Evaluation of advanced soy products in diets fed to Florida pompano *Trachinotus carolinus*

by

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ABSTRACT

It has been suggested that soybean meal could be utilized to substitute the use of animal meal in the development of practical diets for majority of aquaculture species. However, the presence of anti-nutritional factors may limit the wider use of conventional soybean meal (SBM) and play a role in decreasing the growth performance of fish, alter the serum and enzyme activities, and induce the inflammation in the distal intestine and the liver of the fish. Currently, different advanced processing technique, such as fermentation, combination of non-alcohol and enzymatic treatment, and alcohol extraction technique, have been reported to be an effective method to denature and lowering the level of anti-nutritional factors, improve the nutritional value, and reduce the molecular weight of soy-protein. Therefore, this study was conducted to explore the potential use of this advanced soy products, especially fermented soybean meal (FSBM) and enzyme-treated soy (ESBM), as the protein sources in formulating the practical diets for Florida pompano *Trachinotus carolinus*. In this study, the potential use of squid products as an attractant as well as the protein source for pompano also becomes the main parameter of interest to be included in pompano diet.

The first study was designed to evaluate the efficacy of commercially produced FSBM known as PepSoyGen^{TM} to replace conventional SBM in the development of plant-based diet for Florida pompano. In this study, the utilization of FSBM as much as 0, 206, 309 and 410 g kg^{-1} to approximately replace 0, 50, 75 and 100% of SBM had no effects in growth parameters of pompano. The gradual replacement also did not have significant differences in total protein,

albumin, glucose, cholesterol, bile acids, plasma alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase activities in all treatments of pompano. However, glycogen granulation, inflammation and nuclear change condition in the liver of pompano were better as the inclusion of fermented product increased. Based on the results, further research regarding the optimum inclusion level of FSBM to satisfy the nutrient requirement of pompano might be needed to improve the efficacy of fermented soy products.

The second study investigated the potential use of commercial ESBM (NutriVance^{TM}) to reduce the inclusion level of fish meal (FM) in the development of practical diet for Florida pompano. There are two series of trials conducted in this study and the decreasing growth of pompano as the inclusion level of FM was further replaced by soy-protein were confirmed. The results also indicate that the gradual replacement of dietary FM with ESBM had no effect to the majority of serum and enzyme activities of pompano across all trials. The histomorphological structure of liver and distal intestine were slightly affected by lower inclusion level of FM. Overall, the results showed that ESBM could be used as a potential dietary protein sources to replace FM in the development of practical diet for pompano.

The third study was designed to investigate and compare the effects of diets containing high inclusion level of advanced soy products (ESBM and FSBM) in combination with porcine meal (PM) to completely replace poultry by-product meal (PBM) on growth performance, body composition and distal intestine histology of Florida pompano *Trachinotus carolinus*. Under the experimental conditions, fish fed ESBM performed equally well in terms of growth parameters in comparison to fish fed PBM. Meanwhile, fish fed FSBM had significantly lower feed intake,

indicating a palatability issue with the high use of FSBM. Interestingly, the results indicate that the dietary treatments had no effect in whole-body proximate and mineral composition. Moreover, the high inclusion of advanced soy products had no significant effect to the histomorphological conditions of distal intestine of the fish. Based on these results, 451 g kg^{-1} ESBM supplemented with 38 g kg^{-1} PM can be utilized to develop a practical diet for juvenile Florida pompano without impacting growth, nutritive parameters and several distal intestine health parameters.

The fourth study investigated the inclusion effects of squid products, namely squid hydrolysates (SH) and squid meal (SM) to improve the palatability and nutritional value of soy-based diet. The squid products were added to the basal diets, to produce diets containing 1, 2 and 4% of SH and SM. Under the experimental conditions, the addition of 4% SH improved the response of basal diet and did not show any significant difference in terms of growth performance as compared to the reference diet (15% PBM). Meanwhile, the inclusion of SM into the basal diet were not able to induce better growth of pompano. At the end of growth trial, there were no significant effect to the whole body proximate, amino acids composition, enzyme and serum biochemistry activities of the fish. Regarding to the histology analysis, fish fed with basal diet showed disordered vacuolization in the liver and decreased the lamina propria thickness in the intestine. The inclusion of 4% SH partly prevented the alteration of liver and distal intestine of Florida pompano and findings were similar to fish fed with PBM. Based on these results, combination of ESBM and 4% SH has the potential to serve as an alternative protein source and attractant to improve the efficacy of plant-based diet for pompano.

The fifth study aimed to conduct a quantitative review on the effect of various inclusion size of FSBM as a potential ingredient to replace dietary FM on the growth and food conversion ratio (FCR) of several aquaculture species. The effect size of 53 comparisons data between FSBM inclusion level in diet formulation and a control condition was -3.75 [95 % CI -4.49 to -3.01] for final weight and 1.26 [95 % CI 0.58 to 1.94] for FCR. According to meta-regression analysis, FSBM inclusion level of 8–40 % improves the final weight of fish. Meanwhile, inclusion level of FSBM higher than 40 % will likely decrease the final weight of fish compared to fish that received high percentage of FM. On the other hand, the inclusion of FSBM is more effective at the level of 15–44 % to improve the FCR of the diet and inclusion levels out of this range would produce various effects to the FCR. This study provides a useful information on the optimum inclusion level of FSBM to replace dietary FM in formulating practical diets based on the selected study.

We showed that for future aquaculture, the development of sustainable and economically sound practical diet will depend on the reduction of animal meal and the increased inclusion of sustainable protein sources. Further treatment of SBM could produce better nutrition profile in the resulted meal and growth performance of the fish. Therefore, it is important for researcher and stake holders to properly evaluate the utilization of advanced soy products and determine the optimum inclusion level of these advanced soy products to replace the use of conventional SBM and animal meal that still support the optimum growth and health status of fish.

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Chapter I

INTRODUCTION

Global production of aquaculture, as one of the fastest-growing agriculture sectors, expanding rapidly from about 3.5 million tons in 1970 to about 110.2 million tons in 2016, while total capture fisheries production appears to have reached a plateau in recent years (Asche *et al.*, 2008, FAO, 2018). Of this, 54.1 million tons obtained from finfish production and 12.2 % coming from marine aquaculture sector (FAO, 2018). Indeed, since the demands of a growing populations tend to increases, combine with increased average income that shift the diets toward more nutritious and higher quality foods, while at the same time, wild fish stock in the oceans being exploited above the sustainable level, fish supply from aquaculture have to grow accordingly (Tidwell and Allan, 2001, Gjedrem *et al.*, 2012). As the future expansion will most likely take place in the marine environment (Gjedrem *et al.*, 2012) and their expansion may be constrained by increasing dependence on low-value marine "trash fish" and fish meal (New and Wijkström, 2002, Edwards *et al.*, 2004), research in feed formulation and alternative ingredient to replace dietary fish meal will play an important role to ensure the sustainability of marine aquaculture production (Glencross *et al.*, 2007)

Florida pompano, *Trachinotus carolinus* L., is one of the most highly desired marine aquaculture species primarily due to the excellent flavor, acceptance of pelleted feeds, high monetary value and can be cultured in sea water and low salinity environment (Berry and Iversen, 1967, Finucane, 1969b, Bellinger and Avault Jr, 1971, Weirich *et al.*, 2009). In United States, the

history of pompano farming industry was started back since the early 1960s by both public institutions and private company using pond culture system (Berry and Iversen, 1967). Recently, intensive recirculating system and cage farming has been operated to produce marketable size of pompano year-round to fulfill the consumer demands (Weirich *et al.*, 2006, Weirich *et al.*, 2009, Rossi Jr, 2011). However, from the aquaculture standpoint, nutrient requirement and feeding management become one of the major concern to overcome the production limitation (Weirich *et al.*, 2006).

1. Biology and food habits

Florida pompano, also named common pompano or Atlantic pompano, is a member of the family Carangidae (Main *et al.*, 2007), and widely distributed in coastal waters from Cape Cod, Massachusetts, to south-eastern Brazil and throughout the Gulf of Mexico (Gilbert, 1986, Watanabe and Main, 1995). The body are short, compressed and deep with coloration varies from blue-greenish silver in the dorsal part and silver to yellow on the ventral surfaces (Fig. 1). The head profile sloping to a blunt snout, the eye is small and the upper jaw very narrow at the end and extending to below mid-eye (Gilbert, 1986). Pompano possess subterminal and protrusible mouthparts with small and weakly developed teeth during the juvenile stage and tend to disappear or be covered over in the adult phase (Bellinger and Avault Jr, 1971). Depend on season, ecology and size of fish, pompano can probably be considered as an opportunistic feeders that primarily feed on benthic and pelagic invertebrates such as amphipods, bivalve mollusks, crab larvae, copepods and isopods during the early age and become more selective grazers as they grew larger (Berry and Iversen, 1967, Finucane, 1969a, Bellinger and Avault Jr, 1971). In the wild, pompano can grow up to 63.5 cm in length, and 3.6 kg in body weight, and has a life span around 3 – 4 years (Berry and Iversen, 1967, Main *et al.*, 2007).

The digestive tract of pompano can be divided into four topographical divisions (Figure 2): *first,* the head-gut starting from oral opening to the posterior edge of the gill structures. According to Bellinger and Avault Jr (1971) the gill rakers of pompano are fairly short and widely spaced with function to separate food particle from sand and detritus. *Second,* the fore-gut or the fore-intestine, mainly comprises with *esophagus* and *stomach.* Pompano only have short *esophagus* which function to direct the food to the sac-shaped *stomach* (Bellinger and Avault Jr, 1971, Gothreaux, 2008). Based on food habit and the shape of the stomach, pompano could be categorized as an omnivorous species (Lagler *et al.*, 1962). *Third,* the mid-gut, with specific formation of *pyloric caeca* which aid in digestion and absorption by increasing the intestinal surface area (Gothreaux, 2008). The mid-gut could be considered as the true intestine since this site become the final stages of the chemical breakdown of food particle and the resorption of its product (Harder, 1975). *Lastly,* the hind-gut, or the terminal portion of the digestive tract. In pompano, it is quite difficult to differentiate the border between mid-gut and hind-gut. The posterior border of the hind-gut directly connected to the *anus*, which is enlarged by a circular fold of the mucous membrane.

Based on personal observations and study from Gothreaux (2008), Florida pompano intestine considered to be relatively short. As the consequences, the transit time of feed through the gut only occur in 3 hours (Williams *et al.*, 1985) and could have a significant impact to the digestibility of formulated diet. Considering this condition, the development of practical diet for pompano need to meet the specific requirement and contain with readily available nutrients and energy that can be digested easily.

2. Feed formulation for Florida pompano

A number of studies to determine the nutritional requirement, feeding management, limiting amino acids as well as palatability of the diet for Florida pompano have been conducted and presented (Lazo *et al.*, 1998, Groat, 2002, Weirich *et al.*, 2006, Gothreaux *et al.*, 2010, Lech and Reigh, 2012, Quintero *et al.*, 2012, Salze *et al.*, 2016, Rhodes *et al.*, 2017b). From these studies, Florida pompano known to require 40 – 45% crude protein level to support their optimum growth and feed utilization (Lazo *et al.*, 1998, Rossi Jr and Davis, 2012). In addition, based on the lipid-deficient study, the optimum level of fish oil within the diet formulation appears to be between 4 and 8 % (Williams *et al.*, 1985).

Other than to define the optimal protein and lipid contents of the diet, great efforts has also been put into defining the limiting amino acid requirements for Florida pompano. Quantitative requirements of taurine have been estimated at the level of 0.54 – 0.65% (Salze *et al.*, 2014, Rossi Jr and Davis, 2012). Taurine plays a role in modulation of intracellular free calcium concentration and wide variety of functions in the central nervous system (Ripps and Shen, 2012). Moreover, the conjugation of bile acids with taurine makes this substance become more soluble in the water and play an important role in fat emulsification (Thrall *et al.*, 2012). This function promotes both the digestion and absorption of fat as well as fat-soluble vitamins (Thrall *et al.*, 2012). Another limiting amino acids, especially lysine and total sulfur amino acid has also been quantified at the level of 1.5% and 2.4%, respectively (Patro *et al.*, 2011, Riche, 2011). However, since the requirements of the last two limiting amino acids coming from grey literature, there will be a need for further studies to confirm these requirements, especially for the methionine that has essential function as a precursor in the protein synthesis (National Research Council, 2011)

Figure 1. Above. Average size of Florida pompano at the end of trial, and Below, illustration of pompano intestine size to the body of pompano.

Aside from nutritional requirements, feeding management could also influence the growth performance of fish. According to Weirich *et al.* (2006) late afternoon feeding time and distribution of the diet in two or more feedings per day relatively increase the weight gain of pompano. Feed intake could also be induced by using attractants, especially when a substantial amount of fish meal is replaced by plant-protein sources, Several attractants or palatability enhancers, such as krill meal (Gaber, 2005), blue mussel meal (Nagel *et al.*, 2014), tuna by-product meal (Hernández *et al.*, 2011), algal meal (Kissinger *et al.*, 2016), nucleotides (Barnard, 2006) and chemo-attractants derived from hydrolysis process of seafood waste and by-products (Refstie *et al.*, 2004, Barry *et al.*, 2017) could be included to enhance the palatability and feed intake of plant-based diet. Previous research indicated that with four times feeding per day and the use of 5% squid hydrolysates (SH) supplemented into the animal free diet, pompano had significantly higher final weight, percentage weight gain and thermal growth coefficient (TGC) in comparison to fish fed with plant-based diet, 5% chemical attractant mix and 5% poultry by product meal (PBM) (Rhodes *et al.*, 2017a).

3. Alternative protein sources to fish or animal meal

Fish meal has traditionally been considered as the preferred protein source for aquatic animal feeds because of its nutrient characteristics as the excellent source of essential amino acid, fatty acid, vitamins, mineral, palatability, content of unidentified growth factors and lack of anti-nutritional factors (ANFs) (Swick et al., 1995, Samocha et al., 2004, Miles and Chapman, 2006, NRC 2011). Historically, the development of cost-effective practical diet to support the optimum growth of pompano has been initiated by using 30% of FM (Lazo *et al.*, 1998). However, as the margin decreases, feed manufactures and fish producers prefer to use alternative protein sources to produce more cost-effective diet (Sookying *et al.*, 2013). Several alternative protein sources has

been evaluated to replace fish meal and other animal proteins allowing increased levels of plant based proteins, including soybean meal, cotton seed meal, and corn protein concentrate (Cook *et al.*, 2016, Lech and Reigh, 2012, Quintero *et al.*, 2012, Rhodes *et al.*, 2013, Rhodes *et al.*, 2015, Riche, 2015, Riche and Williams, 2011, Rossi Jr and Davis, 2012, Rossi and Davis, 2014). From the pompano study, number of researches demonstrated that this fish has a wide range tolerance to the variety of plant-based protein sources without causing significant deleterious effect to their growth and nutrient utilization.

Among alternative protein sources, processed soybean (*Glycine max*) possesses many qualities that can replace dietary fish meal as an economically and nutritious alternative ingredient (Lech and Reigh, 2012). Crude soybean has a favorable amino acid profile, widely available, highly digestible, easily shipped and stored, and priced competitively with other plant-based food sources (Davis and Arnold, 2000, Gatlin *et al.*, 2007, Amaya *et al.*, 2007). In addition, soybean meal has a favorable amino acid profile with exception of lysine and total sulfur-based amino acids (methionine + cystine) and reported to have an apparent energy and protein digestibility for Florida pompano of 67.4 and 84.3%, respectively (Gothreaux *et al.*, 2010). However, as mentioned earlier, wider use of SBM may be hindered by negative effects associated with the presence of anti-nutritional factors, such as lectins, phytic acid, saponins, phytosterols, and allergens (NRC, 2011), which is responsible for the decreased growth performance (Tibaldi *et al.*, 2006), feed efficiency (Olli *et al.*, 1995) and histomorphological change in the distal intestinal (Rumsey *et al.*, 1994, Nordrum *et al.*, 2000) of some species of fish. Consequently, there will be most likely a need to carry out further processing technique to the conventional soybean meal to produce more nutritious and digestible soy products.

Today, there are several commercial soy products that has been further processed or known as advanced soy products that can be readily used in diet formulation, such as fermented soybean meal (FSBM) and enzyme-treated soybean meal (ESBM). The use of these products might have the potential as a promising alternative protein source to improve the efficacy of soy protein and substitute the use of animal meal in the diet formulation (Lim and Lee, 2011, Barnes *et al.*, 2014). Fermentation process which allows microorganisms to degrade macromolecules to low molecular weights have been reported to contain numerous benefits including the degradation of soy immunoreactivity (Song *et al.*, 2008), lower levels of anti-nutritional factors (Lim *et al.*, 2010, Mukherjee *et al.*, 2016), decrease the molecular weight of soya protein (Feng *et al.*, 2007), improve the nutritional quality and fibrinolytic enzyme activity of the commercial SBM (Bi *et al.*, 2015). Moreover, the use of FSBM as the protein sources in the diet could partly prevent the alterations in the intestinal and liver morphologies in some species of fish (Yamamoto *et al.*, 2010a, Wang *et al.*, 2016).

Likewise, combination of non-alcohol extraction process and enzymatic treatment to produce ESBM has been shown to improve the protein level and reduce the anti-nutritional factors and oligosaccharide level contained in SBM (Amezquita and Arana. 2015). Moreover, according to the information obtained from manufacturer (NutriVance^{TM}, Midwest Ag Enterprises, Marshall, MN, USA), the process was able to decrease the stachyose, raffinose and trypsin inhibitor activity level become 0.50%, 0.20%, and 2.31 mg/g, respectively. Despite research articles describing the use of ESBM in aquaculture species are limited, several technical reports acknowledge that ESBM could be included up to 17.4% in the diet for striped bass *Morone saxatilis* (Amezquita and Arana 2015) and 26.4% for spotted rose snapper *Lutajnus guttatus* (Amezquita *et al.* 2013) without affected the growth parameters.

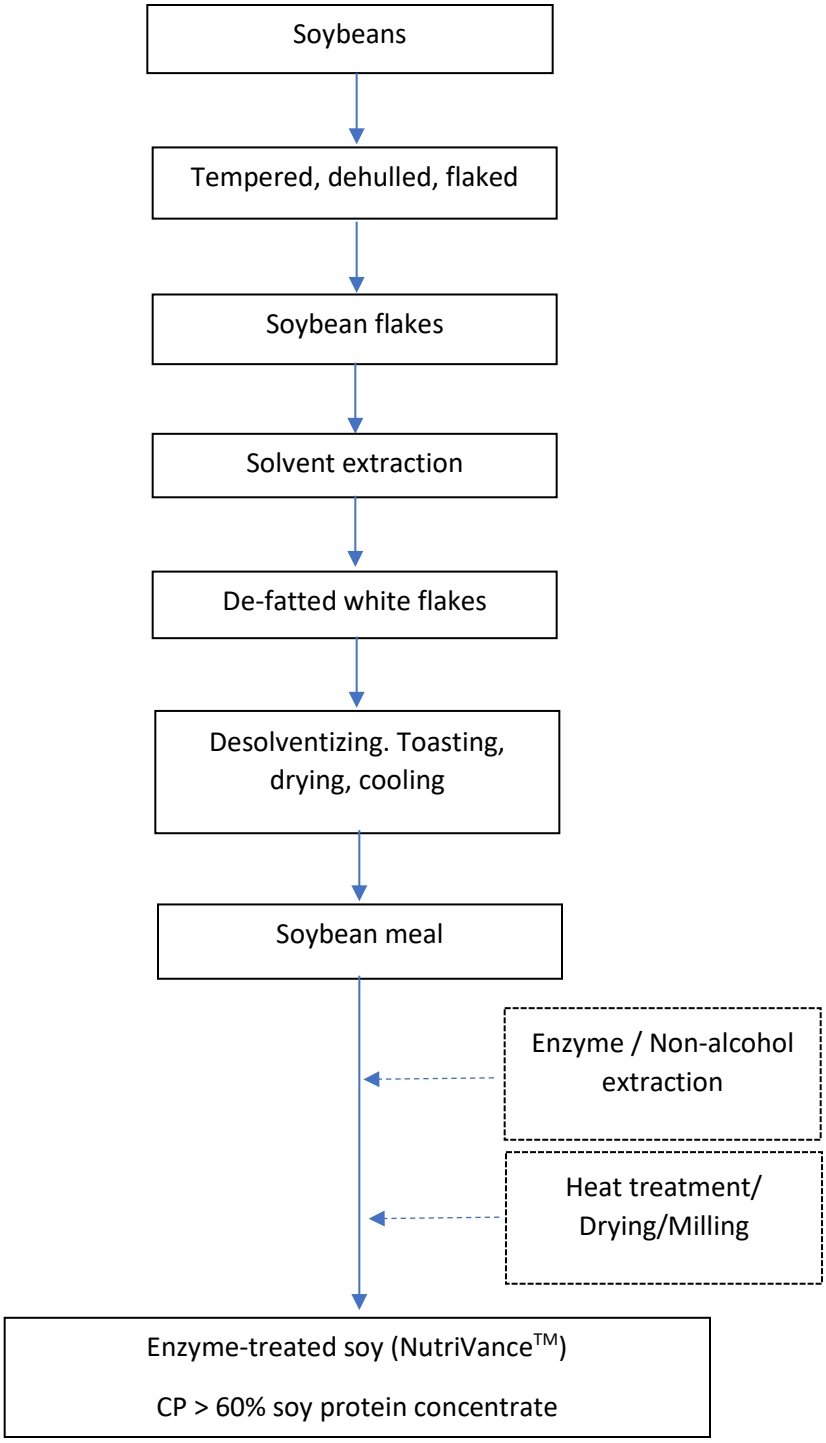

Figure 2. The production flow of enzyme-treated soy, Nutrivance™ (Figure provided by Midwest Ag Enterprises, Marshall, MN, USA)

4. Serum biochemistry and histology

Other than growth performance, health and physiological condition become the major concern when replacing fish meal with plant-protein sources. Since liver play significant role in many body process, including protein, fat and carbohydrate metabolisms, detoxification and excretion of waste, the administration of diet with different treatments can produce several alterations in clinical biochemistry (Thrall *et al.*, 2012). In measuring the clinical biochemistry, serum samples for the respective parameters need to be properly collected and prepared. Serum can be obtained by using centrifugation process to separate the cellular components from the fluid phase of the blood, thus avoid any possibility of cells to metabolize certain chemical components in the serum (Thrall *et al.*, 2012). The same author also suggests several parameters to be included in clinical biochemistry analysis, including the leakage enzyme activities (alanine aminotransferase and aspartate aminotransferase), induce enzyme activities (alkaline phosphatase), total protein, glucose, cholesterol and bile acids concentration.

The spectrum and potential magnitude of changes in the clinical biochemistry results, not only could be used as an indicator for the state of liver function, but also can reveal the causative factors for the change in the liver morphology. Study from Mahmoud *et al.* (2014) indicated that the changes in several parameters of clinical biochemistry positively correlated with several alterations in liver histology of Nile tilapia fed with SBM to completely replace FM in the diet. Likewise, the change in total biliary bile acid also effect the liver morphological features of Rainbow trout *Oncorhynchus mykiss* fed with different soy-source protein (Matsunari *et al.*, 2010). Interestingly, using *Bacillus subtilis* E20-FSBM, abnormalities in fish liver histomorphology could be avoided as the inclusion of fermented soy in diets increased up to 20% (Shiu *et al.*, 2015).

Other than liver, significant number of studies demonstrated that the use of high inclusion level of low-processed SBM will induces the intestinal inflammation in the hindgut of some species of fish (Baeverfjord and Krogdahl, 1996, Ingh *et al.*, 1996, Krogdahl *et al.*, 2003, Bakke-McKellep *et al.*, 2000). Gut inflammation or soy-induced enteritis have been described as widening of *lamina propria* (LP) of mucosal folds, infiltration of inflammatory cells in the LP, reduced number or even absence of supranuclear vacuoles in the absorptive epithelium, elevated number of goblet cells and levels of lysozyme in the gut mucosa (Merrifield *et al.*, 2011, Trushenski, 2015) that might cause disturbance in growth process, digestive system, decreased feed efficiency and liver dysfunction of fish (National Research Council, 2011). According to Sanden *et al.* (2005) trypsin inhibitors and lectins might responsible for the morphological change in the distal intestine of Atlantic salmon *Salmo salar* L. However, Knudsen *et al.* (2007) argues that the gut inflammation in Atlantic salmon might be induced by saponins alone or in combination with other factors, such as the intestinal microflora or the antigenic soybean proteins. Despite the health effects of ESBM to the liver and distal intestine histomorphological conditions is limited, several authors acknowledge the utilization of fermentation technique can eliminate several ANFs that are responsible for the intestinal morphological changes (Yamamoto *et al.*, 2010b, Barnes *et al.*, 2014). Given the background knowledge that using fermentation technique or combination of non-alcohol extraction process and enzymatic treatment should improve the nutritional value of conventional SBM and might have beneficial effect to the health status of the fish, therefore the objective of this research is to evaluate the utilization of commercial fermented soybean meal and enzyme treated soy as an alternative protein sources to replace dietary animal meal on the growth parameters, body composition, serum biochemistry and the histology of the liver and distal intestine of Florida pompano *Trachinotus carolinus.*

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Chapter II

EVALUATION OF FERMENTED SOYBEAN MEAL AS PARTIAL OR TOTAL REPLACEMENT OF SOYBEAN MEAL IN PRACTICAL DIETS FOR FLORIDA POMPANO *Trachinotus carolinus*

Abstract

This study evaluated the suitability of commercially produced fermented soybean meal (FSBM) known as PepSoyGen^{TM}, in a plant-based diet for Florida pompano, *Trachinotus carolinus* fingerlings. An 8-week growth trial was conducted to evaluate the effect of four iso-nitrogenous and iso-lipidic diets containing 0, 206, 309 and 410 g kg^{-1} FSBM, replacing approximately 0, 50, 75 and 100% SBM (designated as Basal, FSBM 50, FSBM 75, and FSBM 100, respectively) on growth performance, body composition, serum biochemistry and morphological condition of liver and distal intestine of Florida pompano. There were no significant differences in final mean weight, percentage weight gain, thermal unit growth coefficient and feed conversion ratio in all treatments. For serum biochemistry analysis, there were no significant differences in total protein, albumin, glucose, cholesterol, bile acids, plasma alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase activities in all treatments. Cellular infiltration, presence of goblet cells and the width of lamina propria in the distal intestine were partly improved in fish fed FSBM 75 and 100 compared to the fish fed FSBM 50 and basal diet. Glycogen granulation, inflammation and nuclear change condition in the liver of pompano were better as the inclusion of fermented

product increased. Results of this study indicate that FSBM can be utilized as an alternative protein source and microbial fermentation process could improve the functional properties of SBM.

Key words: fermented soybean meal, *Trachinotus carolinus,* growth performance, serum biochemistry, histology

1. Introduction

Florida pompano *Trachinotus carolinus* exhibit many favorable characteristics for aquaculture such as excellent flavor, high market value, acceptance of pelleted feeds, relatively fast growth and they can be cultured in seawater and low salinity environments (Lazo *et al.*, 1998, Weirich and Riley, 2007, Riche and Williams, 2010, Salze *et al.*, 2016). Several studies have reported the effectiveness of plant-based diets for pompano culture (Lazo *et al.*, 1998, Rossi Jr and Davis, 2012). Moreover, Quintero *et al.* (2012) have indicated that fish meal (FM) can be reduced from 30 to 15% and replaced with combinations of solvent extracted soybean meal (SBM) and soy protein concentrate (SPC) without compromising the growth performance of Florida pompano. However, reduced performance of pompano has been observed when less than 15% of animal proteins e.g. FM, poultry by product meal or meat and bone meal, included in the diets (Rossi Jr and Davis, 2012). Nonetheless, other studies have suggested that the decreased performance in low animal meal feeds can be partially remediated through the use of amino acid supplements and to a limited degree of attractants (Gaber, 2005, Lian *et al.*, 2005, Rhodes *et al.*, 2017). The complete elimination of animal meal could, in part, be related to the nutritional characteristics of plant-based ingredients such as SBM which is used at increased levels in low animal protein diets. Hence, there is an opportunity to improve the performance and utilization by shifting the characteristics of these ingredients.

The use of microorganisms for solid state fermentation of SBM has been proven to be an alternative method to improve the functional properties and nutritional quality of SBM, reduce the anti-nutrients, and increase the content of soybean peptides (Papagianni *et al.*, 1999, Hong *et al.*, 2004, Bi *et al.*, 2015). Zhuo *et al.* (2016) indicated that fermentation process with *Lactobacillus* spp can improve the crude protein digestibility and dry matter of SBM for grouper *Epinephelus*

coioides. In addition to *Lactobacillus* spp, other microorganisms such as *Aspergillus ficuum* and *Aspergillus oryzae* (Chen *et al.*, 2016), *Bacillus subtilis* (Azarm and Lee, 2014), *Lactobacillus plantarum* P8 (Wang *et al.*, 2016) or the combination of *Bacillus* spp and *Aspergillus* spp (Barnes *et al.*, 2014) have been used to facilitate the fermentation process and decrease the anti-nutrients contained in SBM. Furthermore, Lee *et al.* (2016) also indicated that fermentation process may be more cost-effective than various advanced processing strategies to improve the acceptance and utilization of soy protein for aquaculture species.

To date, relatively few studies have monitored the complementary effects of fermented products to the serum biochemical parameters and enzyme activities in fish. However, the limited resources available have led to contradictory results (Lin *et al.,* 2013). Considering that the evaluation of these parameters was helpful in narrowing the causative factors of metabolic disorders, such as deficiency in amino acids (Li *et al.,* 2014) and could be used as indicators of nutritional quality (Metón *et al.* 1999), serum samples must be evaluated in the study of plant-based diet. In addition, determining the histomorphological change that may occur in the liver and distal intestine of fish fed with high inclusion of soy protein has been suggested to be essential for fish health and nutritional efficiency of the diet (Baeverfjord and Krogdahl, 1996, Baeza-Ariño *et al.,* 2016). With regard to the use of fermented product, Yamamoto *et al.* (2010) found that with proper fermentation and inclusion level, FSBM could prevent various physiological abnormalities in the distal intestine of rainbow trout *Oncorhynchus mykiss*.

To our knowledge, limited studies have been reported on the use of FSBM to gradually replace SBM in Florida pompano diets. Thus, the objective of this study was to evaluate the growth performance, serum biochemical characteristics, and intestinal and liver histological condition of

juvenile Florida pompano in response to several inclusion levels of FSBM to replace traditional SBM.

2. Materials and methods

2.1 Experimental diets

This experiment was conducted using a commercial product of FSBM known as PepSoyGen (Nutrafrema, North Sioux City, SD, USA) produced via fermentation process using *Aspergillus oryzae* and *Bacillus subtilis*. Four iso-nitrogenous and iso-lipidic (400 g kg^{-1} protein and 80 g kg^{-1} lipid) diets were formulated using poultry by-product meal (PBM, Griffin Industries, Inc. Mobile, AL, USA), de-hulled solvent extracted soybean meal (SBM, Bunge Limited, Decatur, AL, USA), fermented soybean meal (FSBM, PepSoyGen^{TM}, Nutrafrema, SD, USA) and corn protein concentrate (Empyreal 75^{TM}, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA) as the dietary protein sources. The experimental diets were formulated to contain 0, 206, 309 and 410 g kg^{-1} FSBM, with FSBM replacing approximately 0, 50, 75 and 100% of the solvent extracted SBM (designated as Basal, FSBM 50, FSBM 75, and FSBM 100, respectively). 15% PBM which has been run in numerous trials for Florida pompano were included in all dietary treatments. Taurine was supplemented in all diets to match the calculated levels in basal diet and to meet the requirement of pompano. All diets were produced in the Laboratory of Aquatic Animal Nutrition, School of Fisheries, Aquaculture and Aquatic sciences, Auburn University, AL, USA, using standard procedures for Florida pompano. Briefly, diets were made by mixing preground dry ingredients and fish oil in a food mixer (Hobart, Troy, OH, USA) for approximately 15 min. The mixture was then blended with boiling water to attain appropriate condition for pelleting. The moist mash from each diet was passed through a 3 mm die in a meat grinder, and the pellets were

then placed into a fan-ventilated oven (<45 ºC) overnight to attain a moisture content less than 10%. The diets were stored at - 20 ºC, and, prior to use, each diet was ground and sieved to an appropriate size. The compositions of the experimental diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate analysis (Table 1) and amino acid profile (Table 2).

Table 1. Composition (g kg-1, *as is*) of diets containing various levels of fermented soybean meal (FSBM) fed to juvenile Florida pompano for 8 weeks.

	Diet code			
	Basal	FSBM 50	FSBM 75	FSBM 100
Ingredient (g kg^{-1}, *as is*)				
Poultry by-product meal[1]	150.0	150.0	150.0	150.0
Soybean Meal[2]	472.1	235.4	116.8	0.0
Fermented Soybean Meal[3]	0.0	206.0	309.0	410.7
Corn protein concentrate[4]	63.0	63.0	63.0	63.0
Menhaden Fish Oil[5]	47.4	49.0	49.7	50.5
Corn Starch[6]	7.0	37.2	52.4	67.0
Whole wheat[6]	220.0	220.0	220.0	220.0
Trace Mineral premix[7]	2.5	2.5	2.5	2.5
ASA Vitamin premix w/o choline[8]	5.0	5.0	5.0	5.0
Choline chloride[6]	2.0	2.0	2.0	2.0
Stay C 35%[9]	1.0	1.0	1.0	1.0
CaP-dibasic[6]	20.0	19.0	18.7	18.5
Lecithin (soy commercial)[10]	5.0	5.0	5.0	5.0
Taurine[6]	5.0	4.9	4.9	4.8
Proximate analyses (g kg^{-1}, as is)[11]				
Crude Protein	423.5	416.0	416.9	418.9
Moisture	47.5	60.9	64.3	65.1
Crude Fat	99.6	93.2	95.9	95.8
Crude Fiber	26.2	32.3	30.1	27.9
Ash	67.4	65.7	64.9	64.0

1 Griffin Industries, Inc., Mobile, AL, USA

2 De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA

3 PepSoyGen™, Nutraferma, Protein and Biotech Products, Sioux City, IA, USA

4 Empyreal 75™, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA

5 Omega Protein Inc., Houston, TX, USA

6 MO Biomedicals Inc., Solon, OH, USA

7 ASA Premix (g 100 g^{-1} premix): cobalt chloride, 0.004; cupric sulfate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α cellulose 81.826

8 ASA Premix (g kg^{-1} Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.

9 Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA

10 The Solae Company, St. Louis, MO, USA

11 Analyses conducted by the University of Missouri-Columbia, Agricultural Experiment Station Chemical Laboratory, MO, USA

Table 2. Amino acid (AA) composition (g kg^{-1}) of experimental diets utilized in the trial

	Diet code			
AA (g kg^{-1}, dry matter)	Basal	FSBM 50	FSBM 75	FSBM 100
Taurine	7.3	6.9	6.7	6.6
Hydroxyproline	3.1	3.5	3.0	3.8
Aspartic Acid	37.1	37.1	37.2	38.0
Threonine	14.7	14.8	14.9	15.1
Serine	17.8	18.3	18.0	18.8
Glutamic Acid	75.3	75.3	74.7	76.4
Proline	24.7	23.7	23.8	23.9
Lanthionine	0.3	0.4	0.5	0.4
Glycine	21.4	21.7	21.3	21.9
Alanine	21.5	21.7	21.7	22.0
Cysteine	5.9	5.7	5.7	5.7
Valine	19.7	19.6	19.8	20.2
Methionine	7.4	7.4	7.3	7.2
Isoleucine	17.8	17.7	18.0	18.1
Leucine	35.1	34.9	35.3	35.6
Tyrosine	15.2	14.8	12.8	15.3
Phenylalanine	20.1	19.9	20.0	20.2
Hydroxylysine	0.9	0.8	0.8	0.7
Ornithine	0.00	0.1	0.1	0.00
Lysine	22.0	20.9	20.8	21.3
Histidine	9.5	9.4	9.4	9.5
Arginine	25.7	25.1	24.1	25.2
Tryptophan	4.7	4.6	5.0	4.5

2.2 Experimental fish and growth trial

Florida pompano fingerlings were purchased from Troutlodge Marine Farms LLC, (Proaquatix) Vero Beach, Florida, USA and nursed in an indoor recirculating system facility at Claude Peteet Mariculture Development Center (CPMC), Gulf shores, Alabama, USA. Pompano were fed with commercial diet (FF Starter, Zeigler Bros., Inc. Gardners, PA, USA) until they reached the suitable size. The trial was conducted in a semi-recirculating system consisted of twelve open top tanks equipped with a reservoir tank, biological filter, supplemental aeration (provided using a central line, regenerative blower and air diffusers) and a circulation pump. At

the start of experiment, twenty uniform-sized fish (mean initial weight 17.01 ± 0.07 g) were stocked into each tank and assigned to triplicate tanks in a completely randomized design. Fish were maintained under a natural photoperiod throughout the trial. Water temperature (28.64 ± 1.59 ^{0}C), salinity (31.17 ± 3.92 ppt), pH (8.05 ± 0.38), and dissolved oxygen (5.89 ± 0.49 mg L^{-1}) were measured two times daily with a water quality multiparameter (ProPlus, Yellow Spring Instruments Co., Yellow Springs, OH, USA). In addition, total ammonia nitrogen (0.13 ± 0.23 mg L^{-1}) was measured two times per week using an ion-selective electrode (Orion 4-Star Plus pH/ISE, Thermo Fisher Scientific, Waltham, MA, USA) and nitrate (7.10 ± 5.90 mg L^{-1}) was measured once a week using colorimetric test kits (La Motte Chemicals, Chestertown, MD, USA). Fish were fed four times per day and the daily ration was adjusted to apparent satiation weekly throughout the trials. Additionally, feed inputs were calculated on a two-week basis after each sampling to adjust for growth and mortalities. The growth trial lasted for 8 weeks. At the end of growth trials, fish were grouped and individually weighed to obtain the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR) and thermal unit growth coefficient (TGC), calculated as follows:

$$\text{PWG} = \frac{(average\ individual\ final\ weight - average\ individual\ initial\ weight)}{(average\ individual\ initial\ weight)} \times 100$$

$$\text{FCR} = \frac{(average\ individual\ dry\ matter\ feed\ intake)}{(average\ individual\ weight\ gain)}$$

$$\text{SR} = \frac{(final\ number\ of\ fish)}{(initial\ number\ of\ fish)} \times 100$$

$$\text{TGC} = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} \times 100$$

where FBW is final body weight, IBW is initial body weight, T is water temperature (^{0}C) and D is number of trial days

2.3 Body composition analysis

Twenty fish from the initial stock population were sampled, and four fish from each tank (twelve fish per treatment) were randomly sampled at the end of the trial and stored at – 80 ^{0}C for body composition analysis. Prior to proximate analysis, dried whole fish were rigorously blended and chopped in a mixer according to methods described by Association of Official Analytical Chemists (AOAC, 1990). All parameters were analyzed at Agricultural Experiment Station Chemical Laboratories, University of Missouri (Columbia, MO, USA) and the mean of each treatment were taken.

2.4 Serum biochemical analysis

At the end of growth trial, twelve fish per treatment (four fish per replicate) were immediately euthanized with Tricaine-S (MS-222, tricaine methanesulfonate salt, Western Chemical, Inc., Ferndale, WA, USA), and blood samples were taken from the caudal vein after 12 h fasting. Serum was obtained by centrifugation at 3,000 rpm for 10 min and stored at – 80 ^{0}C until serum biochemical analysis. Biochemical parameters in the serum samples were analyzed for total protein, albumin, glucose, cholesterol, bile acid concentration, activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) using an automated chemistry analyzer (Cobas C311, Roche Diagnostics, IN, USA) following protocol described by Salze *et al*. (2016).

2.5 Histopathology Analysis

Fish samples for histopathological studies were selected randomly and individually anesthetized in a solution of Tricaine-S (MS-222, tricaine methanesulfonate salt, Western Chemical, Inc., Ferndale, WA, USA). Liver and distal intestine of fish was preserved in Bouin's solution (picric acid-formalin-acetic acid mixture, Ricca Chemical, Arlington, TX, USA) for 20 h at room temperature and then transferred to 70% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological techniques. The blocks of designed sample, dehydrated through a standard ethanol series to 100%, were embedded in paraffin wax and sectioned at 4 μm intervals for staining with Hematoxylin-Eosin (H&E) stain (Merck, Darmstadt, Germany). For estimations, double blinded evaluation with a grading scale of 1 to 5 was used. Score 1 was considered as the normal condition and subsequent scores accounted for increasing levels of histopathological alteration compared to the normal condition. The following parameters were taken into account for liver sections: glycogen granulation, inflammation and nuclear change. While intestinal samples were evaluated for cellular infiltration, presence of goblet cells and widening of the lamina propria within the intestinal folds. Images were acquired by using a digital imaging microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

2.6 Statistical analysis

Statistical analyses were conducted using SAS system (V9.3, SAS Institute, Cary, NC, USA). All data except for histopathological analysis were analyzed using one-way analysis of variance to determine the significant difference ($P < 0.05$) among the treatment means followed by the Tukey's multiple comparison test to determine difference between treatments means in each trial. The pooled standard errors are presented across growth trials, proximate composition, serum levels and enzyme activities, as the variance of each treatment is the same. Histopathological scores were treated as categorical data, tested for normality and homoscedasticity and subsequently analyzed using Welch's one-way analysis of variance followed by Games-Howell *post hoc* tests to determine significant differences between treatments. The results for histopathological evaluation are shown as mean ± standard deviation.

3. Results

3.1 Growth performance

Nutrient composition of the experimental diets and fish growth performance are presented in Table 2 and 3. No significant differences were observed in final mean weight, percentage weight gain, TGC, feed intake and FCR across all dietary treatments (Table 3). However, fish fed with basal diet without any inclusion of FSBM had significantly higher survival rates as compared to fish fed with various inclusion levels of FSBM (Table 3).

Table 3. Growth performance of juvenile Florida pompano (Mean initial weight 17.01 ± 0.07 g) offered fermented soybean meal at varying levels for 8-wk.

	Diets	Final mean weight (g)	PWG (%)	TGC	Feed Intake (g fish^{-1})	FCR[1]	Survival (%)
1	Basal	72.44	324.81	0.1016	94.99	1.72	93.3[a]
2	FSBM 50	68.25	301.68	0.0960	89.75	1.78	78.3[b]
3	FSBM 75	73.48	335.18	0.1025	85.78	1.54	80.0[b]
4	FSBM 100	69.10	305.95	0.0979	82.33	1.58	73.3[b]
	P-value	0.7531	0.7857	0.7788	0.0791	0.3119	0.0027
	PSE[2]	3.9755	26.4259	0.0105	3.0026	0.0952	2.5000

[1] FCR = Feed conversion ratio

[2] PSE = Pooled standard error

3.2 Proximate composition of whole fish

Body composition of the fish fed with diets containing various levels of FSBM is shown in Table 4. No significant differences were identified in the crude protein, moisture, fat, ash and phosphorus content among the treatments ($P > 0.05$). Crude fiber content of fish fed diets FSBM 100 was lower compared to FSBM 50 ($P = 0.0335$).

Table 4. Proximate composition (g kg^{-1}) of whole body of pompano fed experimental diets for 8-wk.

	Diets	Crude protein	Moisture	Fat	Crude Fiber	Ash	Phosphorus
1	Basal	184.4	713.3	69.5	0.6^{ab}	31.5	5.8
2	FSBM 50	189.8	711.2	69.7	0.9^{b}	32.9	6.1
3	FSBM 75	186.5	707.9	72.3	0.8^{ab}	32.8	5.8
4	FSBM 100	184.8	713.7	68.5	0.5^{a}	34.0	6.3
	P-value	0.3772	0.8030	0.9480	0.0335	0.5356	0.2198
	PSE	0.2194	0.4577	0.4723	0.0111	0.1189	0.0175

3.3 Serum levels and enzyme activities

There were no significant differences in plasma total protein (g dL^{-1}), albumin (g dL^{-1}), glucose (mg dL^{-1}), and bile acid (g dL^{-1}) level among the treatments (Table 5). Plasma ALP (U L^{-1}), ALT (U L^{-1}) and AST (U L^{-1}) activities did not differ among the fish groups fed with various inclusion level of FSBM to replace dietary SBM.

Table 5. Effect of different diets on serum levels and enzyme activities in Florida pompano.

	Diets	Total protein ($g\ dL^{-1}$)	Albumin ($g\ dL^{-1}$)	ALP[1] ($U\ L^{-1}$)	ALT[2] ($U\ L^{-1}$)	AST[3] ($U\ L^{-1}$)	Glucose ($mg\ dL^{-1}$)	Cholesterol ($mg\ dL^{-1}$)	Bile acid ($mg\ dL^{-1}$)
1	Basal	4.06	1.30	35.50	17.33	222.0	251.67	173.67	9.80
2	FSBM 50	4.29	1.42	42.93	10.67	100.67	237.67	184.00	8.93
3	FSBM 75	4.05	1.33	38.30	18.00	161.33	240.00	179.67	5.80
4	FSBM 100	4.17	1.41	44.23	11.50	96.33	246.67	176.67	3.83
	P-value	0.3927	0.1079	0.0540	0.2778	0.2515	0.9227	0.5686	0.5623
	PSE[4]	0.1093	0.0288	2.0475	3.6855	45.9682	16.1328	5.2005	3.2368

Note:

[1] ALP = Alkaline phosphatase

[2] ALT = Alanine transaminase

[3] AST = Aspartate transaminase

[4] PSE = Pooled standard error

3.4 Liver and intestine histopathology

The histopathological examination of fish liver (Figure 2A and Table 6) indicated that fish fed with basal diet showed a higher score for glycogen granulation, inflammation, and nuclear change compared to other dietary treatments (Table 5 and Fig 2A). As the inclusion level of FSBM to substitute SBM increases, liver condition of pompano was better with lower numerical value for glycogen granulation, inflammation, and nuclear change. At the end of the growth trial, no incidence of green liver was observed in fish across all dietary treatments. The administration of experimental diet was significantly affecting the morphology of intestines. In distal tract of intestine (Figure 1), fish fed with basal diet and FSBM 50 showed an increase number of goblet cells (GC) compared to FSBM 75 and 100. The width of the lamina propria (LP) with cellular infiltration in the distal intestine of fish fed basal diet and FSBM 50 is wider compared to FSBM 75 and FSBM 100.

Table 6. Diagnostic features of liver and distal intestine of fish based on comparison of dietary treatments. Results presented as mean ± standard deviation (n= 12). Results in the same row with different superscript letter are significantly different ($P<0.05$) based on Welch's one-way analysis of variance followed by Games-Howell *post hoc* tests to determine significant differences between treatments.

Feature	Experimental diet				*P*- value
	Basal	FSBM 50	FSBM 75	FSBM 100	
Intestine					
Goblet cells	4.2±0.4^{a}	3.8±0.5^{a}	2.4±0.5^{b}	2.3±0.5^{b}	< 0.0001
Cellular infiltration	3.8±0.4^{a}	3.7±0.5^{a}	2.4±0.5^{b}	2.0±0.4^{c}	< 0.0001
Lamina Propria width	3.8±0.5^{a}	3.8±0.4^{a}	2.4±0.5^{b}	2.1±0.4^{b}	< 0.0001
Liver					
Glycogen granulation	4.1±0.4^{a}	3.6±0.5^{b}	2.4±0.5^{c}	2.0±0.0^{d}	< 0.0001
Inflammation	3.9±0.3^{a}	3.3±0.5^{b}	2.4±0.4^{c}	1.8±0.2^{d}	< 0.0001
Nuclear change	3.8±0.1^{a}	3.2±0.4^{b}	2.3±0.5^{c}	1.8±0.3^{d}	< 0.0001

Figure 1. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestines from Florida pompano after 8-wk of being fed with (A) basal diet (B) FSBM 50 (C) FSBM 75, and (D) FSBM 100

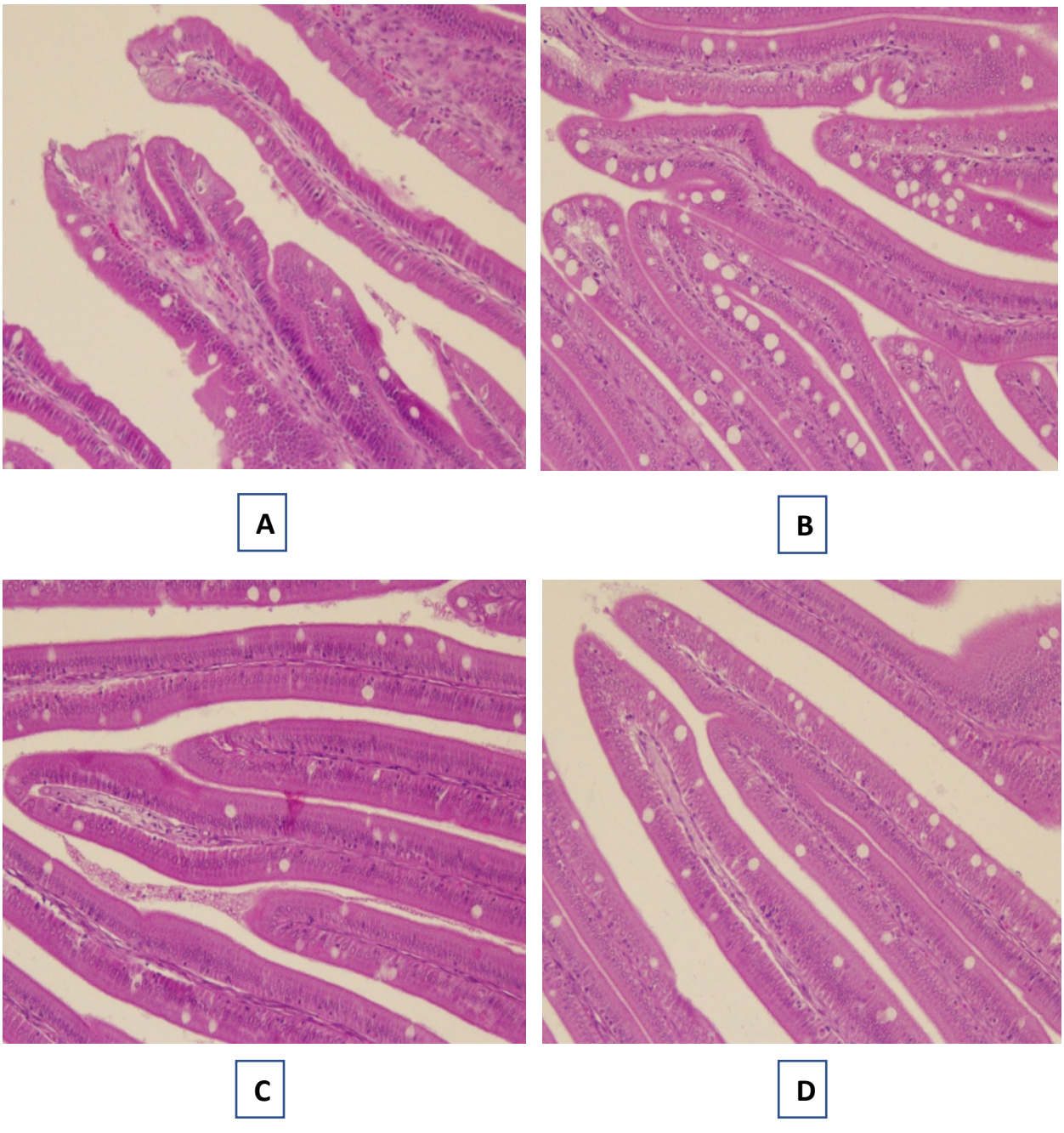
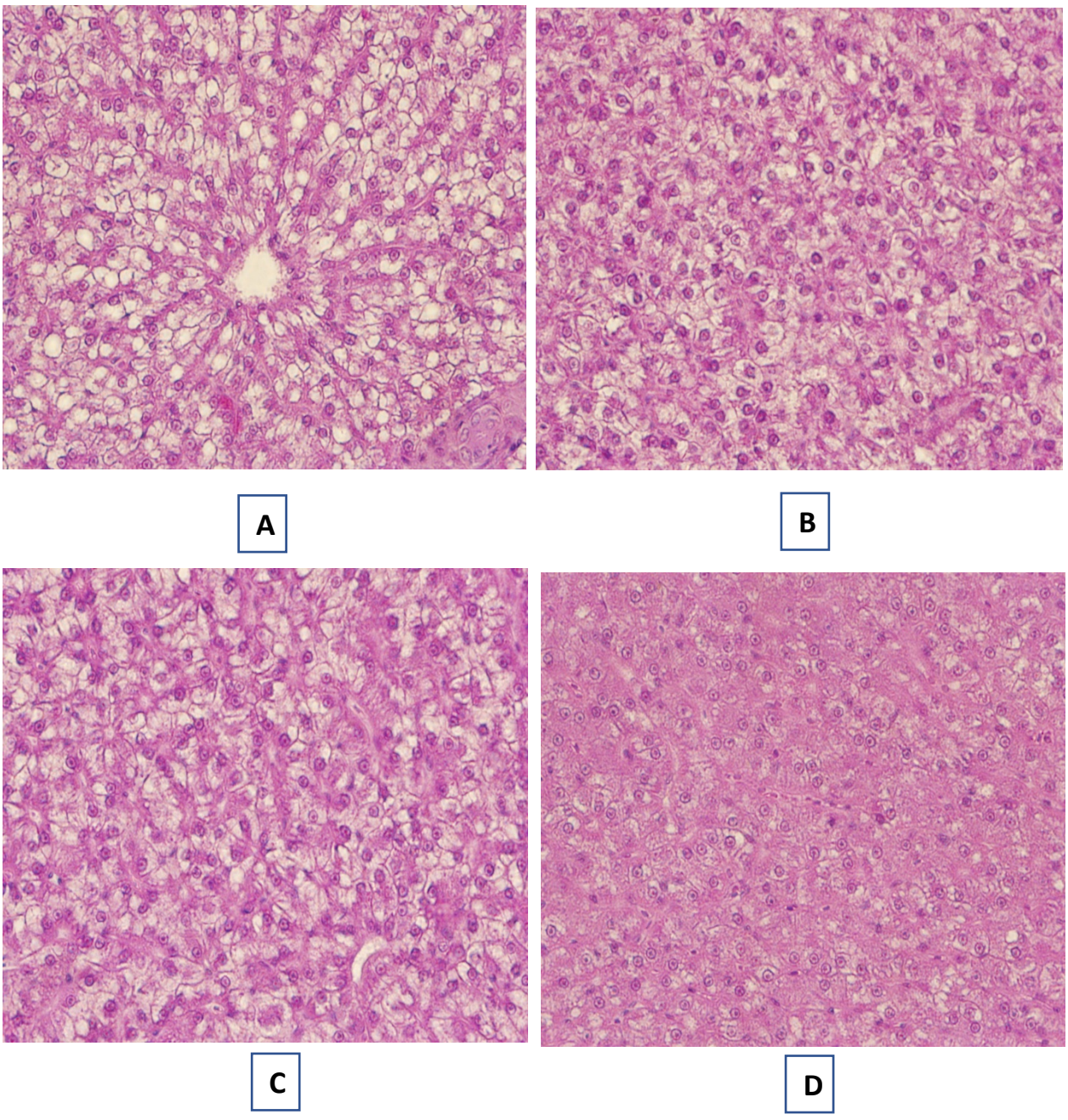


Figure 2. Representative histopathological images of hematoxylin and eosin-stained sections of liver from Florida pompano after 8-wk of being fed with (A) basal diet (B) FSBM 50 (C) FSBM 75, and (D) FSBM 100.



4. Discussion

A previous study indicated that the basal diet consist of 50% SBM and 15% PBM supplemented with taurine can be used as a good alternative ingredients for animal meal without any negative impact on growth performance of juvenile Florida pompano (Rossi and Davis, 2012). To improve the efficacy of soy-based protein, we utilized FSBM to incrementally (0, 50, 75, and 100%) replace SBM on an iso-nitrogenous basis. Our results indicated that the use of commercially produced fermented product, PepSoyGen, to replace the traditional SBM had no effect on growth performance and the proximate composition of Florida pompano. Our results corroborate the study of Choi *et al.* (2016) which reported that the use of various level of FSBM processed with *Phaffia rhodozyma* to replace traditional SBM had no effect on growth performance, feed utilization and proximate composition of juvenile rainbow trout. Similarly, other studies have reported no significant effect on the growth performance of Nile tilapia, *Oreochromis niloticus* (Lim and Lee, 2011) and white shrimp, *Litopenaeus vannamei* (Lin and Mui, 2017) when 50 or 100% of SBM was replaced by FSBM.

Fermentation process enhances the bioavailability of nutritious components (Chi and Cho, 2016) and reduces the immunoreactivity and allergic reactions caused by soy products (Frias *et al.*, 2007). However, depending on the type of fermentation and environmental condition, the use of different microorganisms might significantly decrease some amino acids (Song *et al.*, 2008). According to Lim and Lee (2011), microbial fermentation with *A. oryzae* decreased lysine and threonine concentration in SBM by 19 and 16%, respectively, while *Bifidobacterium lactis* or *Lactobacillus plantarum* significantly decreased the level of methionine and cysteine in SBM (Song *et al.,* 2008). In the present study, taurine was supplemented in the basal diet at the level of

5 g kg^{-1} and into the FSBM diet at a range of 4.8 to 4.9 g kg^{-1} to meet the estimated nutrient requirements for Florida pompano. However, fermentation process seems to have an effect to the taurine profile in the final fermented product, as indicated by the low level of taurine in the FSBM diet (6.6 to 6.9 g kg^{-1}) compared to basal diet (7.3 g kg^{-1}). There is growing evidence that taurine is essential for certain life stages in Florida pompano and reduced growth performance were observed in this fish when taurine requirement level is not met (Rossi Jr and Davis, 2012). In addition, lysine level in the presence of fermented product (20.8 to 21.3 g kg^{-1}) was also lower compared to basal diet (22 g kg^{-1}). Albeit lysine level remains in the predicted requirement (Riche and Williams, 2011), deficiency of this amino acid (AA) has been shown to cause a reduction in growth performance, feed efficiency and health issues (Ketola, 1983, NRC, 2011). As a result, it could be said that the degradations of AA influence the growth performance of pompano. Hence, to improve the efficacy of soy-source protein, high inclusion of fermented product in the diet formulation will either need to be balanced with proper level of AA in its purified form or supplemented with other protein sources rich in AA, especially taurine and lysine.

Fermentation process provides several benefits, such as reduction of soy allergic, immunoreactivity and enhanced the bioavailability of nutritious components to improve the physiological status of the fish (Frias *et al.*, 2007, Chi and Cho, 2016). The results of the present study showed that the total protein, albumin, glucose, cholesterol, bile acid, ALP, ALT and AST were not significantly affected, indicating that the inclusion of FSBM did not cause any serious alteration in all clinical variables compared to traditional SBM. Several studies have been carried out to determine the effect of fermented product on the serum and blood condition of fish. Study from Lim and Lee (2011) reported that plasma glucose, cholesterol, and plasma total protein of Nile tilapia were not affected using FSBM to replace the traditional SBM. Moreover, Murashita *et*

al. (2013) indicated that in comparison with traditional SBM, the use of fermented soy as the primary protein source did not significantly improve the total protein, cholesterol, glucose and total bile acid concentration of rainbow trout. On the contrary, Kader *et al.* (2012) found that the total serum protein of Japanese flounder (*Paralichthys olivaceus*) tended to increase with the increasing of FSBM inclusion levels in the diets. Moreover, Yamamoto *et al.* (2012) reported that the hemoglobin content of rainbow trout *Oncorhynchus mykiss* fed diet FSBM was significantly higher than in fish fed diet SBM. Thus, based on these findings, collection of blood samples at different time points after the last feeding are needed to clarify whether the inclusion of fermented soy might influence the hematological conditions and other enzyme activities in Florida pompano.

In some species, SBM cause several physiological abnormalities in liver (Murashita *et al.*, 2013) and distal intestinal of cultured fish, such as the abnormal vacuolization, widening of central stroma and inflammatory cells infiltration in lamina propria (LP) (Baeverfjord and Krogdahl, 1996, Burrells *et al.*, 1999). Study from Yamamoto *et al.* (2012) showed a morphological changes in the distal intestine of rainbow trout, including an increase of connective tissue in the submucosa and few vacuoles in the cytoplasm of hepatocytes in fish fed SBM. In the present study, the distal intestine of Florida pompano seems to be mildly sensitive to the dietary SBM. The LP with cells infiltration in fish fed with basal diet is significantly wider compared to FSBM 75 and FSBM 100. It has been reported that the use of high inclusion level of SBM might induces the cellular infiltration of the submucosa and LP in the Egyptian sole *Solea aegyptiaca* and Gilthead sea bream *Sparus aurata* (Bonaldo *et al.*, 2006, Bobadilla *et al.*, 2005). However, Yamamoto *et al.* (2010) found that the inclusion of fermented product produced via fermentation process using as compound bacteria predominantly *Bacillus* spp for 10 h could prevent the abnormalities in the LP of mucosal folds caused by SBM and showed normal conditions as shown in fish fed with FM-

based diet. With regard to the presence of goblet cells (GC), the lowest number was found in fish fed with FSBM 75 and 100 than in fish fed FSBM 50 and basal diet. According to Marchetti *et al.* (2006), the emergence of GC associated with the production of mucus in order to protect the mucous membrane from chemical and mechanical damage. In addition, Baeza-Ariño *et al*. (2016) suggest that the increased presence of GC could also be related to the unsatisfactory of protein digestion process. Thus, based on these findings, the use of high inclusion level of fermented soy may serve as a promising ingredient to partly prevent various physiological abnormalities that occur in the distal intestine of pompano fed with plant-based diet.

In this study, histopathological examination of the liver in fish fed basal diet showed a modest amount of glycogen vacuolization, inflammation and nuclear change. Martínez-Llorens *et al.* (2012) suggest that providing plant-based diet for certain period of time will cause a metabolic dysfunction, indicated with glycogen deposition in the liver. The use of FSBM 50 showed better condition than basal diet in terms of glycogen granulation, inflammation, and nuclear change. In addition, liver condition of fish fed with FSBM 75 presented less morphological change than FSBM 50, and liver condition of fish fed with FSBM 100 appeared to be much better than other dietary treatments. However, it is remains unclear whether the better conditions of liver especially in FSBM 100 resulted from the reduction of ANFs contain in the fermented product or other beneficial factors resulted from microbial fermentation process. Several authors reported that microbial fermentation of SBM could enhance the bioavailability of potential antioxidants, production of enzymes, carbohydrase and proteinase, leading to improved digestibility and health condition of fish (Aidoo *et al.*, 1994, Chou, 1995, Pham and Lee, 2007, Kim *et al.*, 2009, Kim *et al.*, 2010, Lim and Lee, 2011). Although the fermentation process could be an important factor for the improvement of physiological condition in the liver and distal intestine of pompano, other

ANFs that still remain in the final fermented product and responsible for the morphological changes and growth performance of fish need to be carefully evaluated.

The observation made in the present study suggest that FSBM produced via fermentation process using *Aspergillus oryzae* and *Bacillus subtilis* may serve as a good source of protein, and the inclusion of 309 and 410 g kg^{-1} FSBM to replace approximately 75 and 100% SBM were able to partially prevents the development of marked histological alteration in the liver and distal intestine of Florida pompano compared to basal and the inclusion of 206 g kg^{-1} FSBM to replace approximately 50% traditional SBM. Based on the results of the growth trial, further research is required to clarify whether the supplementation of essential amino acids to satisfy the nutrient requirement of fish fed with high inclusion level of FSBM may have beneficial effects on growth performance and physiological conditions of Florida pompano.

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Chapter III

PARTIAL OR TOTAL REPLACEMENT OF FISH MEAL BY ENZYME-TREATED SOY IN DIETS FOR FLORIDA POMPANO *Trachinotus carolinus*

Abstract

A series of trials were conducted to evaluate the efficacy of commercial enzyme-treated soy (ESBM) to replace the use of fish meal (FM) in practical diets for Florida pompano *Trachinotus carolinus*. The reference diet which has been run in numerous trials for Florida pompano was formulated using 150, 466 and 80 g kg^{-1} of FM, soybean meal (SBM) and corn protein concentrate (CPC), respectively. In trial 1, test diets were produced by replacing FM with 30.8, 61.4 and 92.1 g kg^{-1} of ESBM. In trial 2, test diets were produced by replacing FM with 28.9, 89.8, 120.1 g kg^{-1} of ESBM. Triplicate group of fish in trial 1 (mean weight = 13.05 ± 0.34 g) and trial 2 (mean weight = 18.45 ± 0.49 g) were fed these diets to apparent satiety for eight weeks. Growth performance was affected as the dietary FM level reduced and replaced with ESBM. In trial 1, final weight (FW), percentage weight gain (PWG) and thermal growth coefficient (TGC) were lower in 6% inclusion level of FM compared to 15%, while feed conversion ratio (FCR) significantly higher in fish fed the lowest inclusion level of FM. In trial 2, FW was significantly lower when FM completely replaced by ESBM and no significant differences in other growth parameters. In all trials, no significant differences were observed in terms of crude protein, moisture, fat, crude fiber, dry matter and ash content. For the serum levels and enzyme activities analysis, no significant differences in total protein, albumin, alkaline phosphatase, alanine

transaminase, aspartate transaminase, glucose and bile acids level across all trials. However, in trial 1, plasma cholesterol level was higher in fish fed 15% FM compared to other dietary treatments. The histomorphological structure of liver and distal intestine were slightly affected by lower inclusion level of FM. Overall, the results showed that ESBM could be used as a potential dietary protein sources to replace FM in the development of practical diet for pompano.

Key words: Growth parameters, serum, enzyme activities, liver, distal intestine, enzyme-treated soybean meal, Florida pompano

1. Introduction

Efforts to formulate economical feed with low level of fish meal (FM) in various aquaculture species have achieved considerable success (Boonyaratpalin *et al.*, 1998, Davis and Arnold, 2000, Kaushik *et al.*, 2004, Hardy, 2010, NRC, 2011). Several alternatives have been used to substantially decrease dietary level of FM in the feed formulation, including the use of plant-protein sources that appear to be the most sustainable and renewable ingredient (Alexis and Nengas, 2001, Kaushik *et al.*, 2004, Hardy, 2010). Among the plant-protein sources, soybean meal (SBM) is one of the most promising alternatives because of its constant availability, favorable amino acid profile, cost effective and high digestibility properties (Suárez *et al.*, 2009, NRC, 2011, Lemos *et al.*, 2000). However, deficiency in sulfur amino acid and the presence of anti-nutrients, such as proteinase inhibitors, phytic acid, saponins, anti-vitamins and phytosterols, limit the use of this ingredient and may have detrimental effects to the digestive process and growth of fish (Tibaldi *et al.*, 2006, NRC, 2011). Therefore, the use of highly processed soy-protein sources that can meet the dietary requirement of pompano appear to be more practical approach at this time.

Studies conducted with Florida pompano *Trachinotus carolinus* indicated that the combination of solvent-extracted SBM with soy protein concentrate (SPC) were able to reduce the FM level from 30 to 15% without deleterious effect on growth performance (Quintero *et al.*, 2012). The result suggest that blending of SBM with advanced soy-products is likely to be a viable strategy to improve the nutritional value of plant-based diet (Rombenso *et al.*, 2013, Lin and Mui, 2017). Today, other than SPC, several advanced products of soy-protein, such as fermented soybean meal (FSBM) or enzyme-treated soybean meal (ESBM) has been available in the market contain with higher levels of protein, low oligosaccharides and minimal levels of soy anti-

nutritional factors (ANFs) (Novriadi and Davis, 2018). Thus, proper inclusion of highly processed SBM could be considered as an alternative to reduce the dietary level of FM in diet formulation.

According to Quintero *et al.* (2012) and Rossi Jr and Davis (2012), 15% of FM protein may be considered as the minimum inclusion level in the development of practical diet that still support the optimum growth of Florida pompano. Continued reduction in FM levels will likely reduce the growth performance of this species (Cook *et al.*, 2016). Similarly, partial replacement of FM with conventional SBM reduced weight gain and specific growth rate (SGR) of red sea bream *Pagrus major* (Biswas *et al.*, 2007). Interestingly, the later author also indicated that when phytase was added into the SBM based diet, fish had better feed consumption and SGR and yielded similar growth with fish fed FM-based diet. Moreover, Tibaldi *et al.* (2006) and Refstie *et al.* (1998) indicated that the use of ESBM as one of the advanced soy products to replace dietary FM in the practical diet for European sea bass *Dicentrarchus labrax* and Atlantic salmon *Salmo salar,* respectively, resulted in better growth performance and nutrient utilization than the conventional SBM. In pompano, deficiency in some amino acid have been considered as the major factors responsible for the slow growth when fish fed with high inclusion level of plant-protein sources (Rossi Jr and Davis, 2012).

Other than growth parameters, hematological and histopathological condition of liver and distal intestine of fish can provide valuable information about the physiological effects of specific ingredient during the evaluation of alternative protein sources to replace dietary FM (Krogdahl *et al.*, 2010, Lin and Luo, 2011, Bonvini *et al.*, 2018, Zhang *et al.*, 2018). Present knowledge indicates that fish fed the diet with more than 50% replacement of FM with SBM had lower glutamyl oxaloacetic transaminase (GOT) and glutamyl pyruvic transaminase (GPT) in the liver than fish fed with FM-based diet (Lin and Luo, 2011). In contrast, there were no significant differences in

the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) when Japanese flounder *Paralichthys olivaceus* fed with various level of SBM to replace the dietary FM (Ye *et al.*, 2011). The results showed an inconsistency in enzyme activities when dietary FM replaced by SBM. In addition, the presence of several anti-nutritional factors (ANFs) in SBM could cause alterations in the histomorphological condition of fish liver (Robaina *et al.*, 1998, Kokou *et al.*, 2015, López *et al.*, 2015) and intestine (Krogdahl *et al.*, 2003, Heikkinen *et al.*, 2006). Although previous studies characterized the serum biochemistry, liver and distal intestine histology of Florida pompano as the effect of poultry by-product meal (PBM) and conventional SBM replacement with advanced soy products (Novriadi *et al.*, 2017b, Novriadi *et al.*, 2017a), to our knowledge, this study is the first report to evaluate the physiological effect of dietary FM substitution with advanced soy product. Thus, the objective of the present study was to analyze the effect of partial and complete replacement of dietary FM with various inclusion levels of ESBM on growth performance, proximate composition of the whole body, amino acids profile, serum and enzyme activities, and histomorphological condition of liver and distal intestine of Florida pompano.

2. Materials and Methods

2.1 Experimental diets

All diets were formulated to contain approximately 400 g kg^{-1} crude protein and 80 g kg^{-1} lipid. The reference diet in trial 1 was produced utilizing 150 g kg^{-1} of FM, 466.0 g kg^{-1} defatted SBM and 80 g kg^{-1} corn protein concentrate (CPC) based on previous work in developing the low FM diet for this species. In trial 1, three experimental diets were formulated to utilize increasing levels (30, 60 and 90 g kg^{-1}) of ESBM to replace dietary FM and labelled as 12, 9 and 6% FM

(Table 1). In trial 2, based on the results of trial 1, four experimental diets were produced to included increasing levels (30, 90, 120 and 150 g kg^{-1}) of ESBM to partially and completely replace dietary FM in the diet (Table 3). All experimental diets were produced at the aquatic animal nutrition laboratory at the School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University (Auburn, AL, USA) using the standard procedures for the pompano feeds. Briefly, diets were made by mixing pre-ground dry ingredients and fish oil in a food mixer (Hobart, Troy, OH, USA) for approximately 15 min. Boiling water was then blended into the mixture to help ensure the appropriate consistency of the mix for pelleting. The mash from each diet was passed through a 3 mm die in the meat grinder, and the pellets were then placed in forced air-drying oven (< 45^{o}C) overnight until moisture content of each diet less than 10%. Subsequently, the diets were crumbled, packed in the plastic bags, properly labelled, and stored in freezer (-20 ^{o}C) until needed. The composition of experimental diets was analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate analysis (Table 1 and 3) and amino acid profile (Table 2 and 4).

Table 1. Composition (g kg^{-1} *as is*) of diets containing various levels of enzyme-treated soy (ESBM) used in the first growth trial

Ingredients (g kg^{-1}, *as is*)	15% FM	12% FM	9% FM	6% FM
Menhaden Fishmeal[1]	150.0	120.0	90.0	60.0
Soybean meal[2]	466.0	466.0	466.0	466.0
Enzyme-treated soy[3]	0.0	30.8	61.4	92.1
Corn protein concentrate[4]	80.0	80.0	80.0	80.0
Menhaden Fish Oil[1]	50.0	52.9	55.8	58.7
Corn Starch[5]	38.5	31.4	24.5	17.5
Whole wheat[5]	180.0	180.0	180.0	180.0
Trace Mineral premix[6]	2.5	2.5	2.5	2.5
ASA Vitamin premix w/o choline[7]	5.0	5.0	5.0	5.0
Choline chloride[5]	2.0	2.0	2.0	2.0
Stay C 35%[8]	1.0	1.0	1.0	1.0
CaP-dibasic[5]	15.0	18.0	21.0	24.0
Lecithin (soy commercial)[9]	5.0	5.0	5.0	5.0
Methionine[5]	0.0	0.2	0.4	0.6
Taurine[5]	5.0	5.2	5.4	5.6
Proximate analyses (g kg^{-1}, *as is*)				
Phosphorus	13.2	12.0	12.3	12.5
Crude Protein	398.2	385.0	395.0	413.2
Moisture	64.1	96.1	80.9	81.7
Crude Fat	96.9	82.4	84.8	82.9
Crude Fiber	28.9	25.5	26.5	28.8
Ash	78.6	75.6	71.3	69.3

1 Omega Protein Inc., Huston TX, USA
2 De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA
3 Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA
4 Empyreal 75™ Cargill Corn Milling, Cargill, Inc., Blair, NE, USA
5 MP Biomedicals Inc., Santa Ana, Ca, USA
6 ASA Premix (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α-cellulose 81.826.
7 ASA Premix (g/kg Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
8 Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA
9 The Solae Company, St. Louis, MO, USA

Table 2. Amino acid (AA) profile (g kg^{-1}, *as is*) of experimental diets for first trial

Composition	15% FM	12% FM	9% FM	6% FM
Taurine	7.3	6.1	7.0	6.5
Hydroxyproline	2.2	1.8	3.8	1.2
Aspartic Acid	36.1	36.8	37.1	39.8
Threonine	14.1	14.2	14.1	14.8
Serine	15.7	15.5	16.0	17.0
Glutamic Acid	71.4	70.6	73.1	76.8
Proline	24.3	22.5	24.2	24.8
Glycine	19.2	18.7	18.6	18.4
Alanine	21.7	20.9	20.6	20.3
Cysteine	5.1	5.2	5.4	5.7
Valine	19.3	19.2	19.4	20.1
Methionine	7.3	7.3	7.5	7.4
Isoleucine	16.8	16.8	17.2	18.1
Leucine	34.4	33.6	34.2	35.1
Tyrosine	12.3	10.2	12.8	13.6
Phenylalanine	19.0	19.1	19.4	20.4
Hydroxylysine	0.8	0.7	0.7	0.5
Ornithine	0.3	0.5	0.3	0.3
Lysine	21.3	21.1	20.9	22.0
Histidine	9.7	9.5	9.7	10.1
Arginine	23.2	22.6	24.1	25.6
Tryptophan	5.1	5.0	5.2	5.6

Table 3. Composition (g kg^{-1}, *as is*) of diets containing various levels of enzyme-treated soy (ESBM) used in the second growth trial

Ingredients (g kg^{-1}, *as is*)	12% FM	6% FM	3% FM	0% FM
Menhaden fishmeal[1]	120.0	60.0	30.0	0.0
Soybean meal[2]	466.0	466.0	466.0	466.0
Enzyme-treated soy[3]	28.9	89.8	120.1	150.5
Corn protein concentrate[4]	80.0	80.0	80.0	80.0
Menhaden fish oil[1]	50.2	54.7	57.0	59.3
Corn Starch[5]	36.0	23.8	17.3	11.3
Whole wheat[5]	180.0	180.0	180.0	180.0
Trace Mineral premix[6]	2.5	2.5	2.5	2.5
ASA Vitamin premix w/o choline[7]	5.0	5.0	5.0	5.0
Choline chloride[5]	2.0	2.0	2.0	2.0
Stay C 35%[8]	1.0	1.0	1.0	1.0
CaP-dibasic [5]	18.0	24.0	27.5	30.5
Lecithin (soy commercial)[9]	5.0	5.0	5.0	5.0
Taurine[5]	5.2	5.6	5.8	5.9
Methionine[5]	0.2	0.6	0.8	1.0
Proximate analyses (g kg^{-1}, *as is*)				
Phosphorus	11.0	10.8	10.8	10.9
Crude Protein	417.2	418.8	431.6	425.0
Moisture	75.9	77.8	60.7	63.9
Crude Fat	96.3	80.8	87.3	87.4
Crude Fiber	28.2	28.2	29.7	31.8
Ash	71.8	67.3	67.0	65.0

1 Omega Protein Inc., Huston TX, USA
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3 Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA
4 Empyreal 75™ Cargill Corn Milling, Cargill, Inc., Blair, NE, USA
5 MP Biomedicals Inc., Santa Ana, Ca, USA
6 ASA Premix Mineral (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α-cellulose 81.826.
7 ASA Premix Vitamin (g kg^{-1} Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
8 Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA
9 The Solae Company, St. Louis, MO, USA

Table 4. Amino acid (AA) profile (g kg^{-1}, *as is*) of experimental diets for second trial

Composition	12% FM	6% FM	3% FM	0% FM
Taurine	7.1	7.1	7.3	7.1
Hydroxyproline	1.5	1.9	1.5	0.8
Aspartic Acid	37.5	38.3	40.2	40.2
Threonine	14.9	14.7	15.2	15.2
Serine	17.1	17.5	18.3	18.4
Glutamic Acid	76.2	77.4	80.3	80.9
Proline	25.7	25.3	25.4	25.5
Glycine	19.1	17.3	17.4	16.7
Alanine	22.1	20.8	20.8	20.7
Cysteine	5.2	5.5	5.7	5.7
Valine	20.5	20.3	21.1	20.3
Methionine	7.1	7.2	7.3	7.1
Isoleucine	18.1	18.2	18.8	18.8
Leucine	37.7	37.2	38.0	38.5
Tyrosine	15.2	14.6	15.3	15.6
Phenylalanine	21.2	21.4	22.2	22.4
Hydroxylysine	1.1	0.9	0.8	0.8
Ornithine	0.3	0.3	0.2	0.2
Lysine	22.4	21.9	22.7	22.2
Histidine	10.0	10.0	10.3	10.2
Arginine	24.7	24.9	26.2	26.0
Tryptophan	5.1	5.2	5.6	5.3

2.2 Growth trials

Two growth trials were carried out at the Claude Peteet Mariculture Development Center (CPMC), Gulf Shores, AL, USA. Florida pompano fingerlings were obtained from Troutlodge Marine Farms LLC, (Proaquatix) Vero Beach, Florida, USA, nursed in an indoor recirculating system facility of CPMC, and fed with commercial diet (FF Starter, Zeigler Bros., Inc. Gardners, PA, USA) until reaching a suitable size. All trials were conducted in in a semi-recirculating system consisted of 12 culture open-top tanks equipped with reservoir tank, biological filter, supplemental aeration (provided using a central line, regenerative blower and air diffusers) and circulation pump. At the start of the trial, twenty fish with average initial weight of 13.05 ± 0.34 g for the first trial

and 18.45 ± 0.49 for the second trial were stocked into each tank and each trial consisted of four treatments each with three replicates in a completely randomized design. Fish from all trial were maintained under a natural photoperiod for 8-weeks and a subsample of fish from the initial stocking was retained for proximate, amino acid and mineral profile analysis. During the trial, fish were fed four times per day and the daily ration was adjusted based on a percentage of body weight after sampling the fish every two weeks. During the sampling period, fish were dipped in Chloroquine phosphate (MP Biomedicals, Solon, OH) as a bactericide at the concentration of 60 mg L^{-1} followed with freshwater dip for approximately one minute to reduce any possibilities for parasitic infection. For water quality observation in all trials, dissolved oxygen (DO), pH, temperature, and salinity were measured twice daily by using a water quality multi-parameter instrument (ProPlus, YSI Inc., Yellow Springs, OH, USA). total ammonia nitrogen was measured two times per week using an ion-selective electrode (Orion 4-Star Plus pH/ISE, Thermo Fisher Scientific, Waltham, MA, USA) and nitrate-nitrogen was measured once a week using colorimetric test kits (La Motte Chemicals, Chestertown, MD, USA). At the end of the growth trial, fish were fasted overnight, then counted and batched weighed to calculate the final biomass, final weight, percentage weight gain (PWG), feed conversion ratio (FCR), percentage survival (SR), voluntary feed intake (VFI), protein efficiency ratio (PER) and thermal unit growth coefficient (TGC).

$$\text{PWG} = \frac{(average\ individual\ final\ weight - average\ individual\ initial\ weight)}{(average\ individual\ initial\ weight)} \times 100$$

$$\text{FCR} = \frac{feed\ given\ (g)}{alive\ weigh\ gain\ (g)}$$

$$\text{SR} = \frac{final\ number\ of\ fish}{initial\ number\ of\ fish} \times 100$$

$$\text{VFI} = \frac{feed\ intake\ (g)}{fish}$$

$$\text{TGC} = \frac{FBW^{1/3} - IBW^{1/3}}{\sum TD} \times 100$$

where FBW is final body weight, IBW is initial body weight, T is water temperature (^{0}C) and D is number of trial days

2.3 Body composition analysis

Upon termination of all growth trials, four fish from each tank or twelve fish per dietary treatment were randomly sampled and stored at – 80 ^{0}C for body composition analysis. Prior to proximate and AA analysis, dried whole fish were rigorously blended and chopped in a mixer according to the standard methods established by Association of Official Analytical Chemists (AOAC, 1990). Proximate composition and amino acids profile of whole pompano body was analyzed by Midwest Laboratories (Omaha, NE, USA) and summarized in Table 6 and 7.

2.4 Serum biochemistry analysis

At the end of all growth trials, twelve fish per treatment (four fish per replicate) were immediately euthanized with Tricaine-S (MS-222, tricaine methanesulfonate salt, Western Chemical, Inc., Ferndale, WA, USA), and blood samples were taken from the caudal vein after an overnight starvation. Blood samples were collected from basal diet, PBM, 4% SH and 4% SM treatment using anticoagulant-free centrifuge tubes. Serum was obtained by centrifugation of blood at 3.000 rpm for 10 min and stored at – 80 ^{0}C until the analysis. Biochemical parameters in the serum samples were analyzed using an automatic chemistry analyzer (Cobas C311, Roche Diagnostics, IN, USA) for total protein, albumin, activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cholesterol, and bile acid concentration.

2.5 Histological analysis

Histological analysis only carried out in trial 1 and samples were randomly collected after an overnight fast with three fish per each treatment tank or nine fish per dietary treatment. Fish were individually euthanized in a solution of Tricaine-S (MS-222, tricaine methanesulfonate salt, Western Chemical, Inc., Ferndale, WA, USA) and dissected to collect the distal intestine tissue. Liver and distal intestine samples of approximately 0.5 cm were immediately fixed in Bouin's solution (picric acid-formalin-acetic acid mixture, Ricca Chemical, Arlington, TX, USA) for 20 h at room temperature and then transferred to 70% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological analysis procedures. The blocks of designed sample were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax and sectioned at 4μm intervals for staining with Hematoxylin-Eosin (H&E) stain (Merck, Darmstadt, Germany). Double blinded evaluation with a grading system of 1 (healthy) to 5 (degraded) was used to evaluate the distal intestine condition according to Novriadi *et al.* (2017b). The following parameters were taken into account for distal intestine analysis: the appearance of goblet cells (GC), cellular infiltration (CI), and widening of the lamina propria within the intestinal folds (WLP). Meanwhile, for the liver, evaluation was focused on hepatocyte vacuolization, nuclear change and glycogen accumulation. Histomorphological images were acquired by using a microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

2.6 Statistical analysis

Possible significant differences in growth performance, proximate composition and amino acids profile of the whole body, serum levels and enzyme activities between dietary groups for both trials were analyzed using one-way analysis of variance to determine the significant differences among treatments followed by Tukey's multiple comparison test to determine the difference between treatment means in each trial. Score data for histomorphological condition of the liver and distal intestine were treated as categorical data, tested for normality and homoscedasticity and subsequently analyzed using Welch's one-way analysis of variance followed by Games-Howell *post hoc* tests to determine significant differences between treatments. Linear regressions were performed to investigate the relationship between FM replacement level and final weight (g) and TGC. All statistical analyses were conducted using SAS system (V9.4. SAS Institute, Cary, NC, USA).

3 Results

3.1 Water quality

For water quality data (mean ± standard deviation) in trial 1, temperature, D.O. salinity, pH, TAN, and nitrate-nitrogen were maintained at 27.07 ± 1.99 °C, 5.52 ± 0.55 mg L^{-1}, 33.73 ± 2.58 ppt, 7.76 ± 0.25, 0.13 ± 0.23 mg L^{-1}, and 57.69 ± 48.48 mg L^{-1}, respectively. In trial 2, temperature, D.O, salinity, pH, TAN, and nitrate-nitrogen were maintained at 28.65 ± 1.15 °C, 5.53 ± 0.39 mg L^{-1}, 33.04 ± 2.56 ppt, 7.83 ± 0.19, 0.05 ± 0.05 mg L^{-1}, and 74.80 ± 29.84 mg L^{-1}, respectively.

3.2 Growth trials

For trial 1, survival was over 90% and no significant differences in terms of feed intake. However, fish fed the lowest inclusion level of FM (FM 6%) had a significantly lower FW, PWG, and TGC. In addition, fish fed 6% FM had the highest value for FCR (Table 5). For trial 2, there were no significant differences in terms of survival, PWG, TGC and FCR. However, as the inclusion level of FM decreases, the VFI tend to decrease and complete replacement of dietary FM resulted with the lowest final weight (Table 5).

Table 5. Growth performance of juvenile Florida pompano fed experimental diets for 56 d for the first trial (Mean initial weight 13.05 ± 0.09 g) and second trial (Mean initial weight 18.45 ± 0.49 g). Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test.

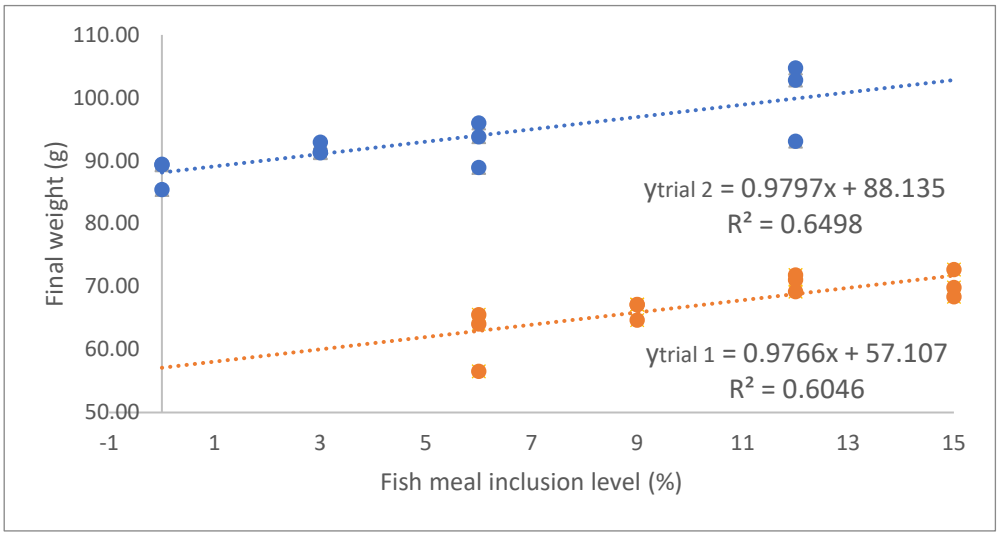
Diet	Final weight (g)	Weight Gain (%)	TGC[1]	Feed intake (g fish^{-1})	FCR[2]	Survival (%)
Trial 1						
15% FM	70.34^{a}	432.73^{a}	0.1183^{a}	96.39	1.69^{a}	98.33
12% FM	70.73^{a}	444.13^{a}	0.1188^{a}	97.07	1.68^{a}	100.00
9 % FM	66.33^{ab}	411.98^{ab}	0.1100^{ab}	94.51	1.77^{a}	91.67
6 % FM	62.04^{b}	376.45^{b}	0.1070^{b}	90.74	1.86^{b}	95.00
P-value	0.0177	0.0201	0.0206	0.1947	0.0445	0.2272
PSE[3]	1.6356	12.2549	0.0022	2.0130	0.0401	2.7639
Trial 2						
12% FM	100.24^{a}	442.41	0.1230	138.55^{a}	1.70	98.33
6% FM	92.92^{ab}	405.65	0.1163	132.80^{b}	1.78	100.00
3% FM	91.88^{ab}	393.16	0.1145	132.65^{b}	1.81	100.00
0% FM	88.08^{b}	382.90	0.1118	128.03^{b}	1.83	98.33
P-value	0.0260	0.2056	0.1092	0.0014	0.3644	0.5957
PSE[3]	2.2011	18.7670	0.0054	1.1389	0.0516	1.1785

Note: [1] TGC = Thermal growth coefficient; [2] FCR = Feed conversion ratio; [3] PSE = Pooled standard error.

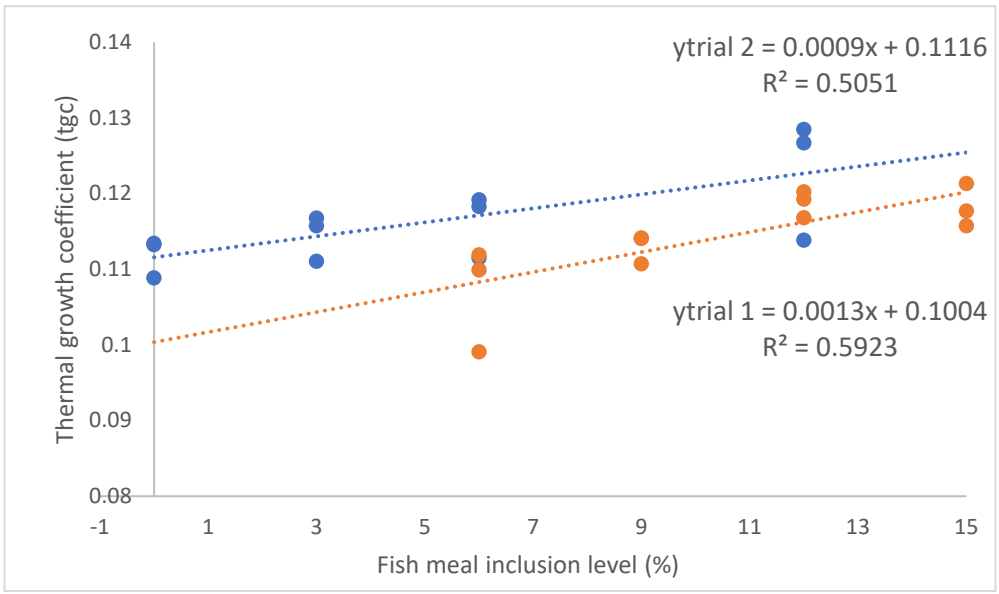
Replacement of dietary FM positively correlated with FW and TGC in trial 1 and 2. The regression line for FW in trial 1 described by $y = 0.9766x + 57.107$ (R^2 = 0.6046, p-value = 0.0177) and trial 2 $y = 0.9797x + 88.135$ (R^2 = 0.6498, p-value = 0.0260) (Fig 1a). Meanwhile for TGC, regression line for trial 1 described by $y = 0.0013x + 0.1004$ (R^2 = 0.5923, p-value = 0.0206), and trial 2 with $y = 0.0009x + 0.1116$ (R^2 = 0.5051, p-value = 01092) (Fig 1b).

Figure 1. Relationship between final weight (y) (Fig 1a) and thermal growth coefficient (Fig 1b) to the replacement level of FM (x) in both trials.

a



b



3.3 Body composition analysis

There were no significant effects of dietary FM replacement in crude protein, fat content, crude fiber, dry matter, moisture and ash content for both trials (Table 6). For amino acids profile in trial 1, as the dietary FM decreased, the level of proline in the whole body of pompano tend to increase (Table 7).

Table 6. Proximate composition (g kg^{-1}, *as is*) of whole body of pompano fed experimental diets for 8 weeks. Results in the same columns with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test (n. p = analysis was not performed).

Treatment	Crude protein	Moisture	Fat	Crude Fiber	Dry matter	Ash
First trial[1]						
15% FM	180.5	709.8	67.5	0.60	n. p[3]	30.5
12% FM	179.1	707.8	76.6	0.60	n. p	0.20
9% FM	175.4	716.9	64.3	0.90	n. p	34.8
6% FM	179.8	712.3	64.0	1.00	n. p	33.4
P-value	0.6479	0.6573	0.4512	0.2713	-	0.3389
PSE	0.3011	0.5239	0.5967	0.0181	-	0.2149
Second trial[2]						
12% FM	178.0	700.0	70.3	n. p	30.00	36.8
6% FM	196.0	708.7	70.1	n. p	29.13	23.5
3% FM	178.7	723.3	66.1	n. p	27.67	25.4
0% FM	194.7	707.0	60.7	n. p	29.30	29.9
P-value	0.0727	0.5037	0.6545	-	0.5037	0.4526
PSE	0.5313	1.0621	0.6017	-	1.0621	0.5994

[1] Analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA)

[2] Analyzed at Midwest Laboratories, Inc. (Omaha, NE, USA)

[3] n. p = analysis was not performed

Table 7. Amino acids analysis (g kg^{-1}, *as is*) of whole fish from trial 1. Values represent the mean of four replicates. Results in the same row with different superscript letter are significantly different (*P<0.05*) based on analysis of variance followed by the Tukey's multiple comparison test.

Composition[1]	Initial	15% FM	12% FM	9% FM	6% FM	*P*-value	PSE
Taurine	1.2	3.7	3.8	3.8	3.8	0.5570	0.0050
Hydroxyproline	3.2	3.4	3.9	4.0	4.5	0.2185	0.0323
Aspartic Acid	13.0	15.5	15.5	15.0	15.0	0.4076	0.0268
Threonine	6.0	7.2	7.2	6.9	7.0	0.3319	0.0117
Serine	5.4	6.3	6.4	6.2	6.2	0.6996	0.0091
Glutamic Acid	19.1	22.5	22.7	22.1	21.9	0.4737	0.0388
Proline	7.7	8.4^{b}	9.4^{ab}	9.1^{ab}	9.8^{a}	0.0325	0.0268
Glycine	13.4	13.2	15.1	14.4	16.4	0.0925	0.0752
Alanine	9.6	11.1	11.8	11.3	12.0	0.2267	0.0310
Cysteine	1.3	1.5	1.5	1.5	1.5	0.6265	0.0041
Valine	6.9	8.5	8.6	8.2	8.2	0.1723	0.0139
Methionine	4.0	4.7	4.7	4.6	4.7	0.6996	0.0091
Isoleucine	6.1	7.3	7.2	6.9	6.8	0.1904	0.0155
Leucine	10.0	12.3	12.0	11.6	11.5	0.1370	0.0234
Tyrosine	33.	5.3	4.9	4.9	4.8	0.1942	0.0164
Phenylalanine	5.7	6.8	6.7	6.5	6.5	0.2428	0.0099
Hydroxylysine	0.5	0.5	0.6	0.6	0.6	0.2679	0.0033
Lysine	10.9	13.4	13.4	13.0	12.9	0.3267	0.0250
Histidine	2.9	3.8	3.7	3.6	3.5	0.0639	0.0058
Arginine	9.1	10.9	11.3	11.0	11.5	0.2590	0.0230
Tryptophan	1.1	1.7	1.7	1.6	1.6	0.9375	0.0053

[1]Analysed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA)

3.4 Serum biochemistry analysis

Table 8 shows results of serum levels and enzyme activities as the effect of dietary FM replacement in both trial. In trial 1, there were no significant effects of dietary FM replacement with ESBM to the level of total protein in the serum, albumin, glucose, bile acids, ALP, ALT and AST activities. However, the 15% FM had the lowest total cholesterol level compared to 9% FM. In trial 2, partial and complete replacement of FM with ESBM did not affect the serum levels and enzyme activities.

Table 8. Effect of different diets on serum levels and enzyme activities in Florida pompano in all trials. Values represent the mean of four replicates.

Diets	Total protein ($g\ dL^{-1}$)	Albumin ($g\ dL^{-1}$)	ALP[1] ($U\ L^{-1}$)	ALT[2] ($U\ L^{-1}$)	AST[3] ($U\ L^{-1}$)	Glucose ($mg\ dL^{-1}$)	Cholesterol ($mg\ dL^{-1}$)	Bile acid ($mg\ dL^{-1}$)
First trial								
15% FM	4.61	1.52	46.07	<5.00	69.00	214.67	236.33[a]	4.10
12% FM	4.21	1.36	35.53	<5.00	65.00	239.67	217.33[ab]	3.73
9% FM	3.92	1.27	40.70	<5.00	30.00	232.67	192.67[b]	3.80
6% FM	4.29	1.39	41.57	<5.00	87.00	215.00	211.67[ab]	3.67
P- value	0.1800	0.1295	0.2438	>0.0000	0.0764	0.7811	0.0230	0.9603
PSE	0.1982	0.0638	3.3131	>0.0000	13.0356	20.9649	7.6014	0.6183
Second trial								
12% FM	4.33	1.37	28.87	15.67	99.67	219.00	176.33	3.47
6% FM	4.27	1.33	26.67	10.33	87.33	198.33	166.33	3.20
3% FM	4.37	1.33	25.00	12.00	113.00	207.00	158.00	3.93
0% FM	4.33	1.33	28.17	19.67	174.67	231.33	170.00	5.60
P- value	0.9672	0.9578	0.4414	0.2825	0.2773	0.6158	0.4760	0.6442
PSE	0.1453	0.0527	1.7166	3.3666	31.2352	18.1082	8.0037	1.4136

Note: [1]ALP= Alkaline phosphatase; [2]ALT= Alanine transaminase; [3]AST= Aspartate transaminase; [4] PSE = Pooled standard error

3.5 Histological analysis

Sections of the liver and distal intestine of Florida pompano fed with various replacement level of FM with ESBM for 8 weeks are shown in Figure 2 and 3, respectively. The fish fed with the lowest level of dietary FM (6% FM) showed an inflammation, glycogen granulation, and nuclear change in the liver compared to other treatments (Table 9). Fish fed with 9% and 6% FM showed a significant increase in the number of goblet cells, cellular infiltration and width of lamina propria than fish fed 15% FM. However, no significant effect of FM reduction from 15 to 12% in terms of liver and distal intestine histomorphological condition.

Table 9. Diagnostic features of liver and distal intestine of fish based on comparison of dietary treatments in trial 1. Results presented as mean ± standard deviation (n= 12). Results in the same row with different superscript letter are significantly different ($p<0.05$) based on Kruskal-Wallis test followed by Tukey's multiple comparison test for the ranked data.

Diets	Liver			Distal intestine		
	Glycogen granulation	Inflammation	Nuclear change	Number of goblet cells	Cellular infiltration	Lamina propria width
15% FM	2.65 ± 0.48^{b}	2.68 ± 0.53^{b}	2.81 ± 0.41^{b}	2.95 ± 0.57^{b}	2.90 ± 0.34^{c}	2.94 ± 0.23^{b}
12% FM	2.73 ± 0.51^{b}	2.78 ± 0.46^{ab}	2.67 ± 0.47^{ab}	2.85 ± 0.61^{b}	2.95 ± 0.52^{bc}	3.03 ± 0.39^{b}
9% FM	2.78 ± 0.56^{ab}	2.98 ± 0.60^{ab}	2.86 ± 0.57^{ab}	3.25 ± 0.46^{a}	3.28 ± 0.40^{ab}	3.58 ± 0.48^{a}
6% FM	3.00 ± 0.50^{a}	3.13 ± 0.31^{b}	3.03 ± 0.31^{a}	3.38 ± 0.52^{a}	3.48 ± 0.46^{a}	3.42 ± 0.45^{a}
p - value	0.0194	0.0055	0.0524	0.0018	<0.0001	<0.0001

Figure 2. Representative histopathological images of hematoxylin and eosin-stained sections of liver from Florida pompano in trial 1 after 56 d of being fed with (A) 15% FM, (B) 12% FM, (C) 9% FM, and (D) 6% FM

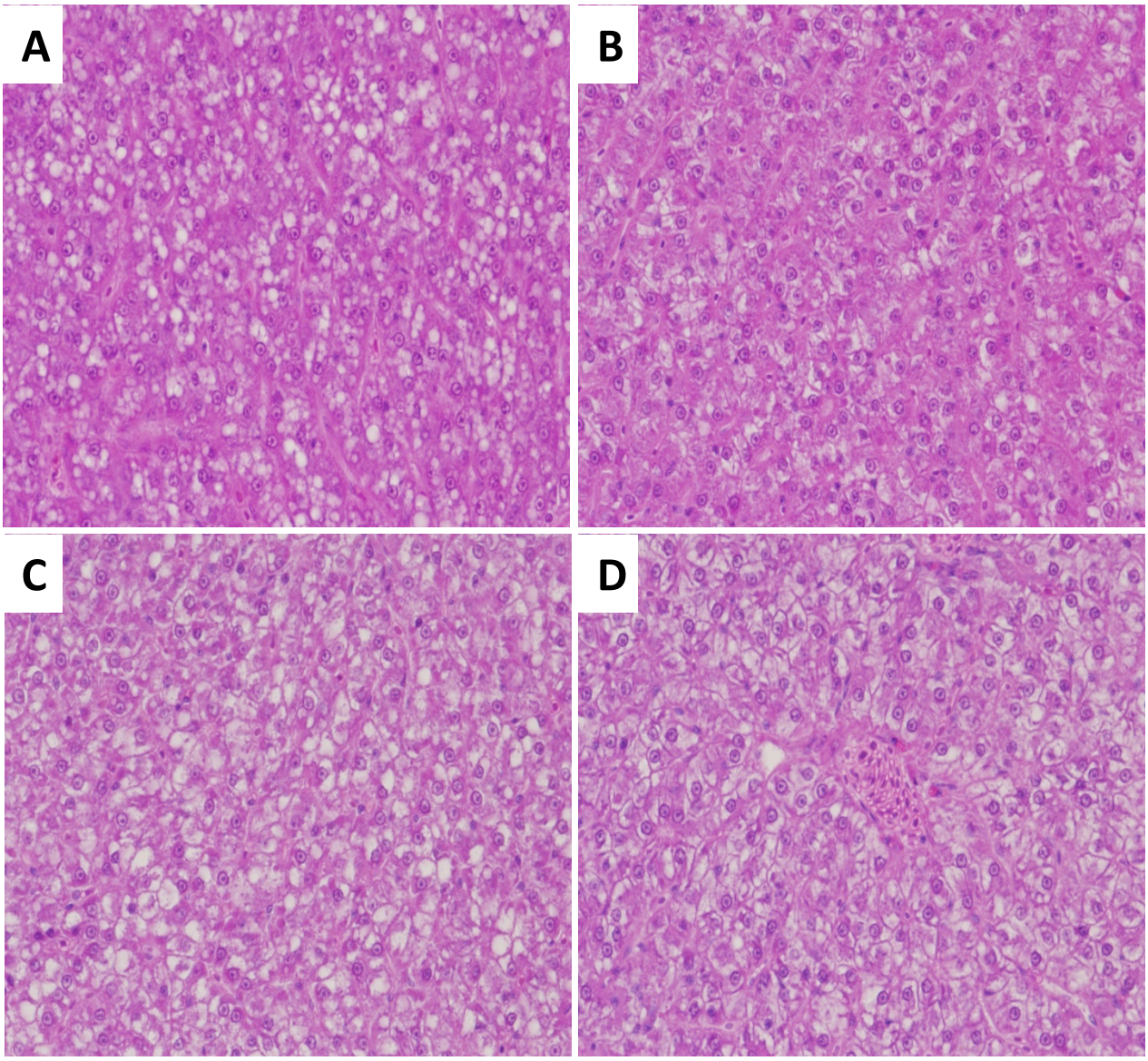
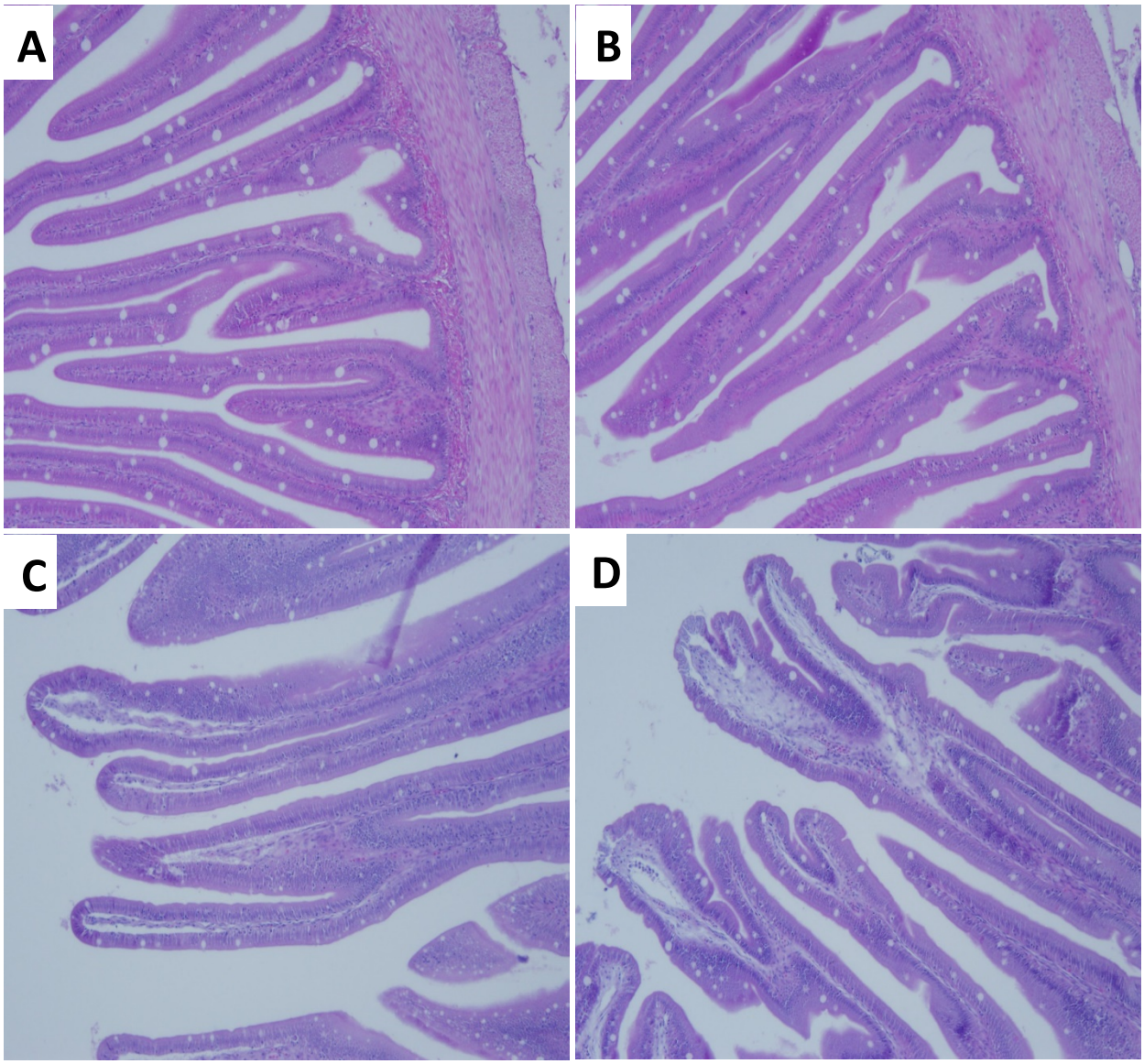


Figure 3. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestine from Florida pompano in trial 1 after 56 d of being fed with (A) 15% FM, (B) 12% FM, (C) 9% FM, and (D) 6% FM



4 Discussion

The use of ESBM produced via a proprietary process combining extraction and enzymatic treatment of soybeans in this study to replace dietary FM had no significant effect to induce better growth of pompano compared to the reference diet (15% FM). In trial 1, a gradually reduction in final weight is consistent with decreasing number of TGC and higher FCR. The regression linear confirmed this decreasing trend as the fish meal inclusion level reduces below 15%. In addition to trial 1, based on the growth prediction relative to the water temperature or known as TGC, higher inclusion level of FM also reduced the growth curves of the fish. Again, the linear regression confirms this result (Fig 1a). Meanwhile, in trial 2, significant reduction only observed in final weight when dietary FM was completely replaced with ESBM. High variation in growth outcomes resulting in an absence of significant differences among the dietary treatments, including TGC. The linear regression supports the findings with high degree of variations in TGC (Fig 1b). The results from both trial support the findings from Quintero *et al.* (2012) where further substitution of FM with SPC produced via aqueous ethanol or methanol extraction process below 15% resulted in reductions in growth parameters. Together, the present findings confirm the reduction in growth of fish as FM further replaced by soy-protein sources (Chou *et al.*, 2004, Shiu *et al.*, 2015, Dawood *et al.*, 2015, Kokou *et al.*, 2015).

According to NRC (2011), reduction in growth performance is often associated with the lower feed intake as the effect of increasing plant-protein sources in the diet. However, in the present study, further reduction of FM only caused a significant reduction in feed intake in trial 1 when dietary FM was reduced from 12% to 6, 3 and 0% but not for trial 1 when dietary FM reduced from 15% to 6%. The results suggest that at certain level, proper combination of ESBM and SBM still provide good palatability to the diets. Interestingly, we could observe a discrepancy in both

growth results, where in trial 1 the decreasing trend occur as the inclusion level of FM was decreased below 6%, while in trial 2, the significant reduction, especially in final weight, only occur when dietary FM completely replaced by ESBM. According to Dabrowski *et al.* (1989) initial size of the fish could influence the fish growth rate and optimum utilization of soy-protein during the growth trial. In their trial, larger fish favors the utilization of SBM compared to small fish. Therefore, the difference in stocking size in the present study could explain the different growth response of pompano to the to the high inclusion level of SBM during the growth trial.

Several alternative protein sources have been evaluated in Florida pompano diet to gradually replace dietary FM, such as cottonseed products (Cook *et al.*, 2016) and poultry by-product meal (Rossi Jr and Davis, 2012), but none of them showed a significant differences in terms of crude protein, crude fat, moisture and ash content in the whole body of pompano. In line with previous study, various levels of dietary FM substitution with soy-products in this study also do not showed any significant differences in proximate composition of the whole body of pompano. Moreover, the dietary treatment in this study also do not cause any significant effect to the amino acid composition. Despite the fact that most of experimental diet was designed to be isonitrogenous and isolipid, the use of advanced soy products with low molecular weight and high nutritional content induce an optimum utilization and retention. However, the effect of plant-protein sources to the nutritional quality of fish beyond the typical growth period for pompano nutrition study requires some further investigations.

Blood biochemical evaluation nowadays received considerable attention in the development of aquafeed, especially for the clinical assessment of specific novel ingredients (Ilham and Fotedar, 2017, Wang *et al.*, 2018, Norag *et al.*, 2018). Using the history results with two advanced products of soy in the development of practical diet for pompano, the serum levels

and enzyme activities of fish fed with various levels of ESBM (102.2– 148 g kg^{-1}) to replace 150 g kg^{-1} dietary PBM supplemented with squid hydrolysates and squid meal did not showed any significant clinical differences for total protein, albumin, glucose, cholesterol, bile acids, ALP, AST and ALT enzyme activities among the dietary treatments (Novriadi *et al.*, 2017c). Additional study with the replacement of conventional SBM with various level of commercial fermented soybean meal (FSBM) also did not showed any significant differences in all observed serum and enzyme activities parameters (Novriadi *et al.*, 2017b). Our results in the present study largely confirm those of previous studies where the various replacement of dietary FM with ESBM did not cause any significant effect to the total protein, albumin, ALP, ALT, AST, glucose and bile acids contents. The only different only observed in the plasma cholesterol level where fish fed 15% dietary FM had significantly higher plasma cholesterol level than fish fed higher inclusion level of soy protein. According to Tocher *et al.* (2008) and Zhu *et al.* (2017) the use of plant-protein sources to replace the animal meal will greatly reduce the level of cholesterol supplied by the feed. In addition, there was a tendency towards the inhibition of cholesterol intestinal absorption due to the presence of phytosterols, which are predominant sterol in plant ingredients, and thus lead to the lower cholesterol level in the plasma (Piironen *et al.*, 2000, Thrall *et al.*, 2012). In addition, a similar result was obtained by Romarheim *et al.* (2006) where plasma cholesterol level were lower in rainbow trout *Oncorhynchus mykiss* fed diets containing SBM and white flakes as an intermediate product in the production of SPC and soy protein isolates than in fish fed dietary FM. Based on these facts, lower cholesterol level in fish fed with high inclusion level of soy protein should not come as a surprise. However, in the second trial, none of these parameters showed any clinical differences among the dietary treatment, indicating that the utilization of ESBM in the

practical diet to replace considerable amounts of FM did not cause any obvious alterations in the blood and serum composition of Florida pompano.

Several fundamental studies indicated that the higher inclusion of plant-based diet will probably causes morphological changes in the intestine and liver of farmed fish (Bureau *et al.*, 1998, Ostaszewska *et al.*, 2005, Sitjà-Bobadilla *et al.*, 2005, Trushenski, 2015). In this study, as the fish received diets with high inclusion level of soy-protein, there were marked increases in the frequency of glycogen granules, liver inflammation and nuclear change. In terms of distal intestine structure, there were slightly increased in the number of goblet cells, cellular infiltration and width of lamina propria. These results ties well with previous studies wherein the complete replacement of 15% PBM with ESBM slightly increase the glycogen deposition and inflammation with nuclear change (Novriadi *et al.*, 2017b). In addition, still from the same study, the number of goblet cells slightly higher in the distal intestine of pompano fed with plant-based diet with an increase of cellular infiltration compared to fish fed 15% PBM. However, the nutritional effect of soy protein to the distal intestine of pompano is not clear since the complete replacement of animal meal and conventional SBM with ESBM in the comparative effect study did not cause any significant alterations compared to the reference diet (Novriadi *et al.*, 2018). We suggest that the use of proper or high inclusion level of ESBM will probably not cause any significant inflammatory reaction.

5 Conclusion

Integrated assessment of novel ingredients by means of growth trial, proximate and amino acid composition of the whole body, serum and enzyme activities in the blood of fish together with microscopical methods for liver and distal intestine tissue analysis probably the most accurate indicator to investigate the nutritional effect of dietary treatment. In this study, 30.8 to 61.4 g kg^{-1} ESBM protein could be used to substitute dietary FM without compromising growth, nutritional and health status of pompano. By using bigger fish, substitution of FM with ESBM even did not cause any significant effect in all of the observed parameters. However, further studies are required aiming to investigate the inclusion effect of ESBM to replace dietary FM for long-term growth period beyond the current growth trial.

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Chapter IV

COMPARATIVE EFFECT OF ADVANCED SOY PRODUCTS WITH PORCINE MEAL ON GROWTH, BODY COMPOSITION AND DISTAL INTESTINE HISTOLOGY OF FLORIDA POMPANO *Trachinotus carolinus*

Abstract

The present study was designed to investigate and compare the effects of diets containing high inclusion level of advanced soy products (enzyme-treated soy and fermented soy) in combination with porcine meal (PM) to completely replace poultry by-product meal (PBM) on growth performance, body composition and distal intestine histology of Florida pompano *Trachinotus carolinus*. Four experimental diets were formulated to be iso-nitrogenous and iso-lipidic, to contain 400 g kg^{-1} crude protein and 80 g kg^{-1} lipid. A reference diet (PBM) contained 150 g kg^{-1} poultry by-product meal (PBM) and 495 g kg^{-1} soybean meal (SBM), and three test diets were formulated replacing PBM with 15 g kg^{-1} of CPC (CPC) or replacing all SBM and PBM with 535 g kg^{-1} fermented soy (FSBM) or 451.3 g kg^{-1} enzyme-treated soy (ESBM). All test diets were supplemented with porcine meal (PM, 38 g kg^{-1} of the diet) to serve as a hydroxyproline source. Diets were fed to apparent satiation to triplicate groups of Florida pompano juveniles (mean weight 8.06 ± 0.22 g). After 8 weeks of feeding, fish fed CPC and ESBM performed equally well in terms of final body weight, thermal growth coefficient and percentage weight gain in comparison to fish fed PBM. Voluntary feed intake (VFI) of fish fed FSBM was significantly lower compared to other treatments. Protein retention efficiency, whole-body proximate composition, phosphorus, sulfur, potassium, magnesium, calcium, sodium, and zinc contents were not significantly influenced by

the dietary treatments. The results obtained in the present histological study showed no significant differences in the thickness of serous layer, muscular layer, and submucosal layer of the intestine among treatments. Fish fed CPC showed a significant widening of the LP with an increase of cellular infiltration and higher presence of goblet cells compared to other dietary treatment. Based on these results, 451 g kg^{-1} ESBM or combination of 150 g kg^{-1} of CPC and 495 g kg^{-1} SBM supplemented with 38 g kg^{-1} PM can be utilized to develop a practical diet for juvenile Florida pompano without impacting growth, nutritive parameters and several distal intestine health parameters.

Keywords: Enzyme-treated soy, fermented soy, porcine meal, corn protein, growth, distal intestine, Florida pompano

1. Introduction

The development of practical diets for juvenile Florida pompano *Trachinotus carolinus* have been evaluated by using several plant protein sources, such as soybean meal (SBM), corn gluten meal and cottonseed meal products, and led to an optimized crude protein and lipid level of 40 – 44 % and 7 – 12%, respectively (Williams *et al.* 1985, Lazo *et al.* 1998, Lech and Reigh 2012, Riche 2014, Cook *et al.* 2016, Rhodes *et al.* 2017). Proper combinations of these plant protein sources supplemented with limiting amino acids also reduces the inclusion of animal meal up to 15% without negatively affecting the growth parameters of pompano (Quintero *et al.* 2012). However, as the inclusion of plant-protein sources, especially SBM, continues to increase and levels of animal meals decrease, the wider inclusion is generally hindered by some limitations associated with the imbalanced of amino acid (AA) profiles (Fagbenro and Davies 2001, Aragao *et al.* 2003) and the presence of anti-nutritional factors (ANFs), such as lectins, phytic acid, saponins, phytosterols, and possible allergens (NRC. 2011). The cumulative effects can be responsible for the decreased growth performance (Tibaldi *et al.* 2006), feed efficiency (Olli *et al.* 1995) and possible histomorphological change in the distal intestine of some species of fish (Rumsey *et al.* 1994, Nordrum *et al.* 2000).

Recent work with various advanced soy products, such as fermented soybean meal (FSBM) and enzyme-treated soybean meal (ESBM), seems promising due to their ability to improve nutritional value of soy protein and substitute for the use of animal meal in fish diet formulation (Lim and Lee 2011, Barnes *et al.* 2014). Fermentation process which allows microorganisms to degrade macromolecules to low molecular weights have been reported to have numerous benefits including the degradation of soy immunoreactivity (Song *et al.* 2008), lower levels of ANFs (Lim *et al.* 2010, Mukherjee *et al.* 2016), improve the nutritional quality and fibrinolytic enzyme activity

of the commercial SBM (Bi *et al.* 2015). Likewise, combination of non-alcohol extraction process and enzymatic treatment to produce ESBM has been shown to improve the protein level and reduce the level of ANFs and oligosaccharide contained in SBM (Amezquita and Arana. 2015). Our recent study with Florida pompano showed that the combination of 47.2 g kg^{-1} SBM and 102 g kg^{-1} ESBM supplemented with 40 g kg^{-1} squid hydrolysates was effective to reduce dietary animal meal and partly prevented the alteration in the distal intestine of pompano (Novriadi *et al.* 2017a). Meanwhile, even though the inclusion of 206, 309 and 410 g kg^{-1} FSBM to incrementally replace 50, 75 and 100% of solvent extracted SBM did not improve the growth, higher amounts of FSBM were able to partially prevent the histological alteration in the liver and distal intestine of pompano (Novriadi *et al.* 2017b). Since 150 g kg^{-1} PBM was still included in all diets, the cause for insignificant growth effects and better liver and distal intestine condition found in FSBM study remain unclear (Novriadi *et al.* 2017b). In this context, it was of interest to further explore the efficacy of advanced soy product to completely replace animal meal on the growth and distal intestine morphology of fish.

Other than soy sources, corn protein concentrate (CPC) as the dried protein fraction obtained after removal of the majority of the non-protein components of the corn by using enzymatic solubilization, is often utilized in plant-based diets for fish due to the ability to balance the AA profile of SBM, resulting in diets that are highly digestible and rich in methionine and cysteine (Phillips and Sternberg 1979, Gatlin *et al.* 2007, Robinson and Li 2008, Hardy 2010, Khalifa *et al.* 2017). Recently, CPC at the level of 80 g kg^{-1} has been frequently used to improve the nutritional quality of soy-based diet for pompano (Cook et al. 2016, Rhodes et al. 2017, Novriadi *et al.* 2017b). Interestingly, in juvenile white seabass *Atractoscion nobilis*, higher inclusion of CPC at the range of 117.9 – 119.9 g kg^{-1} to complement the use of 150 g kg^{-1} SBM or FSBM in combination with

175 – 186 g kg^{-1} soy protein concentrate (SPC) was effective to reduce the inclusion of FM and yielded similar performance with fish fed 480 g kg^{-1} FM (Trushenski *et al.* 2014). Hence, these results suggest that it may be possible to increase the inclusion level of CPC to more than 80 g kg^{-1} in the development of practical diet for pompano.

From nutritive value stand-point, porcine meal (PM) derived from fat trimmings of fresh pork carcasses is a good source of protein supplement for fish and shrimp (Hernández *et al.* 2010, Hernández *et al.* 2008, Wang *et al.* 2012), due to the considerable levels of hydroxyproline (*Hyp*) which is limited in plant protein sources (Aksnes *et al.* 2008, Wu *et al.* 2011). Although described as a non-essential AA that can be derived from the post-translational hydroxylation of proline (Aksnes et al. 2008), supplemental *Hyp* may play a role in inducing the gustatory response (Marui *et al.* 1983, Hara 2012), maintaining structure and function of cells (Wu et al. 2011) and in the biological performance of some species of fish (Aksnes *et al.* 2006). However, to the best of our knowledge, the potential nutritive value of PM as a protein ingredient in Florida pompano diet has not been adequately studied. Therefore, based on all the background information, the objective of the present study was to evaluate the nutritive value of two commercially available advanced SBM (enzyme-treated soy and fermented soy) or CPC fortified with PM on growth performance, body composition and distal intestine morphology of Florida pompano compared to the reference diet.

2. Materials and methods

2.1 Experimental diets

Four diets were formulated to be iso-nitrogenous and iso-lipidic to contain approximately 400 g kg^{-1} protein and 80 g kg^{-1} lipid as a combination of two or more protein sources: de-hulled solvent extracted soybean meal (SBM, Bunge Limited, Decatur, AL, USA), enzyme-treated soybean meal (ESBM, NutriVance^{TM}, Midwest Ag Enterprises, Marshall, MN, USA), fermented soybean meal (FSBM, PepSoyGen^{TM}, Nutrafrema, Protein and Biotech Products, Sioux City, IA, USA), poultry-by product meal (PBM, Griffin Industries, Inc., Mobile, AL, USA), corn protein concentrate (CPC, Empyreal 75^{TM}, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA), and porcine meal (PM, Innomax^{TM} MPI, Sonac LLC, Maquoketa, IA, USA) (Table 1). A reference diet (PBM) which has been run in numerous trials was produced by utilizing 150, 495, and 70 g kg^{-1} of PBM, SBM, and CPC, respectively. Three experimental diets were produced by completely replacing PBM with 150 g kg^{-1} CPC (designed as CPC) or replacing PBM and SBM with 535 g kg^{-1} fermented soy (FSBM) or 451.3 g kg^{-1} enzyme treated soy (ESBM). All experimental diets were supplemented with 38 g kg^{-1} of PM and three limiting amino acids: L-lysine (*Lys*), DL-methionine (*Met*) and taurine (*Tau*) (MP Biomedicals Inc., Santa Ana, CA, USA) to match the calculated levels in PBM as the reference diet. All diets were produced at the Laboratory of Aquatic Animal Nutrition, School of Fisheries, Aquaculture and Aquatic sciences, Auburn University, AL, USA, using standard procedures for pompano. Briefly, diets were prepared by mixing of pre-ground dry ingredients along with Menhaden fish oil (MFO) in a food mixer (Hobart, Troy, OH, USA) for approximately 15 min. Boiling water was then blended into the mixture to attain an appropriate consistency for pelleting. The moist mash from each diet was passed through a 3.0 mm die in a grinder. Wet diets were then placed into a fan-ventilated oven (<45^{o}C) overnight in order to attain

a moisture content of less than 10%. Diets were stored at -20 ºC. Proximate and AA profile of the diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories and summarized in Table 1 and Table 2, respectively.

Table 1. Composition (g kg^{-1} *as is*) of diets fed to juvenile Florida pompano for 8 weeks

	Diet code			
	PBMD	CPCD	FSBMD	ESBMD
Ingredient (g kg^{-1}, *as is*)				
Poultry by product meal[1]	150.0	0.0	0.0	0.0
Soybean Meal[2]	495.0	495.0	0.0	0.0
Fermented Soybean Meal[3]	0.0	0.0	535.0	0.0
Enzyme treated soy[4]	0.0	0.0	0.0	451.3
Corn protein concentrate[5]	70.0	150.9	70.0	70.0
Porcine meal[6]	0.0	38.0	38.0	38.0
Menhaden Fish Oil[7]	49.0	70.0	70.0	70.0
Corn Starch[8]	14.5	20.1	61.0	148.7
Whole wheat[8]	160.0	160.0	160.0	160.0
Trace Mineral premix[9]	2.5	2.5	2.5	2.5
ASA Vitamin premix w/o choline[10]	5.0	5.0	5.0	5.0
Choline chloride[8]	2.0	2.0	2.0	2.0
Rovimix Stay C 35%[11]	1.0	1.0	1.0	1.0
CaP-dibasic[8]	35.0	35.0	35.0	35.0
Lecithin (soy commercial)[12]	5.0	5.0	5.0	5.0
L-lysine[8]	1.0	5.5	5.5	1.0
DL-methionine[8]	5.0	5.0	5.0	5.0
Taurine[8]	5.0	5.0	5.0	5.0
Proximate analyses (g kg^{-1}, *as is*)[13]				
Crude Protein	423.4	421.9	405.6	409.0
Moisture	71.5	86.1	128.9	78.7
Crude Fat	94.7	87.1	60.5	84.7
Crude Fiber	28.4	29.9	30.0	32.7
Ash	78.4	61.4	65.2	59.8

1 Griffin Industries, Inc., Mobile, AL, USA
2 De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA
3 PepSoyGen™, Nutraferma, Protein and Biotech Products, Sioux City, IA, USA
4 NutriVance™, Midwest Ag Enterprises, Marshall, MN, USA
5 Empyreal75™ Cargill Corn Milling, Blair, NE, USA
6 Innomax™ MPI, Sonac USA LLC, Maquoketa, IA, USA
7 Omega Protein Inc., Houston, TX, USA

[8] MP Biomedicals Inc., Santa Ana, Ca, USA

[9] ASA Premix (g 100 g^{-1} premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α cellulose 81.826

[10] ASA Premix (g kg^{-1} Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.

[11] Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA

[12] The Solae Company, St. Louis, MO, USA

[13] Analyses conducted by the University of Missouri-Columbia, Agricultural Experiment Station Chemical Laboratory, MO, USA

Table 2. Amino acid (AA) composition (g kg^{-1}, *dry matter basis*) of experimental diets utilized in the trial

	Diet Code			
AA (g kg^{-1}, dry matter)	PBMD	CPCD	FSBMD	ESBMD
Taurine	7.0	6.3	5.5	6.8
Hydroxyproline	2.4	2.8	3.0	3.2
Aspartic Acid	37.8	35.4	37.6	37.0
Threonine	15.2	14.3	14.4	14.3
Serine	17.8	18.3	17.7	17.9
Glutamic Acid	73.5	77.3	71.3	74.3
Proline	22.6	28.9	22.2	25.0
Glycine	20.5	19.8	19.7	20.9
Alanine	21.9	23.7	20.1	21.3
Cysteine	5.9	6.1	5.5	5.6
Valine	19.6	18.5	18.5	18.2
Methionine	12.2	11.3	10.3	9.7
Isoleucine	18.4	17.6	17.6	17.4
Leucine	35.4	40.1	33.5	34.9
Tyrosine	15.4	15.9	14.4	14.6
Phenylalanine	20.7	21.5	20.2	20.5
Hydroxylysine	1.0	1.1	0.9	1.2
Ornithine	0.2	0.2	0.2	0.2
Lysine	23.9	23.5	24.3	21.1
Histidine	9.7	9.3	9.2	9.4
Arginine	26.3	23.6	24.3	25.4
Tryptophan	5.0	4.6	5.6	5.3

2.2 Fish and experimental design

The growth trial was carried out at the Claude Peteet Mariculture Development Center (CPMC), Gulf shores, AL, USA. Florida pompano (*T. carolinus*) fingerlings were obtained from commercial hatchery (Proaquatix, Vero Beach, FL, USA), and acclimatized for 3 weeks to the experimental facilities and fed with commercial diet (FF Starter, Zeigler Bros., Inc. Gardners, PA, USA) until reaching a suitable size. At the start of the trial, twenty fish (mean individual weight = 8.06 ± 0.22 g) were stocked into each tank and assigned to triplicate tanks in a completely randomized design. An eight-week feeding trial was carried out in a semi-recirculating system consisted of 12 culture open-top 1000 L tanks equipped with reservoir tank, biological filter, supplemental aeration (provided using a central line, regenerative blower and air diffusers) and circulation pump. During the trial, fish were fed four times per day and the daily ration was adjusted to apparent satiation weekly throughout the trials. Additionally, feed inputs were calculated on a two-week basis after each sampling to adjust for growth and mortalities. The culture systems were located in a greenhouse which provided a natural photoperiod throughout the trial. Mean water quality parameters ± Standard deviation of water temperature (27.91 ± 1.52 °C), salinity (25.36 ± 3.16 ppt), pH (7.81 ± 0.19) and dissolved oxygen (6.03 ± 0.61 mg L^{-1}) were monitored two times daily with a multiparameter (ProPlus, YSI Inc., Yellow Springs, OH, USA), total ammonia nitrogen (0.03 ± 0.05 mg L^{-1}) was measured two times per week using an ion-selective electrode (Orion 4-Star Plus pH/ISE, Thermo Fisher Scientific, Waltham, MA, USA) and nitrate-nitrogen (24.93 ± 23.51 mg L^{-1}) was measured once a week using colorimetric test kits (La Motte Chemicals, Chestertown, MD, USA). A subsample of fish from the initial stocking was frozen for body composition analysis. At the end of feeding period, all fish were group and individually weighed to calculate the final biomass, final weight, percentage weight gain (PWG),

feed conversion ratio (FCR), percentage survival (SR), voluntary feed intake (VFI), protein retention efficiency (PRE) and thermal unit growth coefficient (TGC) as follows:

$$\text{PWG} = \frac{(\boldsymbol{average\ individual\ final\ weight - average\ individual\ initial\ weight})}{(\boldsymbol{average\ individual\ initial\ weight})} \times \mathbf{100}$$

$$\text{FCR} = \frac{\boldsymbol{feed\ given\ (g)}}{\boldsymbol{alive\ weigh\ gain\ (g)}}$$

$$\text{SR} = \frac{\boldsymbol{final\ number\ of\ fish}}{\boldsymbol{initial\ number\ of\ fish}} \times \mathbf{100}$$

$$\text{VFI} = \frac{\boldsymbol{feed\ intake\ (g)}}{\boldsymbol{fish}}$$

$$\text{PRE} = \frac{(\boldsymbol{final\ total\ body\ protein - initial\ total\ body\ protein})}{\boldsymbol{total\ dietary\ protein\ fed}}\ \boldsymbol{x}\ \mathbf{100}$$

$$\text{TGC} = \frac{\boldsymbol{FBW^{1/3} - IBW^{1/3}}}{\sum \boldsymbol{TD}} \times 100$$

Where FBW is final body weight, IBW is initial body weight, T is water temperature (^{0}C) and D is number of trial days

2.3 Body composition analysis

Upon termination of the trial, four fish from each tank or twelve fish per dietary treatment were randomly sampled and stored at – 60 ^{0}C for body composition analysis. Prior to proximate and AA analysis, dried whole fish were rigorously blended and chopped in a mixer according to the standard methods established by Association of Official Analytical Chemists (AOAC, 1990). Proximate composition and mineral contents of whole pompano body was analyzed by Midwest Laboratories (Omaha, NE, USA).

2.4 Histological section

At the termination of feeding trial, three fish per each treatment tank or nine fish per dietary treatment were randomly sampled after an overnight fast for histological analysis. Fish were individually euthanized in a solution of Tricaine-S (MS-222, tricaine methanesulfonate salt, Western Chemical, Inc., Ferndale, WA, USA) and dissected to collect the distal intestine tissue. Distal intestine samples of approximately 0.5 cm were immediately fixed in Bouin's solution (picric acid-formalin-acetic acid mixture, Ricca Chemical, Arlington, TX, USA) for 20 h at room temperature and then transferred to 70% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological analysis procedures. The blocks of designed sample were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax and sectioned at 4μm intervals for staining with Hematoxylin-Eosin (H&E) stain (Merck, Darmstadt, Germany). Histopathological evaluation for the distal intestine was performed following the score system described in Novriadi et al. (2017b). The following parameters were taken into account for distal intestine analysis: serous layer (SL), muscular layer (ML), submucosal layer (SML), the appearance of goblet cells (GC), cellular infiltration (CI), and widening of the lamina propria (LP) within the intestinal folds. In total, 108 distal intestine sections were evaluated with three sections per fish. Histomorphological images were acquired by using a microscope (Olympus BX41, Olympus Optical Co., Ltd., Tokyo, Japan).

2.5 Statistical analysis

Growth, body composition, protein retention efficiency and distal intestine histological scores were analyzed using one-way ANOVA to determine the significant differences ($P < 0.05$) among treatments followed by Tukey's multiple comparison test to determine the difference between treatment means. Histological scores were treated as categorical data, tested for normality and homoscedasticity prior to analysis. All statistical analyses were conducted using SAS system (V9.4. SAS Institute, Cary, NC, USA).

3. Results

3.1 Experimental diets

The crude protein values ranged from 405.6 to 423.4 g kg^{-1} and were fairly constant among the experimental diets. The crude fat showed a comparative value among PBM, CPC and ESBM with 94.7, 87.1, and 84.7 g kg^{-1}, respectively (Table 1). Dietary taurine (*Tau*) was lower in FSBM (5.5 g kg^{-1}) compared to PBM (7.0 g kg^{-1}), ESBM (6.8 g kg^{-1}), and CPC (6.3 g kg^{-1}). Supplementation of *Lys* induced a higher level in FSBM (24.3 g kg^{-1}) compared to PBM (23.9 g kg^{-1}), CPC (23.5 g kg^{-1}), and ESBM (21.1 g kg^{-1}). The use of corn protein concentrate (150.9 g kg^{-1}) into SBM increased the level of cysteine in the CPC (6.1 g kg^{-1}) compared to cysteine in PBM (5.9 g kg^{-1}) and advanced soy product diets (5.5 – 5.6 g kg^{-1}). The use of porcine meal (38 g kg^{-1}) increased the level of hydroxyproline (*Hyp*) in all test diets (2.8 to 3.2 g kg^{-1}) compared to PBM (2.4 g kg^{-1}) (Table 2).

3.2 Growth performance

The dietary treatments affected the growth performance of the fish (Table 3). Fish fed CPC and ESBM performed equally well in terms of final body weight, thermal growth coefficient (TGC) and percentage weight gain (PWG) in comparison to fish fed PBM as the reference diet ($P < 0.05$). Voluntary feed intake (VFI) of fish fed FSBM was significantly lower in comparison to that of fish fed PBM, CPC and ESBM. No significant differences in feed conversion ratio (FCR) were seen among dietary treatments and the lowest survival was observed in fish fed ESBM ($P = 0.0043$). Results of Tukey's multiple comparison test indicate that there is no significant difference in terms of protein retention efficiency (PRE) among dietary treatments.

Table 3. Growth performance of juvenile Florida pompano (Mean initial weight 8.06 ± 0.22 g) fed experimental diets for 8-wk. Values represent the mean of three replicates. Results in a row with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

	Experimental diets				*P*-value	PSE
Items	PBMD	CPCD	FSBMD	ESBMD		
FBW (g)	51.49[a]	49.45[a]	32.39[b]	51.55[a]	0.0151	3.6238
TGC	0.1036[a]	0.0922[a]	0.0601[b]	0.0857[a]	0.0018	0.0050
PWG (%)	538.97[a]	517.78[a]	300.02[b]	535.72[a]	0.0115	43.0379
FCR	1.61	1.53	2.21	1.46	0.0676	0.1962
Survival (%)	100.00[a]	96.67[a]	93.33[a]	80.00[b]	0.0043	2.7638
VFI (g)	69.85	62.76	49.79	58.472	0.0020	2.3328
PRE	34.17	29.23	34.35	47.84	0.0589	4.1091

3.3 Body composition

Proximate composition and mineral content of whole pompano body are presented in Table 4. No significant differences were observed in the protein, fat and ash content in whole-body of pompano across all treatments. Manganese (Mn) level of fish fed with CPC was significantly higher compared to other dietary treatment (P = 0.0007). No significant effect was observed in phosphorus (P), sulfur (S), potassium (K), magnesium (Mg), calcium (Ca), sodium (Na), and zinc (Zn) contents of whole pompano body across all the treatments.

Table 4. Proximate composition and mineral contents of whole Florida pompano body in dry weight basis offered experimental diets for 56 d.

Compositions	Treatments				*P*-value	PSE
	PBMD	CPCD	FSBMD	ESBMD		
Proximate composition (%)						
Protein	64.20	60.70	60.77	59.83	0.5639	2.2644
Fat	30.10	28.80	28.67	31.23	0.7406	1.8561
Ash	9.86	9.98	10.34	9.41	0.9139	0.9285
Macro-elements (%)						
Sulfur	0.89	0.81	0.88	0.86	0.3581	0.0292
Phosphorus	1.97	1.92	2.19	2.26	0.1984	0.1173
Potassium	1.16	1.08	1.16	1.13	0.3874	0.0337
Magnesium	0.13	0.12	0.14	0.13	0.2341	0.0067
Calcium	2.71	2.64	3.18	3.30	0.2131	0.2416
Sodium	0.32	0.30	0.31	0.32	0.8098	0.0154
Micro-elements (mg kg^{-1})						
Iron	40.73	36.13	42.37	37.27	0.3237	2.5038
Manganese	8.83^{b}	19.50^{a}	11.30^{b}	12.93^{b}	0.0007	1.0904
Zinc	51.80	50.63	60.93	52.27	0.2107	3.4489

3.4 Distal intestine histology

Representative histopathological images of Florida pompano distal intestine are shown in Figure 1. 2 and 3. There were no significant differences in the thickness of SL, ML and SML among the dietary treatments. The sections in fish fed CPC showed a significant widening of LP with an increase of cellular infiltration and higher presence of goblet cells compared to other dietary treatment, while no significant difference in these histological features was seen in fish fed PBM, FSBM and ESBM.

Figure 1. Representative histopathological image of Florida pompano distal intestine. (a) Measurement of the serous layer (SL), muscular layer (ML), and submucosal layer (SML) with 40 x magnification. (b) Measurement of the villi length (VL), villi thickness (VT), goblet cells (GC) and lamina propia (LP) with 40 x magnification measurements.

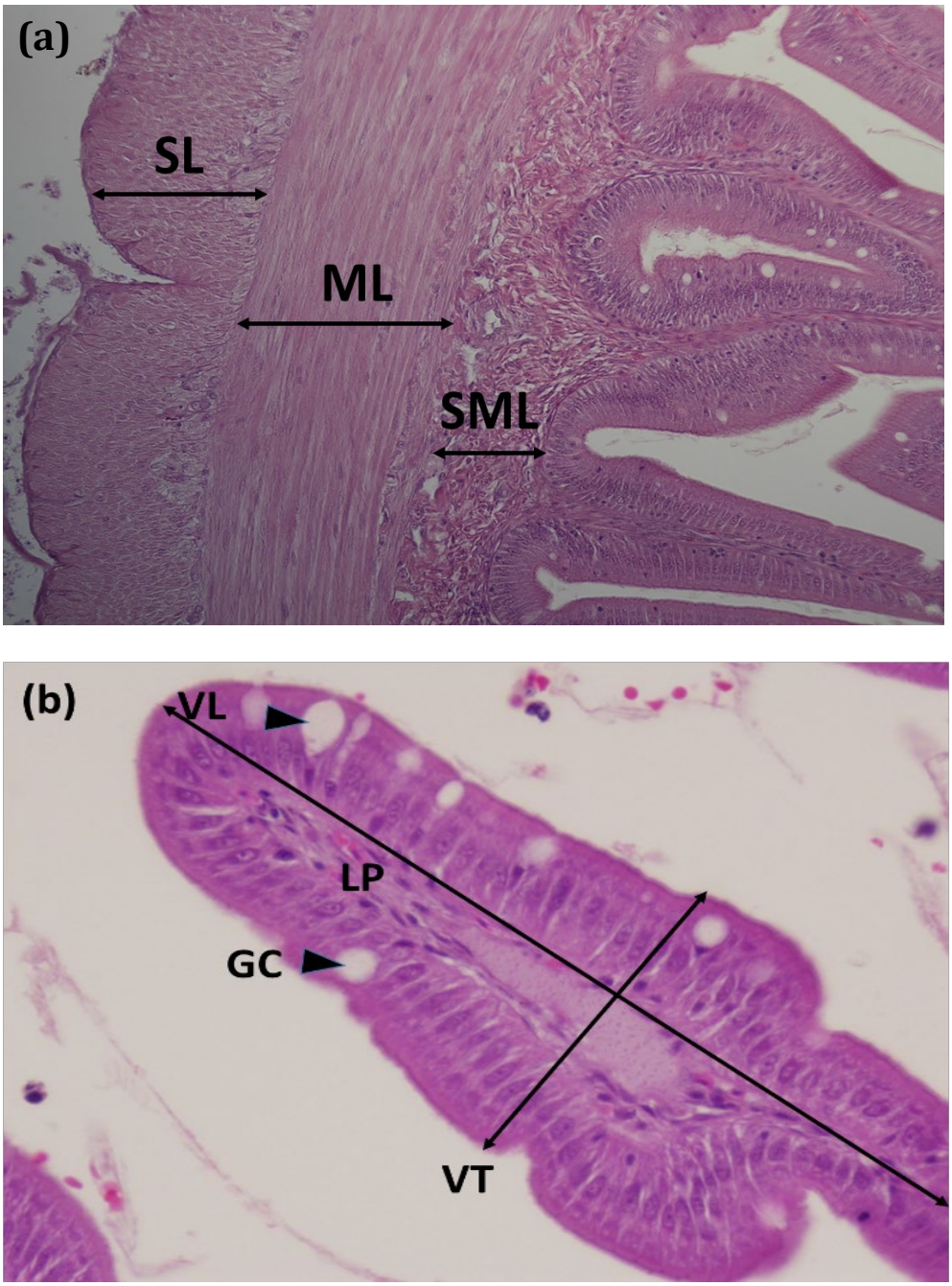


Figure 2. Representative histopathological images of hematoxylin and eosin-stained sections for detail of thickness on distal intestine layers (20 x magnification) from Florida pompano after 56 d of being fed with (A) PBMD, (B) CPCD (C) FSBMD, and (D) ESBMD

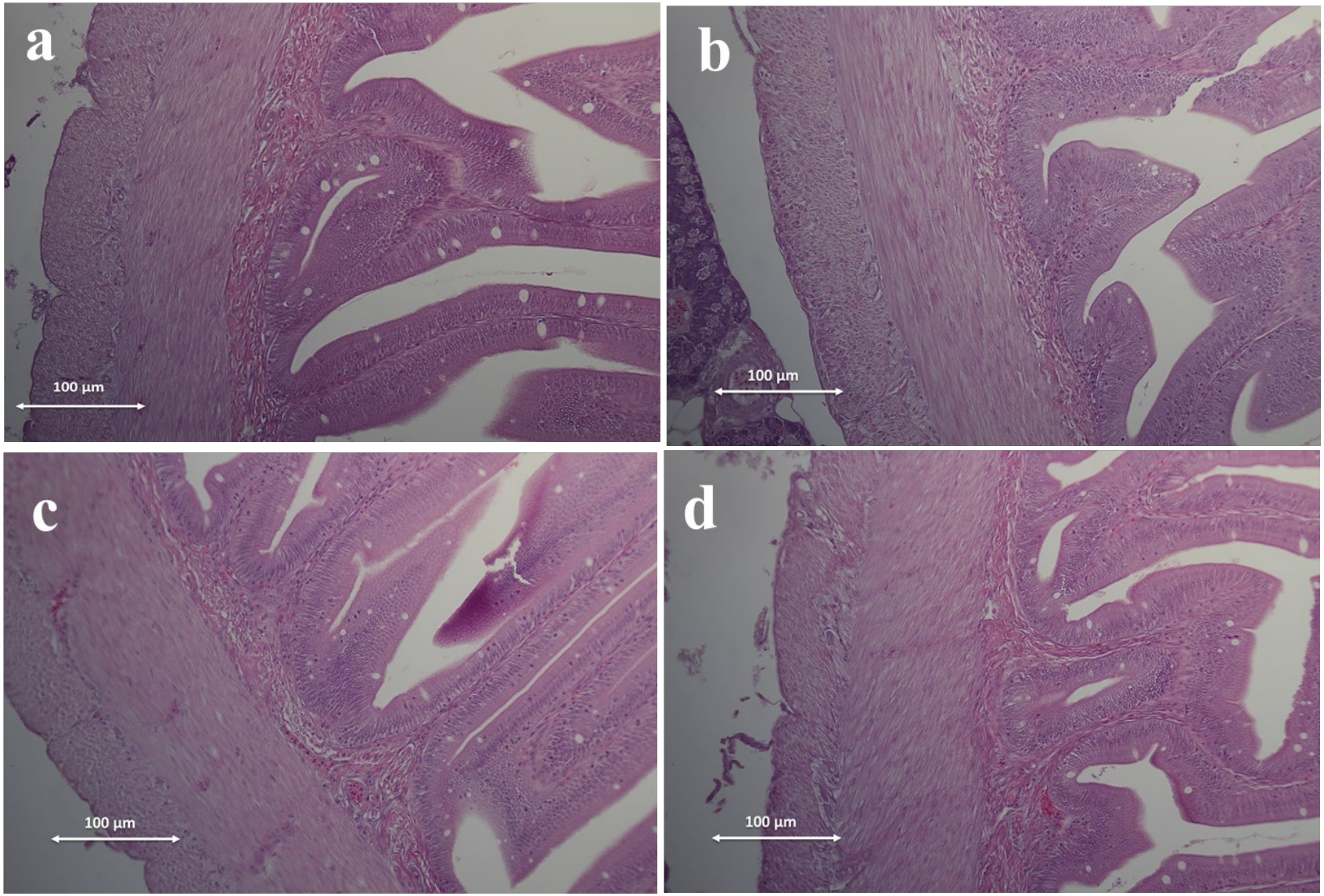
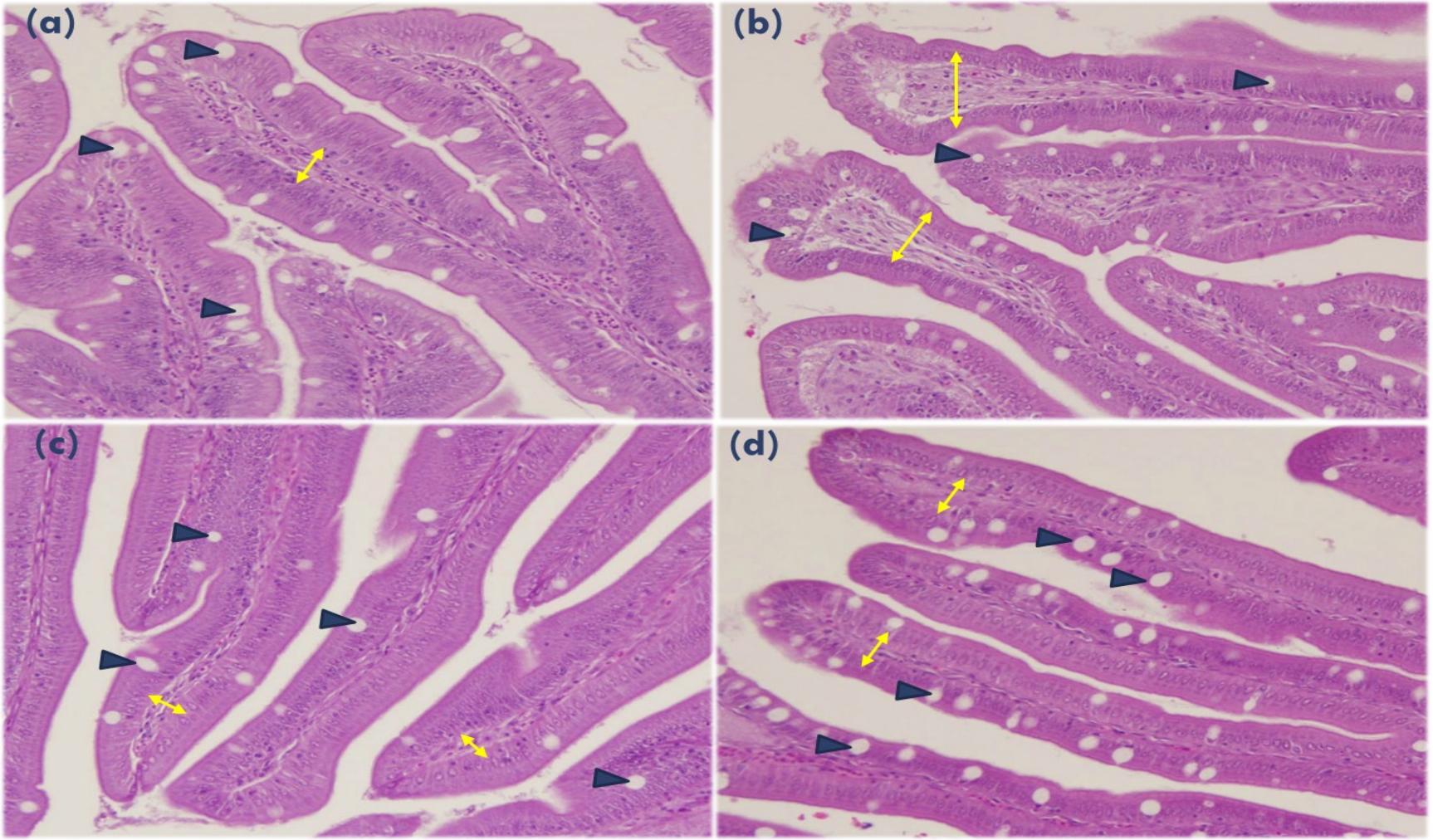


Figure 3. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestines (20 x magnification) from Florida pompano after 56 d of being fed with (A) PBMD, (B) CPCD (C) FSBMD, and (D) ESBMD. (▶) Goblet cells and (↔) = LP thickness.



4. Discussion

In previous studies, complete removal or lowering the reference level of animal meal in the diet resulting with significant reduction in growth performance (Rossi Jr and Davis 2012, Rhodes et al. 2017). Recent study also showed that the average final weight, biomass, TGC and feed intake were significantly lower when reference level of PBM was completely replaced with 150 g kg^{-1} ESBM (Novriadi et al. 2017a). However, in the present study, fish fed both high protein content of enzyme-treated soy (451.3 g kg^{-1}) as the primary protein sources and moderate level of corn (150.9 g kg^{-1}) supplemented with 38 g kg^{-1} of PM yielded similar growth performance in comparison to the group of fish fed with PBMD as the reference diet. Observation using moderate level of CPC has been carried out in red drum *Sciaenops ocellatus,* where fish fed soy-based diet containing 150 g kg^{-1} CPC had comparable growth performance as those fish fed a FM based diet (Rossi *et al.* 2015). In addition, a comparative study performed by Minjarez-Osorio *et al.* (2016) demonstrated that up to 50% FM can be replaced using CPC supplemented with AA without any negative impact to the growth performance of the Sciaenids red drum *Sciaenops ocellatus* and Shortfin corvina *Cynoscion parvipinnis*. Refer to the nutritional content of ESBM and beneficial complementarity in terms of limiting AA between corn and soy (Hertrampf and Piedad-Pascual 2012), comparable growth of fish fed ESBM or CPC with PBM would be due to the nutrient availability and synergistic interactions with PM as well as with other AA supplementation.

On the other hand, even also fortified with CPC and PM, fish fed with FSBM showed the lowest final individual body weight, thermal growth coefficient (TGC) and percentage weight gain (PWG) compared to other dietary treatments. The main reason for unfavorable outcomes with FSBM diet could be attributed to the low feeding activity compared to other dietary treatments. This is in line with our previous findings (Novriadi *et al.,* 2017b), where with an increasing dietary

inclusion level of FSBM to replace traditional SBM, feed intake and growth performance of pompano tend to decrease. The reduction in VFI with high inclusion level of FSBM has been also reported in rainbow trout *Oncorhynchus mykiss* (Yamamoto *et al.* 2010) as well as in Chinese sucker *Myxocyprinus asiaticus* (Yuan *et al.* 2013). According to Kader *et al.* (2011) growth depression and decreased feed efficiency with high inclusion levels of FSBM is most likely due to the small quantitative contribution of fermentation process to completely eliminate the ANFs within the final product. Based on observations made in this study, feeding pompano with diets containing high inclusion level of fermented product should be carefully considered and appropriate supplementation level of limiting AA is needed to support an optimum growth for Florida pompano.

In the present study, no significant differences were observed in the protein, fat, and ash content in whole-body of pompano across all treatments. This is parallel with our previous findings where the proximate composition of pompano fed with ESBM supplemented with various level of squid products did not show any significant differences with reference diet (Novriadi et al. 2017b). Moreover, pompano fed with diets containing various levels of FSBM to replace traditional SBM did not show any significant differences in the level of crude protein, moisture, fat, ash and phosphorus (Novriadi *et al.* 2017b). With regard to the use of other advanced soy product, Rhodes *et al.* (2017) reported that plant-based diet containing 350 g kg^{-1} SBM and 300 g kg^{-1} soy protein concentrate (SPC) supplemented with valine, glycine and histidine also did not result any differences in terms of crude protein, crude fat, moisture, fiber and ash content in the whole-body of pompano compared to fish fed with 294 g kg^{-1} PBM.

A number of studies acknowledge that high inclusion level of low-processed SBM induces the intestinal inflammation in the hindgut of some species of fish (Baeverfjord and Krogdahl 1996,

Ingh *et al.* 1996, Krogdahl *et al.* 2003, Bakke-McKellep *et al.* 2000). Gut inflammation or soy-induced enteritis have been described as widening of LP of mucosal folds, infiltration of inflammatory cells in the LP, reduced number or even absence of supranuclear vacuoles in the absorptive epithelium, elevated number of goblet cells and levels of lysozyme in the gut mucosa (Merrifield *et al.* 2011, Trushenski 2015). According to Sanden *et al.* (2005) trypsin inhibitors and lectins might responsible for the morphological change in the distal intestine of Atlantic salmon *Salmo salar* L. However, Knudsen *et al.* (2007) argues that gut inflammation in Atlantic salmon might be induced by saponins alone or in combination with other factors, such as the intestinal microflora or the antigenic soybean proteins. Since soybean-derivative products can contain a variety of ANFs, the primary causative agent for enteritis problem remains unclear.

Advanced processing techniques may effectively remove some ANFs, but not all (Trushenski 2015). Fermented product, for example, still contains low levels of tannin, phytate, trypsin inhibitor and protease inhibitor (Adeyemo and Onilude 2013). In our previous study, inclusion of a low level of ESBM to replace PBM in combination with high percentage of SBM still produced gut inflammation characteristics, such as widening of LP with heavy infiltration of inflammatory cells as well as high number of GC. However, this inflammation was partly prevented with the addition of 40 g kg^{-1} squid hydrolysate into the diet and fish fed the experimental diet maintained growth and physiological condition similar to a group of fish fed with 150 g kg^{-1} PBM (Novriadi *et al.* 2017c) . In addition, the morphology of LP and number of GC become more normal as the inclusion of FSBM to replace traditional SBM increased, indicating the more favorable outcome with high inclusion level of fermented products (Novriadi *et al.* 2017b).

In the present study, fish fed CPC displayed increased infiltration of inflammatory cells in the LP and a greater number of GC, as well as widening of LP compared to fish fed FSBM, ESBM

and PBM. The elevated number of GC, especially in the distal intestine, suggest an unsatisfactory process of protein digestion (Baeza-Ariño *et al.* 2016) while the cell infiltration into LP indicates the presence of inflammation (Kiron *et al.* 2015). Meanwhile, morphological features of the distal intestine in fish fed FSBM and ESBM were partly improved similarly to the fish fed PBM. Considering all information obtained from Novriadi *et al.* (2017a,b), the efficacy of ESBM and FSBM in combination with PM to partly prevent histomorphological alteration could be caused by the synergistic effect of these ingredients to improve the nutritional and functional quality of the diet. Regarding the inferior outcome in fish fed CPC, morphological change could be attributed to the presence of high level of plant-protein sources in the diet. Despite having changes in the distal intestine morphology, fish fed CPC has equal growth performance with reference diet and ESBM, suggesting that these changes did not significantly influence the growth of pompano.

Regarding the distal intestine layers that are responsible for nutrient circulation in the epithelium through the blood vessels (Al-Hussaini 1949, Baeza-Ariño et al. 2016), individual variations led to non-significant differences in the thickness of the SL, ML and SML among dietary treatments. Baeza-Ariño et al. (2016) also reported that the different inclusion level of pea protein concentrates and rice protein did not significantly affect the ML and SML thickness in the distal intestine of sea bream *Sparus aurata*. In addition, Martínez-Llorens *et al.* (2012) only found differences in SL of fish fed 339 g kg^{-1} of carob seed meal (CSM) and greater thickness in ML on fish fed 339 and 518 g kg^{-1} of CSM, while no significant differences were detected in SML of fish among the dietary treatments.

5. Conclusion

Under the experimental conditions, enzyme-treated soy (ESBM) at inclusion levels up to 451 g kg^{-1} or inclusion of CPC at 150 g kg^{-1} in a soy-based diet formulation supplemented with 38 g kg^{-1} of PM can be utilized in the development of practical diets for Florida pompano without any adverse effects on growth and nutritive parameters. The histological study of the distal intestine provides useful information regarding the effects of a high inclusion level of advanced soy product or moderate level of CPC fortified with PM.

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Chapter V

THE USE OF ENZYME TREATED SOY SUPPLEMENTED WITH VARIOUS LEVELS OF SQUID PRODUCTS IN THE DIET OF FLORIDA POMPANO *Trachinotus carolinus*

Abstract

The purpose of this study was to evaluate the effects of squid products supplementation into plant-based diets with various levels of enzyme-treated soybean meal (ESBM) on the growth, proximate composition, serum biochemistry, and liver and intestinal histological alterations in Florida pompano (*Trachinotus carolinus*). A 15% poultry by-product meal (PBM) based practical diet was used as a reference and basal diet was produced by replacing PBM with ESBM. Basal diet was then modified to contain varying levels of squid products. Squid hydrolysate (SH) and squid meal (SM) were added to basal diets, to produce diets containing 1, 2 and 4% of the squid products. A total of eight experimental diets were each fed to quadruplicate groups of 20 pompanos (mean initial wt = 7.68±0.1 g) in a recirculating rearing system for 56 d. Results from the growth trial indicate that fish fed with basal diet exhibited significantly lower growth performance and feed utilization as compared to fish fed with PBM. The addition of 4% SH improved the response of basal diet and did not show any significant difference in terms of growth performance as compared to PBM. Whole body proximate and amino acids composition of fish were not significantly different among fish reared on any of the diets. Data on mineral composition of fish showed that the content of phosphorus and calcium were significantly higher in fish fed with basal diet compared to other treatments. Total protein and cholesterol level of fish fed PBM were

significantly lower compared to basal diet. Total albumin, alanine transaminase, aspartate transaminase, and bile acids were similar among the dietary treatments. Fish fed with basal diet showed disordered vacuolization in the liver and decreased the lamina propria thickness in the intestine. The inclusion of 4% SH partly prevented the alteration of liver and distal intestine of Florida pompano and findings were similar to fish fed with PBM. Based on these results, combination of ESBM and 4% SH has the potential to serve as an alternative protein source and attractant to improve the efficacy of plant-based diet for pompano.

Key words: Enzyme-treated soybean meal, Squid products, Growth performance, Body composition, Histology, Serum biochemistry, Trachinotus carolinus

1. Introduction

Considerable research has been conducted to evaluate the effect of soybean meal (SBM) inclusion to partially or completely replace the use of animal meal in the aquatic animal feed formulations (Hertrampf and Piedad-Pascual, 2000; Sales, 2009). However, it has been suggested that when a substantial amount of fish meal (FM) is replaced by SBM and other plant protein sources, feed intake generally declines (Morales et al., 1994) and affecting the growth performance of fish (Nunes et al., 2006; Watanabe et al., 1998). Supplementation with attractants or palatability enhancers, such as krill meal (Gaber, 2005), blue mussel meal (Nagel et al., 2014), tuna by-product meal (Hernández et al., 2011), algal meal (Kissinger et al., 2016), nucleotides (Barnard, 2006) and chemo-attractants derived from hydrolysis process of seafood waste and by-products (Barry et al., 2017; Refstie et al., 2004) may enhance the palatability and feed intake of plant-based diet. However, according to De la Higuera (2001) low inclusion level of animal meal is still needed to increase the feed intake in some fish species.

Previous findings in Florida pompano *Trachinotus carolinus* highlighted that reducing animal protein from 15% to 0% in the diet resulting a linear decrease in the growth performance of fish (Rhodes et al., 2017; Rossi Jr and Davis, 2012). In addition, with 100% replacement of animal meal, supplementation of glycine, histidine and fish protein concentrate did not significantly affect the growth performance or feed utilization of pompano (Rhodes et al., 2017). However, when 5% squid hydrolysates (SH) was added to the animal free diet, fish had significantly higher final weight, percentage weight gain and thermal growth coefficient (TGC) in comparison to fish fed with plant-based diet, 5% chemical attractant mix and 5% poultry by product meal (PBM) (Rhodes et al., 2017). The increase in feed intake and growth performance might be due to the presence of chemoattractant properties in squid products, such as glycine and

betaine, that have potential impact to activate fish feeding behavior (Meyers, 1986). In addition, well balanced amino acid profile, high protein value and the presence of highly unsaturated fatty acids (HUFA) proportion of total fat in squid products would also be beneficial factors for its use as an ingredient in aquaculture diets (Hertrampf and Piedad-Pascual, 2000; Lian et al., 2005). Hence, there is an opportunity to improve the performance and utilization of the plant-based diet by proper supplementation of these ingredients.

It has also been suggested that when animal meal is replaced by traditional solvent extracted SBM, the antinutritional factors (ANFs) may play a role in decreasing performance (Batal and Parsons, 2003; Iwashita et al., 2008; Krogdahl et al., 1994). Different processing technique have been reported to be an effective method to denature and reduces several ANFs, including fermentation, enzyme treatment and alcohol extraction (Hong et al., 2004; Papagianni et al., 1999; Riche and Williams, 2011). However, little is known about the value of novel enzyme-treated soybean meal (ESBM) produced by using a combination of non-alcohol extraction process and enzymatic treatment. Thus, the aim of this study was to evaluate the use of ESBM and squid products to completely replace PBM on the growth performance, body composition, serum biochemistry and histology of Florida pompano.

2. Materials and methods

2.1 Experimental diets

Diets were designed to be iso-nitrogenous and iso-lipidic (40% protein and 8% lipid). The basal diet contained no animal-based protein sources and was formulated using de-hulled solvent extracted soybean meal (SBM, Bunge Limited, Decatur, AL, USA), enzyme-treated soybean meal (ESBM, Nutrivance^{TM}, Midwest Ag Enterprises, Marshall, MN, USA), and corn protein

concentrate (CPC, Empyreal 75^{TM}, Cargill Corn Milling, Cargill, Inc., Blair, NE, USA) as the dietary protein sources. The next six diets were formulated to contain 1, 2, and 4% squid hydrolysates (SH) or squid meal (SM), designated as 1%SH, 2% SH, 4% SH, 1% SM, 2% SM, and 4%SM, respectively, at the expense of ESBM. Additionally, a poultry-based practical diet which has been run in numerous trials was included as a reference diet and was produced utilizing 15% poultry by product meal (PBM, Griffin Industries, Inc., Mobile, AL, USA) to completely remove ESBM in the basal diet. Test diets were produced in the Aquatic Animal Nutrition Laboratory, School of Fisheries, Aquaculture and Aquatic sciences, Auburn University, AL, USA, using standard procedures for Florida pompano. Briefly, diets were made by mixing preground dry ingredients and fish oil in a food mixer (Hobart, Troy, OH, USA) for approximately 15 min. Boiling water was then blended into the mixture to attain a consistency appropriate for pelleting. The moist mash from each diet was passed through a 3 mm die in a meat grinder, and the pellets were then placed into a fan-ventilated oven (<45ºC) overnight to attain a moisture content of less than 10%. Diets were stored at -20 ºC, and prior to use, each diet was ground and sieved to an appropriate size. The experimental diets were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA) for proximate analysis (Table 1) and amino acid profile (Table 2).

Table 1. Composition (as is g kg^{-1}) of diets containing various levels of squid hydrolysates (SH) and squid meal (SM) added into the basal diet and fed to juvenile Florida pompano for 8 weeks

Ingredients (g kg^{-1} as is)	PBM	Basal	1%SH	2%SH	4%SH	1%SM	2%SM	4%SM
Poultry by product meal[1]	150.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Soybean Meal[2]	472.1	472.1	472.1	472.1	472.1	472.1	472.1	472.1
Enzyme-treated soybean meal [3]	0.0	148.0	136.5	125.1	102.2	136.8	125.6	103.2
Squid hydrolysates [4]	0.0	0.0	10.0	20.0	40.0	0.0	0.0	0.0
Squid meal[5]	0.0	0.0	0.0	0.0	0.0	10.0	20.0	40.0
Corn protein concentrate[6]	63.0	80.0	80.0	80.0	80.0	80.0	80.0	80.0
Menhaden Fish Oil[7]	47.4	63.7	63.5	63.3	62.9	63.3	63	62.3
Corn Starch[8]	7.0	4.7	6.6	8.4	12.1	6.9	8.3	11.9
Whole wheat[8]	220.0	180.0	180.0	180.0	180.0	180.0	180.0	180.0
ASA Trace Mineral premix[10]	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
ASA Vitamin premix w/o choline[11]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Choline chloride[8]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Stay C 35%[12]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
CaP-dibasic [8]	20	31	30.8	30.6	30.2	30.4	30.5	30
Lecithin (soy commercial)[9]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Taurine[8]	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Lysine[8]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Proximate analyses (g kg^{-1} as is)								
Crude Protein	426.0	414.9	411.5	399.4	422.3	417.6	426.3	408.1
Moisture	72.9	83.6	93.1	110.2	76.6	80.5	59.1	108.7
Crude Fat	95.6	82.8	79.5	85.3	89.4	90.8	82.9	80.4
Crude Fiber	27.5	32.4	29.1	28.8	30.8	29.8	31.7	31.3
Ash	64.9	64.4	63.7	61.6	63.9	62.9	64.6	62.2

1 Griffin Industries, Inc., Mobile, AL, USA.
2 De-hulled Solvent Extracted Soybean Meal, Bunge Limited, Decatur, AL, USA
3 Nutrivance™, Midwest Ag Enterprises, Marshall, MN, USA
4 Produced for this research (Lian et al. 2005)
5 Foodcorp S.A., Chile
6 Empyreal 75™ Cargill Corn Milling, Cargill, Inc., Blair, NE, USA
7 Omega Protein Inc., Houston, TX, USA
8 MP Biomedicals Inc., Santa Ana, CA, USA
9 The Solae Company, St. Louis, MO, USA
10 ASA Premix (g $100g^{-1}$ premix): cobalt chloride, 0.004; cupric sulphate pentahydrate, 0.250, ferrous sulfate heptahydrate, 4.0, manganous sulfate anhydrous, 0.650; potassium iodide, 0.067; sodium selenite, 0.010; zinc sulfate heptahydrate, 13.193, and α-cellulose 81.826.
11 ASA Premix (g/kg Premix): thiamin HCL, 0.5; riboflavin, 8.0; pyridoxine HCl, 5.0; Ca-pantothenate, 20.0; niacin, 40.0; biotin, 0.040; folic acid, 1.80; cyanocobalamin, 0.002; vitamin A acetate (500,000 IU g^{-1}), 2.40; vitamin D_3 (400,000 IU g^{-1}), 0.50; DL-α-tocopheryl acetate, 80.0; and α cellulose, 834.258.
12 Stay C®, (L-ascorbyl-2-polyphosphate 35% Active C), Roche Vitamins Inc., Parsippany, NJ, USA.

Table 2. Amino acid (AA) profile (as is g kg^{-1}) of experimental diets

Composition[1]	PBM	Basal	1%SH	2%SH	4%SH	1%SM	2%SM	4%SM
Taurine	6.5	5.9	6.1	6.3	7.5	5.9	6.2	6.1
Hydroxyproline	3.4	1.0	0.5	0.5	0.1	0.5	0.4	0.8
Aspartic Acid	39.1	40.4	38.7	39.6	40.7	40.2	41.9	39.6
Threonine	15.2	14.6	14.2	14.5	15.2	14.8	15.5	14.7
Serine	17.1	16.9	16.3	16.7	17.1	17.4	17.9	16.7
Glutamic Acid	77.2	79.0	76.4	77.2	79.3	80.2	82.0	76.3
Proline	26.1	24.9	24.3	24.8	25.7	26.1	26.1	24.0
Glycine	21.9	17.1	16.9	16.6	17.8	17.2	18.2	17.4
Alanine	21.8	20.0	19.7	19.5	20.9	20.9	21.5	20.2
Cysteine	5.9	5.9	5.7	5.8	6.1	5.8	6.1	5.6
Valine	20.8	20.4	19.7	20.1	20.7	20.5	21.4	20.0
Methionine	7.1	6.5	6.4	6.6	7.1	6.6	7.1	6.7
Isoleucine	18.2	18.5	18.0	18.4	18.9	18.6	19.3	18.1
Leucine	35.7	36.5	35.6	35.9	37.5	38.1	38.9	35.9
Tyrosine	13.6	13.4	13.5	13.8	14.0	14.4	14.6	11.7
Phenylalanine	20.5	21.2	20.4	20.7	21.2	21.5	22.0	20.7
Hydroxylysine	0.8	0.4	0.5	0.5	0.6	0.4	0.5	0.5
Ornithine	0.3	0.3	0.3	0.3	0.6	0.3	0.3	0.5
Lysine	22.2	21.2	20.4	20.8	21.6	20.8	22.1	21.1
Histidine	10.0	10.2	9.8	9.9	10.1	10.1	10.5	9.9
Arginine	26.1	25.3	24.7	25.2	25.5	25.6	26.6	24.8
Tryptophan	5.8	5.8	5.5	5.5	5.8	5.6	5.6	5.1

[1] Ingredients were analyzed at University of Missouri Agricultural Experiment Station Chemical Laboratories (Columbia, MO, USA)

2.2 Preparation of squid products

Dry squid hydrolysates (SH; 71.6% Crude protein, special select, Rhode Island University, RI, USA) produced from enzymatic hydrolysis of squid processing by-products (SPB) (heads, viscera, cut offs, fins and small tubes) and squid meal (SM; 70% Crude protein, Foodcorp S.A., Chile) served as the supplement in the plant-based diet formulation. For dry squid hydrolysates,

preparation of raw material and hydrolysis process were carried out at the Food Science and Nutrition Research Center-Seafood Lab at the Rhode Island University, RI, USA and processed according to Lian et al. (2005).

2.3 Growth trials

The growth and feeding trials were conducted in the Claude Peteet Mariculture Development Center (CPMC), Gulf Shores, AL, USA. Florida pompano fingerlings were purchased from Troutlodge Marine Farms LLC, (Proaquatix) Vero Beach, Florida, USA, nursed in an indoor recirculating system facility of CPMC, and fed with commercial diet until reaching a suitable size. The trial was conducted in a recirculating system consisted of thirty-two 700-L polyethylene open top tanks equipped with reservoir tank, biological filter, supplemental aeration (provided using a central line, regenerative blower and air diffusers) and circulation pump. At the start of experiment, twenty fish (mean weight = 7.68±0.10 g) were stocked into each tank and assigned to quadruplicate tanks in a completely randomized design. Fish were maintained under a natural photoperiod with water temperature, salinity, pH, and the dissolved oxygen concentration of the culture water at 26.6±1.9 ^{0}C, 30.1±2.8 ‰, 7.9±0.2, and 5.9±0.6 mg L^{-1}, respectively. A subsample of fish from the initial stocking was retained for proximate, amino acid and mineral profile analysis. Fish were fed four times per day and the daily ration was adjusted to apparent satiation weekly throughout the trials. Additionally, feed inputs were calculated on a two-weeks basis after each sampling to adjust for growth and mortalities. The growth trial lasted for 56 d. At the end of growth trials, fish were grouped and individually weighed to obtain the final biomass, final weight, feed conversion ratio (FCR) (feed offered/wet weight gain), percentage survival [100 × (final number/initial number)], and thermal unit growth coefficient (TGC), calculated as: $100 \times [FBW^{1/3} – IBW^{1/3}/\sum DT]$, where

FBW is final body weight, IBW is initial body weight, D is number of days and T is water temperature (^{0}C).

2.4 Body composition analysis

Upon termination of the trial, twenty fish per treatment (five fish per replicate) were randomly selected from each group and stored at – 80 ^{0}C for body composition analysis. Prior to proximate, amino acid and mineral analysis, dried whole fish were rigorously blended and chopped in a mixer according to methods described by Association of Official Analytical Chemist (AOAC). All parameters were analyzed at Midwest Laboratories (Omaha, NE, USA) and the mean of each value were taken.

2.5 Serum biochemistry analysis

At the end of growth trial, sixteen fish per treatment (four fish per replicate) were immediately euthanized with MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma), and blood samples were taken from the caudal vein after 12 h starvation. Blood samples were collected from basal diet, PBM, 4% SH and 4% SM treatment using anticoagulant-free centrifuge tubes. Serum was obtained by centrifugation of blood at 3.000 rpm for 10 min and stored at – 80 ^{0}C until the analysis. Biochemical parameters in the serum samples were analyzed using an automatic chemistry analyzer (Cobas C311, Roche Diagnostics, IN, USA) for total protein, albumin, activities of alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), glucose, cholesterol, and bile acid concentration.

2.6 Histological section

Fish selected for histological sampling and external disease evaluation were individually euthanized in a solution MS222 (Ethyl 3-aminobenzoate methanesulfonate salt, Sigma-Aldrich Co. LLC, USA). Liver and distal intestine of fish was excised and preserved in Bouin's solution (picric acid-formalin-acetic acid mixture, Ricca Chemical, Arlington, TX, USA) for 20 h at room temperature and then transferred to 70% ethanol solution (VWR, Radnor, PA, USA) until processed by standard histological procedures. The blocks of designed sample were dehydrated through a standard ethanol series to 100%, embedded in paraffin wax and sectioned at 4μm intervals for staining with Haematoxylin-Eosin (H&E) stain (Merck, cat: 1.05174.1000; Scharlau, cat: EO0025). Double blinded evaluation with scoring system from 1 to 5 was used to evaluate the histological condition. Score 1 was considered as the normal condition and subsequent scores accounted for histopathological alteration compared to the normal condition. Liver section was evaluated for hepatocyte vacuolization, nuclear change and glycogen accumulation. Intestinal samples were evaluated for cellular infiltration, presence of goblet cells and widening of the lamina propria within the intestinal folds. Histopathological images were acquired by using an imaging microscope (ECLIPSE 80i, Nikon, Japan).

2.7 Statistical analysis

All data except for histological data were analyzed using one-way analysis of variance to determine the significant difference ($P<0.05$) among the treatment means followed by the Tukey's multiple comparison test to determine difference between treatments means in each trial. The pooled standard errors were used across growth trials, proximate composition, serum levels and enzyme activities, as the variance of each treatment is the same. Histological scores were treated

as categorical data, tested for normality and homoscedasticity and subsequently analyzed using Welch's one-way analysis of variance followed by Games-Howell *post hoc* tests to determine significant differences between treatments. Statistical analyses were conducted using SAS system (V9.3, SAS Institute, Cary, NC, USA).

3. Results

3.1 Nutrient composition of experimental diets

The proximate compositions of each primary protein sources used in this research are presented in Table 1. Both squid products, SH and SM contained high protein levels (72.19 – 72.22%) and a moderate level of lipid (9.03 – 10. 53%) and moisture (8.16 - 10.19%), but differed in ash content, where SH (6.03%) had lower ash content compared to SM (9.66%). For the soy protein sources, ESBM has higher protein level but lower in moisture, lipid and ash content compared to SBM. Regarding to the amino acid and proximate profile of experimental diets, the compositions reflected the dietary protein source (Tables 2 and 3).

3.2 Growth performance

The growth performance data are presented in Table 3. Mean survival of fish in all treatments was not significantly affected ($P = 0.3956$) by dietary treatments and was typical for research with this species. Differences in performance of pompano were observed when PBM was completely replaced by ESBM. Reductions in final biomass (702.3 to 405.8 g), individual final weight (38.6 to 28.17 g) and feed intake (43.99 to 32.54 g fish^{-1}) were observed in fish fed basal diet as compared to fish fed with PBM, respectively ($P<0.05$). However, performance with PBM was not significantly better when basal diet was supplemented with 4% SH. Feed intake was significantly

different with these diets, as the feed intake of PBM was significantly higher compared to 4% SH (P = 0.0001). FCR ranged from 1.42 to 2.29 and fish fed with 1% SM had the highest FCR compared to other treatments (P = 0.0066). TGC ranged from 0.0522 – 0.0903 (P = 0.0001). PBM had the highest numerical value but did not have any significant difference with 4% SH, 2% SH and 4% SM.

Table 3. Growth performance of juvenile Florida pompano (Mean initial weight 7.68 ± 0.1 g) fed experimental diets for 56 d. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diet	Final biomass (g)	Final mean weight (g)	TGC[4]	Feed intake (g fish^{-1})	FCR[5]	Survival (%)
PBM[1]	702.30^{a}	38.60^{a}	0.0903^{a}	43.99^{a}	1.42^{b}	91.25
Basal	405.75^{bc}	28.17^{bcd}	0.0677^{bcd}	32.54^{bc}	1.69^{ab}	73.75
1% SH[2]	449.47^{bc}	23.65^{cd}	0.0562^{cd}	32.05^{bc}	2.09^{ab}	85.00
2% SH	515.25^{bc}	32.84^{abc}	0.0784^{abc}	36.54^{bc}	1.48^{b}	78.75
4% SH	541.95^{ab}	35.17^{ab}	0.0822^{ab}	38.93^{b}	1.44^{b}	77.50
1% SM[3]	365.35^{c}	21.58^{d}	0.0522^{d}	31.32^{c}	2.29^{a}	85.00
2% SM	431.25^{bc}	27.25^{bcd}	0.0660^{bcd}	32.11^{bc}	1.69^{ab}	80.00
4% SM	512.28^{bc}	30.16^{abcd}	0.0717^{abc}	36.09^{bc}	1.67^{ab}	86.25
P-value	<0.0001	0.0003	0.0001	0.0001	0.0066	0.3956
PSE[6]	28.1200	1.8458	0.0037	1.2579	0.1220	4.0686

Note: [1]PBM: 15% poultry by-product meal or reference diet; [2]SH: squid hydrolysates; [3]SM: Squid meal; [4] TGC = Thermal growth coefficient; [5] FCR = Feed conversion ratio; [6] PSE = Pooled standard error

3.3 Proximate, amino acids and mineral composition of Florida pompano fillet

Proximate and mineral composition analysis of whole fish body are presented in Table 4. No significant differences were observed in percentage of protein (P = 0.2229), fat (P = 0.2124) and ash content (P = 0.1202) across all dietary treatments. Phosphorus (P) and calcium (Ca) were significantly higher in fish fed with basal diet compared to other treatments while iron (Fe) and manganese (Mn) in fish fed with basal diet were significantly higher than PBM. Meanwhile, sodium (Na) was significantly higher in fish fed with basal diet and 2% SM compared to PBM.

Table 4. Proximate and mineral composition (dry weight basis) of whole body of Florida pompano fed experimental diets for 56 d. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diets[1]	Protein (%)	Fat (%)	Ash (%)	S (%)	P (%)	K (%)	Mg (%)	Ca (%)	Na (%)	Fe (ppm)	Mn (ppm)	Zn (ppm)
PBM	65.43	19.68	15.15	0.93	2.82^{b}	1.21	0.15	4.08^{b}	0.44^{b}	37.70^{c}	15.13^{b}	64.33^{ab}
Basal	60.15	19.65	18.50	0.88	3.76^{a}	1.20	0.19	6.08^{a}	0.65^{a}	58.45^{a}	27.95^{a}	76.00^{a}
1% SH	61.50	25.50	12.31	0.99	2.63^{b}	1.26	0.17	3.70^{b}	0.60^{ab}	56.38^{ab}	23.95^{ab}	61.60^{ab}
2% SH	63.65	22.15	12.98	0.96	2.55^{b}	1.22	0.17	3.59^{b}	0.55^{ab}	53.08^{abc}	22.75^{ab}	64.88^{ab}
4% SH	64.65	20.05	13.95	0.94	2.71^{b}	1.24	0.15	3.95^{b}	0.49^{ab}	46.53^{abc}	19.83^{ab}	60.43^{b}
1% SM	64.93	21.23	13.38	0.95	2.76^{b}	1.25	0.18	3.92^{b}	0.54^{ab}	52.15^{abc}	22.40^{ab}	60.18^{b}
2% SM	63.13	23.30	14.17	0.90	2.87^{b}	1.23	0.18	4.17^{b}	0.65^{a}	49.93^{abc}	25.08^{a}	61.80^{ab}
4% SM	69.63	20.60	12.15	1.04	2.61^{b}	1.43	0.16	3.47^{b}	0.52^{ab}	41.50^{bc}	20.33^{ab}	61.55^{ab}
P-value	0.2229	0.2124	0.1202	0.1283	<0.0001	0.1041	0.0579	<0.0001	0.0203	0.0021	0.0090	0.0443
PSE[2]	1.7719	1.2621	1.1316	0.0271	0.0863	0.0401	0.0073	0.1616	0.0329	2.5128	1.5424	2.4817

The amino acid (AA) profile of whole fish body is presented in Table 5. Significantly higher cysteine level was found in fish fed with basal diet ($P = 0.0459$) compared to 4% SH, while other amino acids values were not affected by the dietary treatments.

Table 5. Amino acids analysis (*as is g x kg*$^{-1}$) of whole fish. Values represent the mean of four replicates. Results in the same row with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Composition	Initial	PBM	Basal	4%SH	4%SM	*P*-value	PSE
Taurine	1.2	4.1	3.7	3.7	3.7	0.2939	0.0201
Hydroxyproline	3.2	3.5	2.7	3.9	4.0	0.2475	0.0410
Aspartic Acid	13.0	15.2	15.5	14.4	14.9	0.2564	0.0382
Threonine	6.0	7.0	7.1	6.7	7.0	0.2910	0.0244
Serine	5.4	6.4	6.2	6.0	6.4	0.2303	0.0149
Glutamic Acid	19.1	22.9	23.2	21.6	22.3	0.1456	0.0485
Proline	7.7	8.7	7.8	8.8	9.2	0.1122	0.0341
Glycine	13.4	14.4	12.3	14.2	14.8	0.2064	0.0737
Alanine	9.6	11.3	10.7	11.0	11.4	0.3078	0.0253
Cysteine	1.3	1.5^{ab}	1.6^{a}	1.3^{b}	1.4^{ab}	0.0459	0.0051
Valine	6.9	8.3	8.5	7.8	8.1	0.3014	0.0415
Methionine	4.0	4.7	4.7	4.4	4.6	0.3839	0.0119
Isoleucine	6.1	7.1	7.4	6.7	6.9	0.1711	0.0217
Leucine	10.0	12.0	12.4	11.3	11.6	0.2027	0.0351
Tyrosine	3.3	5.1	5.4	4.8	4.8	0.1596	0.0205
Phenylalanine	5.7	6.6	6.7	6.3	6.4	0.2493	0.0155
Hydroxylysine	0.5	0.6	0.5	0.6	0.7	0.1121	0.0049
Lysine	10.9	13.5	14.0	12.5	12.8	0.0915	0.0398
Histidine	2.9	3.6	3.8	3.4	3.6	0.2027	0.0112
Arginine	9.1	11.0	10.6	10.7	11.0	0.4003	0.0219
Tryptophan	1.1	1.5	1.6	1.4	1.5	0.3377	0.0070

3.4 Serum levels and enzyme activities

Effect of different diets on serum levels and enzyme activities in pompano are presented in Table 6. Total protein (g dL^{-1}) and cholesterol (mg dL^{-1}) levels of fish fed with PBM were significantly lower than in fish fed basal diet ($P<0.05$). Glucose (mg dL^{-1}) level of fish fed with 4% SM was significantly higher in comparison to fish fed with PBM ($p = 0.0198$). Alkaline phosphatase (ALP) activity was significantly lower in fish fed with PBM ($p = 0.0541$). Higher ALP activity was pronounced in fish fed with basal diet supplemented with 4% SM. Serum levels of Alanine transaminase (ALT), Aspartate transaminase (AST) together with the concentration of albumin and bile acid were not significantly different among the dietary treatments ($p>0.05$).

Table 6. Effect of different diets on serum levels and enzyme activities in Florida pompano. Values represent the mean of four replicates. Results in the same columns with different superscript letter are significantly different ($P<0.05$) based on analysis of variance followed by the Tukey's multiple comparison test.

Diets	Total protein (g dL^{-1})	Albumin (g dL^{-1})	ALP[1] (U L^{-1})	ALT[2] (U L^{-1})	AST[3] (U L^{-1})	Glucose (mg dL^{-1})	Cholesterol (mg dL^{-1})	Bile acid (mg dL^{-1})
PBM	2.72^{b}	0.93	28.47^{a}	32.00	221.50	120.25^{b}	143.75^{c}	2.90
Basal	3.75^{a}	1.21	37.13^{ab}	76.75	289.75	155.50^{ab}	175.25^{b}	4.35
4% SH	3.66^{a}	1.24	35.83^{ab}	34.00	321.75	157.25^{ab}	210.50^{a}	4.00
4% SM	3.99^{a}	1.03	43.20^{b}	56.75	335.00	177.75^{a}	193.25^{ab}	4.35
p- value	<0.0001	0.4022	0.0541	0.6430	0.9067	0.0198	<0.001	0.0531
PSE[4]	0.1152	0.1626	3.7933	32.229	137.29	12.540	7.0063	0.4294

3.5 Histology

Figure 1 shows histological sections of fish liver, where the vacuolization and glycogen deposition were higher in fish fed with basal diet compared to other dietary treatments. Meanwhile, even there is no statistically significant difference among all dietary treatments, fish fed 4% SM shows a slight increase of enlarged cell with nuclear change. The supplementation of 4% SH and the inclusion of 15% PBM partly improve the liver condition of fish with lower score for vacuolization, nuclear change and glycogen accumulation. Based on the histological score analysis, liver alterations could not be attributed only to the dietary treatment but also to possible individual variations. Regarding to the distal intestine analysis, typical changes were apparent after 56 d of feeding trial. The lamina propria (LP) thickness of mucosal folds was decreased with an increase of cellular infiltration in the distal intestine of fish fed with basal diet and 4% SM compared to fish fed with 4% SH and PBM diet. Regarding the goblet cells (GC), differences were noted between basal and PBM. Albeit GC are still present in the distal intestine of fish, the number is lower in fish fed with PBM and 4% SH compared to fish fed with basal diet and 4% SM.

Figure 1. Representative histopathological images of hematoxylin and eosin-stained sections of liver from Florida pompano after 56 d of being fed with (A) PBM, (B) basal, (C) 4% SH, and (D) 4% SM

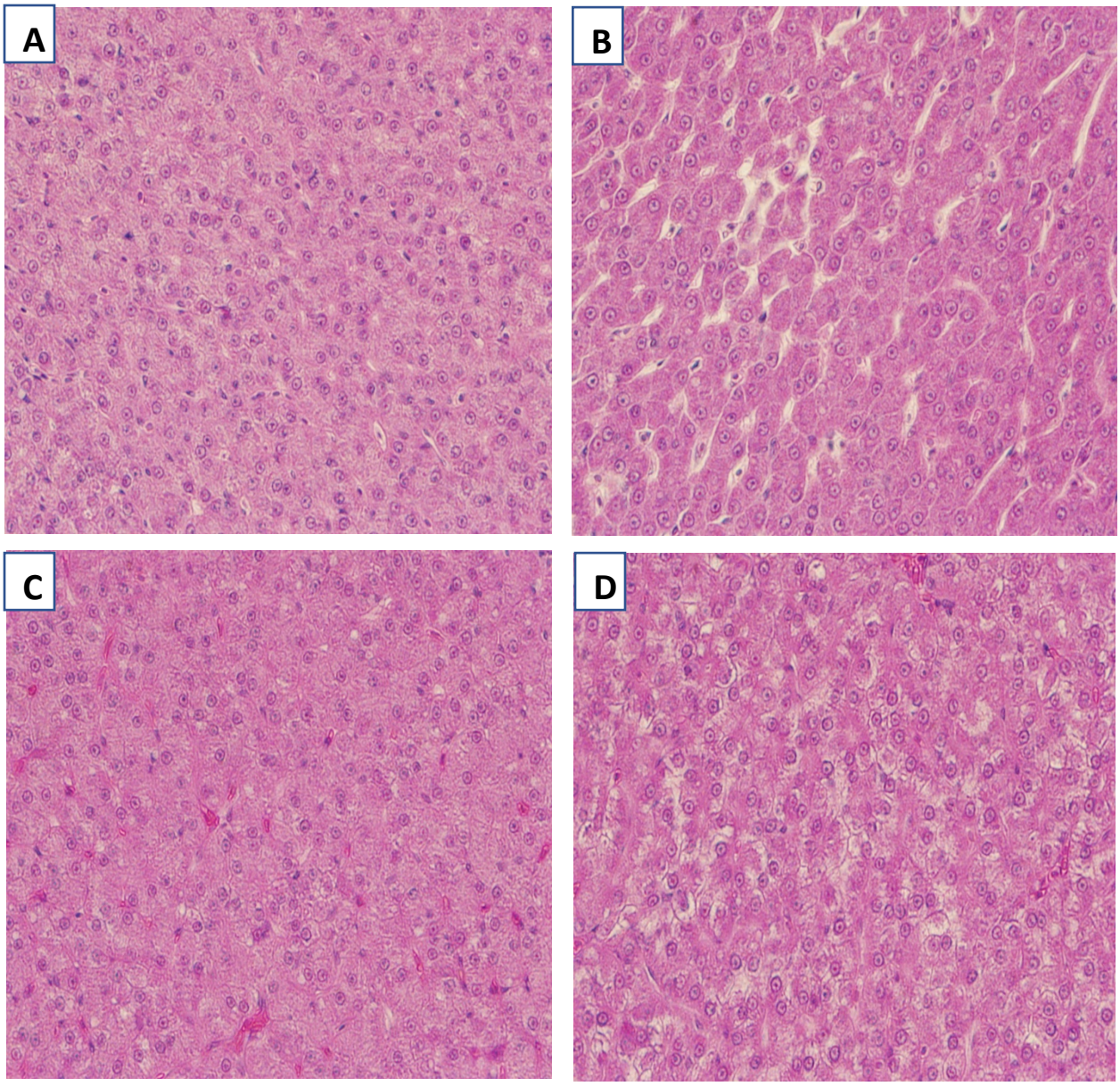
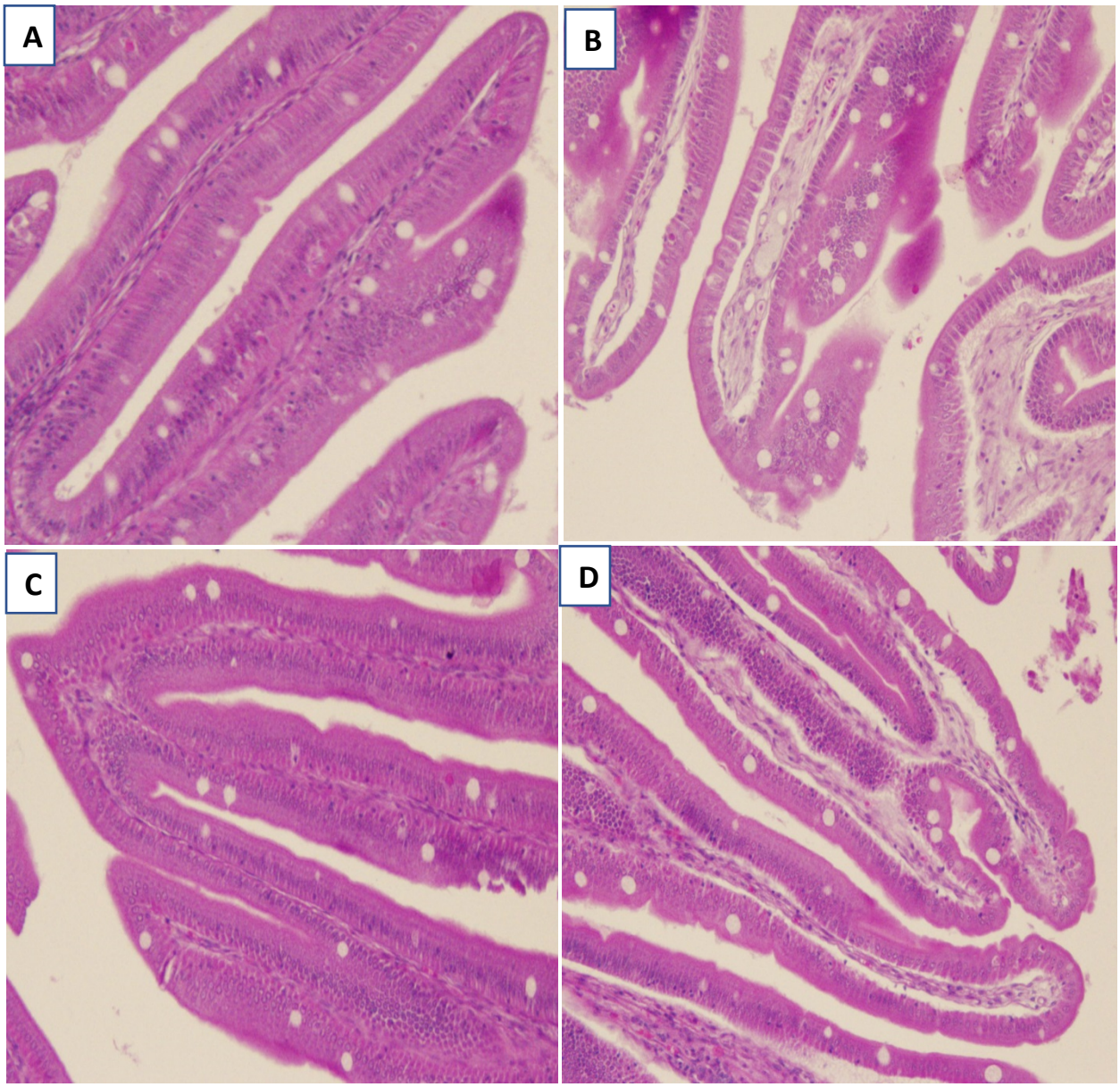


Figure 2. Representative histopathological images of hematoxylin and eosin-stained sections of distal intestines from Florida pompano after 56 d of being fed with (A) PBM, (B) basal, (C) 4% SH, and (D) 4% SM.



4. Discussion

The growth performance and feed utilization of the pompano fed with basal diet in this study was considerably lower than fish fed with 15% PBM, which is consistent with earlier studies (Rhodes et al., 2017; Rossi Jr and Davis, 2012). A number of findings suggest that palatability, digestibility and/or amino acid (AA) availability become problematic when animal meals are removed from diet formulation (Hajen et al., 1993; Rhodes et al., 2017). However, when 4% SH was added to the basal diet, it improved growth performance, TGC and FCR of fish similar to those maintained on the 15% PBM. The positive impact on the use of SH may be explained by the presence of bioactive peptides produced from enzymatic hydrolysis process, high content of essential amino acids and rich in taurine that is needed to improve the growth performance of fish (Kvåle et al., 2009; Lian et al., 2005). In addition, the hydrolysis process also degrades the protein molecule into smaller material and makes the products become more digestible and could be conveniently used as a potential chemoattractant (Carr, 1982) and growth promoter (Lian et al., 2005). Thus, these results may indicate that with proper inclusion level of SH into the plant-based diet, SH could be used as a palatability enhancer and nutrient source, leading to better performance of Florida pompano.

On the contrary, it is unclear why the addition of SM to the basal diet resulted in limited improvements in the growth performance of fish. We expected that with comparable nutritional value to SH, SM would also improve the efficacy of basal diet for pompano. Similarly, Berge et al. (1999) found that there was no significant effect on feed intake and specific growth rate (SGR) of Atlantic halibut *Hippoglossus hippoglossus* when FM and soy protein concentrate (SPC) based diet was coated with SM. Moreover, Kissinger et al. (2016) also indicated that no significant differences were detected in growth performance, hepatosomatic index and survival rate of

yellowtail *Seriola rivoliana* fed with SPC and *H. pluvialis* meal supplemented with various level of SM during partial FM replacement study. According to Cordova Murueta and Garcia Carreno (2001), the growth factors contained in SM are often severed by improper drying process to convert it into meal, lead to the reduction of nutritional value and inability to improve the growth performance of fish. In addition, several factors, such as the quality of SM, different diet formulation, rearing condition and species used for the trial may also influence the results (Zhou et al., 2016). However, it should be noted that final mean weight, feed intake and FCR of pompano in the present study was better as SM inclusion level increased. This suggests that with proper processing technique and inclusion level, SM may have potential to serve as an alternative source of protein and improve the efficacy of plant-based diet.

To further investigate the effect of dietary treatment to the whole-body composition of Florida pompano, twenty fish per dietary treatment (five fish per replicate) were randomly selected to be analyzed for proximate and mineral composition profile. No significant differences in terms of percentage protein, fat and ash content in the dry weight basis across all treatments were detected. The percentage composition of P and Ca were higher in the body of fish fed with basal diet compared to other dietary treatments. Similarly, no significant differences were detected in terms of crude protein, crude fat, moisture, and ash content in pompano when PBM was totally replaced by soy protein concentrate and plant-based diet supplemented with valine, glycine, and histidine (Rhodes et al., 2017). In addition, no significant differences were also observed in the final whole body when various levels of SBM were supplemented at moderate and high levels to replace FM in the diets for red sea bream, *Pagrus major* (Kader et al., 2012). This finding indicated that body composition of fish was not negatively affected by total replacement of animal meal with ESBM.

Screening of serum constituents together with biochemical analysis are important tools for indication of general health condition, physiological stress response and metabolic disturbance diagnosis (Blaxhall and Daisley, 1973; Maita et al., 2002). Results of the present study showed that the dietary treatments influence blood serum composition of pompano. Total protein (TP) values tended to be higher in fish fed with soy source diet with or without squid products compared to PBM. On the contrary, several studies concluded that serum TP levels was decrease with an increase of dietary SBM (Takagi et al., 2001; Ye et al., 2011). These contradictory results may have been caused by the use of ESBM, which contains higher levels of protein, low oligosaccharides and minimal levels of soy ANFs compared to SBM (Amezquita and Arana, 2015), and the use of squid products in the diet. As albumin constitutes most of the serum protein and it is possible to modulate serum protein both qualitatively and quantitatively, it was suggested that the albumin measurement should be performed together with TP levels analysis (Sandnes et al., 1988). Our present results showed that there is no significant difference in terms of albumin level, indicating that none of diets produced an adverse effect on the health status, nutrient intake and liver function of fish.

Dietary SBM is known as the source of sucrose, oligosaccharides, and complex non-starch polysaccharides (Krogdahl et al., 2005). According to Eldridge et al. (1979) glucose concentration in dehulled SBM is about 8.1%. Further processing of SBM still contains similar sugar composition, but the amount of each sugars was less. In our study, results of blood biochemistry analysis showed that fish fed with PBM had the lowest glucose level compared to other dietary treatments. Replacement of animal meal with ESBM slightly increased the glucose level and addition of small amounts of squid products did not reduce the glucose level. In Florida pompano, the effect of soy source protein on glucose homeostasis has been poorly studied. Lovell (1989)

suggests that fish have poorer control over the blood glucose and once it rises, it takes many hours to decrease. Considering the absorption of glucose as the major product of carbohydrate digestion is very efficient (NRC, 2011; Singh and Nose, 1967) and blood samples were taken 12 h after the last feeding, high glucose level in fish fed with carbohydrate-containing feedstuffs should not come as a surprise.

Various studies have investigated whether the use of dietary SBM or other plant-based protein source reduce the body pools of cholesterol and bile acid levels (Kaushik et al., 1995; Krogdahl et al., 2003; Sitjà-Bobadilla et al., 2005), due to the presence of hypocholesteromic effect (De Schrijver, 1990). Likewise, the use of SPC as an advanced product of SBM, to completely replace FM in diet formulation for European seabass (*Dicentrarchus labrax*) had lower total plasma triacylglycerols (TAG) and cholesterol (CHOL) levels compared to fish fed with FM (Kaushik et al., 2004). In contrast, our observations here with pompano do not confirm such relationship, where fish fed with PBM had lower cholesterol level compared to fish fed with basal and soy source protein supplemented with squid products. Data from Ye et al. (2011) also provide evidence that the cholesterol and triglyceride level in Japanese flounder *Paralichthys olivaceus* increased with the elevation of dietary SBM level. It is clear from Table 7, since bile salts have a role to emulsify cholesterols and aid them to be excreted from liver (Yamamoto et al., 2007), the lower value of bile salts in this study might be related to the lower value of cholesterol in fish fed PBM. However, given that all dietary treatments here contained similar amount of SBM, it is difficult to distinguish whether this effect are due to the inclusion of ESBM and squid products into the basal diet or the cholesterol level contain in PBM. In any case, the significance effect of ESBM and squid products to lower the cholesterol and their effects on cholesterol synthesis and metabolism effect in Florida pompano should be studied in more detail.

In the present study, determination of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was also performed to monitor the health status of liver in fish (Maita et al., 1985). Additionally, total alkaline phosphatase (ALP) activity was measured as a good indicator for liver and bone diseases diagnosis (Garnero and Delmas, 1993; Price et al., 1976), especially due to the dietary treatment (Salze et al., 2016). Since ALT and AST are released into blood during organ damage (Tietz and Andresen, 1986), increased levels of these enzymes gives information on organ dysfunction, specifically to the liver cells (Yuangsoi et al., 2014). In our study, there is no significant effect of dietary treatments to the ALT and AST activity, indicating that the dietary treatments did not cause any hepatocellular damage. However, the ALP activity in fish fed with PBM was significantly lower compared to fish fed with basal and soy source protein supplemented with squid products, which indicated a possible failure to maintain the skeletal growth (Salze et al., 2016). Similarly, a study from Xu et al. (2012) reported that the increase of dietary soy protein isolate over 50% to replace dietary FM significantly increased the ALP activity of juvenile Amur sturgeon *Acipenser schrenckii*. According to Salze et al. (2016) low ALP activity related to the decrease in serum protein. Our data confirm this finding, as they clearly show the decrease of serum protein level in fish fed PBM. However, the value of ALP activities might not be associated with dietary treatment and did not cause serious effect to pompano fed with PBM, since the growth performance of this fish was relatively better compared to other dietary treatments.

The intestinal and hepatic structure of fish can be used to evaluate the effect of dietary changes (Boglino et al., 2012) and can be negatively influenced by the inclusion of plant proteins in the diet (Rodiles et al., 2015). In this study, no severe histopathological damage was observed in the liver of fish. The examination of the liver showed a normal pattern in fish fed with PBM and slight

increase of vacuolization and glycogen deposition in fish fed with basal diet. The inclusion of 4% SH in the basal diet seems to partially restore the liver condition to that of fish fed PBM. Knowledge on the effects of high inclusion level of soy protein source in Florida pompano liver is limited. The histopathological change and a metabolic dysfunction in fish liver could be caused by lack of dietary taurine (Salze et al. 2016) or long-term intake of plant-based protein (Martínez-Llorens et al., 2012). It is also possible if that the hepatic vacuolization is evidence of metabolic dysfunction with high inclusion of SBM in the diet, thus affecting the nutrient utilization and growth performance of fish. However, further study is needed to confirm if this occurs in Florida pompano.

The inclusion of a high amount of SBM in formulated diets has been reported to induce inflammation in the distal intestine of fish (Nordrum et al., 2000; Refstie et al., 2000; Urán et al., 2009). The symptoms are characterized by increased amount of connective tissue; a profound infiltration of inflammatory cells in the lamina propria (Krogdahl et al., 2000; Refstie et al., 2000), a reduced number of supranuclear vacuoles in the absorptive epithelium (Trushenski. 2015); and increase in the number of goblet cells (Ng et al., 2005; Oehlers et al., 2011). In the current study, higher GC number was found in fish fed with basal and 4% SM in comparison to fish fed with PBM and 4% SH. The increase of GC could be due to the mechanical irritation and unsatisfactory process of protein digestion (Baeza-Ariño et al., 2016; Franco et al., 2015). The addition of 4% SH into the basal diet significantly reduced the presence of GC and showed a similar score with fish fed PBM. This suggests that the high concentration of peptides produced from enzymatic hydrolysis contained in SH lead to the greater absorption rate (Hou et al., 2017; Hou et al., 2015). Furthermore, in all sections of the intestine, a decrease in LP thickness with an increase of cellular infiltration was observed in fish fed with basal diet and 4% SM. Study from Baeza-Ariño et al.

(2016) showed that as the mixture of vegetable protein concentrate increase to replace FM from 30 to 90%, a decrease in the LP thickness was observed in Sea bream *Sparus aurata* fed with higher FM substitution. In addition, Kokou et al. (2015) noted that the leukocyte infiltration became more obvious as bioprocessed soy inclusion level increase to replace FM in the diet of Gilthead sea bream. In this study, with the inclusion of 4% SH into the basal diet, LP and cellular infiltration did not showed any differences compared to fish fed with PBM. From the point of view of digestibility, the level of peptides and free amino acid generated from hydrolysis process may afford more sufficient digestible protein which may be beneficial to the distal intestine condition of fish.

5. Conclusion

In the present study, the growth performance of Florida pompano fed PBM diets was highest compared to the basal diet. However, the addition of 4% SH into the basal diet improved the nutritional value of plant-based diet and partly prevented the decrease in growth performance, serum biochemistry parameters and the alteration of liver and intestine of pompano. In contrast, the inclusion of 1, 2 and 4% of SM into the basal diet did not significantly improve growth performance of pompano compared to the reference diet. The lack of significant differences in terms of growth performance between fish fed PBM and basal diet supplemented with 4% SH indicated that the combination of ESBM and SH has the potential to serve as an alternative protein source and attractant. Nevertheless, further investigation to determine the proper inclusion level of squid product or other attractants in plant-based diets are needed for pompano.

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Chapter VI

EFFECTS OF FERMENTED SOYBEAN MEAL TO REPLACE DIETARY FISH MEAL ON THE GROWTH PERFORMANCE AND FEED CONVERSION RATIO OF AQUACULTURE SPECIES: A META-ANALYSIS STUDY

Abstract

This study applied a meta-analysis approach to quantify the effect of replacing dietary fish meal (FM) with fermented soybean meal (FSBM) on the final weight and feed conversion ratio (FCR) of fishes. We examined the impact of 14 studies with 53 comparisons between fishes fed with various inclusion levels of FSBM and control treatments. FSBM inclusion levels of 8 % to 60 % resulted in mean effect size of -3.75 [95% confidence interval (CI) -4.49 to -3.01] for final weight and 1.26 [95% CI 0.58 to 1.94] for FCR. FSBM inclusion level greater than 40 % is likely to decrease the final weight of fish compared to the control treatment of the studies. Meanwhile, inclusion of FSBM at the level of 15 % to 44 % improves the FCR of the diet and higher than 44 % produces an inconsistent result. The present study contributes to the FM replacement debate by presenting numerical values and providing strong conclusions compared to the common narrative reviews about partial or total replacement of FM with FSBM.

Keywords: fermented soybean meal, fish meal, meta-analysis, FCR, final weight

1. Introduction

Partial or total replacement of dietary fish meal (FM) protein with a wide variety of plant-based dietary ingredients in fish feed formulation has been widely investigated and debated (NRC 2011; Caruso 2015; Ogello et al. 2017). So far, soybean meal (SBM) has been preferred for FM replacement due to the comparable nutritional value with FM, reasonable balance of amino acid and cost effectiveness (Gatlin et al. 2007; NRC 2011; Watanabe 2012; Qiu and Davis 2016). However, the presence of anti-nutritional factors (ANFs) such as protease inhibitors, tannins, oligosaccharide and phytate; low palatability and deficiency in some amino acids is limiting its use in aquaculture diets (Fowler 1980; Guimaraes et al. 2008; Sales 2009; Phumee et al. 2011; NRC 2011). A high inclusion level of SBM may cause undesirable taste to the diet (Okubu et al. 1992; Ho et al. 2014), induce extensive damage to the intestinal mucosa of the hindgut (Bureau et al. 1998), affect the intestinal microbial communities (Heikkinen et al. 2006), and alter the hepatic morphology (Iwashita et al. 1998). Although several treatments, e.g. heating, alcohol extraction and proper processing technique could eliminate or inactivate the limiting factors in SBM (Masumoto et al. 2001; Lim and Lee 2009; NRC 2011), different sensitivities of fish to SBM inclusion level cause large data variabilities (Chou et al. 2004).

Further processing of SBM, such as fermentation, has recently been proven to prevent the SBM induced abnormalities, eliminate a variety of ANFs, increase the content of soybean peptides and improve the nutritional value of the resulting meals (Papagianni et al. 2000; Hong et al. 2004; Gatlin et al. 2007). For human foods, the fermentation technique has been widely applied in the Far East and Southeast Asia. The fermented products are commonly known as “Dou-Bian-Jiang” in China, “Miso and Natto” in Japan (Lim and Lee 2011), “Thua nao” in Thailand (Chantawannakul et al. 2002) and “Tempeh” in Indonesia (Keuth and Bisping 1994). The

fermentation process, which allows microorganisms such as *Bacillus subtilis* to degrade macromolecules into water-soluble low molecular weight compounds (Kiers et al. 2000), has been utilised to destroy or decrease the ANFs present in SBM (Canella et al. 1984) and improve digestibility (Kiers et al. 2000) and shelf life of the processed foods (Skrede and Nes. 1988). Other than *Bacillus subtilis,* several other bacterial species, e.g *Aspergillus oryzae* (Kim et al. 2009), *Lactobacillus plantarum* P8 (Wang et al. 2016) and *Candida utilis* (Zhou et al. 2011) also play significant roles in fermentation processes. In addition, a commercial product of fermented soy known as PepSoyGen (PSG; Nutrafrema, North Sioux City, South Dakota, USA) manufactured via a proprietary process using *Aspergillus* spp and *Bacillus* spp is readily available as an ingredient to replace FM in fish diet formulation (Barnes et al. 2015; Trushenski et al. 2014).

Initial publication on the use of fermented soy was based on the study of Shimeno et al. (1993), who reported the effects of fermented defatted soybean meal either with *Aspergillus oryzae* or *Eurotium repens* in single moist pellet diets for juvenile yellowtail *Seriola quinqueradiata*. According to Shimeno et al. (1993), the feed efficiency and growth performance of yellowtail fed on FSBM were superior to those fed on unfermented SBM, but they were slightly inferior compared to the group of fish fed with dietary FM without any inclusion of soy-source protein. Recent studies with rainbow trout *Oncorhynchus mykiss* (Walbaum 1792) and black seabream *Acanthopagrus schlegelii* (Bleeker 1854) showed that with proper inclusion level, FSBM may improve the acceptance and utilisation of soy-based diet for carnivorous fish (Azarm and Lee 2014; Barnes et al. 2015). The good proportion of small-sized peptide contain in FSBM becomes one of the beneficial factors to induce better growth in fish, FCR and enhance the nutrient digestibility (Hong et al. 2004; Azarm and Lee 2014; Barnes et al. 2014). Moreover, the microbial species that remain in the final fermented product could also increase the antioxidant activities and non-specific

immune response of fish (Kim et al. 2010). However, several studies also suggest that the use of high inclusion level of FSBM may negatively affect the growth performance of fish (Yuan et al. 2013; Barnes et al. 2015; Wang et al. 2016; Lee et al. 2016). Therefore, this meta-analysis study was undertaken to determine the potential effect and proper inclusion level of FSBM to replace FM in practical diets without compromising the feed efficiency and growth of fish.

In this quantitative review, we employed a structured meta-analysis approach to quantify the effects of FSBM inclusion level on the growth and FCR across different fishes. Meta-analysis is a set of statistical methods combining outcomes across different data sets to examine the response patterns and heterogeneity in outcomes (Koricheva and Gurevitch 2014). Meta-analysis has had a tremendous impact on ecological studies, medicinal research and social science in synthesising particular research questions. In fact, since the 1970s, meta-analysis study has emerged in medical research and its growth has been exponential over time (Chalmers et al. 1977; Haidich 2010). However, to the best of our knowledge, only a few quantitative studies have used meta-analysis to investigate the effect of FM replacement with soy-source protein on growth performance of fish (Sales 2009). Since the sustainable and low-cost protein source is still and always needed to improve farm productivity and efficiency, our study will serve as a catalyst for further development of food formulation and generalize the effect of FSBM inclusion level to the feed efficiency and growth performance of fish.

2. Materials and Methods

2.1 Search strategy and inclusion criteria

The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines were followed (Liberati et al. 2009) and a comprehensive literature search was conducted with the use of Web of Science and several publishers' websites to identify articles published between January 1960 and October 2016 investigating the dietary inclusion effects of FSBM in the growth performance and FCR of fish. The database search used multiple combinations of the following terms: fermented, soybean, fish, fish meal replacement and growth. Thorough literature searches and repeatability became two important aspects for the literature search strategy (Gates 2002; Philibert et al. 2012) to maintain the study objectivity and reduce the possibility for publication bias (Koricheva and Gurevitch 2014). In addition, unpublished results and 'grey literature' (e.g. documents such as theses and dissertations) could also be included in meta-analysis study, especially when the results were coming from reliable sources (Cook 1993; ArchMiller et al. 2015).

Appropriate inclusion criteria were determined prior to the start of the database search to reduce any possible selection bias in the present study, including the clear diet preparation, experimental fish, control group, clearly defined exposure time, and clearly mentioned feeding regime. The evaluation of growth performance as the effect of FSBM inclusion was focused on the final weight for each treatment and FCR. The crude protein level of each diet and type of feeding were also evaluated to gain better consideration for meta conclusion. In addition, studies would only be considered if the article covered the following criteria: (1) the use of pure FSBM in the food formulations without any supplementation. Thus, supplementation with any additional products, such as animal by-products and other attractants to yield a unique taste and improve the

nutritional content of food was not considered; (2) isonitrogenous dietary information particularly on the crude protein ratio; (3) the reduction level of FM as the inclusion effect of FSBM in the dietary formulation; and (4) the assessment of growth performance provided sufficient details for effect size calculation (i.e., Hedges'*d* and 95 % confidence interval [CI]). The study criteria for the present quantitative assessment included:

Feed conversion ratio $\boldsymbol{FCR} = \frac{\textbf{Feed given (g)}}{\textbf{Wet weight gain (g)}}$

Studies fulfilling the above criteria and were considered eligible to be included in this meta-analysis study are presented in Table 1.

2.2 Effect size calculations

We calculated the Hedges'*d* (Gurevitch and Hedges 2001), a metric that has been commonly used in previous meta-analysis for the mean and standard deviation available in the article. The Hedges'*d* transforms all effect size to a common metric to estimate the effect of several FSBM inclusion levels in diet formulation on fish growth performance and FCR. The formula for the sample estimate of *d* is:

$$\boldsymbol{d} = \frac{\mu\mathbf{1} - \mu\mathbf{2}}{\mathbf{SE}}$$

Hedges'*d* compares the effect of size of the mean of one population (μ_1) and the mean for another population (μ_2) scaled by their respective standard errors (SE), so that the differences in *d* could be attributed to different effects on the mean response variable (Preisser et al. 2005). Positive and negative values of the Hedges'*d* indicate the performance of fish in the presence of FSBM in diet formulation. For all study groups, the standardized mean difference, the 95 % confidence interval (95 % CI), and the 95 % prediction interval (95 % PI) were computed. The PI describes the distribution of true effects around the mean, whereas the CI reflects the precision of the mean

effect size. We systematically assessed the heterogeneity among studies in each group by using random effect models and report the I^2 and the chi-squared statistic (Q). I^2 were calculated to assess the heterogeneity of effect sizes as a percentage of total variation and is not affected by low statistical power (Khoury et al. 2013).

2.3 Meta-regression analysis

The determination of which study-level covariates account for the heterogeneity was performed by using meta-regression approaches and FSBM inclusion level as the predictors. From this regression, we were able to assess the relationship between one or more variables (moderators) and the pooled effect size. In this meta-analysis, meta-regressions were performed under the random effects models that allow that the true effect may vary from one study to another (Borenstein et al. 2009). All analysis was performed by computing standardized difference by using Comprehensive Meta Analysis (Borenstein et al. 2009).

3. Results

3.1 Data extraction

We identified 68 publications generated by Web of Science and 85 publications generated by the Google Scholar, including two dissertations that discussed about the use of FSBM. Preliminary searches and coding revealed 21 different fish species and one hybrid fish. The fermentation process involves a simple treatment of soaking the (non-sterile) SBM in distilled water and allowing microorganisms as a reliable source of enzyme to undergo the fermentation process (Bi et al. 2015). This fermentation process may reduce the ANFs such as phytates, protease (trypsin) inhibitors, antigens, lectins, and tannins contain in SBM that affect the growth

performance, protein and mineral utilization, and digestion of the fish (Shiu et al. 2015; Chi and Cho 2016). We carefully assessed the identified publications and applied the exclusion criteria, resulting in 30 studies consisting of 28 publication articles and two dissertations. Of the 30 studies, 14 met our eligibility criteria outlined in the Materials and Methods section. In total, there were 53 independent data sets that were investigated in our meta-analysis study for both final weight and FCR. Details of the studies are summarized in the sources of studies (Table 1).

Table 1. Source of study consist of 53 independent data sets in 14 published articles.

No	References	Common name	Scientific name	Fermentation type	Water type	Sample size (n)	Period (days)	Dietary CP (% DM)	FSBM (%)	FM (%)
1	Azarm & Lee 2014	Black sea bream	*Acanthopagrus schlegeli*	*Bacillus subtilis*	SW	40	56	43.7	0	60
								44.5	8	54
								44.0	16	48
								43.0	24	42
								47.1	32	36
2	Barnes et al. 2014	Rainbow trout	*Oncorhynchus mykiss*	*Bacillus subtilis*	WW	40	205	45.39	0	40
				Aspergillus oryzae				45.95	35	15
								46.96	50	0
3	Lee et al. 2016	Rockfish	*Sebastes schlegeli*	*Bacillus subtilis*	SW	50	56	51.6	0	58
								51.6	8	52
								51.6	16	46
								51.5	24	40
								51.4	32	34

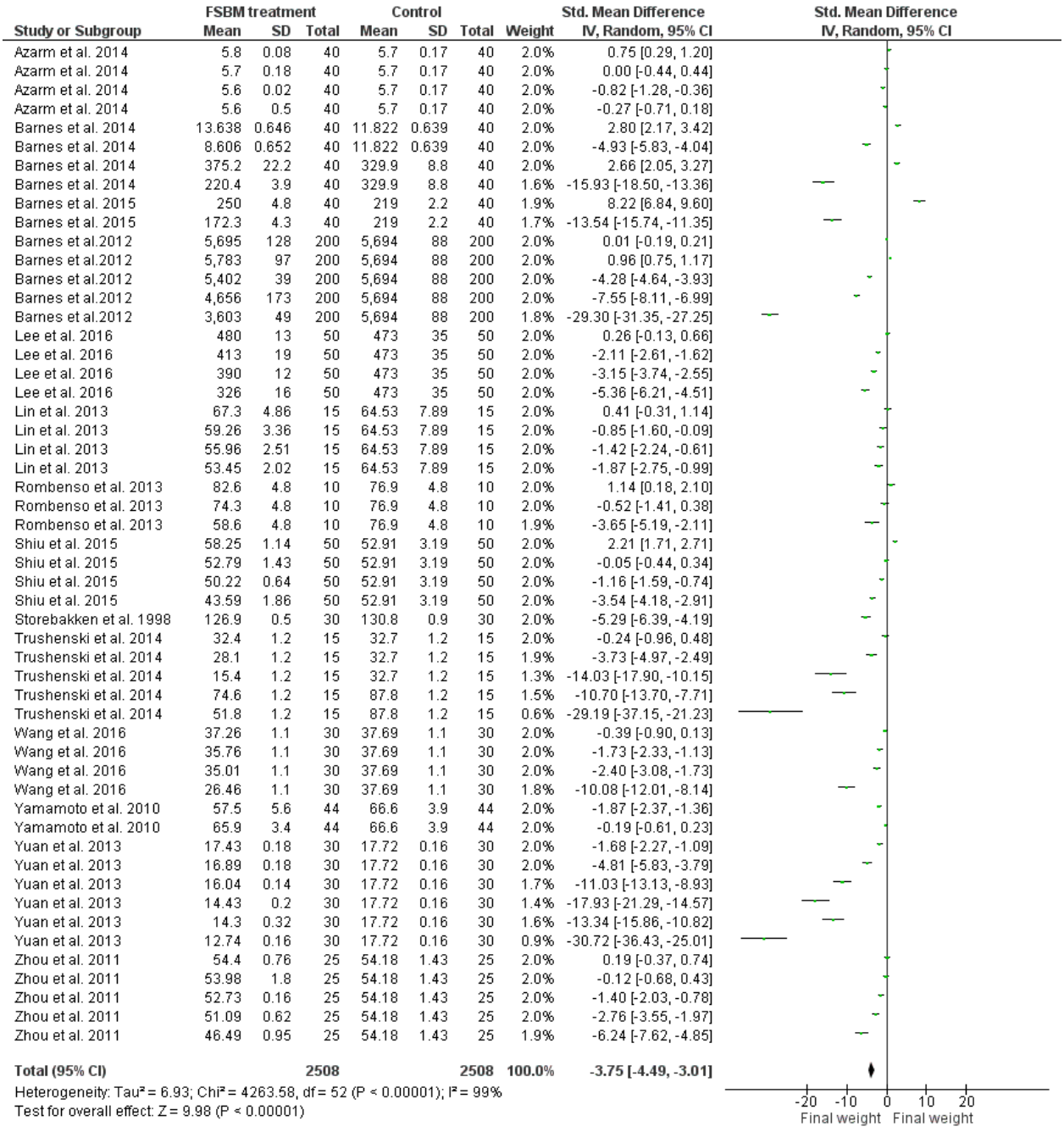
4	Lin et al. 2013	Pompano	*Trachinotus ovatus*	*Bacillus subtilis*	SW	15	NA	46.4 46.2 47.1 47.2 46.6	0 100 200 300 400	65.0 57.3 49.6 41.9 34.2
5	Shiu et al. 2015	Orange spotted grouper	*Epinephelus coioides*	*Bacillus subtilis*	SW	50	84	48.62 48.82 48.69 48.82 48.96	0 9.8 19.6 29.4 39.2	69.6 62.6 55.7 48.7 41.8
6	Storebakken et al. 1998	Atlantic salmon	*Salmo salar*	*Bacillus* spp	FW	30	NA	42.7 41.9	0 20	54.48 34.40
7	Trushenski et al. 2015	White seabass	*Atractoscion nobilis*	*Bacillus subtilis* *Aspergillus oryzae*	SW	15	68	51.1 50.6 50.2 48.6	0 15 25 47.7	48.0 24 12 0
		Yellowtail Jack	*Seriola lalandi*		SW	15	65	50.9 49.3 49.8	0 46.2 52.1	40 20 0
8	Zhou et al. 2011	Black sea bream	*Acanthopagrus schlegeli*	*Candida utilis*	SW	25	56	41.4 41.38 41.3 41.25 41.07	0 7.2 14.4 21.6 28.8	60 54 48 42 36

								41.15	36	30
9	Yamamoto et al. 2010	Rainbow trout	*Oncorhynchus mykiss*	*Bacillus* spp	WW	44	70	44.2 44.0 44.2	0 47.6[1] 47.6[2]	46 0 0
10	Rombenso et al. 2013	Hybrid striped bass	*Morone chrysops* × *M. saxatilis*	*Bacillus subtilis* *Aspergillus oryzae*	FW	10	56	37.3 38.7 39.5 38.9	0 30.3 38.1 46.3	30 10 5 0
11	Wang et al. 2016	Turbot	*Scophthalmus maximus*	*Lactobacillus plantarum* P8	SW	30	66	50.36 50.38 49.50 49.65 50.18	0 11.53 23.08 34.62 46.15	60 51 42 33 24
12	Barnes et al. 2015	Rainbow trout	*Oncorhynchus mykiss*	*Bacillus subtilis* *Aspergillus oryzae*	WW	40	94	45.39 45.95 46.96	0 35 50	40 15 0
13	Yuan et al. 2013	Chinese sucker	*Myxocyprinus asiaticus*	High active microbe[3]	FW	30	56	52.4 52.8 51.1 52.4 53.5 55.1 54.3	0 13 21.7 30.4 39.1 47.8 56.5	65 55.3 48.8 42.3 35.8 29.3 22.8
14	Barnes et al. 2012	Rainbow trout	*Oncorhynchus mykiss*	*Bacillus subtilis* *Aspergillus oryzae*	FW	200	70	52.0 50.5 48.1 46.3 44.1 43.0	0 10 20 30 40 50	50 40 30 20 10 0

3.2 Effect size calculations

The effect size and other statistical characteristics for each study are shown in Table 2 and 3. Studies were divided according to the response of interest, namely final weight and FCR. The overall effect size of the 53 comparisons between FSBM inclusion level in diet formulation and a control condition was -3.75 [95 % CI -4.49 to -3.01] for final weight and 1.26 [95 % CI 0.58 to 1.94] for FCR. Using a random-effect analysis for final weight, negative risk ratio higher than 1.0 indicates that increasing level of FSBM increased the risk for negative growth performance. Meanwhile, the inclusion of FSBM improved the FCR by at least 58 % and possibly as much as 94 %. The level of heterogeneity for final weight is I^2=99 % ($p<0.00001$) and FCR I^2=99 % ($p<0.00001$). The Z-value for final weight is 9.98 ($p<0.00001$) and FCR is 3.64 ($p<0.00001$), which allows us to predict that the slope is probably not zero, and the FSBM inclusion level is more effective when the study is conducted at a closer distance from the equator of meta-regression analysis. Meta-regression is used to relate the size of a treatment effect obtained from a meta-analysis, to a certain inclusion level of FSBM and describe the heterogeneity between studies. Each study for final weight (Fig. 1) and FCR (Fig. 2) in meta-regression, is represented by a circle that shows the actual coordinates (observed effect size by latitude) for that study and the center lines shows the predicted value. From Fig. 1, the study performed relatively close to zero, corresponding to the inclusion level of FSBM ranging from 8 % to 40 %, elicited the best response in terms of final weight. Meanwhile, as the inclusion level of FSBM to replace FM rose higher than 40 %, the final weight of fish tends to decrease. On the other hand, Fig. 2 showed that the inclusion of FSBM is more effective at the level of 15 % to 44 % to improve the FCR of the diet. While with higher inclusion level of FSBM (>44 %), dietary treatments would have a varied effect on the FCR.

Table 2. Standard mean difference, 95 % CI, 95% PI and statistical characteristics of final weight group.



Study or Subgroup	FSBM treatment Mean	SD	Total	Control Mean	SD	Total	Weight	Std. Mean Difference IV, Random, 95% CI
Azarm et al. 2014	5.8	0.08	40	5.7	0.17	40	2.0%	0.75 [0.29, 1.20]
Azarm et al. 2014	5.7	0.18	40	5.7	0.17	40	2.0%	0.00 [-0.44, 0.44]
Azarm et al. 2014	5.6	0.02	40	5.7	0.17	40	2.0%	-0.82 [-1.28, -0.36]
Azarm et al. 2014	5.6	0.5	40	5.7	0.17	40	2.0%	-0.27 [-0.71, 0.18]
Barnes et al. 2014	13.638	0.646	40	11.822	0.639	40	2.0%	2.80 [2.17, 3.42]
Barnes et al. 2014	8.606	0.652	40	11.822	0.639	40	2.0%	-4.93 [-5.83, -4.04]
Barnes et al. 2014	375.2	22.2	40	329.9	8.8	40	2.0%	2.66 [2.05, 3.27]
Barnes et al. 2014	220.4	3.9	40	329.9	8.8	40	1.6%	-15.93 [-18.50, -13.36]
Barnes et al. 2015	250	4.8	40	219	2.2	40	1.9%	8.22 [6.84, 9.60]
Barnes et al. 2015	172.3	4.3	40	219	2.2	40	1.7%	-13.54 [-15.74, -11.35]
Barnes et al.2012	5,695	128	200	5,694	88	200	2.0%	0.01 [-0.19, 0.21]
Barnes et al.2012	5,783	97	200	5,694	88	200	2.0%	0.96 [0.75, 1.17]
Barnes et al.2012	5,402	39	200	5,694	88	200	2.0%	-4.28 [-4.64, -3.93]
Barnes et al.2012	4,656	173	200	5,694	88	200	2.0%	-7.55 [-8.11, -6.99]
Barnes et al.2012	3,603	49	200	5,694	88	200	1.8%	-29.30 [-31.35, -27.25]
Lee et al. 2016	480	13	50	473	35	50	2.0%	0.26 [-0.13, 0.66]
Lee et al. 2016	413	19	50	473	35	50	2.0%	-2.11 [-2.61, -1.62]
Lee et al. 2016	390	12	50	473	35	50	2.0%	-3.15 [-3.74, -2.55]
Lee et al. 2016	326	16	50	473	35	50	2.0%	-5.36 [-6.21, -4.51]
Lin et al. 2013	67.3	4.86	15	64.53	7.89	15	2.0%	0.41 [-0.31, 1.14]
Lin et al. 2013	59.26	3.36	15	64.53	7.89	15	2.0%	-0.85 [-1.60, -0.09]
Lin et al. 2013	55.96	2.51	15	64.53	7.89	15	2.0%	-1.42 [-2.24, -0.61]
Lin et al. 2013	53.45	2.02	15	64.53	7.89	15	2.0%	-1.87 [-2.75, -0.99]
Rombenso et al. 2013	82.6	4.8	10	76.9	4.8	10	2.0%	1.14 [0.18, 2.10]
Rombenso et al. 2013	74.3	4.8	10	76.9	4.8	10	2.0%	-0.52 [-1.41, 0.38]
Rombenso et al. 2013	58.6	4.8	10	76.9	4.8	10	1.9%	-3.65 [-5.19, -2.11]
Shiu et al. 2015	58.25	1.14	50	52.91	3.19	50	2.0%	2.21 [1.71, 2.71]
Shiu et al. 2015	52.79	1.43	50	52.91	3.19	50	2.0%	-0.05 [-0.44, 0.34]
Shiu et al. 2015	50.22	0.64	50	52.91	3.19	50	2.0%	-1.16 [-1.59, -0.74]
Shiu et al. 2015	43.59	1.86	50	52.91	3.19	50	2.0%	-3.54 [-4.18, -2.91]
Storebakken et al. 1998	126.9	0.5	30	130.8	0.9	30	2.0%	-5.29 [-6.39, -4.19]
Trushenski et al. 2014	32.4	1.2	15	32.7	1.2	15	2.0%	-0.24 [-0.96, 0.48]
Trushenski et al. 2014	28.1	1.2	15	32.7	1.2	15	1.9%	-3.73 [-4.97, -2.49]
Trushenski et al. 2014	15.4	1.2	15	32.7	1.2	15	1.3%	-14.03 [-17.90, -10.15]
Trushenski et al. 2014	74.6	1.2	15	87.8	1.2	15	1.5%	-10.70 [-13.70, -7.71]
Trushenski et al. 2014	51.8	1.2	15	87.8	1.2	15	0.6%	-29.19 [-37.15, -21.23]
Wang et al. 2016	37.26	1.1	30	37.69	1.1	30	2.0%	-0.39 [-0.90, 0.13]
Wang et al. 2016	35.76	1.1	30	37.69	1.1	30	2.0%	-1.73 [-2.33, -1.13]
Wang et al. 2016	35.01	1.1	30	37.69	1.1	30	2.0%	-2.40 [-3.08, -1.73]
Wang et al. 2016	26.46	1.1	30	37.69	1.1	30	1.8%	-10.08 [-12.01, -8.14]
Yamamoto et al. 2010	57.5	5.6	44	66.6	3.9	44	2.0%	-1.87 [-2.37, -1.36]
Yamamoto et al. 2010	65.9	3.4	44	66.6	3.9	44	2.0%	-0.19 [-0.61, 0.23]
Yuan et al. 2013	17.43	0.18	30	17.72	0.16	30	2.0%	-1.68 [-2.27, -1.09]
Yuan et al. 2013	16.89	0.18	30	17.72	0.16	30	2.0%	-4.81 [-5.83, -3.79]
Yuan et al. 2013	16.04	0.14	30	17.72	0.16	30	1.7%	-11.03 [-13.13, -8.93]
Yuan et al. 2013	14.43	0.2	30	17.72	0.16	30	1.4%	-17.93 [-21.29, -14.57]
Yuan et al. 2013	14.3	0.32	30	17.72	0.16	30	1.6%	-13.34 [-15.86, -10.82]
Yuan et al. 2013	12.74	0.16	30	17.72	0.16	30	0.9%	-30.72 [-36.43, -25.01]
Zhou et al. 2011	54.4	0.76	25	54.18	1.43	25	2.0%	0.19 [-0.37, 0.74]
Zhou et al. 2011	53.98	1.8	25	54.18	1.43	25	2.0%	-0.12 [-0.68, 0.43]
Zhou et al. 2011	52.73	0.16	25	54.18	1.43	25	2.0%	-1.40 [-2.03, -0.78]
Zhou et al. 2011	51.09	0.62	25	54.18	1.43	25	2.0%	-2.76 [-3.55, -1.97]
Zhou et al. 2011	46.49	0.95	25	54.18	1.43	25	1.9%	-6.24 [-7.62, -4.85]
Total (95% CI)			**2508**			**2508**	**100.0%**	**-3.75 [-4.49, -3.01]**

Heterogeneity: Tau² = 6.93; Chi² = 4263.58, df = 52 (P < 0.00001); I² = 99%
Test for overall effect: Z = 9.98 (P < 0.00001)

Table 3. Standard mean difference, 95% CI, 95% PI and statistical characteristics for feed conversion ratio (FCR) group.

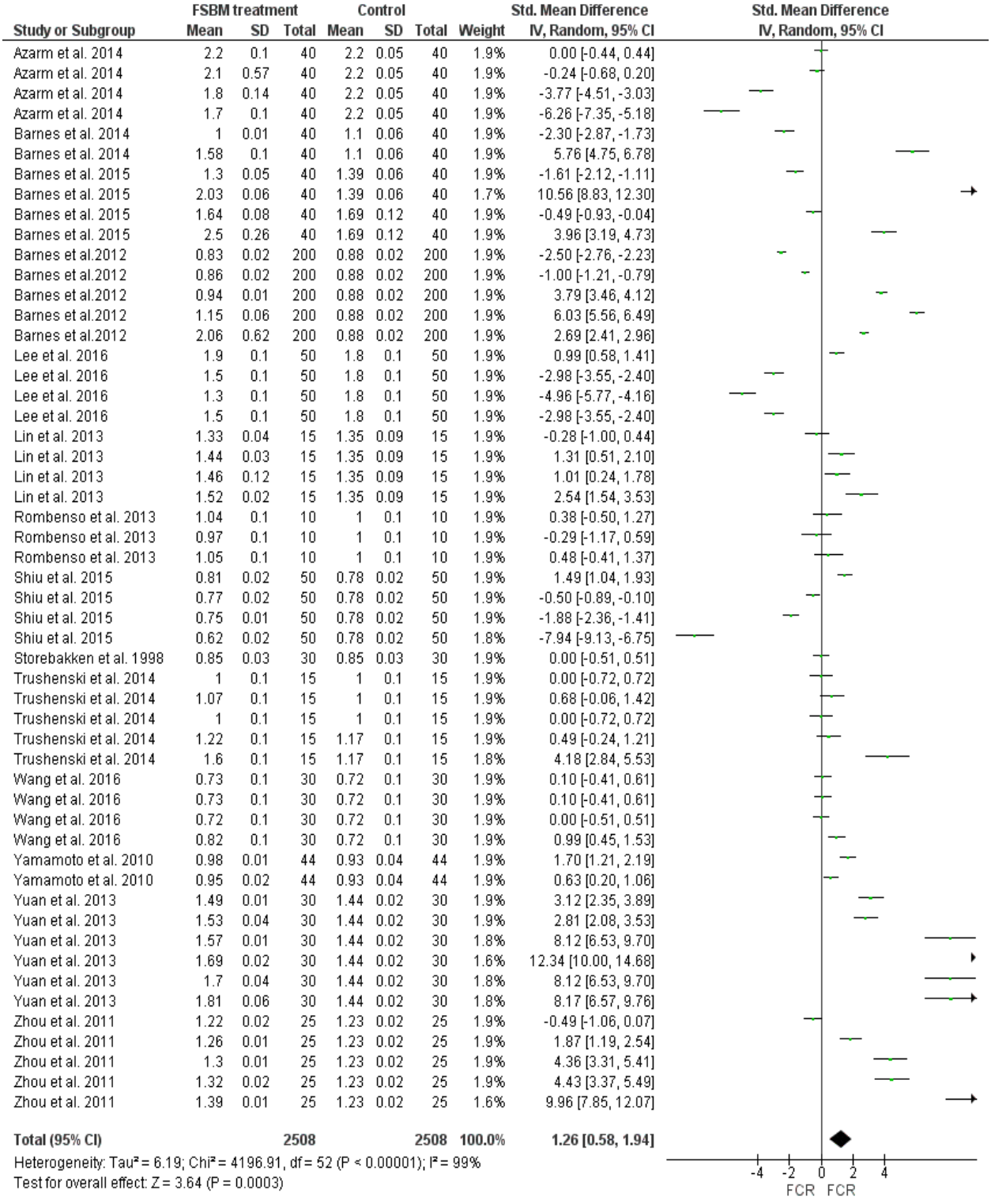


Fig. 1. Random-effect model-regression of inclusion percentage of fermented soybean meal (FSBM) on standard difference (std diff) in means of final weight

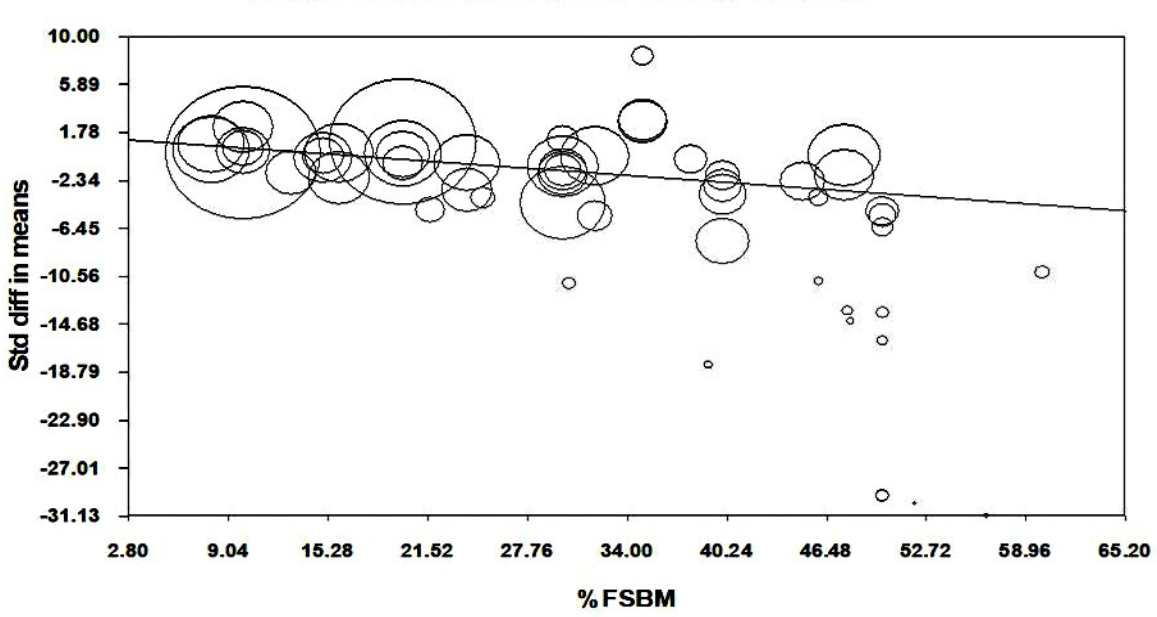
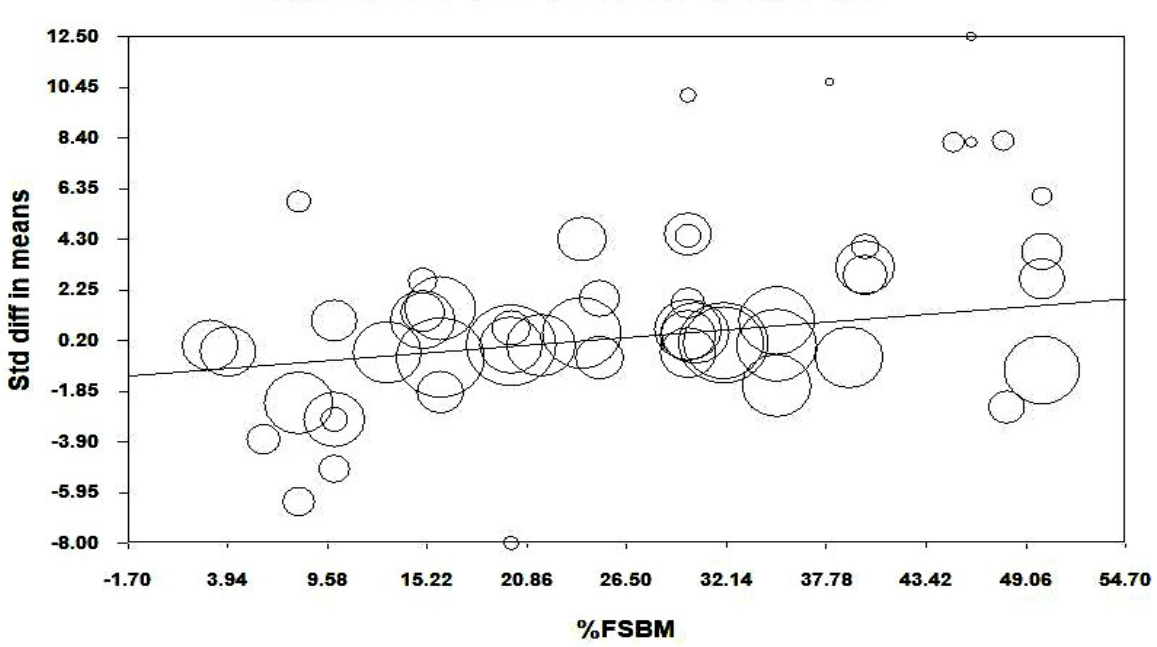


Fig. 2. Random-effect model-regression of inclusion percentage of fermented soybean meal (FSBM) on standard difference (std diff) in means of FCR



4. Discussion

The use of soy-source protein to replace dietary FM in an effort to develop practical diets for fish has been reviewed in several published articles (El-Sayed 1999; Gatlin et al. 2007; Sales 2009) and summarized in a book edited by Lim et al. (2008). The discussions point out that the beneficial effect of SBM depends on fish species, size and the quality of SBM used in the feed formulation (Watanabe 2002). The presence of anti-nutrients, such as proteinase inhibitor, lectins, phytic acid, saponins, phytoestrogens, antivitamins, phytosterols and antigens may limit the higher inclusion of SBM as the primary protein sources (NRC 2011). Sales (2009) suggested that the replacement of FM with defatted SBM at levels higher than 40 % causes negative effects on the growth performance of fish.

Currently, no quantitative review is available on the use of FSBM to improve the growth performance of fish. However, several authors concluded that the use of fungal or bacterial organisms during the fermentation process can lead to the production of enzyme to increase the digestibility (Lio and Wang 2012), reduce the ANFs (Jiao et al. 1992; Kiers et al. 2000), enhance the content of soybean peptides (Hong et al. 2004; Bi et al. 2015) and increase the amino acid content in soybean, such as arginine, serine, threonine, aspartic acid, alanine and glycine by 50.67 %, 45.6 %, 34.55 %, 22.25 %, 21.23 %, and 18.12 %, respectively (Foley et al. 2013). The initial attempt to reveal the efficacy of FSBM without any supplementation in the final product based on the Web of Science and Google Scholar searching method was conducted by Shimeno et al. (1993) and, since then, the investigations concerning the use of fermented soybean meal in fish diet formulations became more popular and finally the commercial products of FSBM were readily available in the market for aquaculture purposes (Barnes et al. 2014; Barnes et al. 2015).

The wide variety of studies, environmental characteristics and type of feeding regime resulting in high heterogeneity and meta-analytics will allow us to clarify some inconsistencies concerning the use of FSBM. The results of effect size calculation and meta-regression analysis in the present study indicated that the use of high inclusion level of FSBM (> 40 %) in diet formulation to partially or totally replace FM will negatively affect the growth of fish. Lee et al. (2016) noted that FM replacement with FSBM up to 20 % improved the growth of rockfish *Sebastes schlegeli* (Hilgendorf 1880), while the replacement for more than 40 % caused an adverse effect on growth of this fish. In addition, Wang et al. (2016) reported that the replacement of FM with FSBM by 60 % significantly reduced growth and feed utilization and lowered the apparent digestibility coefficient of protein in juvenile turbot *Scophthalmus maximus* (Linnaeus 1758). Several previous studies have reported that the use of higher inclusion level of soy-based protein may increase the indigestible carbohydrate levels, poor protein digestibility, imbalanced dietary amino acid concentration and affect the palatability of the diet (Refstie et al. 1998; Francis et al. 2001; Deng et al. 2006). Thus, dietary formulation would need to be modified to improve the efficacy of FSBM and growth of fish.

To improve the efficacy of FSBM, amino acid supplementation and inclusion of attractants could be used in the diet formulation. Nguyen et al. (2015), showed that taurine supplementation in high inclusion level of FSBM, significantly improved the growth and lipid digestibility of yellowtail. Similarly, the combination uses of methionine, lysine and fermented soy improved the growth performance and body protein content of rainbow trout *Oncorhynchus mykiss* compared to fish fed unsupplemented FSBM (Yamamoto et al. 2012). Moreover, the combination of FSBM with attractants or fishery by-products also improves the growth performance of fish through better food palatability (Kader et al. 2011). Thus, the inclusion of attractants and essential amino acids may be needed to enhance the food search (Hartati and Briggs 1993) and the efficacy of FSBM (Novriadi et al. 2017).

In this quantitative review, the inclusion of FSBM showed a positive effect on FCR at the inclusion level ranging from 15 to 44 %. At this level, fermented product appears to improve the nutritional and functional properties of SBM, probably due to the presence of soybean peptides (Min et al. 2009; Rombenso et al. 2013) and inactivation of most anti-nutrients contained in soy-source protein (Lee et al. 2016). It was interesting to observe that with high inclusion level of FSBM for more than 44 % to replace FM produces an inconsistent result to FCR but no adverse effect on feed intake (FI). Wang et al. (2016) reported that no significant differences were observed in FI when juvenile turbot (*Scophthalmus maximus*) was fed diets with graded levels of FSBM ranging from 15 to 60 %, but FCR was significantly increased as the dietary inclusion level of FSBM increases. On the other hand, Zhou et al. (2011) reported that FI was significantly decreased as FSBM inclusion level increased and positively correlated to the FCR. Indeed, it has been suggested that fermented product may still contain ANFs and play a role in the feed efficiency

(Yamamoto et al. 2012). Thus, species-specific sensitivity to the fermented product may partially influence the observed differences in feed efficiency.

5. Conclusion

With different culture settings, fish may react differently when exposed to the diet supplemented with FSBM to replace FM as their protein sources. Here, we systematically reviewed the effect of various inclusion levels of FSBM to replace FM on the growth and FCR of fish. The effect size of 53 comparisons data between FSBM inclusion level in diet formulation and a control condition was -3.75 [95 % CI -4.49 to -3.01] for final weight and 1.26 [95 % CI 0.58 to 1.94] for FCR. According to meta-regression analysis, FSBM inclusion level of 8–40 % may improves the final weight of fish. Meanwhile, inclusion level of FSBM higher than 40 % will likely decrease the final weight of fish compared to fish that received high percentage of FM. On the other hand, the inclusion of FSBM is more effective at the level of 15–44 % to improve the FCR of the diet and inclusion levels out of this range would produce various effects to the FCR. Although FSBM does not appear to be a better protein source than FM, especially when included with high inclusion level, the threshold effect showed that the use of FSBM was able to decrease the levels of FM in diet formulation. Baseline values presented in this study conclude that with proper inclusion level, FSBM could become an excellent candidate as a source of protein in the development of practical diet for aquaculture species.

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Chapter VII

SUMMARY AND CONCLUSION

In response to the expansion of global aquaculture, research efforts to produce proper and economically sound food formulation is very important to improve the production efficiency and the quality of the fish. In case of cost, many researches point out the need to replace marine ingredients in the diet formulation with more sustainable and low-cost protein sources, such as soybean meal (SBM). However, despite SBM possesses many of the qualities required to be a viable alternative to fish meal in formulating aquatic animal feed, phosphorus deficiency, energy density, palatability problem, deficiency in some of essential amino acids, and the presence of anti-nutritional factors, such as proteinase inhibitors, lectin, phytic acid, saponins and phytosterols that responsible for the slow growth, reduced feed intake and histomorphological change in the distal intestinal when the inclusion level of SBM exceeds a certain level in the diet, limit the wider use of this protein source. Thus, further processing technique to the conventional or de-hulled SBM may improve the nutritional value, digestibility and palatability of resulted meal through several additional process, such as extraction, fermentation or enzymatic treatment. Considering the production cost, blending various advanced soy products in combination with high inclusion level of conventional SBM is likely to be a viable strategy to for replacing dietary animal meal.

Today, several commercial advanced processing products of SBM that are considered as a promising alternative for the substitution of animal meal already available in the market, including soy protein concentrate (SPC), enzyme-treated soy (ESBM), and fermented soybean meal (FSBM). The use of SPC produced from high quality, dehulled soybean seeds by removing most

of the oil and water-soluble non -protein constituents have been shown to have the ability to improve the efficacy of plant-based diet. Previous research with advanced soy products in pompano indicated that the inclusion of 120 g kg^{-1} of SPC in combination with 370 g kg^{-1} of conventional SBM could reduce the inclusion level of fish meal from 300 g kg^{-1} to 150 g kg^{-1} without cause any significant differences in growth performances. However, knowledge on the use of ESBM and FSBM in the development of practical diet for Florida pompano are still limited.

Under the experimental conditions, ESBM produced through a series of non-alcohol extraction process and enzymatic treatment from high quality de-hulled soybeans can be utilized to replace the use of animal meal without any adverse effects on growth and nutritive parameters. Two growth trials to analyze the effect of partial and complete replacement of dietary FM with various inclusion levels of ESBM resulted in not significant differences in terms of growth performance (i.e., final weight, percentage weight gain, FCR and thermal growth coefficient) when the inclusion of fish meal (FM) reduced from 150 g kg^{-1} to 120 g kg^{-1} in the diet formulation. Reducing FM content further (60 g kg^{-1} in trial 1 and complete replacement of FM in trial 2) resulted in significant reductions in growth parameters. In addition, no significant differences in terms of crude protein, moisture, ash content, serum levels and enzyme activities as FM was substitute with various inclusion levels of ESBM. However, fish fed with lowest level of FM showed an inflammation, glycogen granulation, and nuclear change in the liver as well as the significant increase in the number of goblet cells, cellular infiltration and width of lamina propria than fish fed 15% FM. Interestingly, none of these alteration in the liver and distal intestine was observed when FM reduced from 150 g kg^{-1} to 120 g kg^{-1}.

In other chapter of research with ESBM, complete replacement of 150 g kg^{-1} poultry by product meal (PBM) exhibited significantly lower growth performance, feed utilization, disorder vacuolization in the liver and reduction in the thickness of the lamina propria in the distal intestine of the fish. However, the addition of 4% squid hydrolysates (SH) into this diet improved the nutritional value of plant-based diet and partly prevented the decrease in growth performance, serum biochemistry parameters and the alteration of liver and intestine of pompano. In contrast, the inclusion of 1, 2 and 4% of squid meal (SM) together with 1 and 2% of SH into the basal diet did not significantly improve growth performance of pompano compared to the fish fed with 150 g kg^{-1} of PBM in their diet formulation. The low quality of the squid and unproper manufacture process to produce the SM may influence the growth factors available in the final product, so that various inclusion levels of SM did not able to induce better growth of fish fed with plant-based diet. Furthermore, other chapter that focus on the comparative effect of advanced soy products showed that increasing the inclusion levels of ESBM up to 451 g kg^{-1} supplemented with 38 g kg^{-1} of porcine meal (PM) to completely replace the inclusion of animal meal and traditional SBM did not cause any adverse effects on growth, nutritive parameters and histomorphological conditions of the distal intestine. Based on field observation, high inclusion levels of ESBM also did not cause any palatability problem to the diet.

Regarding to the use of FSBM, fermentation process with several microorganisms to improve the functional properties and nutritional quality of SBM also has potential to improve the nutritional and health quality of plant-based diet due to the lower level of anti-nutrients, higher content of soybean peptides, better crude protein digestibility and ability to prevent various physiological abnormalities and restores the condition of the lamina propria of mucosal folds in the distal intestine of some species of fish. In this research, as the inclusion of FSBM to replace

the utilization of traditional SBM increases, it is notable that the fermented soy was able to prevent the histological alteration in the liver and distal intestine of Florida pompano. However, increasing the level of FSBM beyond the proper inclusion level lead to the slow growth of the fish and may cause palatability problem to the diet. Based on our comparative effect trial, inclusion of 535 g kg^{-1} FSBM lead to the low feeding activity compared to the high inclusion level of ESBM or moderate level of corn protein concentrate (CPC) fortified with PM. Therefore, feeding pompano with diets containing high inclusion level of fermented product should be carefully considered and appropriate supplementation level of limiting amino acids is needed to support an optimum growth for Florida pompano. Our quantitative review on the use of FSBM confirms these findings. Based on 53 comparisons data proceed with meta-analysis regression analysis, the utilization of FSBM higher than 40 % within the diet formulation will likely decrease the final weight of fish compared to fish that received high percentage of animal meal.

In conclusions, under the experimental conditions, ESBM and FSBM could be used as an acceptable protein source for Florida pompano. The use of these ingredients did not cause any significant alterations in the nutrient profile of the whole body of pompano, serum biochemistry and the histomorphological conditions of the liver and distal intestine of the fish. These results demonstrate that pompano is very tolerant to the soy-based diet and proper supplementation with attractants, limiting amino acids and other bioactive peptides may have the potential to improve the efficacy of plant-based diet.