1.37	1.41	1.16	0.75	1.24
Phe.	2.29	1.75	2.50	2.53	2.44	2.32	2.55	2.07	1.38	2.22
His.	1.72	4.86	1.87	1.60	1.76	1.66	1.81	1.63	1.13	1.70
Lys.	4.02	2.29	2.91	2.84	3.70	3.53	2.99	3.01	2.06	2.94
Arg.	3.11	2.93	2.53	2.53	2.41	2.33	2.52	2.62	1.53	2.62

Table 10. Apparent digestibility coefficients (%, ADC) for dry matter, crude protein and lipid in juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets[1]

	LTFM[2]	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SM	CGM	SOY-T	*P*-values
Dry matter	87.5±2.1^{a}	81.5±0.7^{b}	82±2.8^{b}	83±1.4^{b}	82.5±0.7^{b}	83±2.8^{b}	79.5±0.7^{b}	78.5±2.1^{b}	79.5±0.7^{b}	80.5±2.1^{b}	0.0221
Crude protein	86.3±0.1^{a}	82.4±1.0^{b}	79.1±1.1^{c}	84.1±0.3^{ab}	82.5±1.4^{b}	84.3±1.0^{ab}	78.8±0.3^{c}	75.2±0.3^{d}	68.6±1.5^{e}	78.1±1.9^{c}	0.0001
Lipid	92.9±0.8^{a}	84.8±0.3^{d}	86.9±1.4^{c}	87.3±1.1^{c}	82.8±1.6^{e}	85.8±0.2^{cd}	93.0±0.4^{a}	87.5±0.8^{c}	90.5±0.3^{b}	86.0±0.1^{cd}	0.0001

[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]Reference diet.

Table 11. Apparent digestibility coefficient of ingredients (%, ADI) in juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets[1]

	LTFM[2]	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T	*P*-values
Crude protein	96.5±0.1^{a}	92.1±1.1^{b}	88.4±1.2^{c}	94.2±0.7^{ab}	92.3±1.6^{b}	94.1±1.1^{ab}	88.2±0.3^{c}	84.0±0.3^{d}	76.5±1.3^{e}	87.8±2.8^{c}	0.0001

[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]Reference diet.

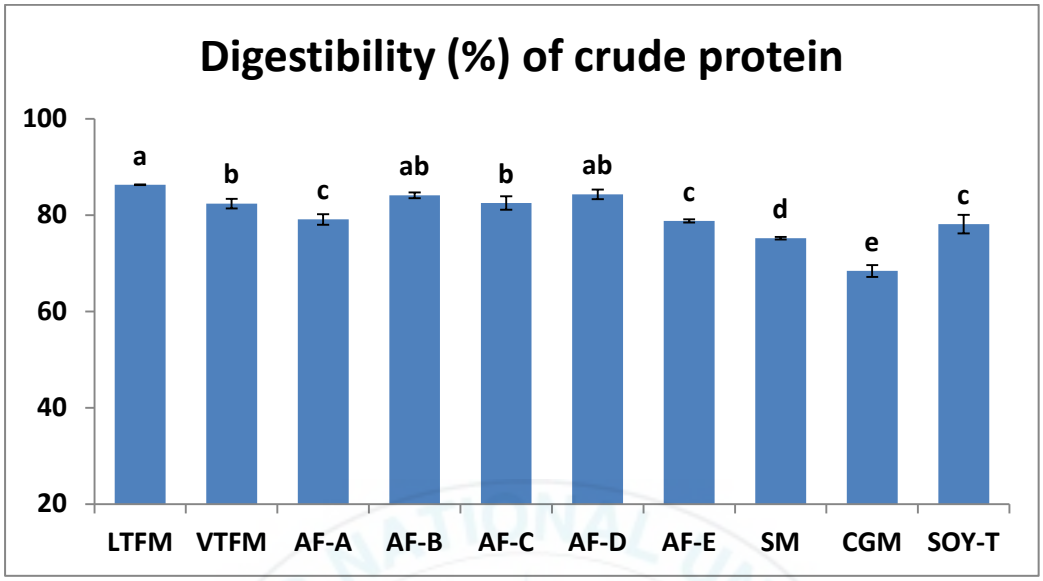

Figure 16. Apparent digestibility coefficients (%) for crude protein of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets ($P < 0.0001$).

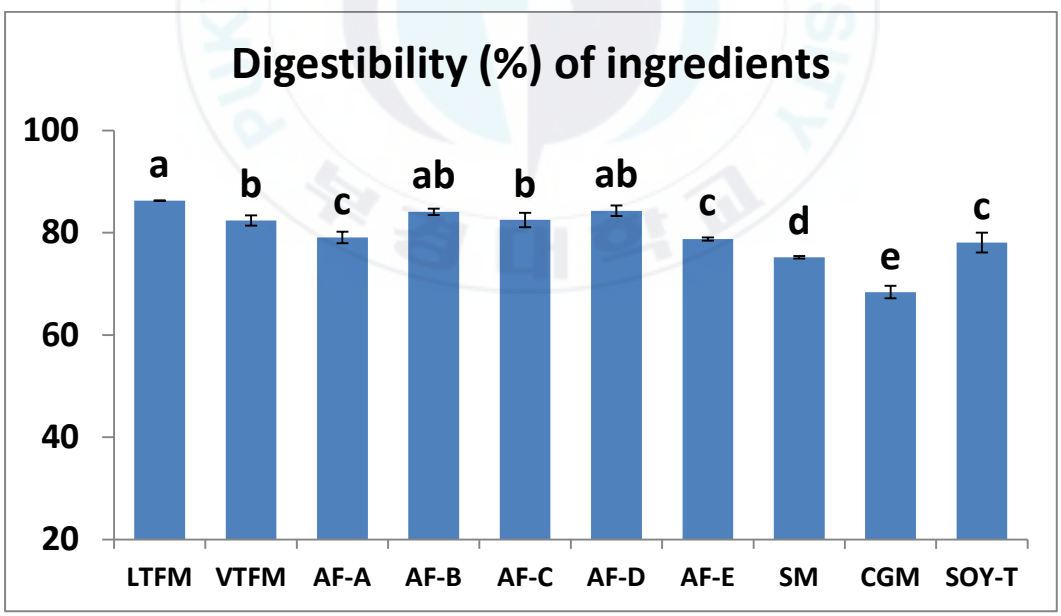

Figure 17. Apparent digestibility of ingredients (%) of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets ($P < 0.0001$).

Table 12. Digestive enzymes activity of juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks[1]

	LTFM	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T	*P*-values
Protease (mU/ml)	1.89±0.2^{a}	1.55±0.1^{b}	1.13^{c}±0.1^{c}	1.07^{c}±0.1^{c}	1.14^{c}±0.1^{c}	1.72±0.1^{ab}	1.56±0.1^{b}	0.83±0.1^{d}	0.67±0.1^{d}	1.70±0.1^{ab}	0.0001
Lipase (mU/ml)	3.43±0.1^{a}	3.73±0.1^{abc}	3.40±0.1^{abc}	3.67±0.1^{ab}	3.43±0.1^{bc}	3.23±0.1^{ab}	3.70±0.1^{ab}	3.47±0.1^{c}	3.77±0.1^{d}	3.47±0.2^{bc}	0.0018
Amylase (mU/ml)	12.8±0.3^{a}	11.8±0.4^{ab}	11.5±0.6^{bc}	8.8±0.3^{d}	12.2±1.1^{abc}	12.5±0.1^{ab}	11.2±0.8^{c}	4.6±0.5^{e}	8.1±0.8^{d}	12.5±1.1^{ab}	0.0001

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

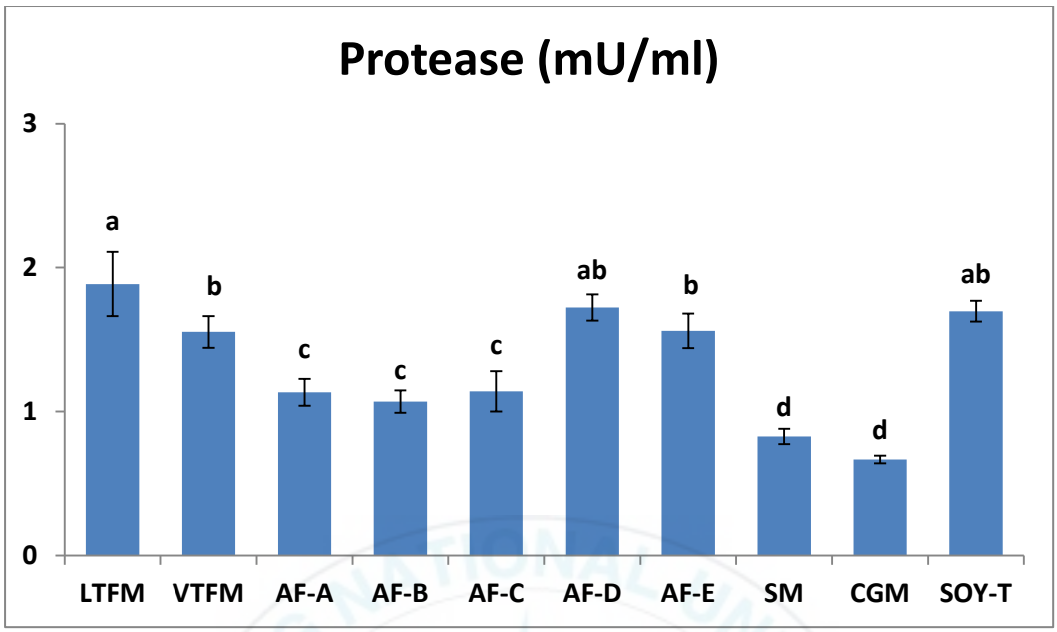

Figure 18. Protease activity (mU/ml) in juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0001$).

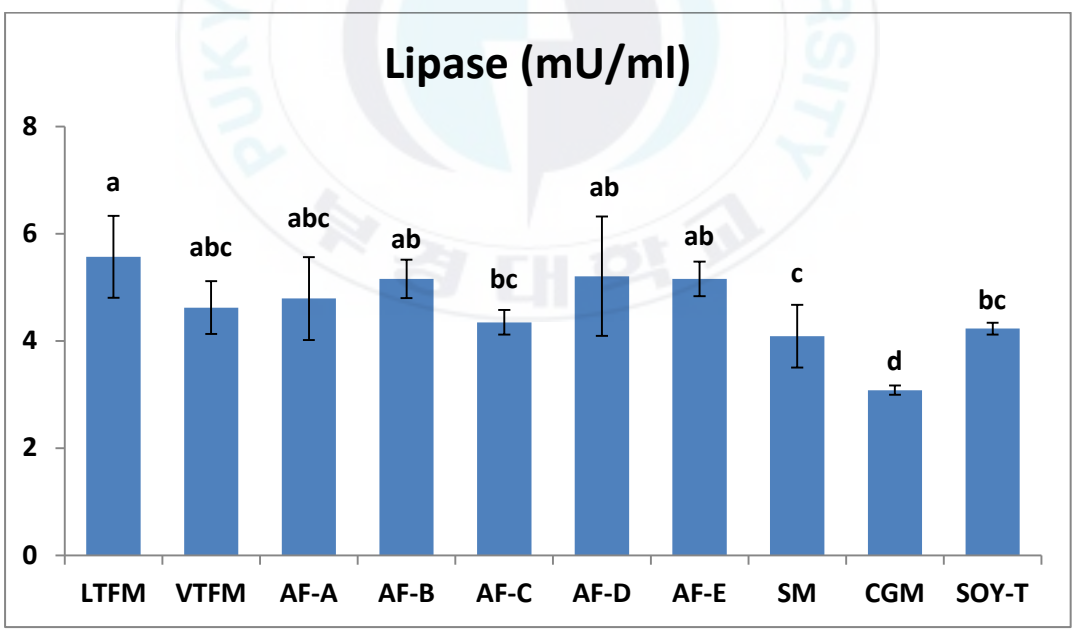

Figure 19. Lipase activity (mU/ml) in juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0018$).

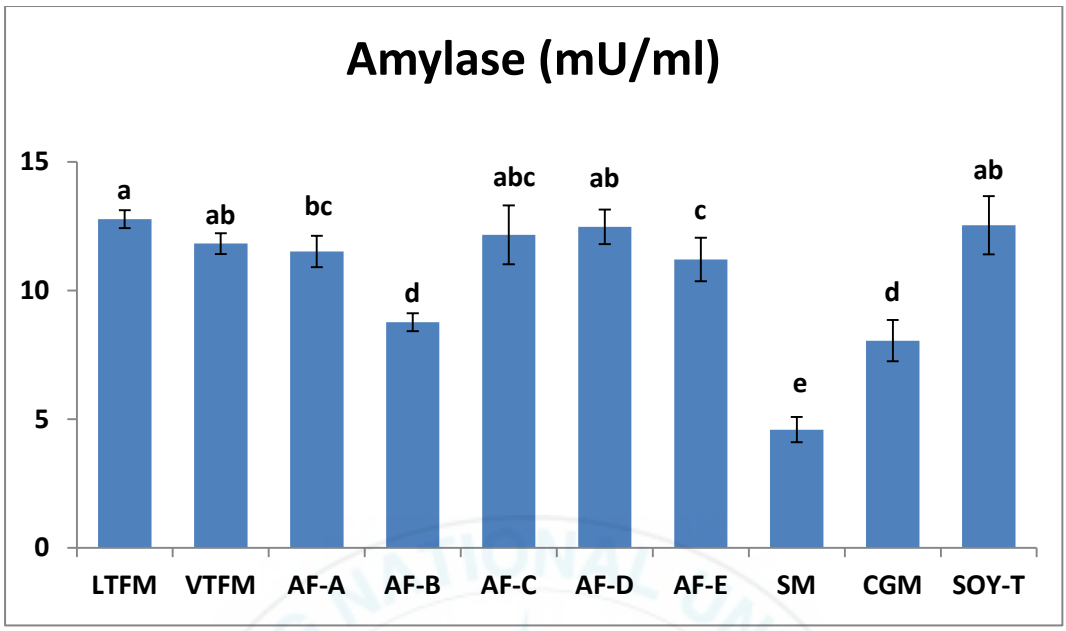

Figure 20. Amylase activity (mU/ml) in juvenile rainbow trout, *Oncorhynchus mykiss* fed experimental diets for 8 weeks ($P < 0.0001$).

CHAPTER 4

Effects of bioprocessed protein concentrates in partial replacement of fish meal on growth, hematology and non-specific immune responses in juvenile whiteleg shrimp, *Litopenaeus vannamei*

1. Introduction

Fish meal (FM) is one of the major sources of protein in the diets of shrimp because of having essential amino acids and its palatability (Davis et al. 2004; Sá et al. 2013). However, demand for fish meal is increasing day-by-day (5% per year) in aqua feed industry which limits the world fish meal resources (Cruz-Suarez et al. 2007). In practical shrimp farming they required near about 25-50% fish meal (FM) in diet (Amaya et al. 2007). Studies have reported that FM can be successfully replaced by soybean meal (SBM) (Alvarez et al. 2007; Smith et al. 2007). However, even though full fat and defatted SBM are good sources of digestible protein, they are low in lysine and methionine (Divakaran et al. 2000; Cruz-suarez et al. 2009), presence of anti-nutritional factors (ANFs) (Francis et al. 2001) and contain poorly digestible non-starch polysaccharides in fish (Refstie et al. 2000). Dersjant-Li (2002) reported that most of the ANFs in SBM can be eliminated by proper processing technique. Kokou et al. (2012), Azarm and Lee (2014) and Kader et al. (2012) reported that bioprocess or fermentation of SBM can increase the FM replacement level in fish.

Corn gluten meal (CGM) is a good source of protein having at least 63% protein and low fat (NRC 2011). However, as a plant protein CGM also contains some ANFs and limiting amino acids (Pereira and Oliva-Teles 2003). It has been reported that mixture of SBM and CGM could increase the FM replacement level in fish (Lunger 2006). Lio and Wang (2012) reported that solid-state fermentation of feed ingredients can increase the nutritive value of aqua feeds. Moreover, the process can increase soluble protein content as well as decrease the ANFs in the diets which may ultimately improve the growth performance and digestibility in fish (Kokou et al. 2012).

The objective of this study was to evaluate the performance of whiteleg shrimp, *Litopenaeus vannamei* fed the four different types of fermented protein

concentrates replacing high fish meal protein (30% and 50% FM replacement level).

2. Materials and methods

2.1. Experimental diets

Ten experimental diets were supplemented with high quality low temperature fish meal based diet (LT-FM) as positive control, low quality Vietnamese fish meal based diet (VT-FM) as negative control, and rest of the eight diets constitutes with four kinds of bioprocessed protein concentrates (BPCs) as alternatives of fish meal (AF-A, B, C, and D) at 30% and 50% replacement levels of high quality fish meal (+) (Table 1). The BPC ingredients were prepared by different processing techniques using soybean meal (SBM) and corn gluten meal (CGM) (1:1). In brief, mixture of SBM and CGM as input or substrate of the processing technique was fermented by using selected *Bacillus* spp. at pasteurization temperature. The important feature of this technique is the fermentation process continued at solid state with minimum adding of water (solid state fermentation, SSF). The AF-A diet was fermented by *Bacillus* spp. only, diet AF-B was pre-treated acid hydrolyzed AF-A, whereas AF-C and D diets were supplemented with AF-A+shrimp soluble extract (SSE) and AF-B+SSE, respectively. The experimental diets were formulated to be isonitrogenous (40% CP) and isocaloric (16.7 kJ/g energy) based on calculation by Garling and Wilson (1976). The actual nutrient contents in experimental diets are shown in Table 13. The experimental diets were prepared followed by Bai and Kim (1997). The dried pellets were stored at -20 ^{0}C until used.

2.2. Experimental shrimp

Juvenile whiteleg were transported from Geoje Marine Hatchery (Geoje, Korea) of National Institute of Fisheries Science (NIFS), Korea to Feeds and Foods Nutrition Research Center (FFNRC), Pukyong National University, Busan,

Korea. Before the start of the experiment, all shrimp were reared in a circular plastic tank with 5,000 L well water and were fed a commercial diet for 2 weeks. For experimental purposes, 30 flow-through aquaria were used for rearing shrimp. After 2 weeks of conditioning period, a group of 15 shrimp with an average initial weight of 3.88±0.05 g (mean±SD) were randomly distributed into aquaria in triplicate according to the ten experimental diets. Shrimp were fed one of the ten isocaloric diets for four times a day (08:00, 12:00, 16:00, 20:00h) at a level of 4% of wet body weight in the first 4 week and 3% in the second 4 week, respectively with apparent satiety. Total shrimp weight in each tank was determined every 2 weeks after anaesthesia with 100 ppm of MS 222 (tricaine methanesulfonate) and the amount of feeds were adjusted accordingly. During the experimental period water flow rate was maintained at 0.8 L/min and water temperature maintained at 27±1℃due to natural fluctuations in seawater temperature. Supplemental aeration was provided to maintain dissolved oxygen levels near saturation.

2.3. Sample collection, analyses and calculations

At the end of the feeding trial, all shrimp were weighed and counted to calculate growth parameters such as percent weight gain (WG), feed efficiency (FE) and specific growth rate (SGR) following the formula:

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

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

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

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

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

Hemolymph parameters such as ALT, AST, T-protein and glucose (Table 16), and non-specific immune parameters (Table 17) were determined. After the final weighing, five shrimp were randomly collected from each aquarium and hemolymph samples were obtained using syringes from the ventral part of carapace and pooled in the vials according to the number of diets. Serum was

collected by centrifuging haemolymph samples at 3,000 × *g* for 20 min and stored at −20°C. The non-specific immune parameters such as superoxide dismutase (SOD) and lysozyme were analyzed by spectrophotometer (Infinite 200 PRO NannoQuant, Tecan, Mannedorf, Switzerland) and hymolymph indexes by blood analyzer (DRI-CHEM 4000i- Fuji Dri-Chem Slide- 3150, Minato-ku,Tokyo, Japan). Crude protein, lipid, moisture and ash of whole-body samples were determined by the AOAC methods (1995). In brief, samples of diets and fish were dried to a constant weight at 135°C for 2 h to determine moisture content. Ash was determined by incineration using muffle furnace at 550°C for 3 hr. Crude lipid was determined by soxhlet extraction unit using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein content analyzed by Kjeldahl method (N × 6.25) after acid digestion.

2.4. Statistical analysis

All the data were analyzed by one-way ANOVA using SAS version 9.1 software (SAS Institute, Cary, NC, USA) to test the effects of dietary protein (Zar 1984). When a significant effect of the treatments was observed, a least significant difference (LSD) test was used to compare means. Treatment effects were considered significant at $P<0.05$.

3. Results and discussion

At the end of feeding trial, shrimp fed AF-B, AF-C, and AF-D diets showed significantly higher WG and SGR at 30% of FM replacement level than those of shrimp fed AF-B at 30% of FM or AF-A, AF-B, AF-C, and AF-D diets at 50% of FM replacement level in juvenile white shrimp (Table 14). However, shrimp fed LT-FM, VT-FM, AF-B, AF-C and AF-D diets showed no significant differences in terms of W

G and SGR at 30% of fish meal replacement level. Yun (2015 unpublished data) reported that 33% of FM could be replaced with soybean meal in white

shrimp reared in biofloc system. However, Qihui et al. (2015) found that extruded soybean meal can reduced up to 20% FM in the diet containing 40% CP and 30% FM in juvenile white shrimp. In addition, Oujifard et al. (2012) reported that 50% FM can be replaced in the diet of whiteleg shrimp using rice protein concentrate (RPC). In this study, FE and PER showed the same trends as WG and SGR. Survivability was not affected by the dietary treatments indicating that whiteleg shrimps have resistance to increasing BPC levels in the diets.

Serum AST, ALT and glucose activity of shrimp fed AF-D at 30% FM replacement showed significantly lower than those of shrimp fed other diets. The results indicate that fish fed AF-D diet at 30% FM replacement possessed better health status than the other diets. Whole body proximate composition of shrimp was not affected by dietary treatments.

In this study, interestingly, we have found that in addition to fermentation process pre-treated acid hydrolysis of ingredients could partially replace FM in the diet of juvenile whiteleg shrimp. Moreover, the study also showed that addition of crystalline amino acids such as methionine and lysine in bioprocessed protein concentrate (BPC) cannot replace 30% of FM which may indicate that acid hydrolysis is an important step to remove ANFs in the ingredient which is in agreement with Gatlin et al. (2007). Furthermore, the results also suggest that without acid hydrolysis of CGM the 30% FM replacement with BPC can be achievable if we add 2% SSE in BPC which beneficial effects has been seen in rainbow trout (Jo et al. 2016). However, in this study, whiteleg shrimp did not perform well at 50% FM replacement level may be due to the presence of higher level of ANFs in the BPCs such as saponins and other ANFs.

In conclusion, the results of the present study indicate that AF-B, AF-C and AF-D diet at 30% FM replacement level seemed to be better performed over other BPC based diets on growth performance and immune parameters in juvenile whiteleg shrimp.

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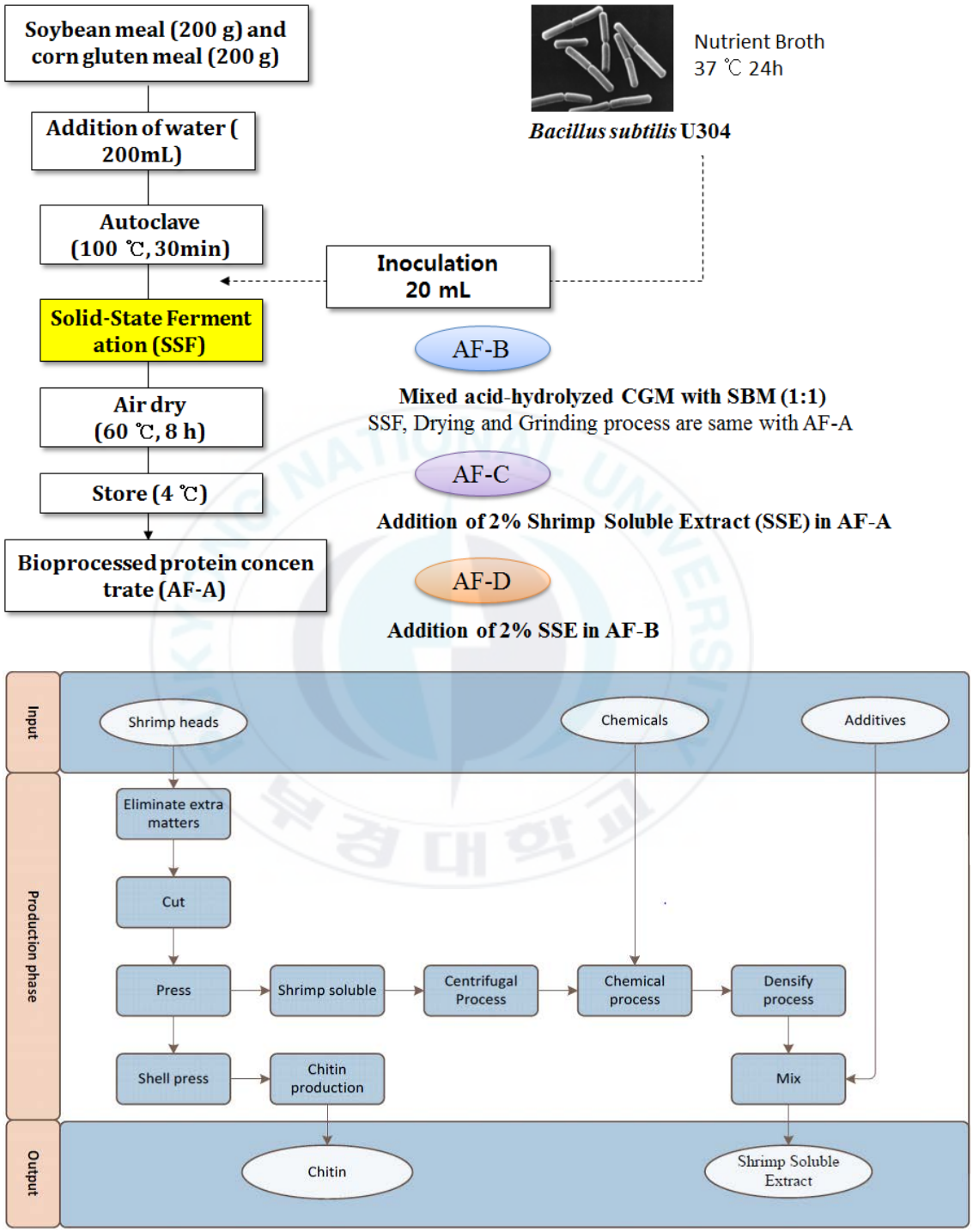

Figure 1. Production process of bioprocessed protein concentrates and shrimp soluble extract

Table 13. Composition of the experimental diets in juvenile whiteleg shrimp, *Litopenaeus vannamei* (% of DM basis)

Ingredients[1]	LTFM	FM-VIE	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)
Fish meal, Denmark LT[2]	27.0	0.00	18.9	18.9	18.9	18.9	13.5	13.5	13.5	13.5
Fish meal, Vietnam	0.00	28.6	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
AF-A	0.00	0.00	9.50	0.00	0.00	0.00	15.2	0.00	0.00	0.00
AF-B[3]	0.00	0.00	0.00	9.50	0.00	0.00	0.00	15.2	0.00	0.00
AF-C	0.00	0.00	0.00	0.00	9.50	0.00	0.00	0.00	15.2	0.00
AF-D	0.00	0.00	0.00	0.00	0.00	9.50	0.00	0.00	0.00	15.2
SBM 44%, South America	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0
Squid liver powder	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Meat, bone meal	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Wheat flour	30.3	30.3	30.3	30.3	30.3	30.3	30.3	30.3	30.3	30.3
Fish oil	1.50	2.30	2.00	2.00	2.00	2.00	2.30	2.30	2.40	2.30
Lecithin powder 97%	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Lysine	0.00	0.00	0.40	0.40	0.00	0.00	0.66	0.66	0.00	0.00
Methionine	0.00	0.00	0.06	0.06	0.00	0.00	0.10	0.10	0.00	0.00
Others[2]	5.20	5.20	5.20	5.20	5.20	5.20	5.20	5.20	5.20	5.20
Total	100	100	100	100	100	100	100	100	100	100

Proximate composition (% of dry matter basis)										
Moisture %	10.5	10.4	10.5	10.4	10.5	10.5	10.7	10.6	10.5	10.9
Crude protein %	40.9	40.2	41.2	41.4	41.0	41.9	41.6	41.5	41.0	40.4
Crude lipid %	8.8	8.9	9.1	9.1	9.0	8.9	9.1	8.8	9.1	9.2
Ash %	8.8	9.5	9.3	9.1	9.1	8.6	9.0	8.9	8.8	8.6

[1]Feedstuffs not mentioned here are the same feed stuffs as the domestic aquaculture feed companies are using currently.

[2]Norse LT-94®, low-temperature dried fish meal, Norsildmel, Bergen, Norway.

[3]AQUATIDE65 (AT-65) provided by CJ CheilJedang Corporation, Seoul, Korea.

[4]Others : Amygluten 1%, Blood meal 1%, Mineral 1%, Vitamin 1.15% , Cholesterol 0.02% and CMC 1% in all the diets.

Table 14. Growth performance of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks[1]

	LTFM	VTFM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	*P*-values
WG	198±7.3^{a}	186±12.4^{ab}	168±13.0^{cd}	189±10.6^{ab}	186±8.1^{ab}	186±9.8^{ab}	154±4.1^{d}	170±3.5^{c}	168±6.2^{cd}	175±4.5^{bc}	0.0002
SGR	2.10±0.1^{a}	2.02±0.1^{abc}	1.90±0.1^{de}	2.04±0.1^{ab}	2.02±0.1^{abc}	2.02±0.1^{abc}	1.79±0.1^{e}	1.91±0.1^{cd}	1.89±0.1^{de}	1.94±0.1^{bcd}	0.0001
FE	44.8±0.2^{a}	42.3±2.4^{ab}	41.3±1.5^{bc}	44.3±2.3^{ab}	42.7±1.3^{ab}	44.1±0.6^{ab}	36.2±2.5^{c}	40.2±2.2^{bc}	39.3±2.6^{bc}	40.8±0.3^{abc}	0.0005
PER	1.09±0.1^{a}	1.08±0.1^{a}	0.98±0.1^{bc}	1.07±0.1^{a}	1.04±0.1^{ab}	1.05±0.1^{ab}	0.86±0.1^{d}	0.94±0.1^{c}	0.93±0.1^{cd}	0.97±0.1^{bc}	0.0001
Sur.	86.7±6.7^{abc}	82.2±3.8^{abc}	86.7±6.6^{abc}	86.7±6.7^{abc}	80.0±6.6^{bc}	88.9±3.8^{ab}	84.4±10.2^{abc}	91.1±3.8^{a}	77.8±3.8^{c}	88.9±3.8^{ab}	0.2016

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

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

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

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

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

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

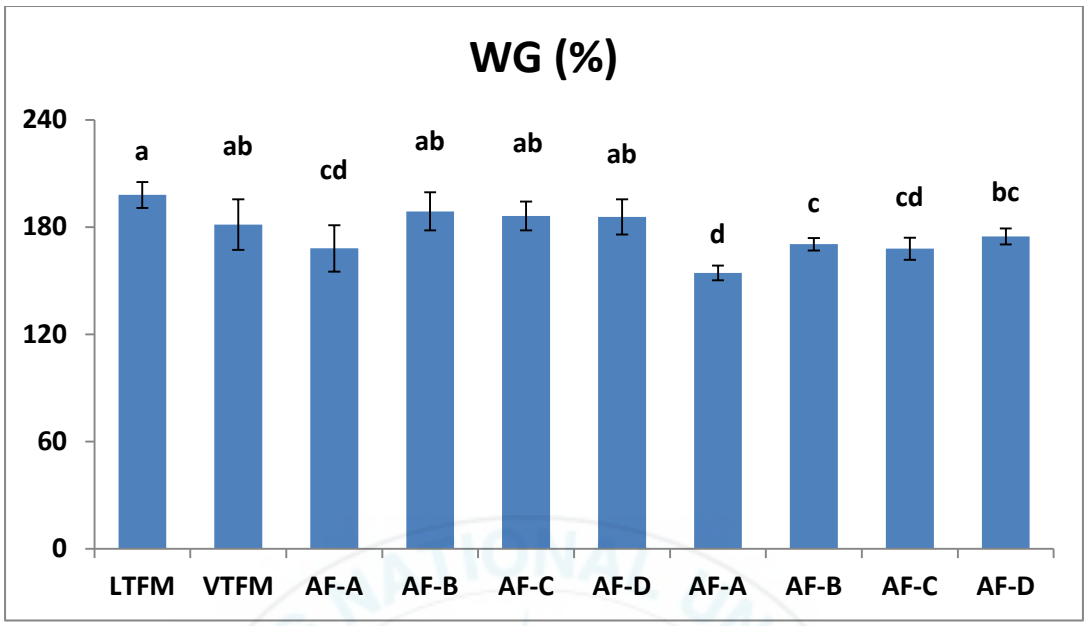

Figure 21. Weight gain (%) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0002$).

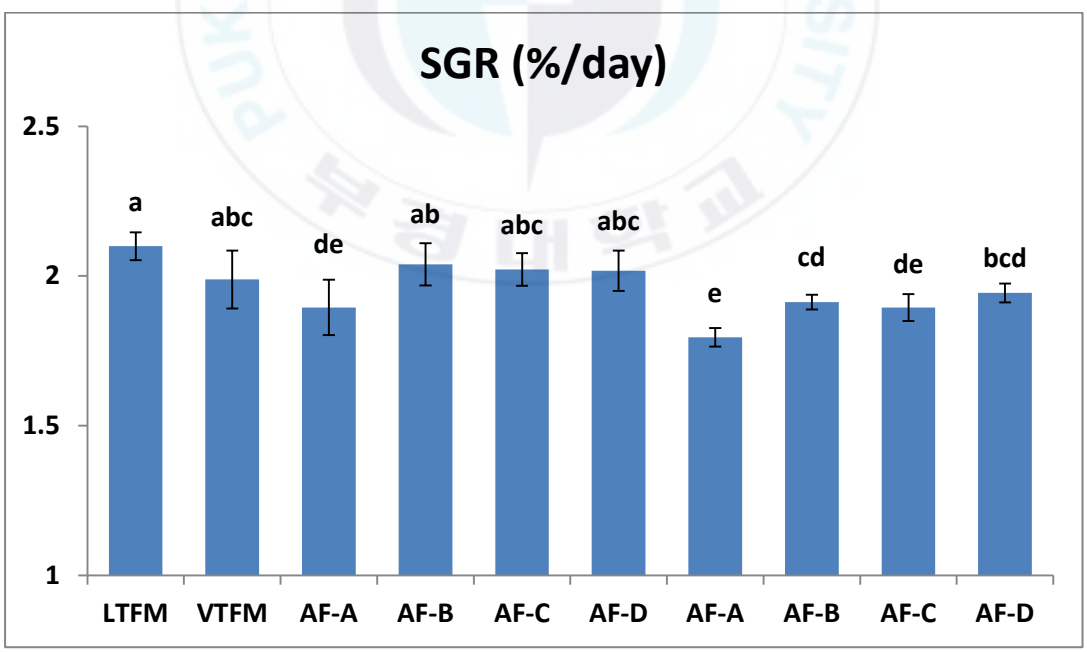

Figure 22. Specific growth rate (%/day) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0001$).

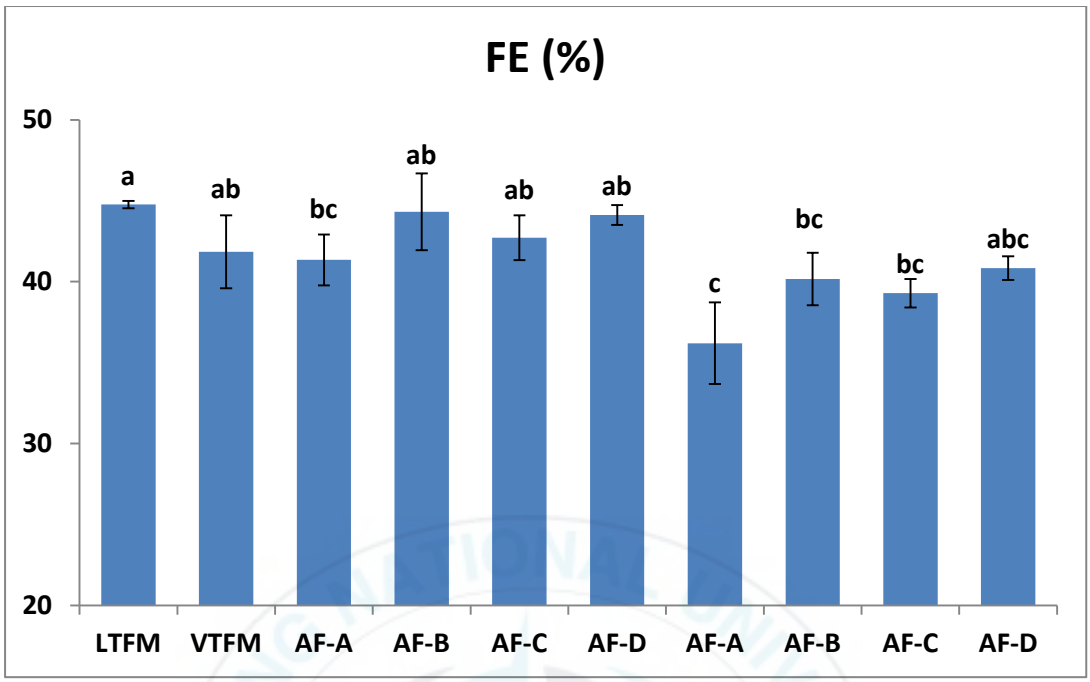

Figure 23. Feed efficiency (%) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0005$).

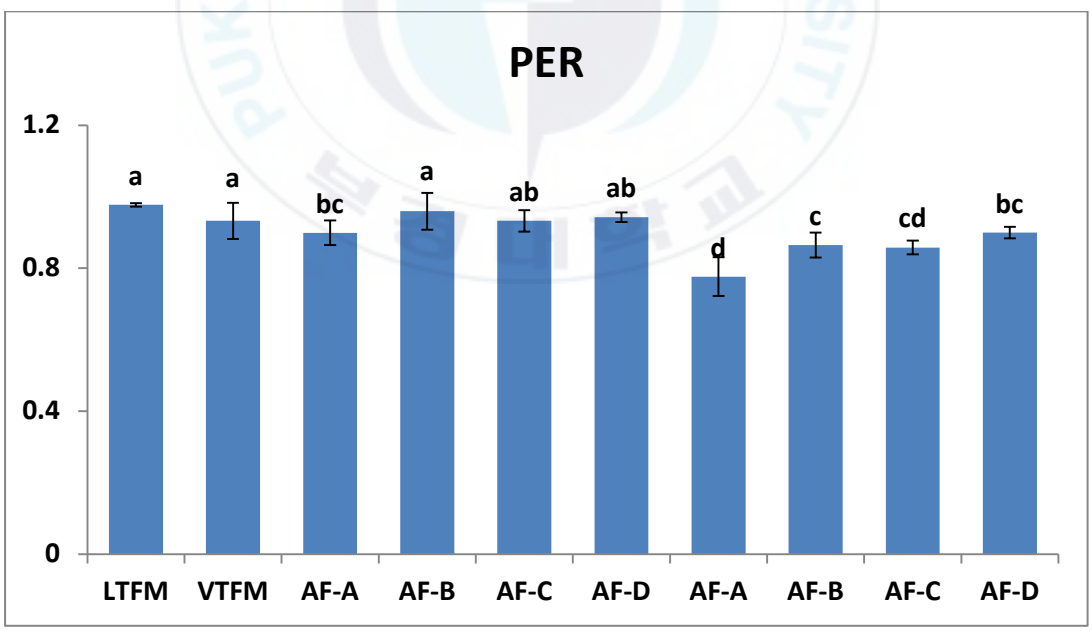

Figure 24. Protein efficiency ratio (%) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0001$).

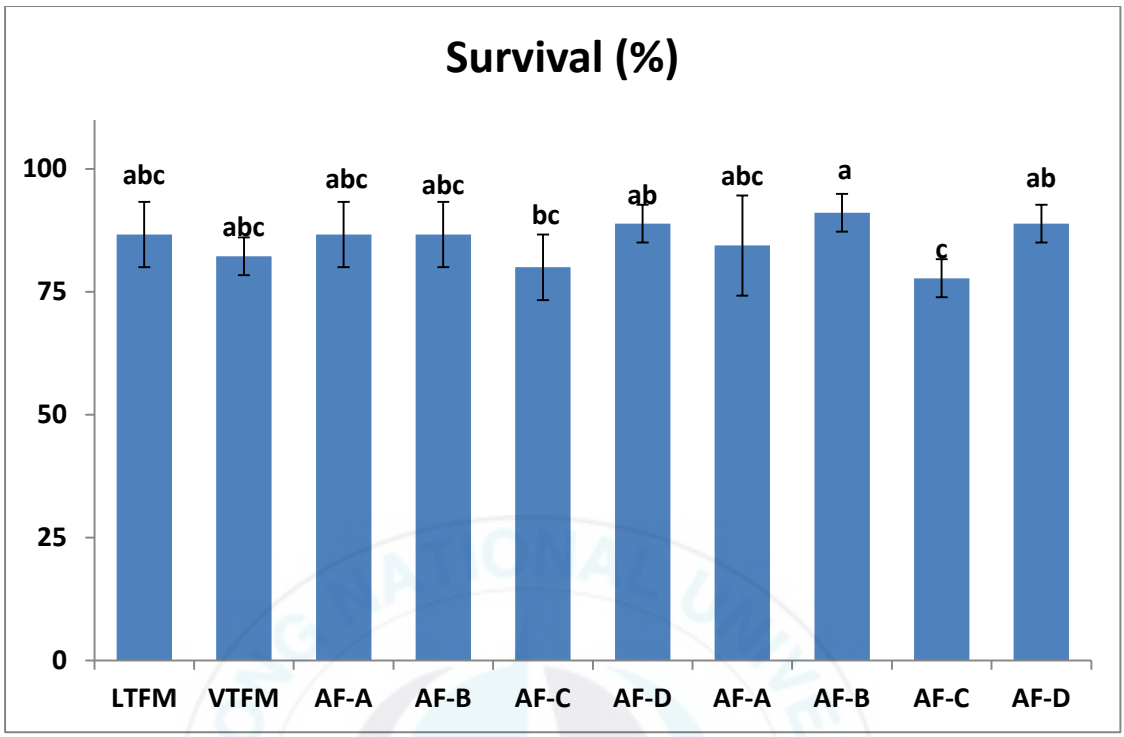

Figure 25. Survival rate (%) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.2016$).

Table 15. Whole body proximate composition of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed the experimental diets for 8 weeks[1]

	Experimental diets										*P*-values
	LT-FM	VT-FM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	
Moisture (%)	75.4±0.1	75.3±0.1	75.5±0.1	75.4±0.1	75.5±0.15	75.7±0.1	75.6±0.3	75.4±0.1	75.6 ±0.2	75.7 ±0.1	0.1896
Crude protein (%)	75.9±1.2	75.4±0.6	75.7±0.7	75.2±1.1	74.8±0.78	75.6±1.2	75.7±0.6	74.9±0.9	75.7±0.8	74.8±0.1	0.7564
Crude lipid (%)	1.94±0.1	1.87±0.1	1.84±0.1	1.84±0.1	1.81±0.07	1.83±0.1	1.83±0.1	1.84±0.1	1.85±0.1	1.83±0.1	0.0113
Ash (%)	13.7±0.1	13.9±0.1	13.7±0.1	13.6±0.1	13.7±0.03	13.8±0.1	13.7±0.1	13.6±0.1	13.7±0.1	13.7±0.1	0.0198

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

Table 16. Hematological analysis of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks[1]

	LTFM	VTFM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	*P*-values
ALT	10.3±1.5^{bcd}	12.3±1.5^{ab}	10.0±1.0^{cd}	11.3±2.1^{abcd}	12.0±1.0^{abc}	9.7±0.6^{d}	11.3±0.6^{abcd}	13.3±1.5^{a}	11.7±0.6^{abcd}	10.3±1.5^{bcd}	0.0493
AST	9.0±1.0^{d}	14.7±1.5^{a}	10.7±1.1^{cd}	10.7±1.5^{cd}	11.3±1.5^{bcd}	9.0±1.0^{d}	12.3±2.5^{abc}	14.0±1.0^{ab}	11.7±2.5^{bcd}	9.0±1.0^{d}	0.0016
TP	2.8±0.4^{b}	3.4±0.3^{ab}	3.2±0.4^{ab}	3.7±0.9^{a}	3.8±0.6^{a}	3.3±0.3^{ab}	3.2±0.1^{ab}	3.7±0.5^{a}	3.6±0.7^{ab}	3.5±0.4^{ab}	0.3995
Glu	21.3±1.5^{d}	28.0±1.0^{abc}	25.7±2.1^{bc}	32.0±2.0^{a}	25.7±2.1^{bc}	21.0±3.6^{d}	29.0±3.6^{ab}	26.0±2.0^{bc}	28.0±2.0^{abc}	24.3±3.2^{cd}	0.0006

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

AST (U/L): Aspartate transaminase

ALT (U/L): Alanine transaminase.

TP (g/dl): Total protein.

Glu (mg/dl): Glucose.

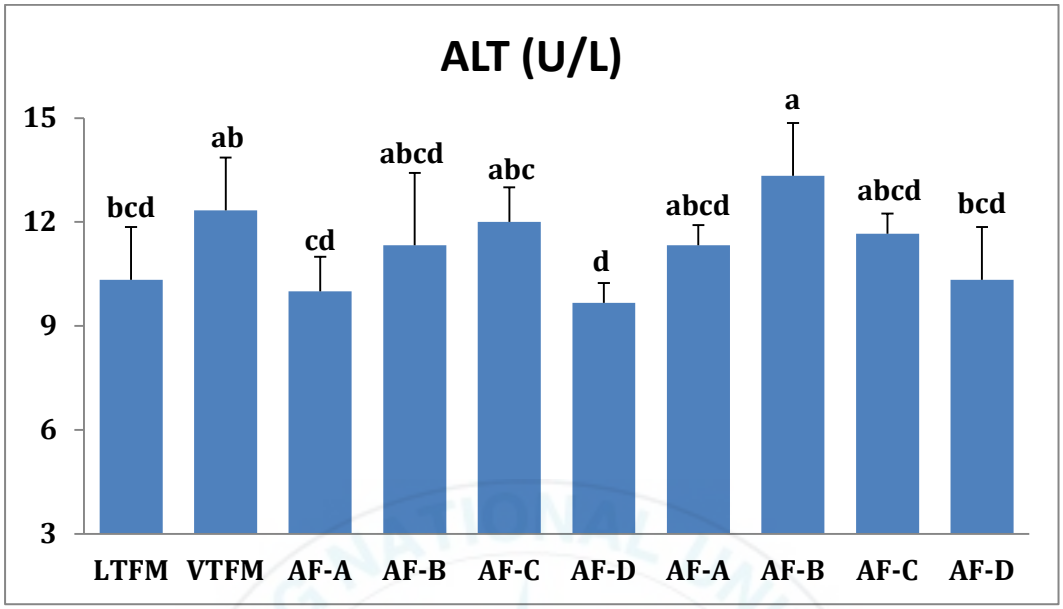

Figure 26. ALT (U/L) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0493$).

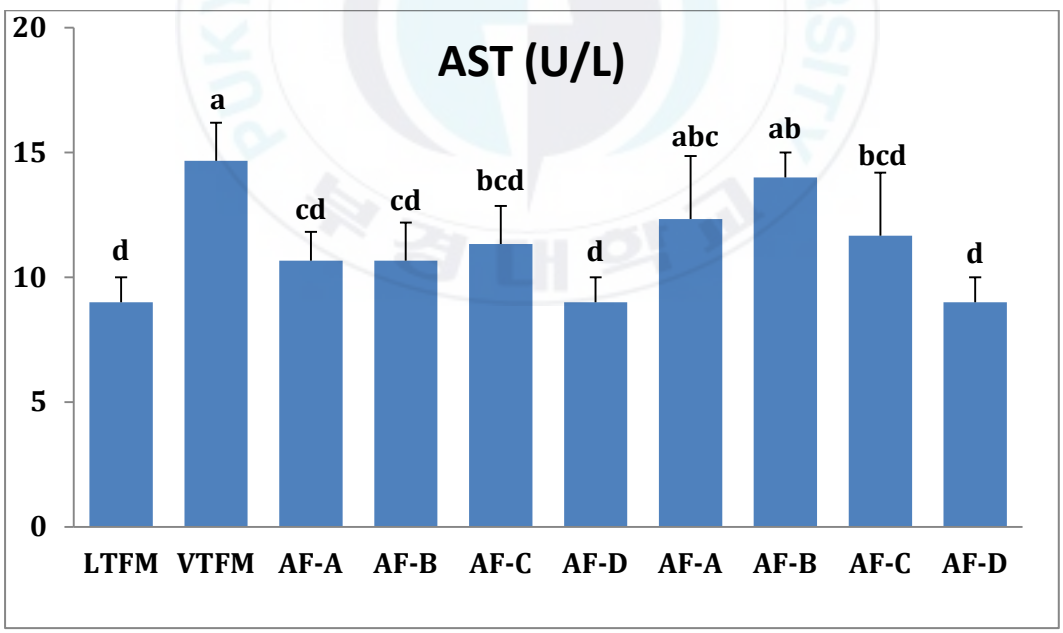

Figure 27. AST (U/L) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0016$).

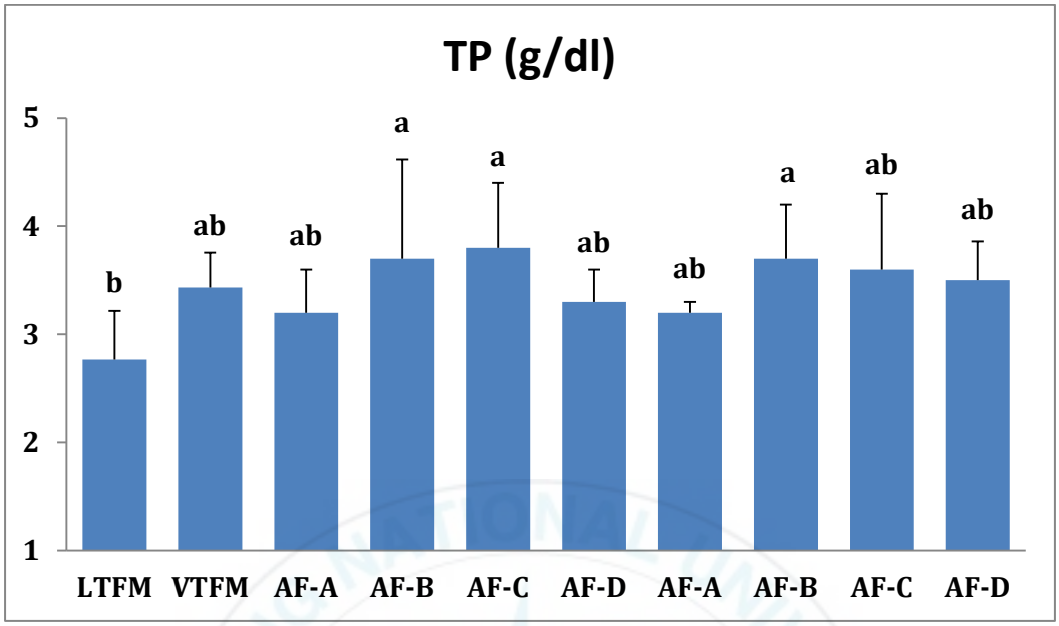

Figure 28. TP (g/dl) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.3995$).

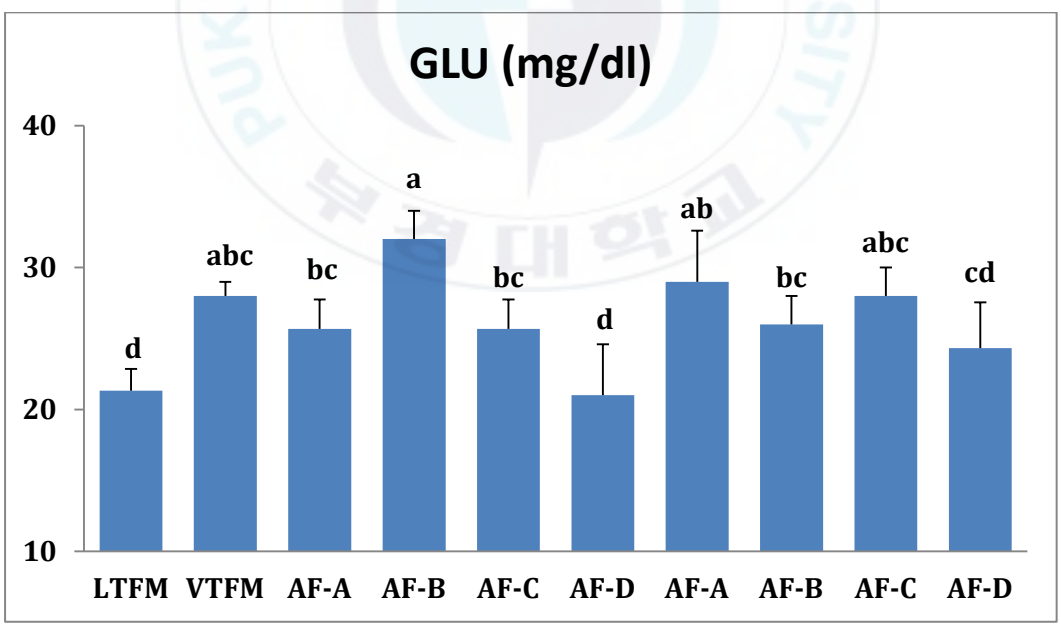

Figure 29. Glucose (mg/dl) of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.0006$).

Table 17. Non-specific immune responses of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks[1]

	LTFM	VTFM	AF-A (30%)	AF-B (30%)	AF-C (30%)	AF-D (30%)	AF-A (50%)	AF-B (50%)	AF-C (50%)	AF-D (50%)	*P*-values
SOD (% inhibition)	65.5±2.7	57.1±9.5	58.8±7.1	60.6±8.2	59.9±7.7	61.6±7.1	56.9±9.7	56.3±8.9	60.6±2.6	62.4±1.0	0.9530
Lysozyme (U/ml)	9.52±1.6	9.55±2.1	10.5±1.9	10.0±2.1	9.89±1.8	10.1±3.1	9.40±1.7	8.74±2.1	8.81±0.9	8.64±0.7	0.9827

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

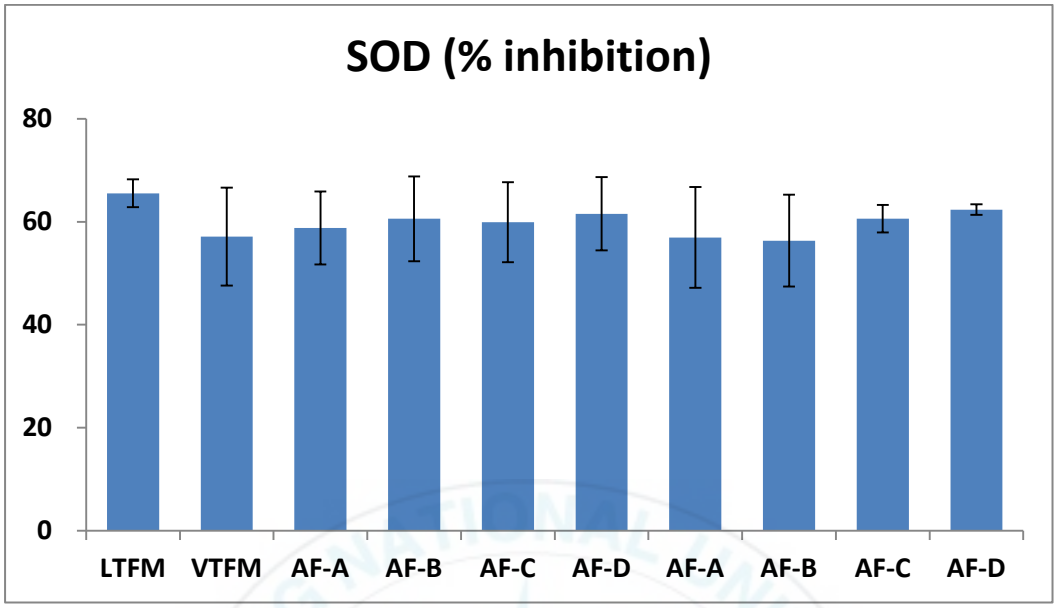

Figure 30. Superoxide dismutase activity (% inhibition) of whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.9530$).

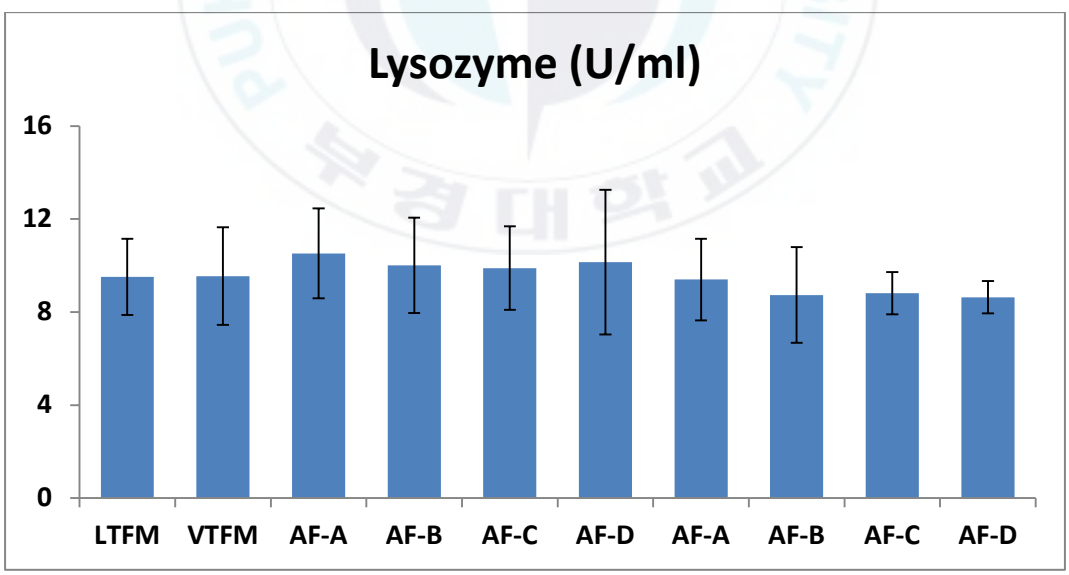

Figure 31. Lysozyme activity (U/ml) of whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.9827$).

CHAPTER 5

Evaluation of different bioprocessed protein concentrates on nutrient digestibility and digestive enzyme activities in juvenile whiteleg shrimp*, Litopenaeus vannamei*

1. Introduction

Fish meal (FM) is the major protein source in the diets of fishes especially carnivore fish. The reason behind is FM contains high quality protein with sound amino acids profile as well as its palatibility in fish. However, as world FM supply is diminishing every year, the fish nutritionists are now looking for alternative sources of protein in terms of nutritional quality, sustainable price, and accessibility (Lim and Dominy 1990; Samocha et al. 2004). Soybean meal (SBM) and corn gluten meals are good sources of plant proteins. However, these ingredients contain some anti-nutritional factors, allergens and limiting amino acids (Francis et al. 2001; Krogdahl et al. 2010). Therefore, mixture of these ingredients can be potential to provide high quality nutrients in fish feeds. Moreover, use of proper processing technique, as for example, fermentation can be a right choice to reduce the ANFs and improve the nutritional quality of the feeds. Some researchers reported that conventional methods of increasing nutritional value of feed ingredients such as solvent extraction or fermentation of ingredients are not enough to completely remove the ANFs (Siddhuraju et al. 2002). In this case, further treatment by more methods such as acid hydolysis or addition of enzymes can effectively reduce the ANFs in the aquafeeds (Siddhuraju and Becker 2005).

Studies on the alternative protein sources are mostly carried on the performance of inredients in terms of fish growth performance, however, little attention is paid on the digestibility and diestive enzyme activities of animal (Oujifard et al. 2012). It has been reported when plant-based protein are included in the diets especially ANFs and carbohydatre fractions may alter the digestion and nutrient utilization (Riche and Williams 2010). Oujifard et al. (2012) postulated that the inclusion of feed ingredients in the diets may contain high amount of nutrient but it may also wasted and deteriorate the culture environment if the diets are not efficiently utilized by fish. Catacutan et al. (2003) opined that

an effective aquafeed can be formulated when we have proper knowledge on the digestibility of the feedstuffs used in the feed fomulation. Therefore, determination of digestibility of ingredients in a diet is the prime concern to evaluate the effective use of an ingredient for fish or shrimp species (Allan et al. 2000). Moreover, the information on the digestive enzyme activities such as protease, amylase, and lipase are also important tools to evaluate the digestibility of the feed ingredients (Zhang et al. 2014).

The present study is undertaken on the evaluation of the variations in nutrient digestibility and the digestive enzyme activities using the four types of bioprocessed protein concentrate in relation to some other plant-based protein ingredients in the diets for white shrimp, *Litopenanaeus vannamei*.

2. Materials and methods

2.1.Experimental diets

Ten experimental diets including five types of bio-processed protein concentrates (BPCs) such as AF-A (only fermented protein concentrate), AF-B (BPC pre-treated with acid hydrolyses), AF-C (AF-A + shrimp soluble extract), AF-D (AF-B + shrimp soluble extract), and AF-E (AF-A + protease enzyme), soybean meal (SM), corn gluten meal (CGM), and a commercial fermented soybean meal (Soytide) were prepared to assess apparent digestibility of diets (ADDs) and apparent digestibility of ingredients (ADIs) in white shrimp by conventional method using 0.5% chromic oxide (Cr_2O_3) in the diets. In the study, one diet with low temperature fish meal (LT-FM) or reference diet and another diet with Vietnam local fish meal (VT-FM) were used as controls. The experimental diets were consisted with 70% reference diet (LT-FM) and 30% test ingredient.

The apparent protein digestibility of diet in shrimp was calculated by:

ADC of protein (%) = (1-(Cr in diet × Protein in feces) /(Cr in feces × Protein in diet)) × 100

The apparent lipid digestibility of diet in shrimp was calculated by:

ADC of protein (%) = [1-(Cr in diet×lipid in feces) / (Cr in feces×lipid in diet)] × 100

The apparent ingredient digestibility in fish was calculated by:

ADC Ingr (%) = ADC Feed+[(ADC Feed-ADC ref.diet) ×
(0.7×Protein in Ref/0.3×Protein in Ingr)]

2.2.Experimental shrimp and sample analyses

Fifteen shrimp with an average initial weight of 6.83±0.32 g (mean±SD) were randomly distributed in each of 30 semi-circulated tanks in triplicates. Shrimp were fed the experimental diets for four times a day to apparent satiation. The feces collection was carried out four times a day by sieving process up to 30 days. Crude protein content in feeds and feces were determined by the AOAC methods (1995). In brief, samples of diets and feces were dried to a constant weight at 135°C for 2 hr to determine moisture content. Ash was determined by incineration using muffle furnace at 550 °C for 3 hrs. Crude lipid was determined by soxhlet extraction unit using Soxtec system 1046 (Foss, Hoganas, Sweden) and crude protein content analysed by Kjeldahl method (N × 6.25) after acid digestion.

For the analysis of digestive enzymes from shrimp intestine, five shrimp were randomly collected from each aquarium and dorsal part of shrimp dissected to collect intestine and pooled in the vials for homogenization. After homogenization, supernatant of intestinal samples were further processed for digestive enzyme analyses such as lipase, amylase and protease following the manufacturers protocols (Biovision, USA).

2.3. Statistical analysis

All the data were analyzed by one-way ANOVA using SAS version 9.1 software (SAS Institute, Cary, NC, USA) to test the effects of dietary protein (Zar

1984). When a significant effect of the treatments was observed, a least significant difference (LSD) test was used to compare means. Treatment effects were considered significant at $P<0.05$.

3. Results and discussion

In digestibility experiment on whiteleg shrimp, the total amino acids (AAs) compositions of the diets were seemed to be lower than the total crude protein content in the diets. The results may occur because some AAs were not reported in present study due to the small amount of feces sample such as tryptophan, taurine etc. Moreover, standard conversion factor (6.25) is too high for plant protein sources; where, the conversion factor for wheat flours expressed between 5.4-5.6 and for whole grain 5.5-5.7 (Mariotti et al. 2008; Nieto-Lopez et al. 2011). Mariotti et al. (2008) reported that in case of compound reference diet the conversion factor should be between 5.4-5.7 for fish and soybean meal instead of using 6.25. Nevertheless, use of conversion factor 5.5-5.6 is better choice for plant protein conversion; in the present study, we used 6.25 as standard conversion factor for analysis of crude protein contents in ingredients, diets and feces to estimate the crude protein digestibility at same value as nitrogen digestibility (Nieto-Lopez et al. 2011).

Digestibility of a feed is an important factor to consider in determining the utilization of the feed (Akiyama et al. 1989), as it reflects the percentage of a feed sample that is absorbed from an animal's intestinal tract (Oujifard et al. 2012). The digestibility study is, therefore, an effective approach to assess the nutritional value of crustacean feeds (Akiyama et al. 1989: Jones and De Silva 1997). In this study, the results demonstrated that apparent digestibility of diets (ADDs) and apparent digestibility of ingredients (ADIs) for crude protein (CP) were significantly higher in shrimp fed AF- B, C, and D diets compared to AF-A, AF-E, soybean meal, corn gluten meal and commercial (Soy-T) diets. However, there

were no significant differences between shrimp fed AF-B and AF-D diets which suggest that shrimp fed pre-treated acid hydrolyzed BPC with supplementation of crystalline AAs (lysine and methionine) and pre-treated acid hydrolyzed BPC with supplementation of 2% SSE had better digestibility than shrimp fed the other plant protein based diets in terms of ADD for protein. Our results also shows that shrimp fed LT-FM and AF-D diets had no significant differences in ADD for protein which is attributed to the similar performance of pre-treated acid hydrolyzed BPC with supplementation of 2% SSE in relation to high fish meal protein. On the other hand, AF-B did not show any significant differences with VT-FM that suggest pre-treated acid hydrolyzed BPC with supplementation of crystalline AAs had similar performance in relation to low fish meal protein. In the present study, ADD for lipid showed the same trend with ADD for protein. In case of ADI for protein, we found same trend in the dietary treatments for ADD for protein. The dry matter and protein digestibility of BPCs in the present study (82-86% and 91-93%) were higher than those reported by Siccardi et al. (2006) and similar to Cruz-Suarez et al. (2009) for full fat soybean meal in whiteleg shrimp, *L. vannamei*.

Determination of digestive enzyme activities in whiteleg shrimp can be a effective tool to understand the digestibility of feedstuffs (Oujifard et al. 2012). In the present study, the digestive enzymes such as protease activity was higher in AF-B diet; whereas, lipase activity was found to be higher in shrimp fed AF-D diet. There were no significant differences in amylase activity among shrimp fed the experimental diets. The results of the enzyme activities were suggested that all the experimental diets are well digested by shrimp; however, considering the higher values of the digestive enzymes LT-FM, AF-B and AF-D showed better performance over the other diets which also supports the ADD and ADI of the diets in the present study.

In conclusion, the results demonstrated that shrimp fed pre-treated acid hydrolyzed BPC with supplementation of lysine and methionine (AF-B) and pre-treated acid hydrolyzed BPC with supplementation of 2% SSE (AF-D) could be recommended as a plant protein based ingredient in the diet of whiteleg shrimp.

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Table 18. Composition of the feeds for digestibility evaluation in juvenile whiteleg shrimp, *Litopenaeus vannamei* (% of DM basis)[1]

Ingredients	LTFM[2]	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T
Fish meal	536	375	375	375	375	375	375	375	375	375
Wheat flour	312	218	218	218	218	218	218	218	218	218
Soybean meal	98.0	68.6	68.6	68.6	68.6	68.6	68.6	68.6	68.6	68.6
Soybean lecithin	24.0	16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.8	16.8
Fish oil	20.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0	14.0
Vitamin premix	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
VTFM		300								
AF-A			300							
AF-B				300						
AF-C					300					
AF-D						300				
AF-E							300			
Soybean meal								300		
Corn gluten meal									300	
Soytide										300
Chromic oxide	5.00	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50	3.50
Total	1000	1000	1000	1000	1000	1000	1000	1000	1000	1000
Proximate composition (% of dry matter basis)										
Moisture %	9.2	8.9	9.8	9.8	9.7	9.7	9.8	9.5	10.1	10.1
Crude protein %	45.8	45.1	44.4	45.4	46.1	45.5	44.9	40.3	42.3	41.3
Crude lipid %	9.5	8.7	7.7	5.9	7.6	7.7	7.3	6.5	6.1	6.4
Ash %	9.4	9.3	6.8	7.1	6.8	7.4	7.1	6.9	5.4	7.1

Table 19. Amino acids profile of the experimental feeds in juvenile whiteleg shrimp, *Litopenaeus vannamei* (% of sample)

Amino acids	LTFM	FM-VIE	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T
Asp.	4.22	3.90	3.71	3.95	3.53	3.80	3.76	3.43	2.44	4.27
Thr.	1.88	1.71	1.60	1.67	1.53	1.62	1.65	1.53	1.09	1.66
Ser.	1.93	1.71	1.91	1.98	1.86	1.94	2.03	1.84	1.24	1.93
Glu.	7.46	7.14	8.01	8.56	7.69	8.36	8.36	7.08	4.98	7.83
Pro.	2.29	2.20	2.53	2.79	11.51	2.71	2.69	1.88	1.70	2.33
Gly.	2.67	2.77	2.03	2.04	0.40	1.98	2.01	1.91	1.47	2.14
Ala	2.69	2.60	2.51	2.72	2.44	2.63	2.65	2.33	1.47	2.17
Val.	2.29	2.17	2.06	2.28	1.99	2.20	2.15	1.54	1.31	2.13
Ile.	2.04	1.91	1.89	2.07	1.83	2.02	1.94	1.69	1.19	1.97
Leu	3.45	3.16	3.85	4.37	3.87	4.25	4.24	3.12	2.03	3.24
Tyr.	1.50	1.11	1.31	1.61	1.22	1.38	1.30	1.06	0.80	1.10
Phe.	1.94	1.80	2.06	2.30	2.05	2.21	2.19	1.57	1.18	2.04
His.	1.31	1.28	1.28	1.28	1.25	1.35	1.40	1.13	0.83	1.39
Lys.	3.32	2.99	2.29	2.34	2.74	3.05	2.29	1.98	1.86	2.53
Arg.	2.79	2.53	2.21	2.32	1.93	2.23	2.31	2.42	1.64	2.44

Table 20. Apparent digestibility coefficients (%, ADC) for crude protein of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets[1]

	LTFM[2]	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T	*P*-values
Dry matter	77.5±0.7^{cd}	76.5±3.5^{d}	82.5±0.7^{bcd}	91.0±1.4^{a}	91.5±2.1^{a}	86.0±1.4^{ab}	81.5±0.7^{bcd}	80.0±4.2^{bcd}	83.0±2.8^{bc}	83.5±4.9^{bc}	0.0028
Crude protein	94.3±0.5^{a}	93.0±0.5^{bc}	91.4±0.3^{d}	93.3±0.5^{bc}	92.7±0.1^{c}	93.6±0.2^{ab}	91.5±0.39^{d}	86.3±0.2^{e}	82.5±0.3^{f}	86.7±0.4^{e}	0.0001
Lipid	95.0±0.3^{a}	93.7±0.6^{b}	87.2±0.4^{c}	93.5±0.6^{b}	93.7±0.8^{b}	94.4±0.1^{ab}	88.0±0.6^{c}	85.4±0.5^{d}	83.5±0.3^{e}	86.8±0.8^{c}	0.0001

[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]Reference diet

Table 21. Apparent digestibility ingredients (%, ADI) for crude protein of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets[1]

	LTFM[2]	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T	*P*-values
Crude protein	97.3±0.6^{a}	94.9±0.5^{cd}	91.3±0.3^{e}	95.7±0.5^{bc}	94.3±0.2^{d}	96.6±0.2^{ab}	91.5±0.6^{e}	82.3±0.1^{g}	76.5±0.4^{h}	83.7±0.6^{f}	0.0001

[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]Reference diet.

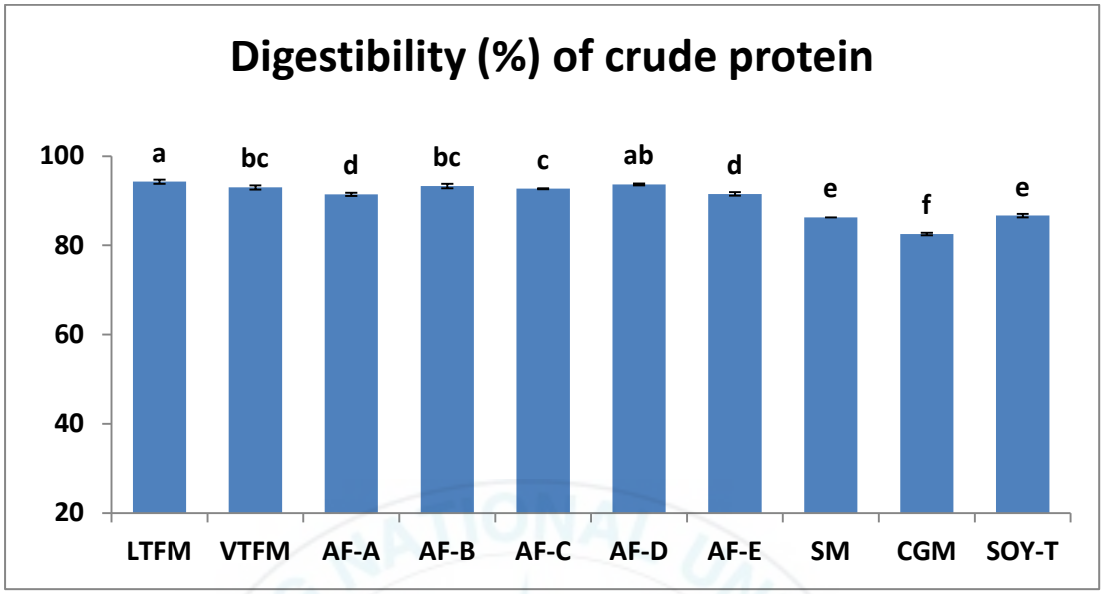

Figure 32. Apparent digestibility coefficients (%) for crude protein of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets ($P < 0.0001$).

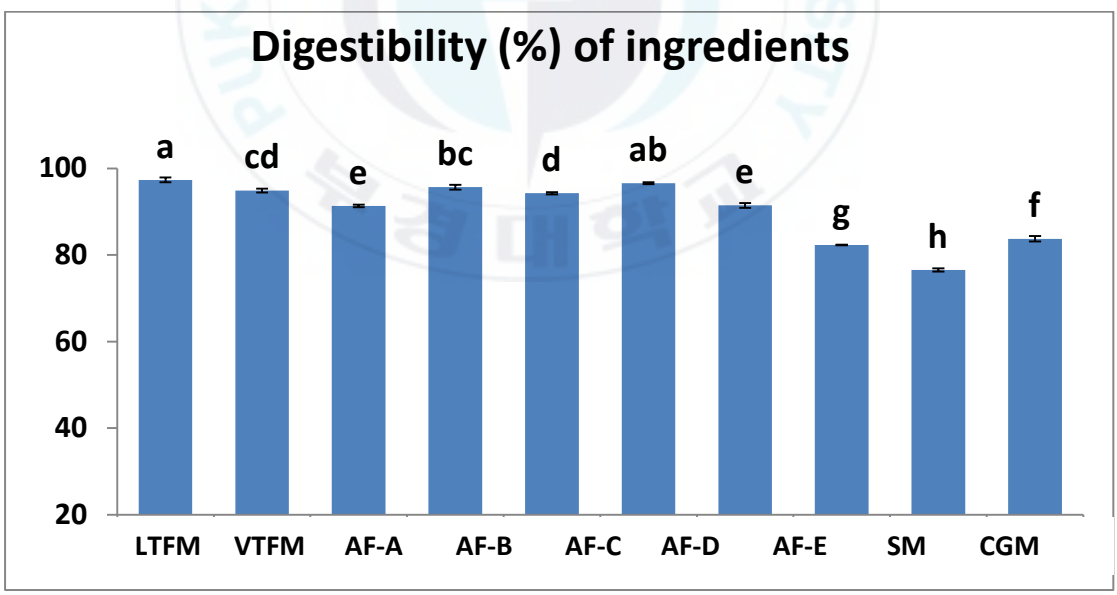

Figure 33. Apparent digestibility of ingredients (%) for crude protein of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets ($P < 0.0001$).

Table 22. Digestive enzymes of juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks[1]

	LTFM	VTFM	AF-A	AF-B	AF-C	AF-D	AF-E	SBM	CGM	SOY-T	*P*-values
Protease (mU/ml)	1.63±0.1^{ab}	1.60±0.1^{ab}	1.69±0.1^{ab}	1.73±0.1^{a}	1.67±0.1^{ab}	1.69±0.1^{ab}	1.53±0.1^{b}	1.60±0.1^{ab}	1.68±0.1^{ab}	1.67±0.1^{ab}	0.01245
Lipase (mU/ml)	1.24±0.1^{ab}	1.20±0.1^{b}	1.26±0.1^{ab}	1.22±0.1^{ab}	1.22±0.1^{ab}	1.44±0.1^{a}	1.23±0.1^{ab}	1.44±0.1^{a}	1.38±0.1^{ab}	1.43±0.1^{ab}	0.01123
Amylase (mU/ml)	2.47±0.2	2.35±0.1	2.55±0.1	2.35±0.1	2.61±0.2	2.38±0.1	2.35±0.1	2.35±0.1	2.50±0.1	2.38±0.1	0.9215

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

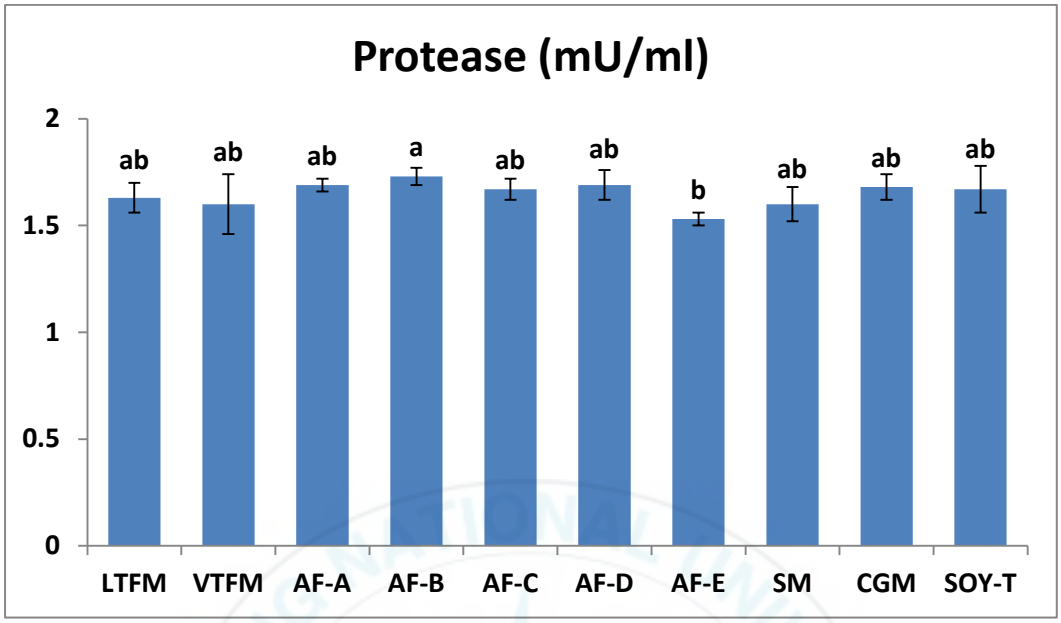

Figure 34. Protease activity (mU/ml) in juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.01245$).

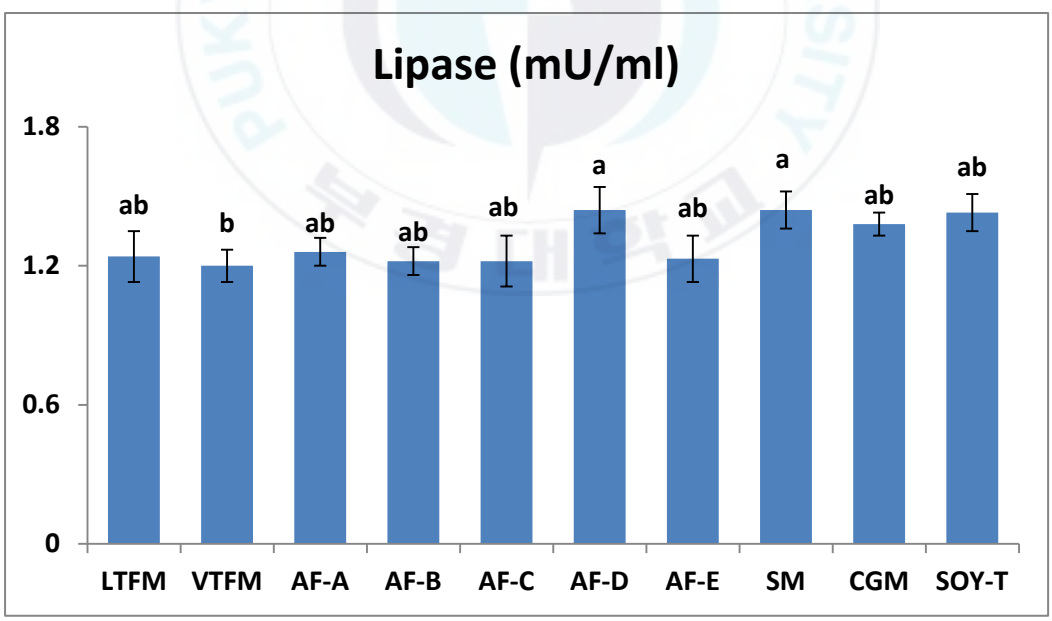

Figure 35. Lipase activity (mU/ml) in juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.01123$).

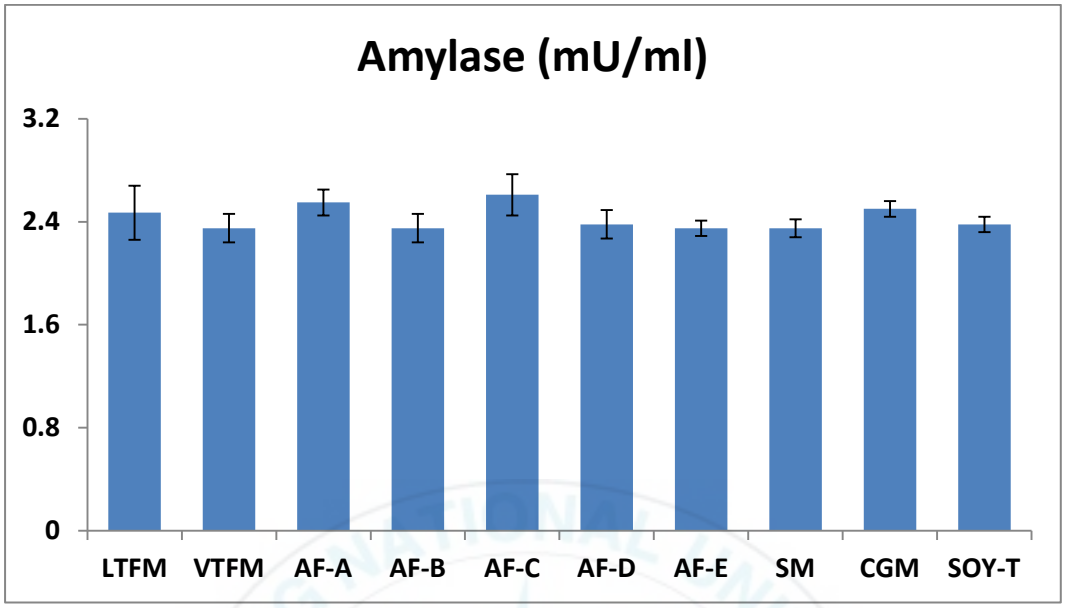

Figure 36. Amylase activity (mU/ml) in juvenile whiteleg shrimp, *Litopenaeus vannamei* fed experimental diets for 8 weeks ($P < 0.9215$).

CHAPTER 6

Conclusions and future research

The results of the present study suggested that:

1. Dietary fish meal (FM) level could be reduced up to 30% by bioprocessed protein concentrates without compromising the growth and health status in rainbow trout.
2. Bioprocessed protein concentrate (BPC) pre-treated with acid hydrolyses and/or added with shrimp soluble extract (SSE) showed better digestibility over other plant protein based ingredients such as soybean meal or protease enzyme treated fermented soybean meal, corn gluten meal (CGM) and commercially produced fermented protein concentrate (Soytide) meal in rainbow trout.
3. Fish meal could be substituted by BPC with acid hydrolyses and/or SSE supplements at 30% replacement level without affecting the health status of white shrimp.
4. BPC with acid hydrolyses and/or added SSE had better digestibility than those of SBM, CGM or enzyme treated BPC diets in whiteleg shrimp.

Future research:

These research works have potential implications in high quality plant protein based feed ingredient development as an alternative of fish meal for sustainable production of two commercially important aquaculture species such as rainbow trout and whiteleg shrimp. More research work should be conducted on the i) identification and characterization of microbiota in intestine of both of the species due to the inclusion of BPC in the diets. ii) understanding the nutrigenomic approaches of the BPCs in rainbow trout and whiteleg shrimp.

CHAPTER 7

Appendix

List of publications

Moniruzzaman, M., **G. Park, H. Yun, S. Lee, Y. Park, and S.C. Bai.** 2015. Synergistic effects of dietary vitamin E and selenomethionine on growth performance and tissue methylmercury accumulation on mercury-induced toxicity in juvenile olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel). Aquaculture Research. doi:10.1111/are.12904 (in press)

Hong, J.W., S. Lee, M. Moniruzzaman, Y. Park, S. Won, H. Jo, S.S.O. Hung, and S.C. Bai. 2016. Dietary eicosapentaenoic acid requirement of juvenile rock bream, *Oplegnathus fasciatus*. Aquaculture Nutrition. DOI: 10.1111/anu.12530 (in press)

Won, S., **M. Moniruzzaman, S. Lee, J.W. Hong, J.K. Park, S. Kim, and S.C. Bai.** 2016. Evaluation of dietary natural mineral materials as an antibiotic replacer on growth performance, non-specific immune responses and disease resistance in rainbow trout, *Oncorhynchus mykiss*. Aquaculture Research (in press).

Jo, H., S. Lee, H. Yun, J.W. Hong, M. Moniruzzaman, S.C. Bai, G. Park S. Chee, and T.E. Jeon. 2016. Evaluation of dietary fishmeal analogue with addition of shrimp soluble extract on growth and nonspecific immune response of rainbow trout, *Oncorhynchus mykiss*. Journal of the World Aquaculture Society. doi: 10.1111/jwas.12355 (in press)

Park, Y., S. Lee, J.W. Hong, D. Kim, M. Moniruzzaman, and S.C. Bai. 2016. Use of probiotics to enhance growth, stimulate immunity and confer disease resistance to *Aeromonas salmonicida* in rainbow trout (*Oncorhynchus mykiss*). Aquaculture Research. doi:10.1111/are.13099 (in press)

Kim, K.W., K.D. Kim, H.S. Han, M. Moniruzzaman, H. Yun, S. Lee, and S.C. Bai. 2016. Optimum dietary protein level and protein-to-energy ratio for growth of juvenile parrot fish, *Oplegnathus fasciatus*. Journal of the World Aquaculture Society. doi: 10.1111/jwas.12337 (in press)

Hong, J.W., S. Lee, H. Yun, M. Moniruzzaman, Y. Park, E. Shahkar, M. Seong, and S.C. Bai. 2016. The optimum dietary docosahexaenoic acid level based on growth and non-specific immune responses in juvenile rock bream, *Oplegnathus fasciatus*. Aquaculture Research. doi:10.1111/are.13167 (in press)

Lee, S., G. Park, M. Moniruzzaman, J. Bae, Y. Song, K.W. Kim, and S.C. Bai. 2016. Synergistic effects of dietary vitamin C and selenium on induced methylmercury toxicity in juvenile olive flounder *Paralichthys olivaceus*. Turkish Journal of Fisheries and Aquatic Sciences. DOI: 10.4194/1303-2712-v17_3_09 (in press)

Park, Y., M. Moniruzzaman, S. Lee, J.W. Hong, S. Won, J.M. Lee, H. Yun, K.W. Kim, D. Ko, and S.C. Bai. 2016. Comparison of the effects of dietary single and multi-probiotics on growth, non-specific immune responses and disease resistance in starry flounder, *Platichthys stellatus*. Fish and Shellfish Immunology 59:351-357.

Lee, J.H., M. Moniruzzaman, H. Yun, S. Lee, Y. Park, and S.C. Bai. 2016. Di etary vitamin C reduced mercury contents in the tissues of juvenile olive fl ounder, *Paralichthys olivaceus* exposed with and without mercury. Enviro nmental Toxicology and Pharmacology 45: 8-14

Park, G., M. Moniruzzaman, H. Yun, S. Lee, Y. Park, and S.C. Bai. 2016. Sy nergistic Effects of Dietary Vitamin C, E and Selenomethionine on Growt h Performance, Tissue Mercury Content and Oxidative Biomarkers of Juv enile Olive Flounder, *Paralichthys olivaceus* (Temminck & Schlegel) Tox ified with the High Dietary Methylmercury. Animal Nutrition and Feed T

echnology 16:155-164.

Kim, K.W., M. Moniruzzaman, K.D. Kim, H.S. Han, H. Yun, S. Lee, and S.C. Bai. 2016. Effects of dietary protein levels on growth performance and bo dy composition of juvenile parrot fish, *Oplegnathus fasciatus*. Internationa l Aquatic Research 8:239-245.

Kim, K.W., M. Moniruzzaman, K.D. Kim, H.S. Han, H. Yun, S. Lee, and S.C. Bai. 2016. Re-evaluation of the optimum dietary protein level for maximum growth of juvenile barred knifejaw *Oplegnathus fasciatus* reared in cages. Fisheries and Aquatic Sciences 19:24.

Lee, S., M. Moniruzzaman, J. Bae, M. Seong, Y. Song, B. Dosanjh, and S.C. Bai. 2016. Effects of extruded pellet and moist pellet on growth performance, body composition, and hematology of juvenile olive flounder, *Paralichthys olivaceus*. Fisheries and Aquatic Sciences 19:32.

Lee, S., M. Moniruzzaman, H. Yun, Y. Park, J. Mann, and S.C. Bai. 2016. Comparative studies on effects of extruded pellets and dough type diets on growth, body composition, hematology and gut histology of juvenile Japanese eel, *Anguilla japonica* (Temminck et Schlegel). Journal of Fisheries and Aquatic Science 11:378-384.

Mizanur, R.M., H. Yun, M. Moniruzzaman, F. Ferreira, K.W. Kim, and S.C. Bai. 2014. Effects of feeding rate and water temperature on growth and body composition of juvenile Korean rockfish, *Sebastes schlegeli* (Hilgendorf 1880). Asian-Australasian Journal of Animal Science 27:690-699.

Raw data:

Expt. 1. Growth performance of juvenile rainbow trout fed the experimental diets for 8 weeks

Diets	Rep.	IBW (g)	FBW (g)	WG (%)	SGR (%/day)	FE (%)
LT-FM	1	15.5	52.7	240.4	2.99	92.1
	2	15.5	53.6	246.3	3.03	93.2
	3	15.4	52.7	241.3	2.99	91.8
VT-FM	1	15.3	50.7	232.5	2.93	87.6
	2	15.5	51.3	231.2	2.92	85.7
	3	15.5	51.4	232.4	2.93	93.6
AF-A (30%)	1	15.6	49.6	218.8	2.83	83.0
	2	15.4	49.6	224.5	2.87	84.6
	3	15.5	49.1	216.9	2.81	84.3
AF-B (30%)	1	15.5	52.5	238.7	2.98	89.8
	2	15.3	49.1	235.7	2.88	89.4
	3	15.4	49.3	236.8	2.84	89.7
AF-C (30%)	1	15.4	53.6	248.3	3.04	93.8
	2	15.5	52.9	241.7	3.00	90.3
	3	15.5	53.0	241.8	3.00	89.4
AF-D (30%)	1	15.4	53.3	246.1	3.03	91.3
	2	15.3	52.0	239.7	2.98	91.6
	3	15.5	54.5	251.9	3.07	93.6
AF-A (50%)	1	15.4	45.1	192.0	2.61	85.1
	2	15.3	48.3	215.1	2.80	83.3
	3	15.4	47.8	210.2	2.76	80.9
AF-B (50%)	1	15.4	47.7	209.3	2.75	79.3
	2	15.4	49.3	221.0	2.84	86.3
	3	15.3	49.0	219.8	2.84	85.8
AF-C (50%)	1	15.4	51.9	237.8	2.97	90.8
	2	15.3	49.6	223.7	2.87	84.0
	3	15.4	49.5	220.9	2.84	84.2
AF-D (50%)	1	15.3	48.2	215.8	2.80	85.7
	2	15.4	52.0	236.9	2.96	89.0
	3	15.4	48.7	215.2	2.80	84.9

Expt. 2. Apparent digestibility of diets and ingredients for protein in juvenile rainbow trout

Diets	Rep	ADD for protein	ADI for protein
LT-FM	1	86.35	96.54
	2	86.25	96.43
VT-FM	1	81.68	91.31
	2	83.09	92.89
AF-A	1	79.89	89.31
	2	78.32	87.57
AF-B	1	83.62	93.49
	2	84.50	94.47
AF-C	1	83.52	93.37
	2	81.55	91.17
AF-D	1	83.61	93.47
	2	85.05	95.08
AF-E	1	79.07	88.40
	2	78.63	87.91
SBM	1	74.97	83.82
	2	75.35	84.24
CGM	1	67.58	75.56
	2	69.67	77.48
SOY-T	1	76.70	85.75
	2	79.42	89.80

Expt. 3. Growth performance of juvenile whiteleg shrimp fed the experimental diets for 8 weeks

Diets	Rep.	IBW (g)	FBW (g)	WG (%)	SGR (%/day)	FE (%)
LT-FM	1	3.6	10.7	198.6	2.10	44.52
	2	3.5	10.7	204.9	2.14	44.95
	3	3.4	9.9	190.4	2.05	44.82
VT-FM	1	3.5	10.6	194.1	2.07	43.13
	2	3.4	10.0	191.4	2.06	44.10
	3	3.5	9.5	171.4	1.92	39.60
AF-A (30%)	1	3.5	9.3	166.6	1.89	41.50
	2	3.5	9.0	155.9	1.81	39.70
	3	3.5	9.8	181.7	1.99	42.84
AF-B (30%)	1	3.5	9.8	178.7	1.97	43.53
	2	3.6	10.7	199.9	2.11	47.00
	3	3.4	9.9	187.8	2.03	42.45
AF-C (30%)	1	3.5	10.2	194.2	2.08	41.53
	2	3.5	9.9	186.6	2.02	44.24
	3	3.5	9.8	178.1	1.97	42.39
AF-D (30%)	1	3.4	10.0	191.6	2.06	44.66
	2	3.4	9.9	191.1	2.05	44.26
	3	3.5	9.6	174.2	1.94	43.44
AF-A (50%)	1	3.5	8.9	152.9	1.78	37.26
	2	3.4	8.9	158.9	1.83	33.31
	3	3.5	8.8	151.1	1.77	38.01
AF-B (50%)	1	3.4	9.4	174.3	1.94	41.75
	2	3.5	9.4	167.6	1.89	38.50
	3	3.5	9.3	169.3	1.91	40.25
AF-C (50%)	1	3.5	9.4	168.5	1.90	39.44
	2	3.4	9.4	173.8	1.94	38.35
	3	3.5	9.2	161.3	1.85	40.09
AF-D (50%)	1	3.4	9.3	170.3	1.91	40.19
	2	3.5	9.8	179.4	1.98	41.63
	3	3.5	9.7	174.5	1.94	40.69

Expt. 4. Apparent digestibility of diets and ingredients for protein in juvenile whiteleg shrimp

Diets	Rep	ADD for protein	ADI for protein
LT-FM	1	93.97	96.95
	2	94.62	97.75
VT-FM	1	92.65	94.57
	2	93.33	95.22
AF-A	1	91.68	91.56
	2	91.19	91.12
AF-B	1	92.94	95.29
	2	93.65	96.03
AF-C	1	92.78	94.42
	2	92.64	94.09
AF-D	1	93.81	96.72
	2	93.49	96.42
AF-E	1	91.82	91.85
	2	91.27	91.05
SBM	1	86.28	82.36
	2	86.25	82.28
CGM	1	82.34	76.25
	2	82.76	76.81
SOY-T	1	86.95	84.20
	2	86.40	83.29