STUDIES ON ALTERNATIVE PROTEIN SOURCES FOR FISHMEAL IN CULTURED MARINE SPECIES

（海産養殖魚における魚粉代替タンパク質に関する研究）

MD. ABDUL KADER

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MD. ABDUL KADER

A thesis submitted in partial fulfillment of the requirements for the degree of

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Dedicated to

My father, mother, sister, brother

MY DEAREST DAUGHTER & WIFE

And all those who inspired me during my entire study life

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ABSTRACT

The success of the further expansion of aquaculture industry will depend in part on reduction in fishmeal use in aquafeed because of its increasing demand, unstable supply and high price. There is need, therefore, to look for sustainable alternatives. This research work was conducted to evaluate the effects of several alternative proteins in the performances of marine fish and find out cost-effective approaches to maximize the utilization of alternative proteins in aquafeeds.

Seafood processing by-products are potential alternative to FM in aquafeed. Soybean meal was mixed with scallop by-products and squid by-products at the ratio of 3:2 and 1:1 respectively, and fermented with combined bacteria (predominantly *Bacillus spp*) to prepare fermented soybean meal and scallop by-product blend (FSSc), and fermented soybean meal and squid by-product blend (FSSq). Two batches of diets were prepared by replacing 15, 30, 45 and 60; and 12, 24, 36 and 48% of fishmeal protein with FSSc and FSSq; and fed to red sea bream (*Pagrus major*) and Japanese flounder (*Paralichthys olivaceus*) for 45 and 56 days respectively. The results demonstrated that weight gain (%) and specific growth rate (SGR, % day^{-1}) of fish were not significantly ($P > 0.05$) different among control, 15 and 30% FSSc containing diets in red sea bream; and control, 12, 24 and 36% FSSq containing diets in Japanese flounder. Feed efficiency ratio (FER) and protein efficiency ratio (PER) were also followed the similar trend as with growth performances of fish. However, growth and feed utilizations were significantly ($P < 0.05$) decreased in fish fed higher levels of fermented products in both the cases. In conclusion, the approach of utilizing fermented products is promising and it could replace at least 30 and 36% fishmeal protein in red sea bream and Japanese flounder diets respectively while ensuring performances of fish.

Second stage of the study was aimed to investigate an approach for improving the utilization of alternative proteins in low fishmeal diets. Diets were prepared by replacing 60% fishmeal protein as follows: soybean protein concentrate (SPC) alone (SP); SPC with 2.5% crystalline amino acids (CAA) (SPAA); SPC with 10% fish soluble (FS) (SPFS); SPC with 10% krill meal (KM) (SPKM); SPC with 10% squid meal (SM) (SPSM) and SPC with a mixture (total 15%) of FS, KM and SM each at 5%, respectively (SPMX). The control diet was fishmeal based diet (FM). Diets were fed to juvenile red sea bream for 56 days. Results showed that weight gain (%) and SGR of fish were both significantly lower in fish fed SP, but those parameters recovered when fed diets with supplementation of ingredients used in the study. The fastest growth was found in fish fed SPMX, followed by SPFS, which values were not significantly different ($P > 0.05$) each other, and those groups grew significantly faster than FM. It can be concluded that supplementation of FS, KM and SM are as effective as CAA to maintain amino acids balance and can act as attractants in high plant protein based diet for maintaining normal feeding behavior, growth performance and health or welfare of juvenile red sea bream.

Based on the earlier findings, two types of non fishmeal diets were formulated by gradually replacing fishmeal with a blend of FS, FSSc and FSSq (2:1:1), and dehulled soybean meal (DSM) respectively. Diets were supplemented with DSM, KM and SM for blend, and FS, KM and SM for DSM, respectively. A commercial diet was also used as a reference diet. Two experiments were conducted independently with juvenile red sea bream for 56 days. It was found that 80% fishmeal protein could be replaced by blend and surprisingly fishmeal could be completely eliminated by DSM without any negative effects on the performances of juvenile red sea bream.

The overall findings of the research suggest that blend of seafood by-product and soybean meal, and subsequent fermentation is an efficient technology to produce new, cost-effective and comparatively balanced dietary ingredients which could partially replace fishmeal from the diets of marine fish. FS, KM and SM are effective supplements with alternative proteins to complement amino acids and act as attractants. A mixture of several marine by-products and soybean meal is effective to replace at least 80% fishmeal protein from red sea bream diet while ensuring performances as well as quality. Further, supplementation of small amount of marine by-products with soybean protein could completely replace fishmeal from red sea bream diet. The increasing use of lower cost by-products and soybean proteins will allow for a significant reduction on the cost of the feeds as costly fishmeal are removed from the formulations, or at least used at more efficient levels.

学 位 論 文 要 旨

氏　名	モハマド　アブドル　カデール
題　目	海産養殖魚における魚粉代替タンパク質に関する研究 （Studies on Alternative Protein Sources for Fishmeal in Cultured Marine Species）

今後の海面養殖業の発展のためには，価格と供給が不安定な魚粉が養魚飼料に占める割合を減らすことが不可欠である。本研究では，魚粉に替わりうる素材を用いて，海産魚の成長に及ぼす効果を検討した。

まず，海産物の加工時に発生するホタテまたはイカ加工副産物と大豆油粕を混合し発酵させた大豆油粕ホタテ発酵物(FSSc,混合比3：2)，大豆油粕イカ発酵物(FSSq,混合比1：1)を用いて，マダイおよびヒラメに対する効果を調べた。魚粉タンパク質の15，30，45，及び60％をFSScで代替した試験飼料をマダイに45日間給餌した結果，15および30％代替区は魚粉単独飼料と同等の増重率と日間成長率を示した。また，FSSqを用いたヒラメに対する飼育試験(代替率：12，24，36，48％)でも36％代替区までが魚粉単独飼料とほぼ同等の成長を示した。代替率が増加するにつれて，成長が低下する傾向がみられたもののFSScとFSSqは有望な代替素材であり，マダイとヒラメ飼料中の魚粉を30％または36％削減可能であることを明らかにした。

次に低魚粉飼料の栄養価改善を目的として，魚粉タンパク質の60％を代替タンパク質で置き換えた試験飼料を用いて，マダイの成長に及ぼす効果を検討した。試験区は，濃縮大豆タンパク(SPC)単独区(SP),SPC＋2.5％結晶アミノ酸区(SPAA)，SPC+10％フィッシュソルブル区(SPFS),SPC+10％オキアミ粉末区(SPKM)，SPC+イカ粉末区(SPSM),SPCにFS,KM,SMをそれぞれ5％ずつ添加した試験区(SPMX)および魚粉単独区の計7区を設定した。56日間の飼育試験の結果，SPMXとSPFSは魚粉単独区に比べ有意に高い成長を示したことから，植物性タンパク質にFS,KMやSMを併用することで飼料の栄養価を改善できることを明らかにした。

これまでの結果を基に，FS,FSSc,FSSq混合物（2：1：1）あるいは脱殻大豆粕ミール（DSM）を用いた2種類の無魚粉飼料を作製した。FS,FSSc,FSSq混合物にはKMとSMを添加し，DSMにはFS,KMおよびSMを添加した。それぞれの無魚粉飼料を用いて魚粉単独飼料及び市販養魚飼料を対照区として，56日間のマダイ稚魚の飼育試験を行った結果，魚粉タンパク質の80％を代替可能であることが明らかになった。

以上の結果から海産物加工副産物と大豆粕を混合した発酵物は，養魚飼料における魚粉の低コストかつ栄養バランスのとれた新規代替素材として優れていることが明らかになった。また，FS，KMおよびSMはアミノ酸の補足に適した素材であり，摂餌誘引効果も高いことを示した。

本研究によって，低コストの副産物と大豆タンパク質を用いて魚粉添加量を減らした養魚飼料の開発が可能であり，養殖魚の生産コストの大きな部分を占める飼料コストの削減が可能であることが示唆された。

LIST OF PUBLICATIONS, SYMPOSIUM PARTICIPATION, PRESENTATIONS AND ACADEMIC AWARDS

A. Publications in Peer Reviewed Journals:

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., Honda, Y., Mamauag, R.E., Laining A., 2011. Growth, nutrient utilization, oxidative condition and element composition of juvenile red sea bream *Pagrus major* fed with fermented soybean meal and scallop by-product blend as fishmeal replacement. Fish. Sci. 77, 119-128.

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B. Publications in Contribution

Kader, M.A., Bulbul, M., Ahmed, G.U. Hossain, M.S., Hossain, M.A. and Koshio, S. Effects of animal proteins either alone or in combination in practical diets on growth and economic benefit of climbing perch, *Anabas testudineus* (bloch) (J. Appl. Aquac. waiting for final decision).

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C. Participation in Conference and Symposium:

(i) "The Present and Future of the Aquaculture Industry", UJNR Aquaculture Panel The 39th Scientific Symposium Kagoshima, Japan page 80, 25 October – 26 October, 2010, Kagoshima, Japan

(ii) The 14th International Symposium on Nutrition and Feeding in Fish, May 31 to June 04, 2011, Qingdao, China.

(iii) International Symposium on Food Function and Safety, March 24, 2010, Kagoshima, Japan.

(iv) Asian-Pacific Aquaculture, November 03 - 06, 2009, Kualalampur, Malaysia.

(v) Inter-University Visiting Programme: Kagoshima University and Texas A&M University, March 01 - 07, 2009, Texas, USA.

(vi) Aquaculture America, February 15 - 18, 2009, Seattle, USA.

(vii) The 5th World Fisheries Congress, October 20 - 24, 2008, Yokohama, Japan.

(viii) World Aquaculture, May 19 - 23, 2008, Busan, Korea.

(ix) International Symposium on Food Function and Safety, December 01, 2008, Kagoshima, Japan.

(x) Seminar on the Management of Inshore Environment and Utilization of Fisheries Resources, November 16 – 18, 2007, Kagoshima, Japan.

(xi) Asian-Pacific Aquaculture, August 6 – 8, 2007, Hanoi, Vietnam.

D. Symposium Abstracts and Presentation:

There were 35 abstracts published in several book of abstracts. The major abstracts were listed below:

2010

Kader, M.A., Bulbul, M., Takahiro, M., Yokoyama, S., Ishikawa, M. and Koshio, S., 2010. Effect of complete replacement of fishmeal with mixtures of soybean meal and sea food processing by-products on the performances of red sea bream, *Pagrus major*. Book of Abstract- The Present and Future of the Aquaculture Industry, UJNR Aquaculture Panel The 39th Scientific Symposium Kagoshima, Japan page 80, 25 October – 26 October, 2010, Kagoshima, Japan

Kader, M.A., Koshio, S., Ishikawa, M. and Yokoyama, S., 2010. Supplemental effects of some crude ingredients in improving feed intake and performances of red sea bream, *Pagrus major* fed high soy protein concentrate diet. Book of Abstract- The 14 th International Symposium on Fish Nutrition and Feeding, page 395, 31 May – 04 June, 2010, Qingdao, China.

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Kader, **M.A.,** Jahan, P., Michael, F.R., Komilus, C.F., Tung, H.T., Traifalgar, R.F., Gao, J., Yokoyama, S., Ishikawa, M. and Koshio, S., 2008. Growth, feed utilization and immunological responses of scorpion fish (*Sebastiscus marmoratus*) by partial substitution of dietary fish meal with fish soluble. Book of Abstract- World Aquaculture, 2008, page 306, May 19-23, 2008, Busan, Korea.

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Kader, M.A., Jahan, P., Michael, F.R., Komilus, C.F., Thu, M., Laining, A., Yokoyama, S., Ishikawa, M. and Koshio, S., 2007. Inclusion of dietary bamboo charcoal with plant by-products mix to improve growth and feed utilization of amberjack (*Seriola dumerili*). Book of Abstract- Asian-Pacific Aquaculture, 2007, page 116, August 6 to 8, 2007, Hanoi, Vietnam.

E. Thesis Titles

Kader, M.A., 2011. Studies on alternative protein sources for fishmeal in cultured marine species. Doctor of Philosophy, Kagoshima University, Japan. 194pp.

Kader, M.A., 2008. Effects of food industry by-products on the performances of marine fish. Master of Science, Kagoshima University, Japan. 92pp.

Kader, M.A., 2003. Comparative study of nutritive value of some commercial fish feeds available in Bangladesh and evaluation of three commercial pangas feeds through growth trial in ponds. Master of Science, Bangladesh Agricultural University, Bangladesh. 96pp.

AWARDS

Award – I:

"Aquaculture CRSP Best Poster Award 2008" (1st place), World Aquaculture 2008 Meeting, May 19-23, 2008, Busan, Korea.

Presenting paper:

Kader, **M.A.,** Bulbul, M., Hossain, M.S., Hossain, M.A., Ahmed, G.U., Komilus, C.F., Yokoyama, S., Ishikawa, M. and Koshio, S., 2008. Evaluation of meat and bone meal in the practical diet for sutchi catfish, *Pangasius hypophthalmus* (sauvage) reared under pond condition.

Award – II:

"Best Paper Presenter Award 2009", The United Graduate School of Agricultural Sciences, Kagoshima University, November 9-11, 2009, Kagoshima, Japan.

Presenting paper:

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Md. Abdul Kader

ABBREVIATIONS AND ACRONYMS

ALP	:	Alkaline phosphatase	HSI	:	Hepatosomatic index
BAP	:	Biological anti-oxidant potential	HUFA	:	Highly unsaturated fatty acid
BUN	:	Blood urea nitrogen	IAA	:	Indispensible amino acid
CAA	:	Crystalline amino acid	KM	:	Krill meal
Cd	:	Cadmium	LDH	:	Lactate dehydrogenase
CF	:	Condition factor	PA	:	Protease activity
COM	:	Commercial diet	Pb	:	Lead
CORT	:	Relative value of cortisol	PER	:	Protein efficiency ratio
Cu	:	Copper	PG	:	Protein gain
DM	:	Dry matter	PR	:	Protein retention
d-ROMs	:	Reactive oxygen metabolites	S.E.M.	:	Standard error of mean
DSM	:	Dehulled soybean meal	SBM	:	Soybean meal
EAA	:	Essential amino acid	SGR	:	Specific growth rate
FAA	:	Free amino acid	SM	:	Squid meal
FE	:	Feed efficiency	SPC	:	Soy protein concentrate
FP	:	Fermented product	T-Bil	:	Total bilirubin
FS	:	Fish soluble	T-Cho	:	Total cholesterol
FSSc	:	Fermented soybean meal and scallop by-product blend	TG	:	Triglyceride
FSSq	:	Fermented soybean meal and squid by-product blend	TP	:	Total protein
Glu	:	Glucose	VSI	:	Viscerotopic index
GOT	:	Glutamyl oxaloacetic transaminase	WG	:	Weight gain
GPT	:	Glutamic-pyruvate transaminase	Zn	:	Zinc
HDL-c	:	High density lipoprotein cholesterol			

CHAPTER I

General Introduction

1. General introduction

1.1 Aquaculture, formulated feed and fishmeal replacement

Aquaculture industry is playing an increasing role to supply comparatively safer animal protein for human consumption, accounted for about 47% of the world's fish food supply (FAO, 2009). The depleted state of wild fish stocks due to overfishing and increasing degradation of aquatic ecosystems and habitats, world aquaculture industry has grown dramatically in the last 50 years at almost 8.5 to 9% per year (Fig. 1.1) and expected to grow more rapidly than other animal food-producing sectors which bring new challenges to sustainable use of aquatic resources and environments (FAO, 2009; *http://web.worldbank.org*).

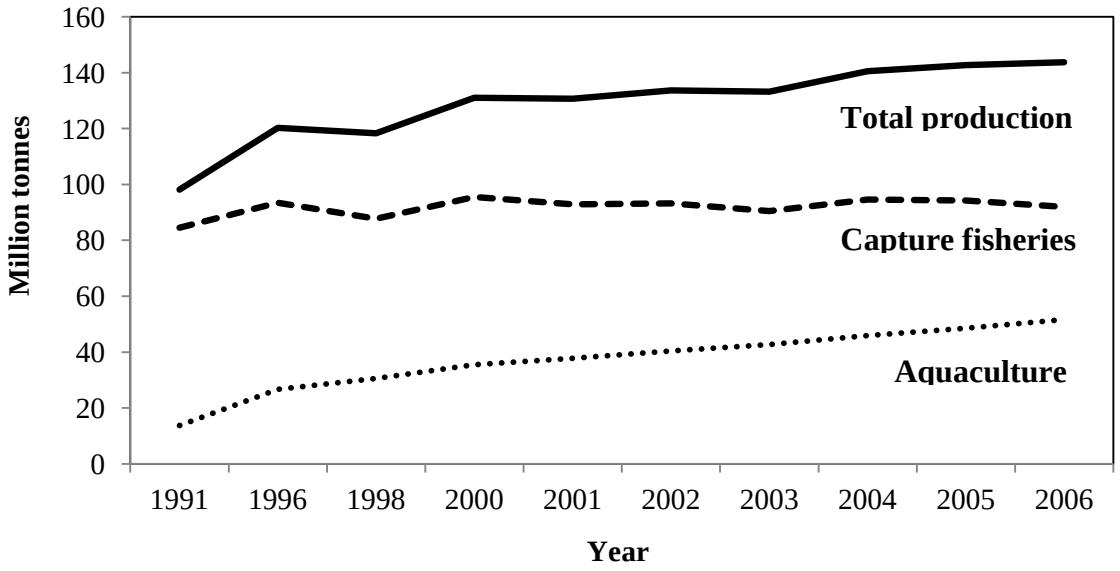

Fig. 1.1: Trend of world aquaculture, capture and total fisheries production (FAO, 2007, 2009)

Nutritionally balanced aquafeed is the most important and crucial factor for the successful aquaculture production. The total aquafeed production estimated at 4% of the total global animal feed production in 2006 accounted for 25.4 million tones (Gill, 2007). It was also reported that 0.65 to 0.80 million tones of dry compound and semi-moist compound feeds

were produced in 2006 in Japan (Tacon and Metian, 2008). To sustain the current growth rate of aquaculture industry, the supply of feed inputs will also have to grow at similar rates so as to meet the demand. Fishmeal has been used traditionally as the sole protein source in compound aquafeed because of its unique nutritional composition. The total amount of fishmeal used in aquafeed is estimated to have grown more than threefold between 1992 and 2006, from 0.96 million tonnes to 3.06 million tones whereas fishmeal production has been remarkably stable or declining trends (Fig. 1.2; FAO, 2009). Therefore, limited access to feed raw materials from the fisheries, combined with a growing demand for fish and other land animal feed has lifted the cost of fishmeal too high in recent years (Fig. 1.3).

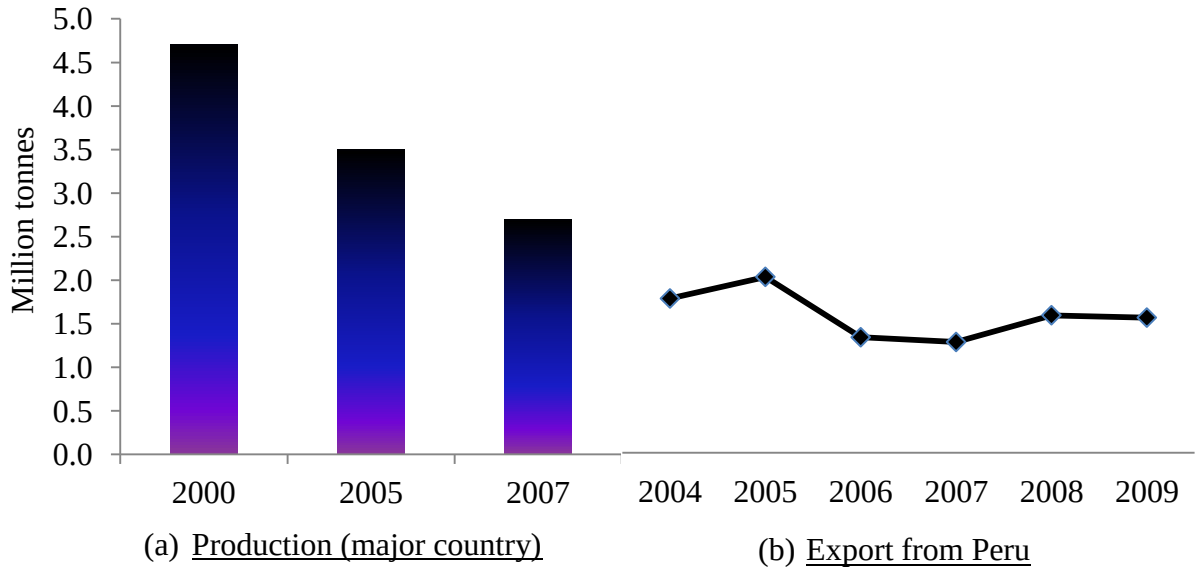

Fig. 1.2: Trend of estimated fishmeal production from major producing countries and export from Peru (FAO, 2007, 2009; http://www.globefish.org)

As feed constitutes 50-60% of the total production cost of carnivorous fish, rising feed cost has a negative impact in the farmer's profitability from aquaculture venture. Therefore, exploration of nutritionally balanced, cost-effective and available alternative feed raw materials to fishmeal is current need for the sustainability of world aquaculture industry.

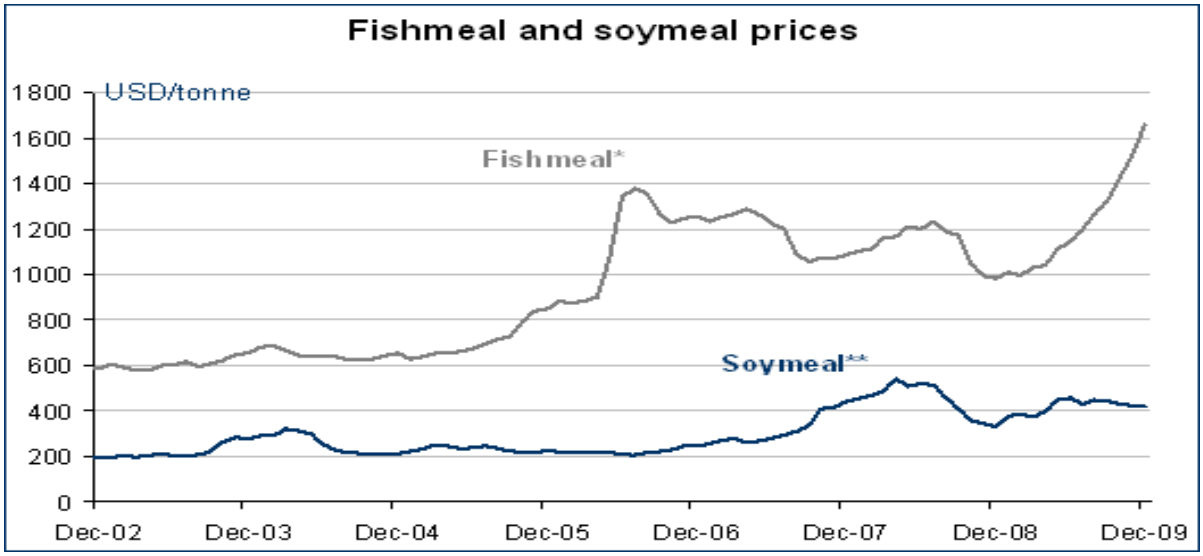

Fig. 1.3: Trend of world fishmeal and soybean meal prices (http://www.globefish.org)

1.2 Alternative protein source

Replacement of fishmeal with plant protein or renewable protein source to develop sustainable and cost-effective aquafeed is one of the major topics in aquaculture nutritional research. Reducing the amount of fishmeal in diet formulations, without reducing fish performance, could have a positive impact on the profitability of commercial fish production (Tidwell *et al*., 2005). A number of studies were stressed on the effectiveness of different alternative proteins of both plant and animal origin in different fish species.

1.2.1 *Plant protein*

Plant proteins are more focused because of their lower price, consistent nutrient composition and availability. Among plant proteins, soybean products have been used extensively for fishmeal replacement in different fish species because of comparatively high protein content, high digestibility, well-balanced amino acids, reasonable price and steady supply (Storebakken *et al*., 2000). It was found that fishmeal protein could be replaced at relatively lower levels with soybean proteins in marine fish, for example 10% in Asian seabass, *Lates calcarifer* (Tantikitti *et al*., 2005); 15% in coho salmon, *Oncorhynchus kisutch*

(Arndt *et al*., 1999); 20% in yellowtail, *Seriola quinqueradiata* (Shimeno *et al*., 1993) and 40% in cobia, *Rachycentron canadum* (Zhou *et al*., 2005). In juvenile red sea bream, *Pagrus major* Takagi *et al*. (1999) reported that 50% fishmeal protein could be replaced with solvent extracted soy protein named as soy protein concentrate (SPC). Kader *et al*. (2010) found that growth performance, feed utilization and physiological condition of red sea bream were significantly depressed with 60% fishmeal protein replacement with SPC. Kissil *et al*. (2000) reported that gilthead seabream, *Sparus aurata* L. are unable to tolerate even 30% fishmeal replacement with soybean protein. Japanese flounder seems to be very sensitive to soybean protein and 20% fishmeal protein could be replaced with dehulled soybean meal (DSM) (Choi *et al*., 2004). More pronounced effect was reported by Deng *et al*. (2006) that growth decreased gradually with increasing dietary SPC, even at the minimum level of 25% substitution of SPC for fishmeal. It was also evident in another flatfish, turbot, *Scophthalmus masimus* L. which could replace only 25% fishmeal protein with SPC (Day and González, 2000). In contrast, comparatively higher replacements (75-94%) were found for Senegalese sole, *Solea senegalensis* post larvae (Aragão *et al*., 2003) and cobia juvenile (Salze *et al*., 2010). The complete replacement of fishmeal with SPC was achieved by Kaushik *et al*. (1995) in rainbow trout, *Oncorhynchus mykiss* and Salze *et al*. (2010) in cobia.

Some other plant proteins are also potential for the partial replacement of fishmeal from aquafeed such as lupin meal (Farhangi and Carter, 2001), rapeseed meal (Francesco *et al*., 2004), cottonseed meal (Rinchard *et al*., 2003), mustard oil cake, linseed and sesame meals (Hossain and Jauncey, 1989), canola meal (Thiessen *et al*., 2004), gluten meal (Francesco *et al*., 2004; Kaushik *et al*., 2004) etc.

1.2.2 *Renewable protein or animal by-products*

In general, animal proteins perform better than plant proteins in diets for carnivorous species (Hardy, 1998). Animal protein sources are mostly form terrestrial animal by-product

such as blood meal (Glencross *et al*., 2003), feather meal (Woodgate, 2004), meat and bone meal (Kader *et al*., in press) and poultry by-product meal (Yang *et al*., 2004) etc. Due to ban of the rendered terrestrial animal meals for farm animal feeds in many European countries and Japan, their use also limited as fishmeal replacement in aquafeeds.

By-catch and by-products/discards from fish processing industries could have significant consequences for populations, food webs and ecosystems. Although no detailed estimation of by-catch is available, a crude estimate suggests that it could be more than 20 million tonnes globally (FAO, 2009). In addition, international fish and shrimp industries through various processing operations also generate a huge amount of proteinaceous by-products including shrimp heads and shells or fish fillet waste as well as a variety of valuable dissolved and particulate organic compounds that are potentially recoverable for feed use (Meyers, 1986). However, by-products and by-catch contains high amount of moisture and these are only available during the fishing season and specific areas. The conversion of fish waste into fishmeal by drying would be a high-cost, complicated process. Therefore, some advanced processing technologies should be applied for converting these by-products as utilization friendly product and increasing the shelf life in storage. For example, fisheries by-products could be converted as hydrolysate, silage and soluble (Refsite *et al*., 2004; Hevroy *et al*., 2005; Bechtel, 2005, Cavalheiro *et al*., 2007; Kader *et al*., 2010). Growth and feed utilization were improved with 15-24% inclusion of fish protein hydrolysate (FPH) in the diets of Atlantic salmon, *Salmo salar* L. (Refsite *et al*., 2004; Hevroy *et al*., 2005) and sea bass, *Lateolabrax japonicus* (Liang *et al*., 2006). Fish soluble (FS) is another potential product from fish processing by-products (Bechtel, 2005). In scorpion fish, *Sebastiscus marmoratus*, feed intake was positively correlated with the increasing levels of FS at 0, 5, 10 and 15% (Kader, 2008). Shrimp head silage could replace fish flour as an ingredient in tilapia feed (Cavalheiro *et al*., 2007). Hernandez *et al*. (2004) reported that co-extruded tuna viscera and corn meal can be included up to 40% in practical shrimp diets. Some marine zooplankton

such as krill meal (KM) and amphipod meal can partially (40-60%) replace fishmeal in diets to Atlantic salmon and Atlantic halibut, *Hippoglossus hippoglossus* L. (Suontama *et al*., 2007).

1.2.3 *Problems associated with alternative protein sources*

The utilization of plant protein is limited by the deficiencies in essential amino acids and minerals and the presence of antinutritional factors, toxins, metabolites and complex carbohydrates (NRC, 1993). Higher levels of plant protein may interfere with the digestive process of fish. Morphological alteration was found in the distal intestine by feeding higher levels of soybean proteins in many fish species such as Atlantic salmon (Refsite *et al*., 2001; Uran *et al*., 2008), rainbow trout (Escaffre *et al*., 2007) and pacu, *Piaractus mesopotamicus* (Ostaszewska *et al*., 2005). There are also some problems associated with the utilization of animal by-products specially seafood processing by-products and by-catch. The nutritional composition of by-products are varied which depend on the landed fish species, season and geographical locations. By-product and discards also need to be preserved to maintain quality as these contain high moisture (Espe and Lied, 1999). Seafood by-products may have been associated with certain food safety issues, as there may be a risk of contaminants like heavy metals through feeds and environments (Mai *et al*., 2006; Ghirimi *et al*., 2008). Overall, in most of the cases, higher or complete replacement of fishmeal with alternative protein sources either plant or animal origin, have detrimental effects: mainly due to imbalance in amino acids, decrease bio-availability, and presence of toxins or anti-nutritional properties. Another key factor is lower feed intake in fish because of decreased diet palatability or acceptability as the level of alternative protein sources increases, especially plant proteins (Kubitza *et al*., 1997; Kissil *et al*., 2000; Chatzifotis *et al*., 2008; Kader *et al*., 2010).

In the context of researching nutritional aspects for aquatic animal, growth performance, feed utilization and whole body composition were the main parameters for evaluation.

Dietary changes often cause no grossly observable signs, but they may severely influence the organism's health status or quality which would not emerge from nutritional parameters. Therefore, obviously, changes of general health parameters, stress condition and fillet quality must be considered as important criteria for evaluating the nutritive value of alternative protein ingredients.

1.3 Approach: new alternative proteins and their utilization

In some instances, seafood processing industries generate by-products more than 50% of the total landing. Therefore, by-products will have a great potential to be used as a fishmeal substitute. Most of them are treated as waste products. In other wards, they are incinerated, dumped into the sea or deposited on land, and then caused a serious environmental crisis (Xu *et al*., 2008). Limited researches have been carried out for the effective utilization of these by-products. On the other hand, due to the recent technological progress, it is now possible to recycle these by-products, rather than disposing them in a less environmentally friendly manner. For example, by-products could be converted to silage or hydrolysate, which has longer storage ability. However, autolysis might occur upon storage and may result in bitter tasting peptides and lipid oxidation (Refsite *et al*., 2004; Hevroy, *et al*., 2005). In addition, these products are liquid or semi liquid forms, which creates the inconvenience on inclusion into the feed (Fagbenro *et al*., 1994). Co-drying of wet fisheries by-products with other dry feed ingredients together with subsequent fermentation might be an alternative approach for the effective utilization of fisheries by-products in aquafeed (Fagbenro and Jauncey, 1995; Sun *et al*., 2007; Mondal *et al*., 2008). The process might also be effective to complement the nutritional composition of plant proteins (Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Tidwell *et al*., 2005; Guo *et al*., 2007). Fermentation is an efficient technique to decrease or eliminate anti-nutritional constituents from oilseeds (Reddy and Pierson, 1994) and improve

the overall nutritional quality (Canella *et al*., 1984). It is also a useful technique for drying wet product with minimal nutrient loss (Yamamoto *et al*., 2004). Furthermore, the fermentation of fish waste is more suitable and convenient for small industries and/or the farmer. Sun *et al*. (2007) reported that fermented fisheries by-products and soybean curd residues mixture could replace 30% fishmeal protein from the diets of Japanese flounder, *Paralichthys olivaceus* while in freshwater fish (*Labeo rohita* and *Heteropneustes fossilis*) 50% fishmeal protein could be replaced with fermented fish offal, mustard oil cake and rice bran mixture (Mondal *et al*., 2007, 2008). Co-dried fermented fish silage and soybean meal was also found to be a suitable protein supplement in the diets of catfish, *Clarias gariepinus* and Nile tilapia, *Oreochromis niloticus* (Fagbenro and Jauncey, 1995; Fagbenro *et al*., 1994). Therefore, blend of fisheries by-product and plant protein together with subsequent fermentation would be an effective approach to utilize fisheries by-products and plant protein more efficiently, which could explore new alternative protein sources for the partial replacement of fishmeal in aquafeeds.

Supplementation of crystalline amino acids (CAA) and synthetic compounds is a common practice to balance amino acids and improve palatability of the formulated aquafeed. Higher or complete replacements of fishmeal by SPC with CAA supplementation were successfully reported in Senegalese sole, cobia and rainbow trout (Aragão *et al*., 2003; Salze *et al*., 2010; Kaushik *et al*., 1995). On the contrary, Takagi *et al*. (2001) reported that inclusion of CAA in higher level of SPC based diet failed to obtain similar performance of red sea bream to that of those fed fishmeal based diet. Thus, use of CAA or synthetic compounds is not yet a practical approach, and not always available, cost-effective especially in developing countries due to the high cost of CAA. Supplementation of crude ingredients would improve the palatability and nutritional quality, especially by improving the amino acids compositions of the high plant protein containing diet, which would help to improve the feed intake as well as the

overall performances of fish (Smith *et al*., 2005). Crude ingredients such as FS, KM and squid meal (SM) are the natural sources for the feeding stimulatory substances and have a good amino acid composition for aquatic animals (Gaber, 2005; Smith *et al*., 2005; Mai *et al*., 2006; Kader *et al*., 2010). These are often used in commercial feeds with small amounts, however the information on the optimal dietary levels of those is unknown, or the comparative evaluation has not been fully made yet. In addition, very few studies were available on the effect of these ingredients or whole meals as crude attractants in high plant protein based diets. As inclusion of whole meal is more practical and economical than the use of CAA, supplementation of small amount of complementary crude ingredients with alternative proteins will be an effective approach to develop low or non fishmeal based diets in a cost-effective and practical manner.

The Japanese flounder and red sea bream are two of the most highly valued marine food fish and commercially important aquaculture species in Japan. These fish are carnivorous, and farmers are mostly used minced whole fish or formulated feeds for their culture practice (Forster and Ogata, 1998). However, formulated feeds contain high amount (50-60%) of fishmeal, which is the most expansive component in aquafeeds. The continuous increasing price of fishmeal, especially last few years (Fig. 1.3) has pressured feed manufacturers to reduce their reliance for use in flounder and sea bream diets. Therefore, finding alternative protein sources and developing low or non-fishmeal diets for those species are urgent areas of study to be conducted immediately. Although several alternative protein sources were reported to partially replace fishmeal from the diets of many fish species, information on Japanese flounder and red sea bream is still limited. Choi *et al*. (2004) found that DSM could replace up to 20% of fishmeal from the diet of Japanese flounder without CAA or attractant supplementation and could replace up to 30% of fishmeal with CAA and/or attractant supplementation. Furthermore, the replacement level of fishmeal protein could be accelerated

to 30-40% when soybean meal (SBM) was blended with cottonseed meal (Pham *et al*., 2007) and supplemented with iron and phosphorus (Lim and Lee, 2008). However, crystalline lysine and methionine were supplemented in both the cases. Similarly, Kikuchi *et al*. (1994b) reported that growth was significantly reduced by 30% fishmeal replacement with defatted SBM and depleted growth was recovered with CAA supplementation. In another study, it was reported that 30-40% fishmeal could be replaced with extruded SBM without CAA supplementation (Saitoh *et al*., 2003). In contrast, Deng *et al*. (2006) observed that growth of Japanese flounder was gradually decreased with the increasing levels of SPC, even at the minimum level of 25% substitution. Previous results also indicated that 20- 40% of fishmeal protein could be replaced from the diets of Japanese flounder by feather meal (Kikuchi *et al*., 1994a), meat and bone meal (Kikuchi *et al*., 1997), and corn gluten meal (Kikuchi, 1999a) with the supplementation of appropriate CAA. Red sea bream is sensitive to soybean proteins specially at juvenile stage (Biswas *et al*., 2007; Takagi *et al*., 1999, 2000b). Juvenile fish, with an initial weight of 19 g showed significant growth retardation when fishmeal was reduced to 40% (control diet contains 65% fishmeal) with 30% SBM (Biswas *et al*., 2007). This was also found in our preliminary study which showed that juvenile fish (2.5 g) cannot tolerate even 15% SBM while 15% fermented SBM exhibited better performances compared to non fermented SBM (Hirakawa, unpubl. data, 2008). On the other hand, Takagi *et al*. (2000b) reported 50% fishmeal protein could be replaced with SBM in yearling fish (260 g). In earlier experiment, Takagi *et al*. (1999) also found that the nutritional value of processed soybean proteins like SPC is superior in red sea bream and can replace 90% fishmeal protein in yearling fish (310 g) and 50% in juvenile fish (33.5 g). It was also reported that when SBM was combined with either corn gluten meal or poultry by-product meal or both, the replacement level of fishmeal protein could be accelerated to 70-90% in the yearling fish (Takagi *et al*., 2000b).

Therefore, it has been reviewed from the previous researches that certain amount of fishmeal could be replaced by different alternative protein sources from the diets of juvenile red sea bream and Japanese flounder. In case of higher fishmeal replacement, the negative effects of alternative proteins, especially imbalance amino acids and lower feed intake were minimized by the supplementation of costly CAA, rather application of cost-effective and practical approaches. In addition, no study was reported for complete fishmeal replacement from these marine species. The present research work was conducted to utilize several seafood or marine by-products and soybean proteins as alternative protein sources in cultured marine species and find out cost-effective approaches to maximize the utilization of alternative proteins in aquafeeds. By the same time, effects of alternative protein sources on the general health condition, oxidative status and fish quality were also evaluated. The overall objectives of the present study were:

1. to investigate new alternative protein sources for marine cultured species by utilizing seafood by-products and soybean proteins.
2. to examine the effectiveness of crude ingredient supplementation instead of CAA in high plant protein based diets for improving feed intake and performances of fish.
3. to explore cost-effective and practical approach for the development of low or non fishmeal based diets for cultured marine species.

CHAPTER II

General Materials and Methods

2. General Materials and Methods

2.1 Preparation of test diets

Dietary ingredients were first ground to a small particle size in a hammer mill and passed through a 100μm mesh sieve. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 30 min. The lipid sources were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. Then required amount of water (35-40% of the dry ingredients), were added to the premixed ingredients and mixed for another 30 min. The pH of the diets was adjusted to 7.0-7.5 with 4N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2 to 2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60°C for 120 min. The test diets were stored at −28°C in a freezer till use.

2.2 Experimental system

All the experiments were conducted in Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The feeding trials were carried out in 100-L polycarbonate tanks (filled with 80L of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. Flow rates of 1.5 L min^{-1} were maintained throughout the experimental periods.

2.3 Feeding protocol

After stocking, fish were fed the respective test diets to the satiation level by hand twice daily, 7 days per week during the feeding trials. Special care was taken to collect uneaten

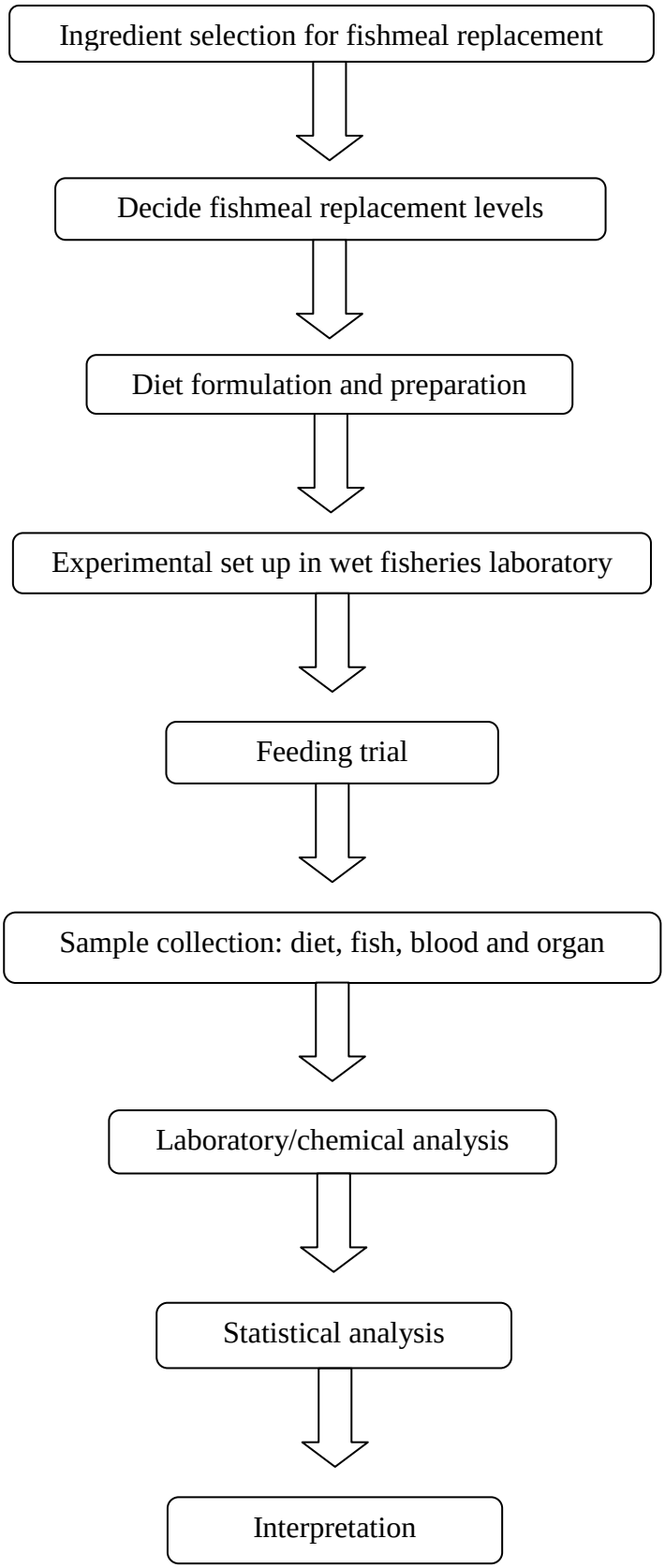

Fig. 2.1: Diagrammatic presentation of the general methodologies applied in the experiments

diets which was freeze dried and finally subtracted from the total amount of supplied test diets to calculate the actual feed intake. All fish were weighted in bulk at certain (10-14 days) interval to determine growth and visually check their health condition. The water quality parameters such as water temperature, pH and salinity were measured and recorded during entire feeding trials.

2.4 Sample collection

The initial samples of 10-15 fish were stored at −20^{o}C for initial whole body analysis. At the end of the feeding trials, all fish were fasted for 24 h prior to final sampling. The total number, individual body weight and length of fish in each tank were measured. Five fish from each replicate tank were randomly collected and stored at −20^{o}C for final whole body analysis. Blood was drawn by puncture of the caudal vein of individual fish. Plasma samples were collected after spinning down the heparinized blood at 3000 × *g* for 15 min at 4^{o}C. Serum was collected after clotting whole blood (non-heparinized) centrifuged at 3000 × *g* for 15 min at 4^{o}C. All the blood samples were kept at −80°C until analysis. Liver was dissected out from three fish in each replicate tank, weighted individually to get heapatosomatic index (HIS), and finally pooled together and stored at −80^{o}C.

2.5 Analytical procedure

Commonly used methodologies for biochemical analysis of ingredients, diets and fish samples are briefly described below.

2.5.1 *Proximate composition*

The ingredients, diets or fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard AOAC methods (AOAC, 1990).

2.5.2 *Moisture*

Fish whole body were dried to a constant weight using the freeze dryer (Eyela freeze dryer FD-1, Tokyo Rikakikai Co. Ltd., Japan). Diet samples were also dried to a constant weight using mechanical convection oven at 135^0C. Loss in weight represented moisture content.

Moisture (%) = {(weight before drying -weight after drying/weight before drying)} × 100

2.5.3 *Ash*

Samples were taken in crucibles with cover slightly ajar and placed in a muffle furnace at 550^0C until sample weight became constant. Ash contents were calculated as follow:

Ash (%) = (weight of ash/ sample weight) × 100

2.5.4 *Crude protein*

Approximately 0.2 g of sample and 2 g of catalizer (K_2SO_4:$CuSO_4$; 9:1) were digested with 10 ml of concentrated H_2SO_4 and 5 ml of 30% H_2O_2 for 90 min at 460^0C. Then digested samples were distilled in 50 ml of 30-40% NaOH using Kjeldahl distilling apparatus (Kjeltec System 1002, Tecator, Sweden). Approximately 150 ml distillate in H_3BO_3 solution mixed with methylene blue and methyl red indicators in ethanol was titrated with 0.1N H_2SO_4 to neutral pH. Percent of N was calculated for obtaining crude protein (%) using the following formula:

Nitrogen (%) = {14.008 × (A-B) × 0.1 × F}/{Sample weight (g) × 10}

Where, A = Amount (ml) of H_2SO_4 solution titrated for sample

B = Amount (ml) of H_2SO_4 solution titrated for blank

F = Factor of 0.1 N sulfuric acid solution

Crude protein (%) = % N × 6.25

2.5.5 *Total lipid*

Total lipids were analyzed according to Bligh and Dyer (1959) method. Approximately 0.2 g samples were homogenized with 1 ml of chloroform and 2 ml of methanol by a polytron homogenizer for 1 minute. After that, another 1 ml of chloroform were added and homogenized for 1 minute. The homogenate were filtered by a Buchner filtration apparatus. The residue on the filter papers was washed with a mixture of chloroform: methanol (1:1 v/v). After two times filtration, the homogenate were transferred to a separatory funnel, added few drops of water, shaken vigorously and stood for complete separation. The volume of chloroform, methanol and water were maintained in the proportions 1:1:0.8, respectively. The lower layer which contained chloroform and lipid fraction was collected and evaporated the chloroform completely under reduced pressure by using a rotary evaporator. Dried lipid was dissolved with chloroform: methanol (1:1 v/v) and transferred to the tarred vial. The total lipid was dried again by flashing with nitrogen and weighed to a constant weight. Total lipid is calculated as follows:

Total lipid (%) = {lipid weight (g) / dried sample weight (g)} × 100

2.4.6 *Amino acids*

Total amino acid (TAA) and free amino acid (FAA) concentrations were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Kyoto, Japan) according to Teshima *et al*. (1986a). To determine TAA, samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzded with 4 N methanesulfonic acid at 110°C for 22 h. The pH of the hydorlysate was adjusted to 2.2, filtered and stored at 4°C. To quantify the free amino acids 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml^{-1} deionized water) and 5 ml 10% trichloroacetic acid (TCA),

homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were then centrifuged at 4°C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4°C. The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column.

2.4.7 *Fatty acids*

Dietary fatty acid contents were analyzed by gas chromatography (GC-17A Shimadzu Co., Japan) following Teshima *et al*. (1986b).

2.4.8 *Blood parameters*

Heparinized disposable syringes were used to collect blood for measuring hematocrit level. Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al*. (2002).

2.6 Statistical analysis

All data were subjected to statistical verification using package super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Duncun's Multiple Range Test or Tukey Kramer test.

CHAPTER III

Utilization of Seafood By-products in Cultured Marine Species

CHAPTER III

Experiment I

Growth, nutrient utilization, oxidative condition and element composition of juvenile red sea bream *Pagrus major* fed with fermented soybean meal and scallop by-product blend as fishmeal replacement

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., Honda, Y., Mamauag, R.E., Laining A., 2011. Fisheries Science 77, 119-128

3.1.1 Abstract

A feeding trial was conducted to evaluate the effect of fermented soybean meal and scallop by-product blend (3:2) (FP) on the performances of juvenile red sea bream. Five isocaloric diets were prepared by replacing 0% (FP0), 15% (FP15), 30% (FP30), 45% (FP45) and 60% (FP60) fishmeal protein with FP, respectively. Triplicate groups of fish (initial mean weight, 2.83g) were fed the test diets for 45 days in a flow-through sea water system. The results demonstrated that growth rates of fish fed FP0, FP15 and FP30 were similar, and significantly higher ($P < 0.05$) than those of FP45 and FP60. Nutrient utilizations were significantly lower in FP60 and no difference was found among the rest. Dietary heavy metal contents were affected by inclusion of FP, reflecting whole body contents of those. In terms of oxidative stress, fish fed FP30 diet was in the best condition, since this fish group showed the least oxidative stressed condition as well as the highest tolerance against oxidation. In conclusion, the approach of utilizing this fermented mixture is promising and it could replace at least 30% fishmeal protein in red sea bream diet without negative effects on the performance, body composition and health of fish.

Keywords: Soybean meal, Scallop by-product, Fermentation, Fishmeal, Growth, Element composition, *Pagrus major*

3.1.2 Introduction

Red sea bream, *Pagrus major* is one of the commercially important aquaculture species in Japan. Most farmers are using commercially manufactured feeds, which contain mostly high amounts of fishmeal, for their operation. On the other hand, limited supply coupled with increasing demand and cost of fishmeal put pressure on feed manufacturers to reduce their reliance on traditional ingredients like fishmeal in sea bream diets. Reducing the amount of fishmeal in diet formulations, without reducing fish performance, could have a positive impact on the profitability of commercial fish production (Tidwell *et al*., 2005). A number of studies have stressed the effectiveness of different alternative proteins of both plant and animal origin. However, their utilization has been limited because of imbalanced amino acid profiles, low protein content and the presence of antinutritional or hazardous compounds.

Japanese are well known for their high consumption of fish and shellfish, which results in large amounts of by-products. Scallops, specially *Mizuhopecten yessoensis* are extensively cultured in the northern part of Japan. The adductor muscle is the only edible part of the scallop and the rest is treated as waste product (Ghimire *et al*., 2008). The huge amounts of these by-products are usually disposed of according to the strict Japanese Government regulations for waste disposal (Ren *et al*., 2008). This is often seen as a threat to the industry as a considerable amount of money is spent on disposal of by-products, even though they are nutrient-rich resources which have the potential to be utilized in aquafeed. But there are problems associated with the utilization of by-products, especially by-product of seafood processing industry, including freshness, quality, availability, higher moisture, indigestible particle and contaminants or toxic metals. Due to the recent technological progress, it is now possible to recycle these by-products, rather than dispose of them in a less environmentally friendly manner. However, these products in liquid or semi liquid form create difficulties in their inclusion to the feed (Fagbenro *et al*., 1994). One possibility for solving this problem is fermentation, which can efficiently decrease or eliminate anti-nutritional constituents from

oilseeds (Reddy and Pierson, 1994) and improve the overall nutritional quality (Canella *et al*., 1984). It is also a useful technique for drying wet product with minimal nutrient loss (Yamamoto *et al*., 2004). Sun *et al*. (2007) reported that fermented fisheries by-products and soybean curd residues mixture could replace 30% fishmeal protein from the diets of Japanese flounder, *Paralichthys olivaceus*, while in fresh water fish (*Labeo rohita* and *Heteropneustes fossilis*) 50% fishmeal protein could be replaced with fermented fish offal, mustard oil cake and rice bran mixture (Mondal *et al*., 2007, 2008). Co-dried fermented fish silage and soybean meal was also found to be a suitable protein supplement in the diets of catfish, *Clarias gariepinus* and Nile tilapia, *Oreochromis niloticus* (Fagbenro *et al*., 1994; Fagbenro and Jauncey, 1995).

Therefore, the present study was conducted to investigate the viability of the utilization of fermented soybean meal and scallop by-product blend (3:2) by evaluating several parameters such as growth rates, nutrient utilization, and health status as well as whole body concentrations of copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb).

3.1.3 Materials and methods

3.1.3.1 *Ingredients and test diets*

All feed ingredients used in this study were commercially obtained. High quality brown fishmeal (Nippon Suisan Co. Ltd. Tokyo, Japan; 71.10% crude protein, 10.25% total lipid and 15.57% ash as dry matter basis) was used as the major protein source and fishmeal protein was replaced with a fermented product (FP) in the other diets. FP is a fermented soybean meal and scallop by-product mixture in the proportion of 3:2, provided by AFCEP, Japan Corp. (Yazu, Niigata, Japan). The condition of fermentation (patent pending Afcep Japan Corp.) was described recently (Yamamoto *et al*., 2010). Appropriate ratio (3:2) of soybean meal and scallop by-product were added with 30% of water and fermented with compound bacteria (predominantly *Bacillus* spp), for 7 h until the temperature reached to

80°C. The resulting FP contains 51.91% crude protein, 7.16% total lipid and 6.44% ash (dry matter basis). The formulation of the experimental diets is shown in Table 3.1.1. Five diets were formulated to replace 0, 15, 30, 45 and 60% of fishmeal protein with FP so that all the diets became nearly isonitrogenous (50% protein), isolipidic (15% total lipid) and isocaloric (23KJ/g). The diets were designated as FP0, FP15, FP30, FP45 and FP60 respectively.

The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 30 min. Pollack liver oil, soybean lecithin and HUFA were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. Finally, water (35-40% of dry ingredients) was added to the premixed ingredients and mixed for another 30 min. The pH of the diets was adjusted to 7.0-7.5 with 4N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate die (1.2 to 2.2 mm) to prepare pellets and dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60°C for 120 min. The test diets were stored at −28°C in a freezer until use.

3.1.3.2 *Fish and feeding protocol*

The experiment was conducted in Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Juvenile red sea bream were purchased from Matsumoto Suisan, Miyazaki Prefecture, Japan and maintained in the laboratory condition for 1 week while being fed a commercial diet (50% crude protein, Higashimaru, Kagoshima, Japan). On the day of initial sampling, juveniles (average body weight 2.83 ± 0.02g) were randomly distributed among fifteen, 100-l polycarbonate circular tanks at a stocking density of 15 fish per tank with triplicates. All the tanks were equipped with a continuous filtered flow through sea water system (flow rate 1.5l/min). Artificial aeration and natural light/dark regime (12h:12h) were also maintained.

Table 3.1.1: Ingredients and chemical composition of the experimental diets

Ingredients (% dry matter basis)	Test diets				
	FP0	FP15	FP30	FP45	FP60
Fishmeal	55.0	46.8	38.5	30.3	22.0
Fermented product	-	11.4	22.9	34.4	45.8
Casein	5.0	5.3	5.5	5.8	6.0
Pollack liver oil[a]	4.0	4.0	4.0	4.0	4.0
Soybean lecithin[a]	2.0	2.0	2.0	2.0	2.0
HUFA[b]	0.5	0.5	0.5	0.5	0.5
Wheat flour	10.0	8.0	6.0	4.0	2.0
Vitamin mixture[c]	3.0	3.0	3.0	3.0	3.0
Mineral mixture[d]	3.0	3.0	3.0	3.0	3.0
Vitamin C ester[e]	0.3	0.3	0.3	0.3	0.3
Activated gluten	5.0	5.0	5.0	5.0	5.0
Carboxymethyl cellulose	1.0	1.0	1.0	1.0	1.0
α-cellulose	11.2	9.7	8.3	6.7	5.4
Proximate composition					
Moisture (%)	10.1	9.9	10.3	9.2	10.2
Crude protein (% DM[f])	50.8	50.8	50.6	50.8	51.3
Total lipid (% DM)	15.4	15.2	15.4	14.6	14.4
Ash (% DM)	11.0	10.6	11.1	10.1	10.0
Gross energy (KJ/ g DM)	23.2	23.3	23.4	23.5	23.8

[a] Riken Vitamin, Tokyo, Japan.

[b] Highly unsaturated fatty acid, Poweash A, Oriental Yeast Co, Ltd., Tokyo, Japan.

[c] Vitamin mixture (g kg^{-1} diet): ß-carotene 0.10; vitamin D_3 0.01; menadione $NaHSO_3.3H_2O$ (K_3) 0.05; DL-α-tochopherol acetate (E) 0.38; thiamine-nitrate (B_1) 0.06; riboflavin (B_2) 0.19; pyridoxine-HCl (B_6) 0.05; cyanocobalamin (B_{12}) 0.0001; biotin 0.01; inositol 3.85; niacine (nicotic acid) 0.77; Ca panthothenate 0.27; folic acid 0.01; choline choloride 7.87; ρ-aminobenzoic acid 0.38; cellulose 1.92.

[d] Mineral mixture (g kg^{-1} diet): $MgSO_4$ 5.07; Na_2HPO_4 3.23; K_2HPO_4 8.87; Fe citrate 1.10; Ca lactate12.09; Al $(OH)_3$ 0.01; $ZnSO_4$ 0.13; $CuSO_4$ 0.004; $MnSO_4$ 0.03; Ca $(IO_3)_2$ 0.01; $CoSO_4$ 0.04.

[e] L-Ascorbyl-2-phosphate-Mg.

[f] Dry matter basis.

All fish were fed the respective test diets to apparent satiation by hand twice daily, 7 days per week for 45 days. Special care was taken to collect uneaten diets (if leftover), freeze dried and finally subtracted from total amount of supplied dry feed to get the actual feed intake. Every 10 days interval, all the fish were weighted in bulk to determine growth and visually checked their physical appearance, movement and activity. The mean (± S.D.) water quality parameters monitored during the feeding trial were: water temperature 28.2 ± 0.8°C, pH 8.0 ± 0.5 and salinity 33.5 ± 0.9 ppt. These ranges are considered within optimal values for juvenile red sea bream.

3.1.3.3 *Sample collection*

At the beginning of the experiment, 20 fish from the stock were sampled for analysis of whole body composition and stored at −20°C. At the end of the experiment, all the fish were anaesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) and the total number, individual body weight and fork length of fish from each tank were measured. Five fish from each replicate tank were randomly collected and stored at −20°C for final whole body analysis. Liver was dissected out from 3 fish per replicate tank, weighted and stored at −80°C. Blood was drawn by puncturing the caudal vein of 4 fish per replicate. Plasma samples were collected after spinning down the heparinized blood at 3000×g for 15 min at 4°C. All the blood samples were kept at −80°C until analysis.

3.1.3.4 *Biochemical analysis*

Proximate composition of feed ingredients, test diets and whole body samples were analyzed by following AOAC (1990). Moisture was determined by drying the sample at 105°C to a constant weight. The Kjeldahl method was used to determine nitrogen levels and crude protein was calculated multiplying by 6.25. Total lipid was analyzed using the Bligh and Dyer (1959) method and ash by combustion at 550°C in a muffle furnace. The gross energy of the diets was determined using a bomb calorimeter (OSK 150, Ogawa Sampling,

Saitama, Japan). Total amino acid (TAA) and free amino acid (FAA) concentration in the ingredients and diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Tokyo, Japan) according to Teshima *et al*. (1986a). The analyses of Cu, Zn, Cd and Pb in diet and whole body fish samples were performed by Atomic Absorption Spectrophotometer (AAS; Hitachi A-2300, Tokyo, Japan) after acid digestion. Plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). To evaluate the oxidative stress of fish, biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer (FRAS4, Diacron International s.r.l., Grosseto, Italy) following Morganti *et al*. (2002) and Kader *et al*. (2010).

3.1.3.5 *Statistical analysis*

All data from the feeding trial, chemical analysis, element composition, blood chemistry and oxidative status were tested using one-way analysis of variance (Package Super-ANOVA, ver, 1.11, Abacus Concepts, Berkeley, CA, USA). The level of significance between individual treatments ($P < 0.05$) was evaluated by Duncan's New Multiple Range Test (DMRT) (Package Super-ANOVA).

3.1.4 Results

The experimental diets showed similar composition with respect to protein, lipid and energy level (Table 3.1.1). Although there were some variations in essential amino acid (EAA) content among the different diets (Table 3.1.2), comparable values were found in FP0, FP15 and FP30, while FP45 and FP60 diets showed comparatively deficient levels of methionine, tryptophan and valine for juvenile red sea bream (Forster and Ogata, 1998). Table 3.1.3 presents the FAA contents in fishmeal, FP and experimental diets. The total FAA

was very low in FP compared to fishmeal, which was reflected in the tendency of total levels of FAA to decrease, as levels of FP increased in the diets.

The effects of the test diets on fish growth performance, feed utilization and somatic parameters after 45 days feeding trial are summarized in Table 3.1.4. High survival (%) was observed in all the dietary treatments without significant difference ($P > 0.05$) among them. Growth performances in terms of weight gain (%) and SGR (% day) of fish in different dietary treatments were decreased with the increased levels of FP in diets. Neither weight gain (%) nor SGR (% day) were significantly different among FP0, FP15 and FP30 which represented 0, 15 and 30% fishmeal protein replacement with FP respectively. However, higher replacement groups (FP45 and FP60) exhibited significantly ($P < 0.05$) lower growth performances. Similarly, feed intake was affected by the dietary treatments and significantly higher feed intake was found in fish fed FP0, FP15 and FP30 diets than that of fish fed FP60 diet. Feed efficiency ratio (FER) and protein efficiency ratio (PER) were also significantly lower in the FP60 group as well. However these parameters (feed intake, FER and PER) were not significantly different among fish fed FP15, FP30 and FP45 diets. There was a decreasing trend in the condition factor (CF) with the increasing level of FP in the diets. No significant difference was found in the hepatosomatic index (HSI) among the treatments.

Table 3.1.2: Essential amino acid contents of fishmeal, fermented product (FP) and test diets (g $100g^{-1}$ dry sample)

Amino acids	Fishmeal	FP	Test diets					Req[a]
			FP0	FP15	FP30	FP45	FP60	
Arginine	4.60	3.16	3.68 (170)[b]	3.44 (161)	3.33 (164)	2.89 (144)	2.28 (115)	(129)
Histidine	2.42	1.89	1.78 (83)	1.89 (88)	1.59 (78)	1.45 (72)	1.62 (82)	(51)
Isoleucine	2.60	1.78	1.69 (78)	1.54 (72)	1.45(71)	1.50 (75)	1.67 (84)	(84)
Leucine	4.98	3.22	3.17 (147)	3.17 (148)	3.10 (152)	3.10 (154)	2.86 (144)	(157)
Lysine	4.75	3.52	3.42 (158)	3.58 (167)	3.62 (178)	3.86 (192)	3.23 (163)	(165)
Methionine	1.50	0.38	1.14 (53)	1.02 (48)	0.88 (43)	0.76 (38)	0.60 (30)	(84)[c]
Phenylalanine	2.58	3.69	2.43 (112)	2.66 (124)	2.74 (134)	3.00 (149)	3.48 (176)	(152)[d]
Threonine	2.69	1.85	1.73 (80)	1.71 (80)	1.71 (84)	1.79 (89)	1.74 (88)	(67)
Tryptophan	0.66	*	0.39 (18)	0.20 (9)	0.17 (8)	0.18 (9)	0.18 (9)	(21)
Valine	3.20	1.73	2.18 (101)	2.20 (103)	1.79 (88)	1.58 (78)	1.09 (55)	(92)

[a] Estimated essential amino acid requirement of juvenile red sea bream (Forster and Ogata, 1998).

[b] Data in parenthesis are expressed: (each essential amino acid/total amount of essential amino acid) × 1000.

[c] Requirement for methionine and cystine; [d] Requirement for phenylalanine and tyrosine.

[*] Trace amount.

Table 3.1.3: Free amino acid contents of fishmeal, fermented product (FP) and test diets (g $100g^{-1}$ dry sample)

Amino acids	Fishmeal	FP	Test diets				
			FP0	FP15	FP30	FP45	FP60
Indispensable							
Arginine	0.17	0.09	0.09	0.08	0.10	0.13	0.09
Histidine	0.07	0.06	0.04	0.04	0.05	0.03	0.08
Isoleucine	0.18	0.02	0.07	0.06	0.05	0.04	0.05
Leucine	0.30	0.01	0.13	0.11	0.08	0.07	0.04
Lysine	0.16	0.08	0.11	0.09	0.09	0.08	0.08
Methionine	0.23	0.09	0.01	0.00	0.00	0.00	0.00
Phenylalanine	0.12	0.04	0.06	0.05	0.04	0.04	0.04
Threonine	0.11	0.08	0.07	0.05	0.02	0.04	0.02
Tryptophan	0.00	0.02	0.00	0.00	0.00	0.00	0.01
Valine	0.24	0.03	0.05	0.04	0.05	0.05	0.06
Dispensable							
Taurine	0.89	0.17	0.51	0.43	0.37	0.33	0.27
Aspartic acid	0.04	0.08	0.02	0.03	0.03	0.04	0.04
Glutamic acid	0.24	0.15	0.13	0.12	0.13	0.13	0.13
Serine	0.03	0.01	0.02	0.02	0.02	0.01	0.01
Proline	0.08	0.00	0.05	0.04	0.03	0.03	0.02
Glycine	0.08	0.12	0.05	0.05	0.06	0.06	0.07
Alanine	0.27	0.08	0.18	0.16	0.13	0.13	0.09
Tyrosine	0.04	0.02	0.01	0.01	0.00	0.00	0.02
Hydroxyproline	0.08	0.04	0.03	0.04	0.02	0.01	0.00
Total	3.35	1.55	1.62	1.41	1.27	1.24	1.11

Values are means of duplicate measurements.

Table 3.1.4: Growth performance, nutrient utilization and somatic parameter in juvenile red sea bream fed test diets for 45 days

Parameters	Test diet				
	FP0	FP15	FP30	FP45	FP60
IBW (g)[a]	2.81 ± 0.02	2.84 ± 0.01	2.82 ±0.01	2.86 ± 0.02	2.82 ± 0.01
FBW (g)[b]	26.35 ± 0.68[c]	24.99 ± 0.93[c]	24.64 ± 0.20[c]	21.92 ± 0.75[b]	19.42 ± 1.01[a]
WG (%)[c]	837 ±29.1[b]	779 ± 33.1[b]	775 ± 6.8[b]	667 ± 24.6[a]	589 ± 35.3[a]
SGR (% day)[d]	4.97 ± 0.07[b]	4.83 ± 0.08[b]	4.82 ± 0.02[b]	4.53 ± 0.07[a]	4.28 ± 0.12[a]
FI (g fish^{-1} 45 days^{-1})[e]	33.4 ± 0.33[c]	32.2 ± 1.08[bc]	32.2 ± 1.05[bc]	29.6 ± 0.24[ab]	28.4 ± 1.25[a]
FER[f]	0.71 ± 0.02[c]	0.69 ± 0.01[bc]	0.68 ± 0.03[bc]	0.64 ± 0.02[ab]	0.58 ± 0.01[a]
PER[g]	1.39 ± 0.03[b]	1.35 ± 0.02[b]	1.34 ± 0.06[b]	1.27 ± 0.04[ab]	1.16 ± 0.02[a]
Survival (%)	99.3 ± 0.7	97.8 ± 1.3	98.5 ± 0.7	98.5 ± 1.5	97.1 ± 0.7
CF[h]	2.31 ± 0.05[c]	2.25 ± 0.07[bc]	2.24 ± 0.04[bc]	2.12 ± 0.05[ab]	2.08 ± 0.04[a]
HSI[i]	1.47 ± 0.10	1.53 ± 0.14	1.41 ± 0.11	1.45 ± 0.31	1.19 ± 0.20

Values are means ± S.E.M. (n = 45). Within a row, means with the same letters are not significantly different ($P > 0.05$).

[a] Mean initial body weight.

[b] Mean final body weight.

[c] Weight gain (%), (final weight – initial weight) × 100 / initial weight.

[d] Specific growth rate, {ln (final weight) – ln (initial weight) / 45 days} × 100.

[e] Feed intake, (dry diet given – dry remaining diet recovered) / no of fish.

[f] Feed efficiency ratio, total live weight gain (g) / total dry feed intake (g).

[g] Protein efficiency ratio, live weight gain (g) / protein intake (g).

[h] Condition factor (%), weight of fish / fork length of fish^3 × 100.

[i] Hepatosomatic index (%), weight of liver / weight of fish × 100.

Whole body proximate composition of fish is reported in Table 3.1.5. Significant ($P < 0.05$) effects were found in dry matter and total lipid content of the whole body at the end of the feeding trial while whole body protein and ash content were unaffected. The fish fed FP60 exhibited significantly lower dry matter and total lipid than those in fish fed FP0, FP15 and FP30 diets. Concentration of Cu, Zn, Cd and Pb in the diets and whole fish body are shown in Table 3.1.6 and Fig. 3.1.1, respectively. It was found that dietary Cu and Cd significantly increased with increasing levels of FP, while no differences were found for Zn and Pb among the diets. At the end of the feeding trial, whole body analysis showed that Cd was significantly increased in fish fed FP45 and FP60 diets and Pb was increased in all the replacement groups, while no significant difference was found in Cu and Zn content compared to the control.

Table 3.1.7 summarized hematocrit levels, plasma chemical parameters and oxidative status of red sea bream after the 45 days feeding trial. Hematocrit levels were highest in the control group, but no significant difference was found among the treatments. Plasma total bilirubin (T-Bil), glucose (Glu), glutamyl oxaloacetic transaminase (GOT), glutamyl pyruvic transaminase (GPT) and blood urea nitrogen (BUN) did not show significant difference but tended to increase in the replacement groups. Plasma triglyceride (TG) was significantly increased in fish fed FP45 and FP60 diets, while total cholesterol (T-Cho) and high density lipoprotein cholesterol (HDL-c) significantly decreased with the increasing levels of FP in the diets. Significantly higher levels of d-ROMs were detected in FP0 group of fish compared to fishmeal replacement groups, while such variations were not found in BAP among different treatments. Fig. 3.1.2 shows the pattern of combined effects of d-ROMs and BAP. FP30, FP45, and FP60 diet groups were located in A, FP0 in B, and FP15 in C zones, respectively.

Table 3.1.5: Proximate analysis (%) of whole body of juvenile red sea bream fed test diets for 45 days

Parameters	Initial[a]	Test diet				
		FP0	FP15	FP30	FP45	FP60
Dry matter	19.6	28.75 ± 0.31^{bc}	29.76 ± 0.59^{c}	28.90 ± 0.26^{bc}	28.23 ± 0.12^{ab}	27.22 ± 0.44^{a}
Crude protein	12.7	15.09 ± 0.14	15.78 ± 0.15	15.09 ± 0.17	15.21 ± 0.06	15.07 ± 0.09
Total lipid	2.31	9.45 ± 0.35^{b}	9.33 ± 0.56^{b}	9.18 ± 0.11^{b}	8.57 ± 0.15^{ab}	7.68 ± 0.35^{a}
Crude ash	4.4	4.43 ± 0.11	4.62 ± 0.14	4.45 ± 0.14	4.32 ± 0.06	4.40 ± 0.13

Values are means ± S.E.M. (n = 3). Within a row, means with the same letters are not significantly different ($P > 0.05$).

Crude protein, total lipid and ash are expressed on a wet weight basis (%).

[a] Initial values were not included in the statistical analysis.

Table 3.1.6: Element concentrations (μg g^{-1}) of the experimental diets containing graded levels of FP

Element	Test diets				
	FP0	FP15	FP30	FP45	FP60
Copper (Cu)	5.11 ± 0.45^{a}	6.45 ± 0.28^{ab}	6.33 ± 1.37^{ab}	8.56 ± 0.63^{bc}	9.92 ± 0.15^{c}
Zinc (Zn)	98.58 ± 6.63	103.4 ± 2.9	106.6 ± 0.8	121.3 ± 18.7	122.2 ± 2.1
Cadmium (Cd)	0.54 ± 0.05^{a}	0.86 ± 0.01^{ab}	1.01 ± 0.24^{b}	1.47 ± 0.02^{c}	1.81 ± 0.01^{c}
Lead (Pb)	0.68 ± 0.09	0.66 ± 0.13	0.57 ± 0.04	0.88 ± 0.17	0.78 ± 0.11

Values are means ± S.E.M. (n = 3). Within a row, means with the same letters are not significantly different ($P > 0.05$).

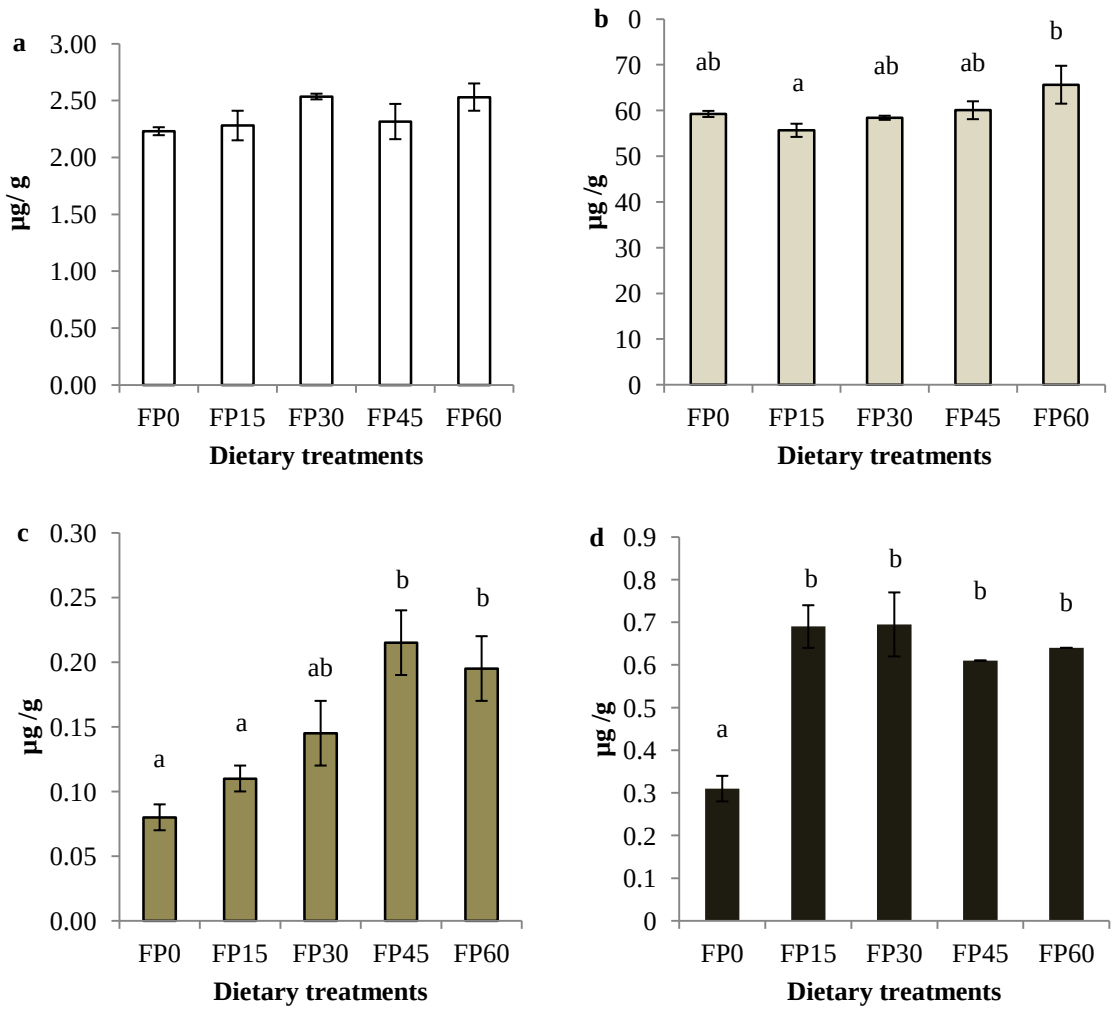

Fig. 3.1.1: Element content of whole body (dry matter) of red sea bream fed test diets for 45 days. Values are means ± S.E.M. (*n* = 3). **a** copper (Cu), **b** zinc (Zn), **c** cadmium (Cd), **d** lead (Pb).

Table 3.1.7: Blood parameters in red sea bream fed test diets for 45 days

Parameters	Test diet				
	FP0	FP15	FP30	FP45	FP60
Hematocrit (%)	40.00 ± 0.76	37.00 ± 0.58	37.00 ± 1.00	37.67 ± 1.20	39.33 ± 0.88
Total protein (g/dl)	4.17 ± 0.18	4.23 ± 0.22	4.23 ± 0.19	4.50 ± 0.25	4.13 ± 0.09
Total bilirubin (g/dl)	0.20 ± 0.00	0.23 ± 0.03	0.23 ± 0.03	0.30 ± 0.06	0.27 ± 0.03
Glucose (mg/dl)	66.33 ± 8.84	59.50 ± 9.50	79.50 ± 2.50	81.00 ± 12.06	89.00 ± 7.21
GOT(IU/l)[a]	39.00 ± 7.00	36.50 ± 15.50	79.00 ± 0.00	77.00 ± 8.00	61.50 ± 10.50
GPT (IU/l)[b]	10.00 ± 0.00	10.00 ± 0.00	10.33 ± 0.33	14.33 ± 3.38	11.00 ± 1.00
LDH (IU/l)[c]	1847 ± 721	1840 ± 673	3048 ± 192	1881 ± 143	1511 ± 137
BUN (mg/dl)[d]	6.67 ± 0.88	7.33 ± 0.33	7.33 ± 0.33	8.33 ± 1.33	8.33 ± 0.88
Triglyceride (mg/dl)	238 ± 21[a]	231 ± 11[a]	227 ± 17[a]	325 ± 5[b]	313 ± 14[b]
T-Cho (mg/dl)[e]	312 ± 18[bc]	321 ± 4[c]	267 ± 16[ab]	273 ± 14[abc]	232 ± 19[a]
HDL-c (mg/dl)[f]	298 ± 18[b]	282 ± 4[b]	216 ± 4[a]	213 ± 19[a]	220 ± 4[a]
Oxidative stress parameters					
d-ROMs (U.Carr)[g]	165.7 ± 4.1[b]	74.3 ± 4.7[a]	46.0 ± 18.2[a]	77.0 ± 37.5[a]	29.7 ± 1.3[a]
BAP (µ Mol/l)[h]	3875 ± 84	3296 ± 13	3836 ± 121	3721 ± 60	3815 ± 404

Values are means ± S.E.M. (n = 3). Within a row, means with the same letters are not significantly different ($P > 0.05$).

[a] Glutamyl oxaloacetic transaminase (GOT); detection limit for GOT is ≥10.00.

[b] Glutamic pyruvate transaminase (GPT); detection limit for GPT is ≥10.00.

[c] Lactate dehydrogenase (LDH), [d] Blood urea nitrogen (BUN), [e] Total cholesterol (T-Cho), [f]High density lipoprotein cholesterol (HDL-c), [g] Value of reactive oxygen metabolites,

[h] Value of biological anti-oxidant potential.

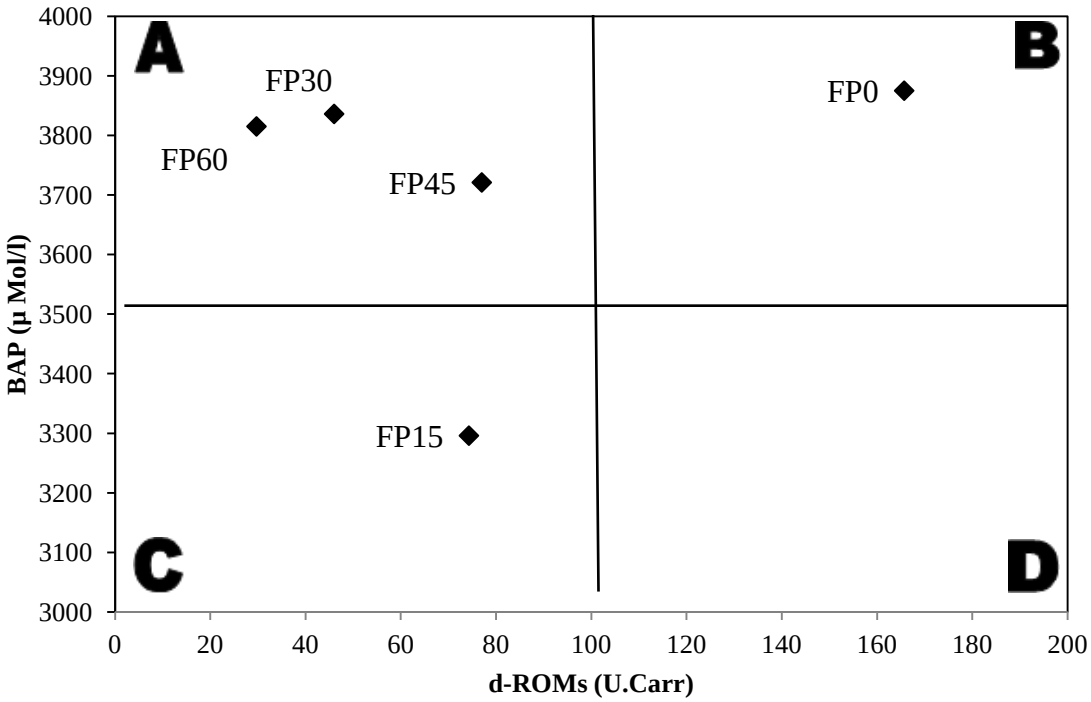

Fig. 3.1.2: Oxidative stress parameters in red sea bream fed test diets for 45 days. Values are means of triplicate groups. Zone **A**: High antioxidant potential and low reactive oxygen metabolites (good condition). Zone **B**: High antioxidant potential and high reactive oxygen metabolites (acceptable condition). Zone **C**: Low antioxidant potential and low reactive oxygen metabolites (acceptable condition). Zone **D**: Low antioxidant potential and high reactive oxygen metabolites (stressed condition).

3.1.5 Discussion

The present study demonstrated that no significant differences ($P > 0.05$) on most parameters investigated concerning fish performances were detected among FP0, FP15 and FP30 diet groups, respectively. This suggests that up to 30% fishmeal protein could be replaced with fermented soybean meal and scallop by-product blend. The result is in harmony with a previous report by Sun *et al*. (2007) who found that fermented fisheries by-products and soybean curd residues could replace up to 30% fishmeal protein in the diet of juvenile Japanese flounder. It has also been reported that 50% fishmeal protein can be replaced with fermented fish offal, mustard oil cake and rice bran mixture in the diets of Indian major carp (*L. rohita*) and freshwater catfish (*H. fossilis*) (Mondal *et al*., 2007, 2008). Co-dried fermented fish silage and soybean meal can also partially replace fishmeal protein in tilapia and catfish diets (Fagbenro *et al*., 1984; Fagbenro and Jauncey, 1995). Therefore, it is evident from the present study that a blend of fisheries by-product and plant protein, and subsequent fermentation is an effective method to utilize alternative protein sources in aquafeeds. Blend of different ingredients is often recommended in achieving balanced nutritional composition, complementing amino acid profiles and masking the unpalatable substances present in feed ingredients (Tidwell *et al*., 2005; Kader *et al*., 2010; Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Guo *et al*., 2007). Fermentation is a technique commonly used in the food industry, which may decrease or eliminate anti-nutritional constituents from oilseeds (Reddy and Pierson, 1994), and increases the bio-availability of nutrients as well as overall nutritional quality (Canella *et al*., 1984). It is also a useful method for drying wet product with minimal nutrient loss, which increases the storage stability (Yamamoto *et al*., 2004).

Since all the diets were isonitrogenous, isolipidic and isocaloric, the reduced growth of the fish fed diets containing higher levels of FP appeared to be due to increasing levels of soybean meal. Significantly ($P < 0.05$) lower growth performances in fish fed diets FP45 and

FP60 might be attributed to the decreased feed intake in these groups of fish. Higher inclusion levels of soybean proteins is known to decrease the diet palatability, which has significant effects on feed intake in red sea bream (Kader *et al*., 2010). It is well known that diet palatability and feed intake of fish will be affected by the amount as well as kind of FAAs (Mackie and Mitchell, 1985; Uyan *et al*., 2007; Chatzifotis *et al*., 2008; Kader *et al*., 2010). In the present experiment, comparatively lower values of FAAs were found in FP than in fishmeal. Decreasing trends of taurine, alanine and total FAAs were found with the increasing levels of FP in the diets and the lowest values were detected in diets FP45 and FP60. It has been reported that taurine is an effective feeding stimulant in high soybean based diets for common dentex *Dentex dentex* (Chatzifotis *et al*., 2008) and an essential element for normal feeding behavior and growth of red sea bream (Matsunari *et al*., 2008). Therefore, lower growth performance in higher fishmeal replacement groups of fish might be influenced by the dietary taurine content (Matsunari *et al*., 2008; Takagi *et al*., 2001). An imbalance of dietary essential amino acids (EAAs) might be another reason for poor growth performance in fish fed FP45 and FP60 diets (Takagi *et al*., 2001). Dietary methionine, tryptophan and valine were comparatively low in FP45 and FP60 diets, which might not satisfy the requirements of the juvenile red sea bream (Forster and Ogata, 1998). FER and PER were also lowest in these groups, which resulted in the reduced growth performance of the fish. The other factors responsible for lower growth performance with higher levels of FP in diets might be the poor digestibility of FP and antinutritional factors probably still present after fermentation although quantitative analyses were not performed in the present experiment that need further clarification. Tidwell *et al*. (2005) mentioned that poor performance of largemouth bass, *Micropterus salmoides* fed on a diet replacing 50% fishmeal with SBM might be due to antinutritional factors rather than the palatability or imbalance amino acids of diets as feed intake was not significantly different.

In the present experiment, both whole body dry matter and lipid content were significantly ($P < 0.05$) decreased in fish fed higher level of FP (higher soybean meal), and this has also been reported for red sea bream (Uyan *et al*., 2007), Japanese flounder (Sun *et al*., 2007) and tin foil barb, *Barbodes altus* (Elangovan and Shim, 2000). Since fishmeal lipid contains a good proportion of both saturated and unsaturated fatty acids, fish fed the higher levels of fishmeal based diets might have better lipid utilization (Dias *et al*., 2009). Moreover, it may be possible that fish fed higher levels of fishmeal might have converted a larger portion of dietary protein to lipid, if amino acid intake was in excess of requirements (Elangovan and Shim, 2000).

Although some metals and their compounds like Cu and Zn are essential for fish metabolism, they are potentially harmful if consumed in higher quantities. Other metals like Cd and Pb have no known role in biological systems and are harmful for the growth performances and health of fish (Canli and Atli, 2003; Moren *et al*., 2006). So far, standard values for the maximum permissible limit of heavy metals are not available for most of the countries. Therefore, dietary and whole body heavy metal contents were compared to the previous studies. Moren *et al*. (2006) reported that dietary composition of heavy metals had no significant effect on the bioaccumulation in fish muscle even at very high levels of Cu and Cd. This might be due to the fact that accumulation of these metals mostly occurs in fish liver and kidney rather than muscle (Canli and Atli, 2003; Berntssen *et al*., 2000). Therefore, variations found in the carcass metal compositions in the present study are possibly due to data of the whole body sample, which is not be the same as those in the muscle sample. Although dietary Cu significantly increased with the increasing levels of FP, this was not reflected in the whole body concentration. Similarly, no significant difference ($P > 0.05$) was found in dietary Pb contents among different treatments, however bioaccumulations of this metal significantly increased in fish fed FP based diets. The reason for this is still unknown.

On the other hand, whole body Cd contents were closely reflected by the dietary levels which are in line with the previous study (Mai *et al*., 2006). This may suggest that the metabolism of heavy metals varies depending on the type of metal. Although this was a preliminary study for the utilization of scallop by-product in aquafeeds, results from the present study will help shedding a light on the heavy metal accumulations in fish tissues when fed the diets containing scallop by-product for a long time.

Blood parameters are important tools for the indication of the physiological stress response as well as the general health condition of fish. Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to the previous findings (Takagi *et al*., 2001; Uyan *et al*., 2007; Kader *et al*., 2010). Accordingly, no serious alteration in the fish health was found in the present study except for those of TG, T-Cho and HDL-c. Plasma TG increased and HDL-c decreased significantly with the increasing levels of FP, correlating with growth retardation which also agrees with the previous findings in red sea bream (Kader *et al*., 2010). Therefore, fish in those groups may be in a slight deteriorated health condition. As all the diets were iso-caloric in the present experiment, the increased triglycerides and decreased HDL-c could be related to abnormal or decreased lipid metabolism of fish and decreased liver function might also be a factor for decreased production of HDL-c (Gaziano *et al*., 1997). Oxidative stress can be generated at high level of reactive oxygen species and/or decreased efficacy of antioxidant system, which is an emerging health risk factor in human or other mammals (Pasquini *et al*., 2008). Recently, in our laboratory, simultaneous analysis of d-ROMs and BAP has been used for the evaluation of the oxidative stress condition of fish. These tests have been applied as a suitable tool for evaluating the oxidative stress in humans, pig, rabbit and dog (Oriani *et al*., 2001; Ballerini *et al*., 2003; Pasquini *et al*., 2008). Based on Kader *et al*. (2010) and some unpublished data, values obtained from the present study are within the range of those

obtained in the previous studies of red sea bream. The present study indicated that d-ROMs value in FP0 group was significantly ($P < 0.05$) higher than in the rest, while similar levels of BAP were maintained among the treatments. As indicated in Fig. 3.1.2, it is interesting to note that FP30, FP45, and FP60 diet groups were located in A zone. This suggests that oxidative stress was reduced in fish fed diets containing FP, although the mechanism that caused reduced oxidative stress for those fish fed fermented ingredients should be further studied. FP30 in particular can be considered the best diet in terms of growth, oxidative condition and tolerance against oxidation.

The present study demonstrated that at least 30% fishmeal protein could be replaced with fermented soybean meal and scallop by-product blend without any negative effects on growth, feed utilization and carcass composition of fish. In addition, element compositions and blood parameters also assured the health and carcass quality of the fish at this recommended inclusion level. The findings of this study will encourage feed manufacturers to utilize plant proteins and sea food processing by-products blend with fermentation more efficiently in generating low-cost and healthy aquafeed. On the other hand, further research is needed to gain more insight into the effects of long term dietary exposure to the recommended feed, on the element composition of fish tissues.

CHAPTER III

Experiment II

Performance responses, nutrient utilizations and body compositions of Japanese flounder, *Paralichthys olivaceus* fed with fermented soybean meal and squid by-product blend as fishmeal replacement

(In contribution: Aquaculture Research)

3.2.1 Abstract

A feeding trial was carried out to examine the efficacy of fermented soybean meal and squid by-product blend (1:1) (FP) as replacement of fishmeal for Japanese flounder, *Paralichthys olivaceus*. Five isonitrogenous (48% crude protein) and isolipidic (14% total lipid) diets were prepared by replacing 0 (FP0), 12 (FP12), 24 (FP24), 36 (FP36) and 48% (FP48) fishmeal protein with FP. The diets were fed to juveniles with initial weight of 3.94 g. Fifteen fish per tank were held in 100L polycarbonate circular tank with triplicates (total 45 fish per treatment). The test diets were hand delivered twice a day at satiation level for 56 days. Weight gain (%) and specific growth rate (% day) of fish were improved with 12% FP substitution, and just a slight decrease of the mentioned parameters was found in FP24 and FP36, respectively. However, no significant ($P > 0.05$) differences were detected relative to the control (FP0). On the other hand, growth performances were significantly ($P < 0.05$) depressed in the highest replacement group (FP48) although dry matter feed intake was not significantly varied among different dietary treatments. Feed efficiency ratio and protein efficiency ratio were also significantly lower in that group as well. There was no significant effect in the proximate composition of whole body of fish compared to the control. However, whole body contents of methionine and phenylalanine were significantly decreased with the increasing levels of FP in diets. Protein retention (% of intake) was significantly decreased in FP48 group while no effect was detected in lipid retention. Retention of most of the amino acids showed a decreasing trend with the increasing levels of FP in diets and significant difference was found in the case of leucine. Dietary treatments did not alter most of the plasma metabolites. No effect was found on the oxidative stress level of fish. Meanwhile, biological antioxidant potential (BAP) significantly increased in the groups of fish fed FP36 and FP48 diets. Serum total protein contents were increased in the replacement groups and significantly higher in FP36 and FP48 groups. Plasma lysozyme activity was improved in all

the replacement groups and FP12 showed significantly higher than others. Antibacterial activity was significantly increased with the increasing levels of FP in diets. Based on the overall performances of fish, it is concluded that FP is a potential candidate for alternative protein ingredient in aquafeed and can replace 36% fishmeal protein in the diet of Japanese flounder.

Key words: Soybean meal, Squid by-product, Fermentation, Fishmeal replacement, Growth, Nutrient retention, *Paralichthys olivaceus*

3.2.2 Introduction

Since feed is the single most important economic factor governing the success of the commercial aquaculture production and fishmeal is the most expansive ingredient in aquafeed; reducing the amount of fishmeal in diet formulations, without reducing fish performance, could have a positive impact on the profitability of commercial fish production (Tidwell *et al*., 2005). The main scientific area in fish feed production is therefore, to find out alternative protein sources, both from plant and animal origin. In some instances, sea food processing industries generate by-products more than 50% of the total landing which have great potential to use as fishmeal substitute. Japanese are well known for their high consumption of fish and shellfish. Squid is a marine cephalopod and is one of the major seafood in Japanese dishes. The world total catches of cephalopods (squid, cuttlefishes and octopuses) were 3.6 - 3.8 millions of tons (FAO, 2009) and the domestic capture of squid was 0.33 millions of tons in Japan (http://www.maff.go.jp). Thus, the industry generated considerably a large amount of marine wastes by-products. The main types of squid by-products are heads, viscera, unclaimed fins, mantles, tentacles and pens or backbones etc. Only a small part of these by-products are utilized for animal feed production. Most of them treated as waste product; incinerated, dumped into the sea or deposition on land and caused a serious environmental crisis (Xu *et al*., 2008). Due to the recent technological improvement, it is now possible to recycle these by-products, rather than dispose of them in a less environmental friendly manner (Hernández *et al*., 2004). For example, co-drying of wet fisheries by-products with another dry feed ingredients and subsequent fermentation might be an alternative approach for the effective utilization of fisheries by-products in aquafeed (Fagbenro and Jauncey, 1995; Sun *et al*., 2007; Mondal *et al*., 2008). The process might also be effective to complement the nutritional composition of plant proteins (Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Tidwell *et al*., 2005; Guo *et al*., 2007). Squid meal or squid by-

product meal are generally used in aquafeed as feeding stimulant (Xue and Cui, 2001; Catacutan and Pagador, 2004). Our previous study suggested that supplementation of small amount of squid meal can improve the utilization of soybean protein and allow comparatively higher level of fishmeal substitution in red sea bream, *Pagrus major* diet (Kader *et al*., 2010). Therefore, squid by-product is a promising alternative protein which could be utilized by blending with plant protein such as soybean meal and fermentation process (Kader *et al*., 2011).

Fermentation is a cost-effective and useful technique for drying wet products with minimal nutrient loss (Yamamoto *et al*., 2004). Fermentation allows the utilization of microorganisms to breakdown complex compounds to yield a unique tasting and aromatic foods. The process could facilitate to decrease or eliminate anti-nutritional constituents from oilseeds (Reddy and Pierson, 1994), improve the overall nutritional quality (Canella *et al*., 1984) and increase the shelf life of the processed food (Skrede and Nes, 1988). Growth performances were significantly improved by feeding fermented meals like sesame, black gram and duckweed leaf meal in Indian major carp, *Labeo rohita* when compared to raw meal (Mukhopadhyay and Ray, 1999; Bairagi *et al*., 2002; Ramachandran and Roy, 2007). Co-dried fermented fish silage and soybean meal (SBM) was found to be a suitable protein supplement in the diets of catfish, *Clarias gariepinus* and Nile tilapia, *Oreochromis niloticus* (Fagbenro *et al*. 1994; Fagbenro and Jauncey 1995). Mondal *et al*. (2007, 2008) reported that fermented fish offal, mustard oil cake and rice bran mixture could replace 50% fishmeal protein from the diets of Indian major carp, *L. Rohita* and catfish, *Heteropneustes fossilis*. In earlier study, it was also found that fermented SBM and scallop by-products blend could replace 30% fishmeal protein in red sea bream diet (Kader *et al*., 2011).

Japanese flounder is a highly popular and commercially important aquaculture species in Japan. Most farmers are using commercially manufactured feeds, which contain mostly high

amounts of fishmeal, for their operation. Although several alternative protein sources were reported to partially replace fishmeal from the diets of many fish species, information on Japanese flounder is limited. It has been reported that 20-40% of fishmeal could be replaced by SBM, corn gluten meal, feather meal and meat and bone meal with the supplementation of deficient amino acids (Kikuchi *et al*., 1994a; Kikuchi *et al*., 1997; Kikuchi, 1999a; Choi *et al*., 2004). However, comparatively lower level (about 20%) of fishmeal substitution was achieved without amino acid supplementation to the reported alternative proteins. Conversely, Kikuchi *et al*. (1999b) indicated that about 45% fishmeal protein can be replaced by SBM in combination with other protein sources without amino acid supplementation. There is no published data available on the utilization of squid by-products or blend of fermented soybean and squid by-product in Japanese flounder. Therefore, the present study was conducted to evaluate the effect of fishmeal replacement with fermented soybean and squid by-product blend (1:1) in growth performance, feed utilization, nutrient retention and health/welfare of juvenile Japanese flounder.

3.2.3 Materials and methods

3.2.3.1 *Test fish and experimental system*

Juvenile Japanese flounder were collected from a local hatchery, in Miyazaki prefecture, Japan and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were reared in 500 l polycarbonate tank and fed a commercial diet (50% crude protein; Higashimaru, Kagoshima, Japan) for two weeks to adjust in the laboratory condition. The experiment was carried out in 100-l polycarbonate tanks (filled with 80 l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. A flow rate of 1.5 l min^{-1} and natural light/dark regime was maintained throughout the experimental period.

3.2.3.2 *Ingredients, formulation and preparation of test diets*

The fermented soybean and squid by-product blend is termed as fermented product (FP) was supplied by a local company AFCEP, Japan Corp. (Yazu, Niigata, Japan). The condition of fermentation (patent pending Afcep Japan Corp.) was described recently (Yamamoto *et al*., 2010). It was a blend of fermented soybean meal and squid by-product in the proportion of 1:1. High quality brown fishmeal was used as the major protein source in control diet and fishmeal protein was gradually replaced with FP. The proximate composition and chemical analysis of the test diets were shown in Table 3.2.1. Five isonitrogenous (48% crude protein) and isolipidic (14% total lipid) diets were formulated to replace 0, 12, 24, 36 and 48% fishmeal protein with FP. The diets were designated as FP0, FP12, FP24, FP36 and FP48 respectively. Pollack liver oil, soybean lecithin and HUFA were supplied as lipid sources, and wheat flour as the carbohydrate or nitrogen free extract sources.

All the dietary ingredients were first ground to a small particle size in a hammer mill and passed through a 100 μm mesh sieve. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 30 min. Pollack liver oil, soybean lecithin and HUFA were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. The required amount of water (35-40% of the dry ingredients) was then added to the premixed ingredients and mixed for another 30 min. The pH of the diets was adjusted to 7.0-7.5 with 4N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2 to 2.2 mm) to prepare pellets which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60^{o}C for 120 min. The test diets were stored at −28^{o}C in a refrigerator till use.

Table 3.2.1: Ingredients and chemical composition of the experimental diets

Ingredients (% dry matter)	Test diets				
	FP0	FP12	FP24	FP36	FP48
Fishmeal[1]	60.00	52.80	45.60	38.40	31.20
FP[2]	-	12.13	24.25	36.38	48.51
Pollack liver oil[3]	2.50	2.25	2.00	1.75	1.50
Soybean lecithin[3]	2.00	2.00	2.00	2.00	2.00
HUFA[4]	0.50	0.50	0.50	0.50	0.50
Wheat flour	10.00	8.00	6.00	4.00	2.00
Vitamin mixture[5]	4.00	4.00	4.00	4.00	4.00
Mineral mixture[6]	3.00	3.00	3.00	3.00	3.00
Vitamin C ester[7]	0.30	0.30	0.30	0.30	0.30
Activated gluten	5.00	5.00	5.00	5.00	5.00
Carboxymethyl cellulose	1.00	1.00	1.00	1.00	1.00
α-cellulose	11.70	9.02	6.35	3.67	0.99
Proximate composition					
Moisture (%)	11.84	11.38	11.62	11.06	11.91
Crude protein (% DM[8])	47.77	47.57	48.54	48.90	48.88
Total lipid (% DM)	14.56	14.23	14.60	14.49	14.14
Ash (% DM)	14.82	13.67	13.16	12.45	12.39
Carbohydrate (% DM)[9]	11.01	13.15	12.08	13.1	12.68
Gross energy (KJ g^{-1})[10]	18.92	19.11	19.30	19.52	19.30

[1] Nippon Suisan Co. Ltd., Tokyo, Japan: proximate composition (% dry matter): moisture, 7.5; crude protein, 67.0; total lipid, 11.4 and ash, 19.8.

[2] AFCEP, Japan: proximate composition (% dry matter): moisture, 18.4; crude protein, 46.3; total lipid, 10.4 and ash, 8.6.

[3] Riken Vitamin, Tokyo, Japan.

[4] Highly unsaturated fatty acid, Poweash A, Oriental Yeast Co, Ltd., Tokyo, Japan.

[5] Vitamin mixture (g/kg diet): ß-carotene 0.10; Vitamin D_3 0.01; Menadione $NaHSO_3.3H_2O$ (K_3) 0.05; DL-α-Tochopherol Acetate (E) 0.38; Thiamine-Nitrate (B_1) 0.06; Riboflavin (B_2) 0.19; Pyridoxine-HCl (B_6) 0.05; Cyanocobalamin (B_{12}) 0.0001; Biotin 0.01; Inositol 3.85; Niacine (Nicotic acid) 0.77; Ca Panthothenate 0.27; Folic acid 0.01; Choline choloride 7.87; ρ-Aminobenzoic acid 0.38; Cellulose 1.92.

[6] Mineral mixture (g/kg diet): $MgSO_4$ 5.07; Na_2HPO_4 3.23; K_2HPO_4 8.87; Fe Citrate 1.10; Ca Lactate12.09; Al $(OH)_3$ 0.01; $ZnSO_4$ 0.13; $CuSO_4$ 0.004; $MnSO_4$ 0.03; Ca $(IO_3)_2$ 0.01; $CoSO_4$ 0.04.

[7] L-Ascorbyl-2-phosphate-Mg.

[8] Dry matter.

[9] Carbohydrate calculated by difference: 100-protein-lipid-ash-moisture.

[10] Calculated using combustion values for protein, lipid and carbohydrate of 236, 395 and 172 KJ/kg, respectively.

3.2.3.3 *Experimental procedure*

At the start of the feeding trial, the fish were fasted for 24 h before weighing. Healthy and homogenous sized juveniles (average initial body weight 3.94 ± 0.05 g) were distributed in previously prepared 15 tanks with 15 fish per tank. Each test diet was randomly assigned to triplicates tanks. All fish were hand fed the respective test diets to apparent satiation twice (08.30 and 16.30) daily, 7 days per week for 56 days. Special care was taken to collect the uneaten diets which were then freeze dried and finally calculated the feed intake. Every two weeks, all the fish were weighted in bulk to determine growth and check health condition. During the experimental period, the water temperature ranged from 18.5 to 22.0^{o}C; pH 7.9 to 8.3 and salinity 33.1 to 34.5.

3.2.3.4 *Sample collection*

The initial sample of 15 fish was stored at −20^{o}C for initial whole body analysis. At the end of the feeding trial, all fish were fasted for 24 h prior to final sampling. The total number, individual body weight and length of fish in each tank were measured. Five fish from each replicate tank were randomly collected and stored at −20^{o}C for final whole body analysis. Blood was drawn by puncture of the caudal vein of individual fish. Plasma samples were collected after spinning down the heparinized blood at 3000 × *g* for 15 min at 4^{o}C. Serum was collected after clotting whole blood (non-heparinized) centrifuged at 3000 × *g* for 15 min at 4^{o}C. All the blood samples were kept at −80°C until analysis. Liver was dissected out from three fish in each replicate tank, weighted individually to get heapatosomatic index (HSI), and finally pooled together and stored at −80^{o}C.

3.2.3.5 *Biochemical analysis*

The ingredients, diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard AOAC methods (AOAC, 1990). Total amino acid (TAA) and free amino acid (FAA) concentration were analyzed using high performance liquid chromatography (HPLC, Shimadzu Corp. Tokyo, Japan) according to Teshima *et al*., (1986a). To determine TAA, samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzded with 4 N methanesulfonic acid at 110^{o}C for 22 h. The pH of the hydorlysate was adjusted to 2.2, filtered and stored at 4^{o}C. To quantify the free amino acids, 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml^{-1} deionized water) and 5 ml 10% trichloroacetic acid (TCA), homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were then centrifuged at 4^{o}C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4^{o}C. The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column. Plasma chemical parameters and serum protein were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically form blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al*. (2002) and Kader *et al*. (2010). Serum lysozyme activity was measured with turbidimetric assays (Takahashi *et al*., 1986). *Escherichia coli* strain IAM11239 was used for the assay of serum bactericidal activity which was performed according to Yamamoto and Iida (1995).

Table 3.2.2: Total amino acid contents of test diets (g $100g^{-1}$ dry sample)

Amino acids	Test diets				
	FP0	FP12	FP24	FP36	FP48
Indispensable					
Arginine	2.51	2.14	2.49	2.98	3.05
Histidine	1.80	1.82	1.80	1.96	1.72
Isoleucine	1.78	1.71	1.83	1.80	1.79
Leucine	3.16	3.10	3.09	3.23	3.50
Lysine	4.37	4.33	4.14	4.05	3.77
Methionine	1.00	0.94	0.86	0.80	0.72
Phenylalanine	1.53	1.58	1.38	1.40	1.40
Threonine	2.04	2.19	2.21	2.35	2.02
Tryptophan	0.23	0.20	0.19	0.17	0.19
Valine	2.09	2.01	1.96	2.00	1.90
Dispensable					
Taurine	0.50	0.48	0.44	0.47	0.43
Aspartic acid	4.96	5.53	5.62	5.34	5.48
Glutamic acid	9.17	9.44	9.33	9.92	9.08
Serine	1.99	2.09	2.02	2.12	2.05
Proline	2.95	2.90	2.84	3.10	3.05
Glycine	2.85	2.83	2.96	2.59	2.69
Alanine	2.09	2.21	1.86	1.84	1.81
Tyrosine	1.14	1.04	0.78	0.62	0.41

Values are means of triplicate measurements.

Table 3.2.3: Free amino acid contents of test diets (g $100g^{-1}$ dry sample)

Amino acids	Test diets				
	FP0	FP12	FP24	FP36	FP48
Indispensable					
Arginine	0.07	0.07	0.07	0.06	0.05
Histidine	0.25	0.18	0.17	0.16	0.17
Isoleucine	0.02	0.04	0.05	0.07	0.10
Leucine	0.04	0.08	0.10	0.14	0.21
Lysine	0.14	0.16	0.12	0.14	0.20
Methionine	0.02	0.04	0.02	0.04	0.04
Phenylalanine	0.08	0.11	0.11	0.14	0.19
Threonine	0.03	0.04	0.05	0.06	0.09
Tryptophan	0.00	0.00	0.01	0.01	0.01
Valine	0.05	0.08	0.08	0.10	0.14
Dispensable					
Taurine	0.42	0.40	0.39	0.37	0.35
Aspartic acid	0.02	0.05	0.09	0.10	0.12
Glutamic acid	0.06	0.12	0.17	0.20	0.24
Serine	0.02	0.03	0.04	0.05	0.08
Proline	0.03	0.05	0.06	0.07	0.10
Glycine	0.03	0.03	0.03	0.04	0.05
Alanine	0.07	0.09	0.10	0.12	0.15
Tyrosine	0.00	0.01	0.05	0.08	0.11
Total	1.34	1.59	1.69	1.94	2.40

Values are means of triplicate measurements.

3.2.3.6 *Statistical analysis*

All data were subjected to statistical verification using Package Super-ANOVA 1.11, Abacus Concets, Berkeley, California, USA. Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

3.2.4 Results

3.2.4.1 *Effects on growth performance*

Table 3.2.4 shows the growth performance, feed utilization and somatic parameters of fish after 8 weeks feeding trial. During the entire feeding trial, only two fish died in two different tanks and no significant difference ($P > 0.05$) was found in survival (%) of fish at the end of the trial. Weight gain (%) and specific growth rates (% day) were improved with 12% FP substitution (FP12), and just a slight decrease of the mentioned parameters was found in FP24 and FP36 groups respectively. Both the parameters were significantly ($P < 0.05$) decreased in fish fed FP48 diet compared to the control group (FP0). However, no difference was found among fish fed FP0, FP12, FP24 and FP36 diets. In general, all the diets were well accepted by fish and no difference ($P > 0.05$) was found in feed intake among different treatments (overall mean 21.74 ± 1.74 g $fish^{-1}$ $56days^{-1}$). Feed efficiency ratio (FER) and protein efficiency ratio (PER) followed the same trend as growth performance with fish fed FP48 diet had significantly lower value than the control group. Condition factor (CF) was also significantly decreased in the same group while no difference was found between control and other groups. Hepatosomatic index (HSI) was significantly increased with the increasing levels of FP in diets.

Table 3.2.4: Growth performance, nutrient utilization and somatic parameter in juvenile Japanese flounder fed test diets for 56 days

Parameters	Test diets				
	FP0	FP12	FP24	FP36	FP48
Mean initial weight (g)	3.93 ± 0.02	3.93 ± 0.02	3.96 ± 0.06	3.93 ± 0.03	3.93 ± 0.01
Mean final weight (g)	28.6 ± 0.35^{ab}	30.2 ± 0.94^{b}	27.9 ± 1.33^{ab}	27.3 ± 1.2^{ab}	23.8 ± 0.57^{a}
WG[1]	631 ± 6^{b}	667 ± 20^{b}	603 ± 23^{ab}	595 ± 35^{ab}	505 ± 16^{a}
SGR[2]	3.6 ± 0.02^{b}	3.6 ± 0.05^{b}	3.5 ± 0.06^{ab}	3.5 ± 0.09^{ab}	3.2 ± 0.05^{a}
FI[3]	21.0 ± 0.5	22.6 ± 0.1	22.6 ± 1.1	22.5 ± 0.7	21.0 ± 0.9
FER[4]	1.18 ± 0.01^{b}	1.16 ± 0.04^{b}	1.06 ± 0.02^{b}	1.06 ± 0.01^{ab}	0.95 ± 0.02^{a}
PER[5]	2.43 ± 0.02^{bc}	2.44 ± 0.07^{c}	2.18 ± 0.03^{b}	2.17 ± 0.03^{ab}	1.94 ± 0.04^{a}
Survival (%)	100	100	97.7	100	97.7
CF[6]	0.90 ± 0.01^{bc}	0.93 ± 0.01^{c}	0.90 ± 0.01^{bc}	0.88 ± 0.01^{b}	0.82 ± 0.01^{a}
HSI[7]	1.23 ± 0.06^{a}	1.26 ± 0.09^{ab}	1.54 ± 0.05^{bc}	1.69 ± 0.06^{c}	1.68 ± 0.09^{c}

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

[1] Weight gain (%), (final weight – initial weight) × 100 / initial weight.

[2] Specific growth rate (% day^{-1}), {ln (final weight) – ln (initial weight)/56 days} × 100.

[3] Feed intake (g fish^{-1} 56 days^{-1}), (dry diet given – dry remaining diet recovered) / no of fish.

[4] Feed efficiency ratio, total live weight gain (g) / total dry feed intake (g).

[5] Protein efficiency ratio, live weight gain (g) / dry protein intake.

[6] Condition factor (%), weight of fish / length of fish^{3} × 100.

[7] Hepatosomatic index (%), weight of liver / weight of fish × 100.

3.2.4.2 *Effects on body composition*

There was no significant difference in whole body moisture, crude protein, total lipid and ash content between fish fed the control (FP0) and diets in which fishmeal protein was replaced with FP at various levels (Table 3.2.5). The whole body methionine and phenylalanine were significantly decreased with the increasing levels of FP in diets (Table 3.2.6). Almost all other indispensable amino acids (IAAs) in whole body were found the lowest value in fish fed FP48 diet. However, dispensable amino acids (DAAs) were not markedly affected by the dietary groups except taurine which showed a decreasing trend with the increasing levels of FP in diets.

Table 3.2.5: Proximate analysis (%) of whole body of juvenile Japanese flounder fed test diets for 56 days

Parameters	Initial[1]	Test diet				
		FP0	FP12	FP24	FP36	FP48
Moisture	79.39	76.8 ± 0.35	76.9 ± 0.27	76.9 ± 0.05	75.6 ± 0.34	76.3 ± 0.15
Crude protein	13.6	15.6 ± 0.36	15.5 ± 0.20	15.8 ± 0.23	16.3 ± 0.05	16.1 ± 0.13
Total lipid	4.26	4.05 ± 0.30	3.96 ± 0.15	4.11 ± 0.17	4.70 ± 0.03	4.68 ± 0.08
Ash	2.55	3.18 ± 0.08^{ab}	3.00 ± 0.17^{a}	3.28 ± 0.02^{ab}	3.38 ± 0.10^{ab}	3.47 ± 0.02^{b}

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

Crude protein, total lipid and ash are expressed on a wet weight basis (%).

[1] Initial values were not included in the statistical analysis.

Table 3.2.6: Total amino acid (g $100g^{-1}$ dry) in whole body of juvenile Japanese flounder after 56 days feeding trial

Amino acids	Test diets					*P value*
	FP0	FP12	FP24	FP36	FP48	
Indispensable						
Arginine	2.20 ± 0.12	2.19 ± 0.03	2.70 ± 0.25	2.72 ± 0.12	2.39 ± 0.38	0.7505
Histidine	1.94 ± 0.2	1.84 ± 0.10	1.49 ± 0.40	1.46 ± 0.07	1.49 ± 0.26	0.2514
Isoleucine	2.55 ± 0.06	2.56 ± 0.10	2.34 ± 0.26	2.24 ± 0.06	2.11 ±0.24	0.3306
Leucine	4.78 ± 0.08^{ab}	4.87 ± 0.12^{ab}	5.30 ± 0.10^{b}	4.62 ± 0.26^{a}	4.44 ± 0.07^{a}	0.0155
Lysine	5.12 ± 0.04	5.48 ± 0.15	4.99 ± 0.08	3.75 ± 0.36	3.60 ± 1.07	0.1650
Methionine	1.09 ± 0.02^{b}	0.98 ± 0.06^{ab}	1.04 ± 0.05^{ab}	0.89 ± 0.08^{ab}	0.77 ± 0.08^{a}	0.0374
Phenylalanine	2.28 ± 0.14^{b}	2.20 ± 0.18^{ab}	1.66 ± 0.27^{ab}	1.55 ± 0.04^{a}	1.48 ± 0.15^{a}	0.0153
Threonine	2.75 ± 0.03	2.86 ± 0.08	2.49 ± 0.36	2.58 ± 0.04	2.31 ± 0.41	0.5046
Valine	3.01 ± 0.05	3.07 ± 0.06	2.46 ± 0.31	2.68 ± 0.03	2.45 ± 0.24	0.0873
Dispensable						
Taurine	0.54 ± 0.01	0.56 ± 0.03	0.49 ± 0.15	0.49 ± 0.01	0.45 ± 0.07	0.8594
Aspartic acid	8.91 ± 0.22	9.37 ± 0.44	9.75 ± 0.78	10.10 ± 0.36	9.37 ± 0.69	0.6155
Glutamic acid	11.25 ± 0.28	11.51 ± 0.40	11.46 ± 0.33	12.20 ± 0.54	11.34 ± 0.73	0.6795
Serine	2.46 ± 0.03	2.60 ± 0.09	2.84 ± 0.22	2.74 ± 0.07	2.72 ± 0.28	0.5640
Proline	2.85 ± 0.05	3.12 ± 0.11	2.99 ± 0.02	2.23 ± 0.19	2.45 ± 0.66	0.3684
Glycine	3.96 ± 0.07	4.57 ± 0.19	3.43 ± 0.98	3.59 ± 0.21	4.20 ± 0.93	0.6317
Alanine	4.44 ± 0.03	4.82 ± 0.16	4.51 ± 0.34	4.59 ± 0.12	4.40 ± 0.61	0.7960

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

3.2.4.3 *Effects on nutrient retention*

Protein retention (PR) was highest for the control group, while there was no significant difference among FP0, FP12, FP24 and FP36 groups (Table 3.2.9 and Fig. 3.2.1). However, PR was significantly decreased in fish fed diet with the highest level of fishmeal replacement group (FP48). Although, lipid retention (LR) was not significantly affected by the dietary treatments, the highest value was found for fish fed FP0 and the lowest FP24 (Fig. 3.2.2). The retention of individual EAAs in fish fed FP48 diet generally showed the lowest value among the dietary treatments and significant differences were only found in the case of leucine retention (Table 3.2.7). However, no difference was found in individual EAAs retention of fish fed FP12, FP24 and FP36 diets compared to the control group.

3.2.4.4 *Effects on blood parameters*

Blood parameters of juvenile Japanese flounder were presented in Table 3.2.8. Overall, dietary treatments had no effect in hematocrit and blood chemical parameters of fish. Oxidative status of fish was analyzed from blood plasma and it showed no significant difference in d-ROMs while BAP values were tended to increase significantly with fishmeal protein replacement (Table 3.2.8). Total serum protein was significantly increased with the increasing levels of FP in diets, while bactericidal activity (quantified as bacterial count in serum) had an opposite trend. Lysozyme activity was significantly increased in fish fed FP12 diet and no difference was found between fish fed FP0 and FP48 diets.

Table 3.2.7: Amino acid retention (% of intake) in juvenile Japanese flounder after 56 days feeding trial

Amino acids	Test diets					*P value*
	FP0	FP12	FP24	FP36	FP48	
Arginine	21.54 ± 3.72	22.36 ± 1.62	22.35 ± 3.13	22.78 ± 4.77	19.11 ± 4.24	0.9095
Histidine	28.47 ± 1.13	25.06 ± 1.78	19.84 ± 3.61	18.63 ± 1.74	18.95 ± 4.21	0.1098
Isoleucine	37.44 ± 1.19	37.17 ± 1.68	33.92 ± 3.49	31.80 ± 2.11	29.68 ± 0.09	0.0852
Leucine	39.53 ± 1.78^{bc}	39.08 ± 1.07^{bc}	42.00 ± 0.66^{c}	36.88 ± 1.11^{b}	28.21 ± 0.24^{a}	0.0001
Lysine	33.07 ± 1.40	35.72 ± 1.16	33.67 ± 0.00	28.35 ± 3.86	30.93 ± 0.29	0.1872
Methionine	29.06 ± 0.84	26.35 ± 1.98	30.54 ± 1.82	29.10 ± 3.88	22.95 ± 2.68	0.3293
Phenylalanine	38.90 ± 4.79	34.16 ± 2.92	27.97 ± 5.55	26.49 ± 1.88	23.76 ± 2.99	0.0726
Threonine	34.77 ± 1.720	32.23 ± 0.99	28.89 ± 7.45	28.03 ± 1.26	27.59 ± 4.51	0.4784
Valine	37.89 ± 1.61	38.25 ± 0.78	32.33 ± 6.84	34.37 ± 1.25	31.80 ± 0.59	0.0873

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

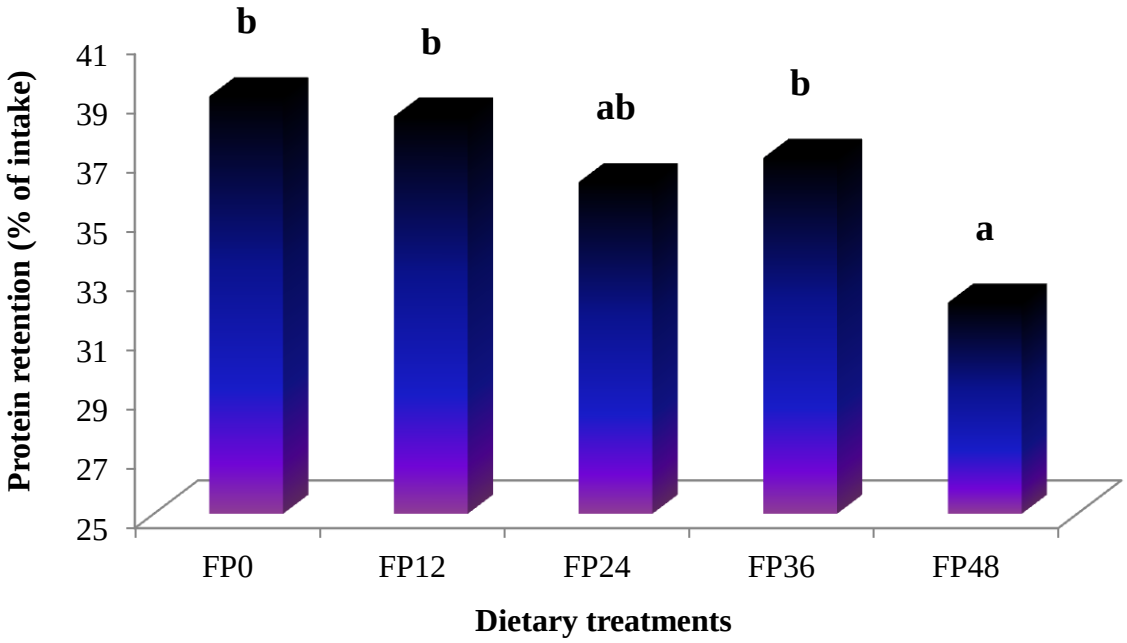

Fig. 3.2.1: Protein retention (% of intake) in juvenile Japanese flounder after 56 days feeding trial

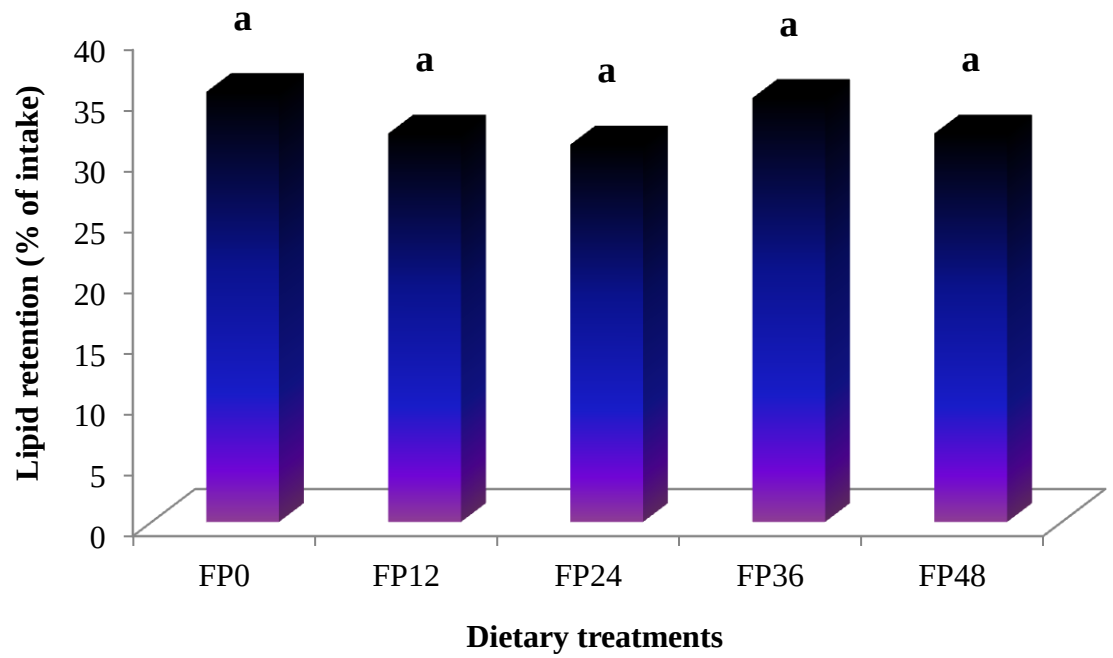

Fig. 3.2.2: Lipid retention (% of intake) in juvenile Japanese flounder after 56 days feeding trial

Table 3.2.8: Blood parameters in juvenile Japanese flounder fed test diets for 56 days

Parameters	Test diet				
	FP0	FP12	FP24	FP36	FP48
Hematocrit (%)	27.1 ± 1.55	32.3 ± 0.28	28.7 ± 0.73	28.1 ± 1.81	27.2 ± 0.88
Total albumin (g/dl)	<1.00	<1.00	<1.00	1.13	1.30
Total bilirubin (g/dl)	0.57 ± 0.03	0.50 ± 0.10	0.40 ± 0.06	0.47 ± 0.09	0.40 ± 0.00
Glucose (mg/dl)	29.0 ± 0.6	32.0 ± 2.1	27.3 ± 1.5	34.0 ± 5.1	34.0 ± 1.5
GOT (IU/l)[1]	<10.0	<10.0	<10.0	15.3	16.7
GPT (IU/l)[2]	<10.0	<10.0	<10.0	<10.0	<10.0
Triglyceride (mg/dl)	494 ± 5.7	461 ± 2.5	463 ± 18.5	462 ± 38.0	487 ± 13.0
Oxidative stress parameters					
d-ROMs (U.Carr)[3]	202 ± 9	207 ± 46	252 ± 27	234 ± 55	208 ± 51
BAP (μ Mol/l)[4]	3919 ± 227[a]	4212 ± 107[a]	4658 ± 200[a]	8997 ± 657[b]	9369 ± 599[b]
Non-specific immune parameters					
TSP (g/dl)[5]	2.87 ± 0.15[a]	2.98 ± 0.12[a]	3.0 ± 0.20[a]	5.17 ± 0.44[b]	5.25 ± 0.35[b]
LA (unit/ml)[6]	36.1 ± 2.8[a]	77.8 ± 7.4[b]	58.8 ± 13.2[ab]	52.8 ± 7.4[ab]	38.9 ± 2.8[a]
BC ($\times 10^6$ CFU)[7]	1.69 ± 0.08[d]	0.70 ± 0.03[c]	0.61 ± 0.01[bc]	0.46 ± 0.13[b]	0.23 ±0.02[a]

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

[1] Glutamyl oxaloacetic transaminase (GOT), [2] Glutamic pyruvate transaminase (GPT),

[3] d-ROMs , reactive oxygen metabolites or oxidative stress parameter, [4] BAP , biological anti-oxidant potential or anti-oxidant level.

[5] TSP, total serum protein, [6] LA, lysozyme activity, [7] BC, bacterial count.

3.2.5 Discussion

Increased aquaculture production costs have increased the interest in utilization of food processing by-products as an alternative protein source in aquafeed. It has several important advantages, these being to diminish dependence on fishmeal, the major protein source and to eliminate the need for costly waste management programs. However, high moisture content in by-products especially seafood processing by-products is a barrier to utilize them in a cost-effective approach as drying in a conventional method is quite expensive (Fagbenro *et al*., 1994). Therefore, alternative processing technologies might be investigated to transform them into utilization friendly product. Co-drying has been reported an effective process whereby dry feedstuffs are added to wet feedstuffs to absorb some portion of the moisture (Fagbenro and Jauncey, 1995). Combination of two feedstuffs might also improve the nutritional properties of the blend (Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Tidwell *et al*., 2005; Guo *et al*., 2007). Fermentation is another approach for drying wet product with minimal nutrient loss (Yamamoto *et al*., 2004). It may decrease or eliminate anti-nutritional constituents from oilseeds (Reddy and Pierson, 1994), improve the bio-availability of nutrients and overall nutritional quality (Canella *et al*., 1984). Therefore the combined technology of co-drying and fermentation might be an effective approach to utilize by-product as well as plant proteins in a cost-effective manner which was also used in our previous study (Kader *et al*., 2011). Co-dried fermented fish silage and SBM can partially replace fishmeal protein in tilapia and catfish diets (Fagbenro *et al*., 1994; Fagbenro and Jauncey, 1995). Nwanna (2003) investigated that fermented shrimp head waste meal can replace 30% fishmeal in African catfish diets. It has also been reported that 50% fishmeal protein can be replaced with fermented fish offal in the diets of Indian major carp and freshwater catfish (Mondal *et al*., 2007; 2008). The fermentation technique allows comparatively higher inclusion levels (30-40%) of oilseed meals, legumes and aquatic

macrophytes compared to non-fermented raw meals (10-20%) in the diets of *L. rohita* (Mukhopadhyay and Ray, 1999; Bairagi *et al*., 2002; Ramachandran and Roy, 2007).

Fermented soybean and squid by-product blend was evaluated as fishmeal substitute in terms of growth performance, nutrient utilization, nutrient retention and general health/welfare of juvenile Japanese flounder. It is evident from the present study that 36% fishmeal protein could be replaced from Japanese flounder diet without any significant effects on the performances of fish. Although growth and nutrient utilization were depleted when fish were fed the FP48 diet, representing 48% fishmeal protein replacement with FP, these parameters for all flounder were within an acceptable range when compared to previous studies for this species in our laboratory (Saitoh *et al*., 2003; Uyan *et al*., 2006). Thus it was suggested that the combination of soybean meal and squid by-product and subsequent fermentation is an efficient technique to develop value added product which could replace at least one third of fishmeal from flounder diet and inclusion level of fishmeal was reduced to about 40% without CAA supplementation. Choi *et al*. (2004) found that dehulled SBM could replace up to 20% of fishmeal from the diet of Japanese flounder without CAA or attractant supplementation and could replace up to 30% of fishmeal with CAA and/or attractant supplementation. Furthermore, the replacement level of fishmeal protein could be accelerated to 30-40% when SBM was blended with cottonseed meal (Pham *et al*., 2007) and supplemented with iron and phosphorus (Lim and Lee, 2008). However, crystalline lysine and methionine were supplemented in both the cases. Similarly, Kikuchi *et al*. (1994b) reported that growth was significantly reduced by 30% fishmeal replacement with defatted SBM and depleted growth was recovered with CAA supplementation. In another study, it was reported that 30-40% fishmeal could be replaced with extruded SBM without CAA supplementation (Saitoh *et al*., 2003). In contrast, Deng *et al*. (2006) observed that growth was gradually decreased with the increasing levels of soy protein concentrate (SPC), even at

the minimum level of 25% substitution. It was also evident in another flatfish, turbot, *Scophthalmus masimus* L. which could replace only 25% fishmeal protein with SPC (Day and González, 2000). Previous results also indicated that 20- 40% of fishmeal protein could be replaced by feather meal (Kikuchi *et al*., 1994a), meat and bone meal (Kikuchi *et al*., 1997), and corn gluten meal (Kikuchi, 1999a) with the supplementation of appropriate CAA. Therefore, it is clear from the present study that blend of squid by-product with SBM provides advantages like nutritional balance and complement amino acids to achieve the similar or even better result for fishmeal replacement level compared to those supplemented with CAA. This is in accordance with the earlier findings by Kikuchi (1999b), who reported that about 45% fishmeal protein can be replaced with defatted SBM in combination with some other protein sources in the diets of Japanese flounder.

Reduction of feed intake with the increasing levels of fishmeal replacement were attributed for the reduced growth performance of Japanese flounder (Uyan *et al*., 2006; Deng *et al*., 2006). In the present experiment, although growth was significantly affected, feed intake was not affected even 48% fishmeal protein replacement with FP. It is well known that feed intake of fish will be affected by the amount as well as the kind of dietary FAA (Mackie and Mitchell ,1985; Uyan *et al*., 2006; Kader *et al*., 2010). Since synthetic FAA was not added to the diets, however dietary total FAA was increased with the increasing levels of FP (Table 3), it is attributed that squid by-products was acted as natural feeding stimulant and improved the diet palatability. Addition of palatability enhancers is an effective approach when developing diets containing high plant protein in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Papatryphon and Soares Jr., 2000; Kissil *et al*., 2000; Kader *et al*., 2010). Squid meal, squid liver meal or squid by-product meal are excellent sources of high quality marine protein which generally used as feeding stimulant in feeds of different fish species (Xue and Cui, 2001; Catacutan and Pagador, 2004). Mai *et al*. (2006)

found a positive relationship between the growth performance of Japanese seabass and 5-10% inclusion of squid viscera meal. Dietary inclusion of 10% squid meal was as effective as CAA in high SPC based diet to improve feed intake and growth of red sea bream (Kader *et al*., 2010). Therefore, diet palatability might not be a factor for reduced growth of fish fed FP48 in the present study. Since all the diets were isonitrogenous, isolipidic and isocaloric, the reduced growth of fish in this group might appeared to be due to imbalance of dietary EAA (Deng *et al*., 2006; Uyan *et al*., 2006). It was found that dietary lysine, methionine, and valine were comparatively low in diets with higher fishmeal replacement groups and a decrease of nearly 28% in methionine concentration occurred in FP48 compared to those in FP0 diet which might not satisfy the requirements of the juvenile Japanese flounder (Forster and Ogata, 1998). Feed efficiency ratio and protein efficiency ratio were also the lowest in these groups, which resulted in the reduced growth performance of the fish. The other factors responsible for lower growth performance with higher level of FP in diet might be the poor digestibility of FP and antinutritional factors are probably still present after fermentation, although quantitative analyses were not performed in the present experiment that need further clarification. Tidwell *et al*. (2005) mentioned that poor performance of largemouth bass, *Micropterus salmoides* that fed on a diet replacing 50% fishmeal with SBM might be due to antinutritional factors rather than the palatability or imbalance amino acids of diets as feed intake was not significantly different. Further, protein and EAA retentions are considered to be an important indicator of the suboptimal supply and utilization of amino acids (Rodehutscord *et al*., 1995; Deng *et al*., 2006). Therefore, decreased protein and EAA retentions in fish fed FP48 diet might partly also be explained for the lower growth performance of fish in this group.

Although growth performance and feed utilization was reduced in fish fed with higher fishmeal replacement groups, dietary treatments have no effects on the whole body proximate

composition of fish at the end of the feeding trial (Kikuchi, 1999b). Most of the dietary amino acids reflected the whole body amino acid composition of fish.

Blood parameters are being increasingly used as indicators of the physiological condition of fish. Fermented product had no observable effect on the hematocrit and hematological parameters of fish in the present experiment and these parameters for all flounder were within a normal range when compared with previous studies for this species (Kikuchi *et al*., 1994b; Kikuchi, 1999 a, b; Jung *et al*., 2003; Pham *et al*., 2007). Accordingly, no serious alteration in the fish health was found in the present study except biological antioxidant potential, serum protein and bactericidal activity of fish, which were significantly increased with the increasing levels of FP in diets. The reason for these increasing activities was not clear. In many biological studies, products made by fermentation have been shown to exhibit immunomodulatory effects. Recently, it has been investigated that fermented SBM exhibited a combined effect of better nutritional status and reduced immunological challenge over the non-fermented SBM in newly weaned piglet (Song *et al*., 2010). Ashida and Okimasu (2005) reported that fermented vegetable product enhanced phagocytic activity and lysozyme activity in Japanese flounder. Fermented shrimp processing waste have been shown to exhibit antioxidant activity (Sachindra and Bhaskar, 2008). Dietary proteins harbour bioactive peptides that are inactive within the sequence of the parent proteins but that can be released during gastrointestinal digestion, food processing or by fermentation (Korhonen and Pihlanto, 2003). During the process of fermentation, the protein is hydrolyzed and produces low molecular weight peptides and amino acids some of which have immunomodulatory effects (Sachindra and Bhaskar, 2008). Protein hydrolysate from seafood processing by-products have been shown immunomodulatrory effects in coho salmon (Murray *et al*., 2003) and Japanese sea bass (Liang *et al*., 2006). Therefore, enhanced health parameters of fish in the present study might partly be associated with the fermentation process. However, the data is

not enough to confirm the result which is another new field of interest for further clarification.

Results from the present study reveal that 36% of fishmeal protein could be substituted by fermented soybean and squid by-product blend without any negative effects on growth, feed utilization, nutrient retention and health/welfare of fish. In this replacement level, inclusion of dietary fishmeal can be reduced to about 40%. The findings of this study will encourage feed manufacturers to utilize plant proteins and seafood processing by-products more efficiently in generating low-cost and healthy aquafeed.

CHAPTER IV

Approach for Improving the Utilization of Alternative Proteins

CHAPTER IV

Experiment I

Supplemental effects of some crude ingredients in improving nutritive values of low fishmeal diets for red sea bream, *Pagrus major*

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., 2010. Aquaculture 308, 136-144.

4.1 Abstract

A feeding trial was conducted to improve nutritional quality of low fishmeal diets by supplementing crude ingredients. Seven isonitrogenous (50% crude protein), isolipidic (12.5% total lipid) and isocaloric (22 KJ/g gross energy) test diets were formulated, where diet 1 was a fishmeal based control diet (FM), and diets 2 to 7 were prepared by replacing 60% fishmeal protein (24% fishmeal contents) as follows: soybean protein concentrate (SPC) alone (SP); SPC with 2.5% crystalline amino acids (CAA) (SPAA); SPC with 10% fish soluble (FS) (SPFS); SPC with 10% krill meal (KM) (SPKM); SPC with 10% squid meal (SM) (SPSM) and SPC with a mixture (total 15%) of FS, KM and SM 5%, respectively (SPMX). Triplicate groups of fish (average initial weight 0.82 ± 0.01 g) were randomly stocked in twenty-one 100-l polycarbonate tanks at a stocking density of 15 fish per tank. The fish were fed to satiation by hand twice daily, 7 days per week for a period of 50 days.

Results showed that weight gain (%) and specific growth rate (SGR) of fish were both significantly lower in fish fed SP, but those parameters recovered when fed diets with supplementation of ingredients used in the study. The fastest growth was found in fish fed SPMX, followed by SPFS, which values were not significantly different ($P > 0.05$) each other, and those groups grew significantly faster than FM. However, no difference was found among FM, KM and SM groups. The growth results were mostly reflected in feed intake. Similarly, feed efficiency ratio, protein efficiency ratio and protein retention (% of intake) were also significantly decreased in fish fed SP while no difference was detected between FM and the rest. Therefore this study demonstrates that the dietary amino acids were balanced by supplementation of FS, KM and SM, and those would have acted as feeding stimulants. Whole body proximate compositions were not markedly influenced by the dietary treatments. No difference was found in the digestive tract and liver protease activity among treatments.

Blood parameters showed that the physiological condition of fish fed SP was significantly depleted, but it was recovered with the supplementation of either CAA or crude ingredients.

Based on the overall performances of fish, it can be concluded that supplementation of FS, KM and SM are as effective as CAA to maintain amino acids balance and can act as attractants in high plant protein based diet for maintaining normal feeding behavior, growth performance and health or welfare of juvenile red sea bream.

Key words: Crude ingredient, Feeding stimulants, Palatability, Feed intake, Low fishmeal feeds, Red sea bream

4.2 Introduction

Because fishmeal replacement is necessary for sustainable aquaculture, it's important to search for the effective approach to utilize the available alternative protein sources other than fishmeal. Fishmeal can be partially replaced in the diets of many fish species, but in most cases, higher or complete replacements have detrimental effects mainly due to imbalance amino acids, decreased bio-availability, and presence of toxins or anti-nutritional properties. Another key factor is lower feed intake in fish because of decreased diet palatability or acceptability as the level of alternative protein sources increases, especially plant proteins (Kubitza *et al*., 1997; Kissil *et al*., 2000; Chatzifotis *et al*., 2008). Commonly recognized feeding stimulants are relatively small soluble molecules, such as certain amino acids (taurine, glycine, arginine, glutamic acid and alanine etc), betaine, nucleotides and organic acids (Grey *et al*., 2009), which are rich in marine organisms like fish, krill, squid, shrimp etc (Gaber, 2005; Smith *et al*., 2005; Mai *et al*., 2006). In comparison, plant proteins contain less of these substances, and this leads to decrease palatability of plant protein rich diets. Therefore, addition of palatability enhancers is an effective approach when developing diets containing high plant protein in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Papatryphon and Soares Jr., 2000; Kissil *et al*., 2000).

Considerable research has dealt with feeding stimulants for different species of fish. A mixture of free amino acids was frequently used as feeding stimulant in many fish species such as sea bass, *Dicentrarchus labrax* (Dias *et al*., 1997), red drum, *Sciaenops ocelletus* (McGoogan and Gatlin, 1997), striped bass, *Morone saxatilis* (Papatryphon and Soares Jr., 2000, 2001) and yellowtail, *Seriola quinqueradiata* (Kofuji *et al*., 2006). However, Kohbara *et al*., (1989) reported that a synthetic mixture is inferior to the natural feeding stimulants as some effective components are absent in the synthetic mixture. Therefore, tissue extracts of marine organisms such as fish (Hidaka *et al*., 2000), shrimp (Mearns *et al*., 1987), squid

(Toften *et al*., 2003; Kofuji *et al*., 2006), krill (Kofuji *et al*., 2006), mussel (Tandler *et al*., 1982) and worms (Fuke *et al*., 1981) are often used as feeding stimulants. In very few cases, the stimulants are supplemented in the form of crude ingredient in feed formulations which is also effective to increase feed intake in fish (Kubitza *et al*., 1997; Kolkovoski *et al*., 2000; Gaber 2005; Mai *et al*., 2006; Kader, 2008).

In juvenile red sea bream, *Pagrus major* Takagi *et al*. (1999) reported that 50% fishmeal protein could be replaced with soy protein concentrate (SPC) without affecting growth performances. Although, SPC has a balanced amino acid composition, sulfur amino acids especially methionine and lysine, are the limiting factors. Higher inclusion levels of SPC resulted in lower feed intake in fish because of decreased attractiveness and palatability of diets (Davis *et al*., 1995; Takagi *et al*., 2001). Therefore, inclusion of palatability enhancer or attractants is recommended in high SPC diets. Although the crude ingredients such as fish soluble (FS), squid meal (SM) and krill meal (KM) etc., which are the natural sources for the feeding stimulatory substances, are often used in commercial feeds with small amounts, the clear effects of those are unknown, or the comparative evaluation was not made yet. In addition, very few studies were available on the effect of these ingredients or whole meals as crude attractants in high plant protein based diets. On the other hand, the use of whole meal is more practical and economical than the use of crystalline amino acids (CAA) or extracts.

Because the studies on the comparative evaluation of CAA, FS, KM and SM in high SPC based diet with low fishmeal level for red sea bream have not been reported, the present study was undertaken to determine their effects on feed intake, growth performances, body composition, enzymatic activity and health or welfare of juvenile red sea bream.

4.3 Materials and methods

4.3.1 Test fish and experimental system

The fertilized eggs of red sea bream were collected from a local hatchery, in Miyazaki prefecture, Japan, and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. Hatched larvae were reared until they reached the desired size of juveniles with feeding live foods and a commercial diet (GemmaMicro, Skretting Ltd., Stavanger, Norway). The feeding trial using juveniles was carried out in 100-l polycarbonate tanks (filled with 80l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

4.3.2 Test diets

Table 4.1, and 4.2 summarize the composition and chemical analysis of the experimental diets. All the dietary components were obtained commercially, except for FS, which was provided by "Makurazaki Fish Processors Cooperatives, Kagoshima, Japan". FS was obtained as a by-product when powdered bonito (“Katsuobushi”in Japanese) is processed in the factories. After processing “Katsuobushi”, the residues are heated at 90^{o}C and the resulting coagulated materials are pressed and passed through a vibrating screen for removing solids followed by centrifugation to separate fish oil and finally evaporated to obtain FS.

Table 4.1: Composition of experimental diet (% dry matter basis)

Ingredients	Diet Group*						
	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
FM[1]	60.00	24.00	24.00	24.00	24.00	24.00	24.00
SPC[2]	-	50.93	45.05	36.54	38.76	34.54	29.45
CAA mixture[3]	-	-	2.50	-	-	-	-
FS[4]	-	-	-	10.00	-	-	5.00
KM[5]	-	-	-	-	10.00	-	5.00
SM[6]	-	-	-	-	-	10.00	5.00
Pollack liver oil[7]	2.00	5.50	5.50	5.50	3.50	5.00	4.50
Soybean lecithin[7]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
HUFA[8]	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Wheat flour	10.00	3.00	5.00	5.00	5.00	5.00	5.00
Vitamin mixture[9]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Mineral mixture[10]	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Vitamin C ester[11]	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Activated gluten	5.00	5.00	5.00	5.00	5.00	5.00	5.00
CMC[12]	1.00	1.00	1.00	1.00	1.00	1.00	1.00
α-cellulose	13.20	1.77	3.15	4.16	3.94	6.66	7.25
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00

*FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1]Nippon Suisan Co. Ltd., Tokyo, Japan: proximate composition (% dry matter): moisture, 8.2; crude protein, 72.1; total lipid, 15.6 and ash 12.0.

[2]SPC: soy protein concentrate; J. Oil Mills, Japan; proximate composition (% dry matter): moisture, 7.7; crude protein, 51.0; total lipid, 3.7 and ash 3.5.

[3]Crystalline amino acid mixture: 0.50% for each of lysine, methionine, taurine, glycine and alanine.

[4]FS: fish soluble; Makurazaki Fish Processors Cooperatives, Kagoshima, Japan; proximate composition (% dry matter): moisture, 50.2; crude protein, 73.4; total lipid, 4.0 and ash, 19.3.

[5]KM: krill meal; Nippon Suisan Co. Ltd., Tokyo, Japan; proximate composition (% dry matter): moistre, 6.9; crude protein, 62.1; total lipid, 23.4 and ash 9.7.

[6]SM: squid meal; Nippon Suisan Co. Ltd. Tokyo, Japan; proximate composition (% dry matter): moisture, 9.3; crude protein, 83.6; total lipid, 9.2 and ash 6.4.

[7]Riken Vitamin, Tokyo, Japan.

[8]Poweash A, Oriental Yeast Co, Ltd., Tokyo, Japan.

[9] Vitamin mixture (g kg^{-1} diet): ß-carotene 0.10; Vitamin D_3 0.01; Menadione $NaHSO_3.3H_2O$ (K_3) 0.05; DL-α-Tochopherol Acetate (E) 0.38; Thiamine-Nitrate (B_1) 0.06; Riboflavin (B_2) 0.19; Pyridoxine-HCl (B_6) 0.05; Cyanocobalamin (B_{12}) 0.0001; Biotin 0.01; Inositol 3.85; Niacine (Nicotic acid) 0.77; Ca Panthothenate 0.27; Folic acid 0.01; Choline choloride 7.87; ρ-Aminobenzoic acid 0.38; Cellulose 1.92.

[10]Mineral mixture (g kg^{-1} diet): $MgSO_4$ 5.07; Na_2HPO_4 3.23; K_2HPO_4 8.87; Fe Citrate 1.10; Ca Lactate12.09; Al $(OH)_3$ 0.01; $ZnSO_4$ 0.13; $CuSO_4$ 0.004; $MnSO_4$ 0.03; Ca $(IO_3)_2$ 0.01; $CoSO_4$ 0.04.

[11]L-Ascorbyl-2-phosphate-Mg.

[12]Carboxymethyl cellulose.

Table 4.2: Chemical analysis of the experimental diets

Ingredients	Diet Group*						
	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Proximate composition (% dry matter basis)							
Crude protein	50.5	49.7	48.8	50.7	49.3	49.1	50.9
Crude lipid	12.3	13.0	12.6	12.6	12.1	12.6	12.8
Ash	12.6	10.1	9.8	12.1	10.3	9.7	11.1
Gross energy (KJ g^{-1})	22.3	23.0	22.9	22.4	22.6	22.8	22.6
Amino acid (AA g $100g^{-1}$ dry sample)							
Arginine	4.53	2.96	3.00	3.78	3.69	3.88	4.27
Histidine	1.91	1.79	1.72	2.02	1.88	1.84	1.94
Isoleucine	1.53	1.54	1.55	1.36	1.32	1.53	1.35
Leucine	3.18	3.19	3.14	2.96	2.92	3.16	3.03
Lysine	3.31	3.09	3.45	2.96	3.01	3.37	3.14
Methionine	1.44	0.89	1.52	0.96	0.94	1.12	1.02
Phenylalanine	2.40	2.69	2.69	2.67	3.04	2.65	2.74
Threonine	1.74	1.69	1.62	1.69	1.69	1.73	1.59
Tryptophan	tr[1]	tr	tr	tr	tr	tr	tr
Valine	2.27	1.71	1.71	1.64	1.69	2.14	1.83
$\sum$ IAA[2]	22.30	19.54	20.40	20.04	20.17	21.42	20.91
Taurine	0.40	0.10	0.62	0.41	0.19	0.20	0.39

*FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1]Trace; [2]$\sum$ IAA, total indispensable amino acids.

Table 4.3: Free amino acid contents of experimental diets (FAA g $100g^{-1}$ dry sample)

	Diet Group*						
Amino acids	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Indispensable							
Arginine	0.04	0.03	0.05	0.05	0.07	0.08	0.09
Histidine	0.09	0.05	0.06	0.38	0.06	0.05	0.23
Isoleucine	0.03	0.01	0.01	0.03	0.01	0.02	0.02
Leucine	0.08	0.01	0.01	0.06	0.01	0.05	0.05
Lysine	0.07	0.06	0.25	0.06	0.07	0.09	0.15
Methionine	0.04	0.01	0.18	0.03	0.02	0.01	0.00
Phenylalanine	0.14	0.07	0.08	0.12	0.38	0.07	0.29
Threonine	0.04	0.01	0.01	0.03	0.01	0.02	0.03
Tryptophan	0.03	0.00	0.00	0.02	0.00	0.02	0.00
Valine	0.09	0.03	0.03	0.06	0.03	0.07	0.04
Dispensable							
Taurine	0.36	0.08	0.54	0.32	0.15	0.16	0.33
Aspartic acid	0.01	0.01	0.02	0.03	0.01	0.03	0.03
Glutamic acid	0.07	0.04	0.04	0.09	0.11	0.13	0.11
Serine	0.01	0.00	0.01	0.02	0.01	0.02	0.02
Proline	0.04	0.01	0.01	0.04	0.06	0.03	0.06
Glycine	0.03	0.01	0.39	0.02	0.03	0.02	0.04
Alanine	0.11	0.04	0.58	0.09	0.05	0.05	0.10
Tyrosine	0.12	0.04	0.05	0.11	0.05	0.05	0.12
$\sum$ FAA[1]	1.40	0.53	2.32	1.56	1.13	0.96	1.69

*FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1]$\sum$ FAA; total free amino acids.

Seven isonitrogenous (50% crude protein), isolipidic (12.5% total lipid) and isocaloric (22 KJ g^{-1} gross energy) diets were formulated, where diet 1 was a 60% fishmeal based control diet (FM). Diets 2 to 7 were prepared as follows, by replacing 60% fishmeal protein with SPC alone (SP); SPC and 2.5% CAA (SPAA); SPC and 10% FS (SPFS); SPC and 10% KM (SPKM); SPC and 10% SM (SPSM) and SPC and a mixture (total 15%) of FS, KM and SM 5% each (SPMX). Pollack liver oil, soybean lecithin and highly unsaturated fatty acids (HUFA) were supplied as lipid sources, and wheat flour as the carbohydrate or nitrogen free extract sources.

All the dietary ingredients were first ground to a small particle size in a hammer mill and passed through a 100μm mesh sieve. The diets were prepared by thoroughly mixing all the dry ingredients in a food mixer for 30 min. Pollack liver oil, soybean lecithin and HUFA were premixed with a sonicator (CA-4488Z, Kaijo Corporation, Tokyo, Japan), added to the dry ingredients and mixed for another 15 min. As FS is sticky and water soluble product, the required amount was mixed with water (35-40% of the dry ingredients), and then added to the premixed ingredients and mixed for another 30 min. The pH of the diets was adjusted to 7.0-7.5 with 4N sodium hydroxide. The mixture was then passed through a meat grinder with an appropriate diameter (1.2 to 2.2 mm) to prepare pellets, which were then dried in a dry-air mechanical convection oven (DK 400, Yamato Scientific, Tokyo, Japan) at 60°C for 120 min. The test diets were stored at −28°C in a freezer untill use.

4.3.3 Feeding protocol

At the start of the feeding trial, healthy and homogenous sized juveniles (average initial body weight 0.82 ± 0.01g) were stocked in previously prepared twenty-one tanks with 15 fish per tank in triplicates per dietary treatment. All fish were fed the respective test diets to the satiation level by hand twice daily, 7 days week per week for 50 days. Special care was taken to collect uneaten diets which was freeze dried and finally subtracted from the total amount

of supplied test diets to calculate the actual feed intake. All fish were weighted in bulk at 10 days interval to determine growth and check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature 27.0 ± 1.2°C; pH 8.1 ± 0.2 and salinity 33.9 ± 0.9 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

4.3.4 Sample collection and biochemical analysis

The initial sample of 20 fish for whole body analysis was stored at −20°C. At the end of the feeding trial, all fish were fasted for 24 h prior to final sampling. All the fish were anaesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg l^{-1}. Then the total number, individual body weight and length of fish from each tank were measured. Five fish from each replicate tank were randomly collected and stored at -20°C for final whole body analysis. Using heparinized syringes, blood was collected from the caudal vein of three fish in each replicate tank and pooled. A small fraction of the heparinized blood was used to analyze the haematocrit and hemoglobin level. Plasma samples were obtained by centrifugation at 3000 × *g* for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80°C. Liver was dissected out from three fish in each replicate tank, weighted individually to get hepatosomatic index (HSI), and finally pooled together and kept at −80°C. Digestive tracts were separated, cut into small pieces, wash with pure water, pooled together and stored at −80°C.

The ingredients, diets and fish whole body were analyzed for moisture, crude protein, total lipid and ash, in triplicate, using standard AOAC methods (AOAC, 1990). Gross energy of the diets was determined using a bomb calorimeter (OSK 150, Ogawa Sampling, Saitama, Japan). Total amino acid (TAA) and free amino acid (FAA) concentration in the ingredients and diets were analyzed using high performance liquid chromatography (HPLC, Shimadzu

Corp. Kyoto, Japan) according to Teshima *et al*. (1986a). To determine TAA, samples were prepared as follows: 2 mg samples were spiked with known amount of norleucine as an internal standard and hydrolyzded with 4 N methanesulfonic acid at 110°C for 22 h. The pH of the hydorlysate was adjusted to 2.2, filtered and stored at 4°C. To quantify the free amino acids 100 mg sample was mixed with 0.9 ml cold deionized water, 0.1 ml internal standard (norleucine, 0.6 mg DL-norleucine 0.1 ml^{-1} deionized water) and 5 ml 10% trichloroacetic acid (TCA), homogenized using a polytron homogenizer (Kinematica, Gmbh LITTAU, Lucerne, Switzerland). Samples were then centrifuged at 4°C, 3000 × g for 15 min and supernatant was repeatedly washed with diethyl ether to remove TCA from homogenate. Finally, pH was adjusted to 2.2 and filtered samples were stored in 4°C. The chromatographic separation and analysis of the amino acids were performed with the HPLC unit with an ion exchange resin column. Protease activity (PA) was analyzed using digestive organ and liver samples according to Sigma's Non-specific Protease Activity Assay (Casein as a Substrate). Hemoglobin and plasma chemical parameters were measured spectrophotometrically with an automated analyzer (SPOTCHEM™ EZ model SP-4430, Arkray, Inc. Kyoto, Japan). Biological antioxidant potential (BAP) and reactive oxygen metabolites (d-ROMs) were also measured spectrophotometrically from blood plasma with an automated analyzer FRAS4, Diacron International s.r.l., Grosseto, Italy by following Morganti *et al*. (2002).

4.3.5 Evaluation of growth performance parameters

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR %, day^{-1}) = {Ln (final weight) – Ln (initial weight) / duration} × 100

Survival (%) = 100 × (final no of fish / initial no of fish)

Feed intake (g $fish^{-1}$ 50 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed efficiency ratio (FER) = live weight gain (g) / dry feed intake (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Protein gain (PG, g kg weight gain^{-1}) = {(final weight (g) × final whole body protein content (%) / 100) – (initial weight (g) × initial whole body protein content (%) / 100)} / (weight gain (g)) × 1000

Protein retention (PR, % of intake) = (protein gain (g kg weight gain^{-1}) × 100) / protein intake (g kg weight gain^{-1})

Condition factor (CF, %) = weight of fish / (length of fish)^3 × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

4.3.6 Statistical analysis

All data were subjected to statistical verification using Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

4.4 Results

4.4.1 Test diet analysis

All the diets contain nearly similar levels of crude protein (50%), total lipid (12.5%) and gross energy (22 KJ g^{-1}) (Table 4.2). However, almost all the IAAs especially arginine, methionine and valine, were found to decrease in all the SPC based diets except for methionine in SPAA, as CAA was supplemented in this diet. Dietary taurine levels were high in SPAA; intermediate in FM, SPFS and SPMX; comparatively low in SPKM and SPSM and very low in the SP. The difference in TAA contents between the highest (FM) and the lowest group (SP) was 2.76 g, and that of other SPC based groups was very small. The dietary contents of free amino acids were shown in Table 4.3. The content of total free amino acids

was the highest in SPAA, followed by SPMX, SPFS, FM, SPKM, SPSM, and SP, respectively. The dietary contents of free histidine and phenylalanine were relatively high in SPFS and SPMX.

4.4.2 Survival and growth performance

Growth performance and feed utilization of the fish are given in Table 4.4. Survival (%) of fish did not differ significantly ($P > 0.05$) between treatments. Final weight, weight gain (%) and SGR of fish fed SPFS and SPMX were significantly higher than those fed the other diets. On the other hand, the growth parameters of fish fed SPAA, SPKM, and SPSM were not significantly different from those of fish fed FM. The poorest growth performance was found in fish fed SP. Similarly, FER, PER and PR were also significantly decreased in fish fed SP while no difference was detected between FM and the rest. However, no difference was detected in PG between FM and other dietary groups.

Feed intake of fish was significantly affected ($P > 0.05$) depending on the dietary treatments. SP (60% fishmeal protein was replaced with SPC alone) was not well accepted by the fish, and the value was significantly lower ($P < 0.05$) than other test diets. On the other hand, feed intake was markedly improved by supplementing CAA and crude ingredients. The significantly higher feed intake was found in fish fed SPFS and SPMX compared to other diet groups. There were no significant differences of feed intake in fish fed SPAA, SPKM, SPSM, and FM. SGR and feed intake of fish are graphically presented in Fig. 4.1. CF followed almost similar trends as with growth performance, where it significantly decreased in fish fed SP compared to FM, SPAA, SPFC and SPMX groups. However, no significant difference was found in CF among SP, SPKM and SPSM groups. On the other hand, dietary treatments showed no significant effects on HSI of fish (Table 4.5).

Table 4.4: Growth parameters and nutrient utilization in red sea bream fed test diets for 50 days*

Parameters	Diet Group**						
	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Initial weight (g)	0.82 ± 0.00	0.82 ± 0.01	0.83 ± 0.00	0.82 ± 0.01	0.83 ± 0.00	0.83 ± 0.01	0.82 ± 0.01
Final weight (g)	23.65 ± 0.31^{bc}	13.73 ± 0.04^{a}	25.71 ± 0.41^{c}	27.91 ± 0.27^{d}	23.15 ± 0.36^{b}	22.97 ± 0.77^{b}	28.38 ± 0.65^{d}
Percent weight gain (%)	2779 ± 34^{bc}	1587 ± 9^{a}	3001 ± 49^{c}	3292 ± 36^{d}	2699 ± 44^{b}	2686 ± 103^{b}	3360 ± 73^{d}
SGR[1]	6.72 ± 0.02^{bc}	5.65 ± 0.01^{a}	6.87 ± 0.03^{c}	7.05 ± 0.02^{d}	6.66 ± 0.03^{b}	6.66 ± 0.08^{b}	7.09 ± 0.04^{d}
FI[2]	21.03 ± 0.48^{b}	13.81 ± 0.22^{a}	23.66 ± 0.67^{b}	26.44 ± 0.65^{c}	20.94 ± 0.71^{b}	20.69 ± 0.57^{b}	27.56 ± 0.49^{c}
FER[3]	1.09 ± 0.01^{b}	0.94 ± 0.02^{a}	1.05 ± 0.01^{b}	1.03 ± 0.02^{b}	1.08 ± 0.02^{b}	1.07 ± 0.01^{b}	1.01 ± 0.00^{ab}
PER[4]	2.15 ± 0.02^{bc}	1.89 ± 0.03^{a}	2.14 ± 0.02^{bc}	2.02 ± 0.05^{ab}	2.27 ± 0.08^{c}	2.18 ± 0.02^{bc}	1.96 ± 0.03^{ab}
PG[5]	158 ± 0.7^{ab}	159 ± 1.1^{ab}	157 ± 1.3^{ab}	159 ± 3.8^{ab}	153 ± 0.7^{a}	156 ± 3.4^{ab}	163 ± 1.7^{b}
PR[6]	33.91 ± 0.21^{b}	29.92 ± 0.20^{a}	33.63 ± 0.53^{b}	32.12 ± 0.30^{ab}	33.42 ± 0.87^{b}	33.84 ± 0.49^{b}	32.00 ± 0.70^{ab}
Survival (%)	95.33 ± 2.33	90.00 ± 3.00	95.33 ± 2.33	97.67 ± 2.33	93.00 ± 0.00	93.00 ± 0.00	97.67 ± 2.33

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

**FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1]SGR: specific growth rate (% day^{-1}), [2]FI: feed intake (g dry diet fish^{-1}50 days^{-1}), [3]FER: feed efficiency ratio, [4]PER: protein efficiency ratio, [5]PG: protein gain (g kg body weight gain^{-1}), [6]PR: protein retention (% of intake).

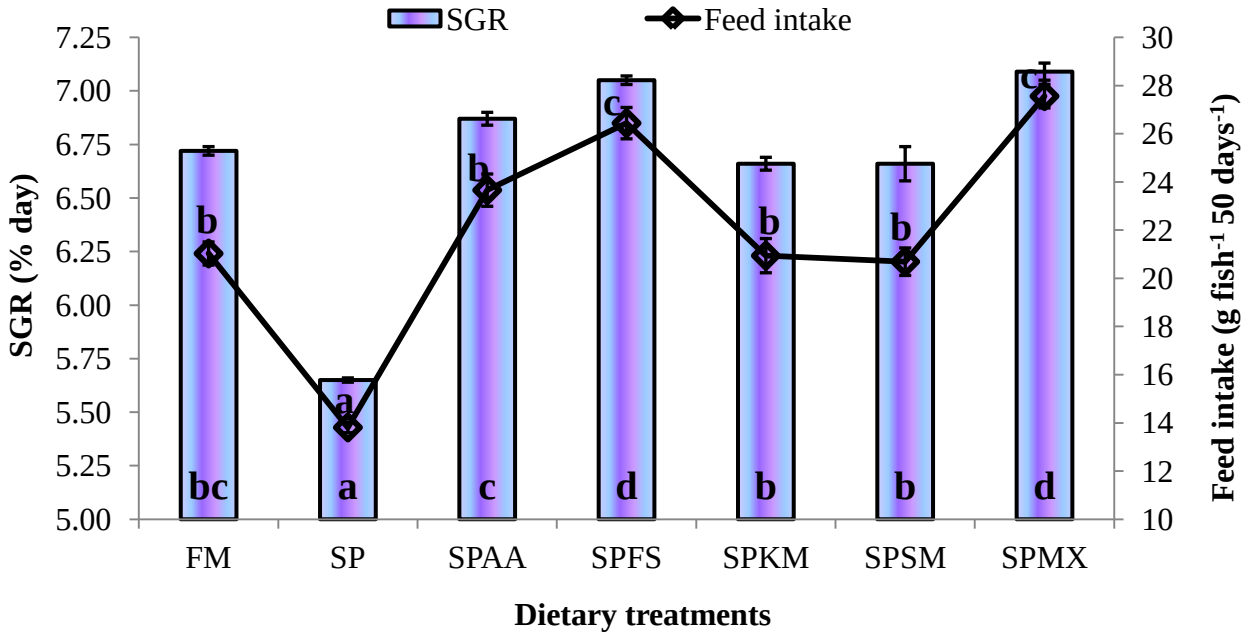

Fig. 4.1: Graphical presentation of specific growth rate (SGR) and feed intake of red sea bream after 50 days feeding trial

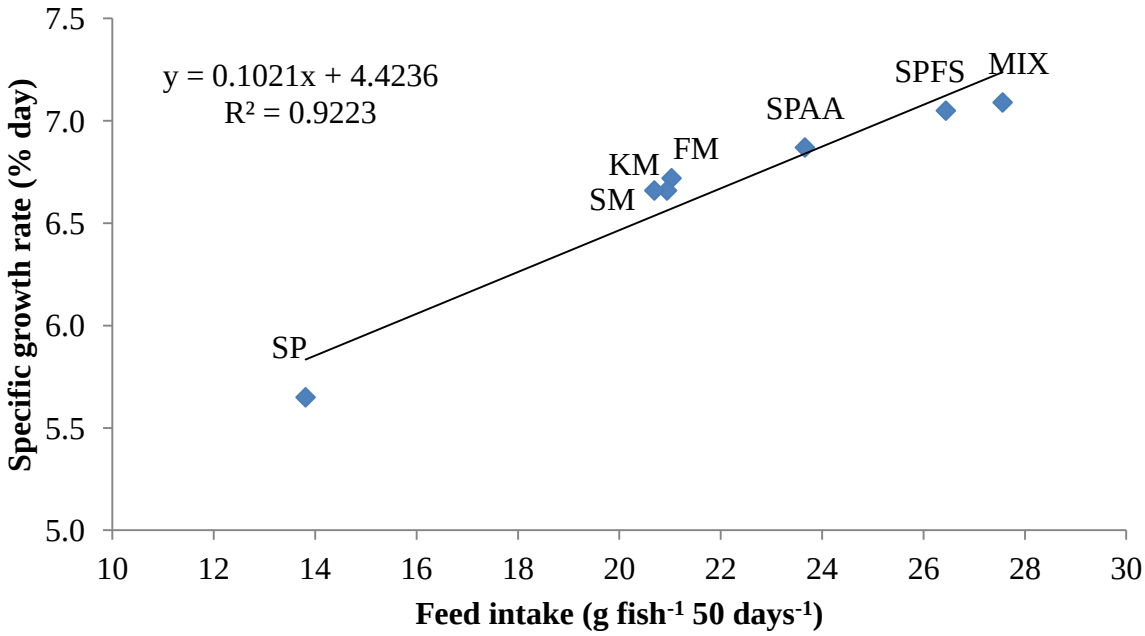

Fig. 4.2: The relationship between specific growth rate (SGR) and feed intake of red sea bream after 50 days feeding trial

4.4.3 Whole body composition

Table 4.5 represents the whole body proximate analysis of fish. In comparison with the control, dietary treatments had no significant influence on the whole body moisture, crude protein and total lipid contents at the end of the feeding trial. On the other hand, whole body ash contents in SP and SPKM groups were significantly lower than that in FM. There were no significant differences among fish fed FM, SPAA, SPFS, SPSM and SPMX groups. The PA in digestive tract was higher than that in liver regardless the dietary treatment (Table 4.6). Although significant differences of PA were not detected between treatments in both organs, there was a trend that the PA in digestive tract was relatively higher in SPAA, SPFS and SPMX compared to those in FM, SP, and SPKM groups while the highest liver PA was found in FM, and the lowest in SPSM.

4.4.4 Blood parameters and oxidative stress condition

Blood chemical parameters in red sea bream are presented in Table 4.7. Statistical significances were detected in five parameters such as total bilirubin (T-Bil), glutamyl oxaloacetic transaminase (GOT), glutamic-pyruvate transaminase (GPT), blood urea nitrogen (BUN), and total cholesterol (T-Cho). Values in SP were higher than those in other groups except T-Cho, where values in SP, SPKM, and SPSM groups were significantly lower than that in FM. T-Cho values in FM, SPAA, and SPFS groups were not significantly different. Values of BUN in all groups except SP were not significantly different.

Oxidative status of fish was also analyzed from plasma (Table 4.7). Although not significant, comparatively higher levels of reactive oxygen metabolites (d-ROMs) were detected in fish fed SP, SPAA, SPFS, SPKM and SPSM groups compared to FM and SPMX groups while biological anti-oxidant potential (BAP) was not affected by the dietary groups.

Table 4.5: Whole body proximate analysis (%) and somatic parameters in juvenile red sea bream fed test diets for 50 days*

Parameters	Initial[1]	Diet Group**						
		FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Moisture	81.1	72.1 ± 0.2	71.5 ± 0.2	70.9 ± 0.5	71.3 ± 0.9	71.9 ± 0.6	71.8 ± 0.4	71.4 ± 0.8
Crude protein	12.1	15.6 ± 0.1	15.7 ± 0.1	15.6 ± 0.1	15.8 ± 0.4	15.2 ± 0.1	15.4 ± 0.3	16.1 ± 0.2
Total lipid	2.9	7.5 ± 0.1	8.4 ± 0.3	8.4 ± 0.1	8.5 ± 0.6	8.7 ± 0.6	8.7 ± 0.2	8.4 ± 0.5
Crude ash	3.2	4.4 ± 0.1^{b}	3.8 ± 0.2^{a}	4.0 ± 0.1^{ab}	4.0 ± 0.1^{ab}	3.9 ± 0.1^{a}	4.0 ± 0.1^{ab}	4.1 ± 0.1^{ab}
CF[2]	-	1.93 ± 0.02^{bc}	1.80 ± 0.01^{a}	1.97 ± 0.01^{bc}	1.98 ± 0.02^{c}	1.87 ± 0.01^{ab}	1.91 ± 0.01^{abc}	1.96 ± 0.04^{bc}
HSI[3]	-	1.12 ± 0.05	1.32 ± 0.10	1.67 ± 0.10	1.61 ± 0.16	1.50 ± 0.13	1.71 ± 0.15	1.70 ± 0.06

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Crude protein, crude lipid and ash are expressed on a wet weight basis.

** FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%), [3]HSI: hepatosomatic index (%).

Table 4.6: Protease activity (unit mg^{-1} protein) in the digestive tract and liver in juvenile red sea bream fed test diets for 50 days*

Protease activity (unit mg^{-1} protein)	Diet Group**						
	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Digestive tract	0.093±0.001	0.093±0.002	0.109±0.000	0.106±0.000	0.093±0.008	0.090±0.003	0.111±0.008
Liver	0.049±0.004	0.042±0.004	0.032±0.002	0.042±0.001	0.038±0.006	0.026±0.003	0.041±0.010

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

** FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

Table 4.7: Blood parameters in juvenile red sea bream fed test diets for 50 days*

Parameters	Diet Group**						
	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Hematocrit (%)	42.5 ± 1.0	44.3 ± 1.3	43.1 ± 0.6	43.1 ± 0.3	42.8 ± 1.6	42.9 ± 0.2	43.8 ± 1.8
Hemoglobin (g/dl)	5.10 ± 0.80	5.35 ± 0.25	4.85 ± 0.35	5.75 ± 0.25	4.75 ± 0.45	5.45 ± 0.15	5.00 ± 0.30
Total protein (g/dl)	4.30 ± 0.06	4.70 ± 0.20	4.53 ± 0.22	4.52 ± 0.12	4.20 ± 0.12	4.15 ± 0.05	4.47 ± 0.09
Total albumin (g/dl)	1.03 ± 0.03	1.20 ± 0.06	1.20 ± 0.06	1.15 ± 0.03	1.07 ± 0.03	1.07 ± 0.07	1.12 ± 0.06
Total bilirubin (mg/dl)	0.63 ± 0.03^{ab}	1.03 ± 0.09^{c}	0.52 ± 0.06^{a}	0.57 ± 0.04^{a}	0.88 ± 0.02^{bc}	0.45 ± 0.10^{a}	0.60 ± 0.06^{ab}
Glucose (mg/dl)	62.3 ± 3.5	69.0 ± 1.7	67.7 ± 2.2	61.7 ± 1.8	67.7 ± 1.7	71.3 ± 1.9	64.7 ± 1.5
GOT (IU/l)[1]	94 ± 4^{ab}	156 ± 28^{c}	91 ± 2^{a}	106 ± 2^{ab}	135 ± 10^{bc}	75 ± 4^{a}	70 ± 8^{a}
GPT (IU/l)[2]	25.7 ± 0.9^{a}	49.7 ± 6.4^{b}	22.7 ± 6.4^{a}	28.3 ± 2.0^{a}	37.7 ± 5.2^{ab}	34.0 ± 4.0^{ab}	29.3 ± 2.3^{ab}
BUN (mg/dl)[3]	6.3 ± 0.3^{ab}	8.3 ± 0.33^{b}	6.3 ± 0.3^{ab}	5.7 ± 0.3^{a}	6.8 ± 0.6^{ab}	6.0 ± 0.6^{a}	5.3 ± 0.3^{a}
Triglycerides (mg/dl)	193 ± 14	248 ± 11	253 ± 30	239 ± 15	188 ± 5	176 ± 27	229 ± 2
Total cholesterol (mg/dl)	334 ± 18^{b}	253 ± 2^{a}	282 ± 9^{ab}	287 ± 7.5^{ab}	243 ± 7^{a}	247 ± 31^{a}	250 ± 4^{a}
HDL-c (mg/dl)[4]	215 ± 20	193 ± 28	215 ± 11	207 ± 15	170 ± 14	267 ± 27	224 ± 2
Amylase (IU/l)	33.3 ± 0.3	42.5 ± 2.5	36.2 ± 6.7	21.7 ± 6.1	48.5 ± 5.5	50.0 ± 19.0	29.7 ± 3.3
Oxidative stress parameters							
d-ROMs (U.Carr)[5]	87.5 ± 1.5^{a}	177.5 ± 18.5^{b}	153.0 ± 13.0^{b}	161.3 ± 6.3^{b}	147.0 ± 1.0^{b}	173.3 ± 10.8^{b}	50.5 ± 1.5^{a}
BAP (μ Mol l^{-1})[6]	3559 ± 270	3453 ± 249	3272 ± 166	3435 ± 2	3469 ± 465	3484 ± 47	3387 ± 256

*Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

** FM: FM based diet (positive control), SP: SPC based diet (negative control), SPAA: SPC with 2.5% crystalline amino acids, SPFS: SPC with 10% fish soluble, SPKM: SPC with 10% krill meal, SPSM: SPC with 10% squid meal, and SPMX: SPC with a mixture of FS, KM and SM at 5%.

[1]GOT: glutamyl oxaloacetic transaminase, [2]GPT: glutamic-pyruvate transaminase, [3]BUN: blood urea nitrogen, [4]HDL-c: high density lipoprotein cholesterol, [5]d-ROMs: reactive oxygen metabolites, [2]BAP: biological antioxidant potential.

4.5 Discussion

The supplemental effects of CAA, FS, KM and SM alone or a mixture of FS, KM and SM in high SPC diets were evaluated in the present experiment. The study was based on the hypothesis that the supplementation would improve the palatability and nutritional quality, especially improving the amino acids compositions of the high plant protein diet, which would help to recover feed intake as well as the overall performance of fish. Recently, an experiment was conducted on comparative evaluation of several crude ingredients as attractants in shrimp diet (Smith *et al*., 2005), but no study has been reported for red sea bream so far. The present study clearly demonstrated that supplementation of CAA or FS, KM and SM in low fishmeal with high SPC diets recovered feed intake, growth performance and health/welfare of the fish. Likewise, the combined effect of FS, KM and SM or even FS alone in high SPC diets significantly improved the performance of fish. It should be noted that the performances of fish fed these diets could get over those of fishmeal based control diet. This indicates that FS, KM and SM acted as natural feeding attractants as well as being effective in balancing amino acids and masking the unpalatable substances present in SPC. Therefore, it would be evident that dietary IAA and FAA contents were improved by the supplementation of FS, KM and SM alone or in combination, respectively.

The lowest growth performances were found in fish fed SP diet from where 60% fishmeal protein was replaced with SPC protein. This suggests that red sea breams are not able to utilize higher SPC in their diet, which agrees with the previous study (Takagi *et al*., 1999), which found that only 50% fishmeal protein could be replaced by SPC protein in diets for juvenile red sea bream. That group also tried to replace higher levels of fishmeal in red sea bream diet with supplementation of lysine and methionine, but failed to obtain similar performance of fish as that in the control group (Takagi *et al*., 2001). Kissil *et al*. (2000) and Deng *et al*. (2006) reported that growth was significantly depressed even at 25-30%

substitution of SPC in the diets of gilthead seabream and Japanese flounder, respectively. Day and González (2000) suggested that 25% fishmeal protein could be substituted with SPC in diets for turbot. In contrast, higher or complete replacements of fishmeal with SPC were reported in Senegalese sole, *Solea senegalensis* (Aragão *et al*., 2003), cobia (Salze *et al*., 2010) and rainbow trout (Kaushik *et al*., 1995). Hence, efficiency on the utilization of soybean proteins varies among different fish species, and this is related to the number of challenges associated with soybean products, such as lower levels of sulfur amino acids like lysine and methionine, less palatability, lower digestibility and the presence of antinutritional factors. Supplementation of lysine and methionine to compensate for the deficiency of IAA and some other amino acids (e.g. glycine, alanine and taurine etc) as attractants, are beneficial in recovering amino acid balance and palatability in high soybean protein based diets (Fuke *et al*., 1981; Takagi *et al*., 2001; Venou *et al*., 2006; Chatzifotis *et al*., 2008). This was also evident in our present study where supplementation of 2.5% CAA significantly recovered growth performance of juvenile red sea bream compared to those fed the SP diet. The improved growth performance might be attributed to the increased feed intake of fish. In the present experiment, only 66% feed intake was found in fish fed the SP diet, relative to FM diet while it was 113% in SPAA diet. Because the only the difference was supplemental CAA, it was proved that imbalance in amino acids was the major factor causing the lower feed intake and subsequently lower growth performance of red sea bream fed diet replacing 60% fishmeal protein with SPC alone.

The supplementation of FS, KM and SM significantly improved the feed intake of fish, to the extent of being approximately equal to or higher than in fish fed FM and SPAA diets. This suggests that small amounts of FS, KM and SM are effective enough to improve the IAA composition of diets and those acted as feeding stimulants. This was also confirmed by the analyzed values of TAA and FAA in test diets (Table 2 and 3). Improved feed intake was

reported with the supplementation of FS in scorpion fish, *Sebastiscus marmoratus* (Kader, 2008), fish protein hydrolysate (FPH) in rainbow trout (Espe *et al*., 1999; Refsite *et al*. 2004), KM in tilapia (Gaber 2005;), krill hydrolysate in yellow perch (Kolkovski *et al*., 2000), squid viscera in Japanese seabass, *Lateolabrax japonicus* (Mai *et al*., 2006) and squid extract in Atlantic salmon, *Salmo salar* L. (Toften and Jobling, 1997; Toften *et al*., 2003). The highest feed intake exhibited by fish fed SPFS and SPMX diets could possibly be attributed to the presence of FS, which is known as a strong feeding stimulant (Kader, 2008). FS is a water soluble compound which is rich in soluble protein, minerals and vitamins; free amino acids, peptides, nucleotides and low molecular weight components such as taurine, creatinine, carnosine etc (Kousoulaki *et al*., 2009). In scorpion fish, feed intake was positively correlated with the increasing levels of FS at 0, 5, 10 and 15% (Kader, 2008). The results also agreed the studies of Espe *et al*. (1999) and Refsite *et al*. (2004), who reported that feed intake increased with increasing level of soluble proteins from fish silage or FPH in Atlantic salmon (*Salmo salar* L.) diets. It was suggested that high level of taurine might be one of the major characteristics as a feeding stimulant in FS, and other FAA help to enhance the stimulating effect. Taurine has been reported to be an effective feeding stimulant in high SPC diet for common dentex (Chatzifotis *et al*., 2008) and an essential element for normal feeding behavior and growth of red sea bream (Matsunari *et al*., 2008) and Japanese flounder (Park *et al*., 2002). The feed intake was highest in SPMX diet, and this might be, in part, due to the highest contents of dispensable FAA in the diet.

Growth performance in terms of weight gain (%) and SGR were directly influenced by feed intake in this study. Supplementation of CAA, FS, KM and SM significantly improved the growth performance of fish. The growth results of SPAA, SPKM and SPSM diets were similar to or those of SPFS and SPMX diets even higher than that of fish fed FM diet. The increased feed intake in fish fed SPFS and SPMX diets appears to explain the significantly

better growth in those groups. This might be due to the fact that higher feed intake would increase the amounts of protein and energy available for increasing fish growth. In addition, feed intake and growth performance of juvenile red sea bream significantly increased with the supplementation of 0.5 to 2.0% taurine and the optimum requirement is assumed to be less than 0.5% (analyzed value was 0.4%) (Matsunari *et al*., 2008). In the present study, dietary taurine level in FM, SPAA, SPFS and SPMX diets were met the recommended requirement level, while the values were very low in SP diet (0.1%). Therefore, dietary taurine might play a significant role in the performance of red sea bream. However, significantly higher feed intake and growth performance in SPFS and SPMX groups might possibly due to the combined effects of taurine, other FAAs and some other minor water soluble components present in the FS. A mixture rather than single compounds are more effective to stimulate feeding behavior of fish (Adron and Mackie, 1978; Papatryphon and Soares Jr., 2000; Kasumyan and Døving, 2003). It has also been reported that a synthetic mixture is inferior to the natural feeding stimulants as some effective components are absent in the synthetic mixture (Kohbara *et al*., 1989). Kubitza *et al*. (1997) found that a diet containing 10% menhaded fishmeal showed the highest feed intake in largemouth bass over synthetic nucleotides and control; while betaine and CAA were ineffective. Another possible reason for higher feed intake in fish fed FS based diets might be due to the higher palatability of FS (Refsite *et al*., 2004). In a recent study, Kousoulaki *et al*. (2009) also found that growth of Atlantic salmon was significantly increased with the addition of FS in the test diets compared to those without supplementation.

The effect of the supplementation of KM and SM were less pronounced than FS in improving feed intake and growth of fish. The difference in FAA contents in ingredients might be the reason for this, which would not be sufficient enough to stimulate the feed intake as happened with FS. However, the results demonstrate that KM and SM were still

good enough to catch up the performance of FM diet. Several studies on fish indicated effective utilization of KM and SM in aquafeeds (Gaber, 2005; Anderson *et al*., 1997; Kolkovski *et al*., 2000; Olsen *et al*., 2006; Suontama *et al*., 2007; Mai *et al*., 2006). On the other hand, Berge *et al*. (1999) didn't find any positive effect on feed intake or growth of Atlantic halibut, *Hippoglossus hippoglossus* with supplementation of 2% squid powder.

The activity of the digestive tract and liver enzymes could provide further insight into the possible effects of different diets on fish performance. In this study, no difference was found in protease activity measured from the digestive tract and liver of red sea bream. This agrees the study of Tibaldi *et al*. (2006), in which intestinal proteolytic enzyme activity of European sea bass was not affected when fed differently processed soybean meal based diets (60% fishmeal protein replacement). Furthermore, it was also found that there was no difference of trypsin activity in digestive tract of rainbow trout fed graded levels of dehulled lupin, while a significant difference was found in growth and feed utilization (Farhangi and Carter, 2001). In contrast, Kofuji *et al*. (2006) reported that dietary inclusion of CAA and krill and squid extracts in a fishmeal based diet improved the protease activity in yellowtail. It has been reported that residual protease inhibitors in soybean or lupin meal could reduce the protease activity only if they are present above a critical level in the diet (Tibaldi *et al*., 2006; Farhangi and Carter, 2001). The alcohol extractions of soybean meal during the process of producing SPC (used in this study), might have deactivated or reduced the protease inhibitor (Kaushik *et al*., 1995) and the level of SPC used in this study might have limited the protease inhibitor below the critical level to affect the protease activity.

Blood parameters serve as reliable indicators for the physiological condition as well as welfare of fish. Plasma bilirubin, GOT (or aspartate aminotransferase, AST) and GPT (or alanine aminotransferase, ALT) were significantly higher in fish fed SP diet compared to others. These parameters are often used for the evaluation of the liver function as they are

released into the blood during injury or damage to the liver cells (Lemaire *et al*., 1991). Higher levels for several blood parameters in fish fed SP diet indicate that the kidneys may not be working as well as they should be. Higher T-Cho was found in fish fed the FM diet and it was significantly lower in fish fed the SPC based diets. A higher BUN and lower T-Cho in this study agreed with the finding of Takagi *et al*. (2001) when red sea bream was fed the high SPC based diets. However, the values of the hematocrit, hemoglobin, total protein, albumin, glucose, triglycerides, high density lypoprotien cholesterol (HDL-c) and amylase were not affected by the dietary treatments, and were found to be within values in the previous studies of juvenile red sea beam (Takagi *et al*., 1999, 2001).

Oxidative stress is an emerging health risk factor involved in many diseases of animals, and it can generate high level of reactive oxygen species (ROS) and/or decrease efficacy of antioxidant system (Pasquini *et al*., 2008). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani *et al*., 2001; Ballerini *et al*., 2003; Pasquini *et al*., 2008). Recently, in our laboratory, these indices have been used for determining the oxidative stress condition of fish species. Based on Komilus (2008) and other unpublished data, the values obtained from the present study are within the range of those obtained in the previous studies of red sea bream. Animals with higher d-ROM values indicate that they are under more oxidative condition. On the other hand, animals with higher BAP values indicate they have more strong tolerance against oxidation. The d-ROMs were significantly lower in fish fed FM and SPMX diets than those in fish fed SP, SPAA, SPFS, SPKM and SPSM diets while maintaining similar levels of BAP in this study. This indicates that the former groups may suffer less oxidative stress than the latter, but the resistant capability against stress was not affected by the different dietary treatment under the condition applied. Since this parameter is still new for fish, more data will be needed to further understand the oxidative stress to fish.

In conclusion, supplementation of CAA, FS, KM and SM could recover the depleted performances of red sea beam. Therefore, the results of this trial provide clear evidence that FS, KM and SM are as effective as CAA to keep amino acids balanced and to act as attractants in formulating high plant protein based practical diets for carnivorous fish such as red sea bream. Among the three crude ingredients, the most promising results came from FS. Furthermore, FS in association with KM and SM gives the best performances of juvenile red sea bream, which is surely a promising finding in the formulation of non-fishmeal cost-effective diet for carnivorous fish in the future.

CHAPTER V

Development of Low/Non Fishmeal Based Aquafeeds

CHAPTER V

Experiment I

Effect of composite mixture of seafood by-products and soybean proteins in replacement of fishmeal on the performances of red sea bream, *Pagrus major*

(In Contribution: Aquaculture)

5.1.1 Abstract

Feeding trials were conducted to evaluate the potential of using a mixture of different protein sources based on seafood by-products and soybean meal to partially or completely replacement of fishmeal in practical diets for red sea bream, *Pagrus major*. Four diets were formulated to be isonitrogenous (50% crude protein), isolipidic (15% total lipid) and isocaloric (20 KJ g^{-1}). The control diet was the fishmeal based diet (FM100). The remaining three diets were prepared by replacing 60 (FM40), 80 (FM20) and 100% (FM0) of fishmeal protein with different proportions of sea food by-products and soybean meal. A commercial diet (19.3 KJ g^{-1} gross energy) was used as the reference diet (COM). The growth trial (Part I) was conducted with fifteen fish (1.35g), stocked in triplicate in each of 15 100-l polycarbonate circular tanks and fed all the experimental diets to satiation by hand twice daily for 56 days. After finished the growth trial, the remaining fish of each treatment were randomly distributed into duplicate tanks and fed the diets prepared with the addition of 0.05% chromium oxide as inert marker to assess the apparent digestibility (Part II). In Part III, dietary effects on the organoleptic characteristics and heavy metal accumulation in fish fillets were evaluated by a long term (120 days) independent feeding trial. Duplicate groups of fish (5.50 g) were stocked in 6, 100-l polycarbonate tanks at the rate of 8 fish per tank and supplied with each of three diets, such as FM100, FM20 and FM0 that were used in the growth trial. At the end of the trial, a group of similar size of red sea bream (fed COM diet) was collected from a commercial farm, Nobeoka, Miyazaki, Japan.

Results of the study indicated that weight gain (%) and specific growth rate (% day) of fish did not differ significantly ($P > 0.05$) with up to 80% fishmeal protein replacement. In this level, growth was also comparable ($P > 0.05$) with the commercial diet (COM). Feed intake and utilization were significantly ($P < 0.05$) depressed in 100% fishmeal replacement group (FM0). However, no difference was found among the rest. Similar trends were also

found for protein gain and retention. Whole body composition was directly influenced by the nutritional composition of the diets. Whole body protein was comparatively higher and total lipid was significantly lower in COM group. On the other hand, fish fed FM0 showed significantly lower ash content. No difference was found in condition factor and hepatosomatic index among treatments. There was no abnormal signs observed in hematological parameters and oxidative stress conditions of fish and it was assumed that all the fish were in good physiological state. Apparent digestibility of dry matter had no significant differences compared to the control whereas apparent protein digestibility was significantly decreased in FM0 group. Dietary treatments had no significant effects on the sensory characteristics and heavy metal accumulation in fish fillet after 120 days feeding trial. It was concluded that 80% of the fishmeal protein in a typical commercial diet could be replaced with a combination of seafood by-products and soybean proteins while confirming fish performance and quality.

Key words: Seafood by-product, Soybean meal, Fishmeal replacement, Composite mixture, Growth, Digestibility, Quality and Red sea bream.

5.1.2 Introduction

Red sea bream, *Pagrus major* is one of the commercially important aquaculture species whose production reaches the second largest in Japan (Koshio, 2002). In raising red sea bream, most farmers use commercially manufactured feeds, which often contain high levels of fishmeal as dietary protein. However, the continuous increasing demand in contrast to limited supply have soared the fishmeal price almost double during last few years (http://www.globefish.org). Therefore, replacement of fishmeal with cost-effective alternative protein sources is the prerequisite for profitable aquaculture venture. Although several alternative protein sources were reported to partially replace fishmeal from the diets of many fish species, limited studies have focused on evaluating the potential to reduce fishmeal level with alternative protein sources combined form plant and animal origin. It has been reported that 30-50% fishmeal protein could be replaced by soybean meal (SBM) (Ukawa *et al*. 1994), malt protein flour (Yamamoto *et al*. 1996), soy protein concentrate (SPC) (Takagi *et al*. 1999) and corn gluten meal (Takagi *et al*. 2000a) in juvenile red sea bream diets. Combined inclusion of plant proteins and terrestrial animal by-products could accelerate the fishmeal replacement levels to 50-60% in juvenile and 70-90% in yearling red sea bream (Aoki *et al*., 1998; Takagi *et al*. 2000b). However in most developed countries including Japan have restrictions to use terrestrial animal by-products in animal feed. In addition, higher or complete replacement of fishmeal with alternative protein sources in juvenile red sea bream was yet reported. Therefore, it is current need to find other alternative feed ingredients and better approaches to reduce or eliminate the inclusion of fishmeal in sea bream diets.

Japan is the largest seafood consumer in the world. Large amounts of seafood waste and deteriorated whole fish and shellfish are discarded daily in the canning industry and fish markets. These by-products are usually disposed of according to the strict Japanese government regulations for waste disposal (Ren *et al*., 2008). This is often seen as detrimental to the industry, as a considerable amount of money is spent on disposal of by-products, even though they are nutrient-rich resources. These by-products could be recycled

as a potential source of high-protein feedstuff in animal feeds (livestock, poultry, and fish). However, problems associated with the utilization of by-products, especially by-products of seafood processing, including freshness, quality, availability, higher moisture content, indigestible particles and contaminants or toxic metals. Conversion of these products by drying would be a high-cost, complicated process. The fermentation is more suitable and convenient for small industries and/or the farmer. It is a useful technique for drying wet product with minimal nutrient loss (Yamamoto *et al*., 2004) which can efficiently decrease or eliminate anti-nutritional constituents from oilseeds (Reddy and Pierson, 1994) and improve the overall nutritional quality (Canella *et al*., 1984). The previous findings showed that fermented soybean meal and scallop by-product (3:2) (FSSc), and fermented soybean meal and squid by-product (1:1) (FSSq) could effectively replace 30-36% of fishmeal protein from the diets of juvenile red sea bream and Japanese flounder respectively (Chapter III). It was also investigated that supplementation of small amounts of fish soluble (FS), krill meal (KM) and squid meal (SM) in high SPC based diets are effective enough to improve the amino acid composition and those acted as feeding stimulants which could facilitate to replace 60% fishmeal protein in juvenile red sea bream diet (Chapter IV; Kader *et al*., 2010). In that study, it was also suggested that FS is the most promising supplement and FS alone or combining with KM and SM supported even significantly higher growth performances than control group without inclusion of any CAA. Hence, blend of different ingredients is often recommended in achieving balanced nutritional composition, complementing amino acid profiles and masking the unpalatable substances present in feed ingredients (Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Tidwell *et al*., 2005; Guo *et al*., 2007; Kader *et al*., 2011), it is therefore potential to replace higher or complete fishmeal form sea bream diet by combining different ingredients used in the previous studies in appropriate proportion. The present experiment was conducted to evaluate the effect of gradually replacing fishmeal by combining different protein sources in growth, nutrient utilization, digestibility, health/welfare and quality of juvenile red sea bream.

5.1.3 Materials and methods

5.1.3.1 Part I: Effects on growth, feed utilization, body composition and blood parameters of fish

5.1.3.1.1 *Test fish and experimental system*

Juvenile red sea bream were purchased from a commercial hatchery (Matsumoto Suisan Co., Miyazaki, Japan) and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The fish were maintained in 500l tank with continuous aeration and flow through sea water, and fed a commercial diet (50% crude protein; Higashimaru, Kagoshima, Japan) for one week to acclimatized with the laboratory facilities. The feeding trial was carried out in 100-l polycarbonate tanks (filled with 80l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. Photoperiod was natural throughout the experimental period and all tanks had similar lighting conditions. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period.

5.1.3.1.2 *Test diets*

Table 5.1.1 and 5.1.2 summarize the formulation and proximate composition of the experimental diets. Dietary components and preparation of diets have been described in the recent publication (Kader *et al*., 2010). In addition, two fermented products named as FSSc and FSSq (AFCEP, Niigata, Japan) were also used in the present study. The condition of fermentation (patent pending Afcep Japan Corp.) was described recently (Kader *et al*., 2011). FS is a skipjack processing by-product, contains 70% crude protein with high amount of free amino acids and could be supplemented in high soybean protein based diets to complement amino acid composition and act as feeding stimulant (Kader *et al*., 2010; Chapter IV). A mixture (termed as MIX) of these three products such as FS, FSSq and FSSc at 2:1:1 ratio

(based on a preliminary study) was used to replace fishmeal in the present experiment. Four diets were formulated to be isonitrogenous (50% crude protein), isolipidic (15% total lipid) and isocaloric (20 KJ g^{-1}). In the control diet, fishmeal was used as sole protein source to provide 45% crude protein (FM100). The remaining three diets were prepared by replacing 60 (FM40), 80 (FM20) and 100% (FM0) of the fishmeal protein with MIX and supplementation of dehulled soybean meal (DSM), KM and SM proteins at 5% each in FM40 and FM20 diets; and 10% each in FM0 diet, respectively. A commercial diet (19.3 KJ g^{-1} gross energy) was used as the reference diet (COM) for comparison. Pollack liver oil, soybean lecithin and HUFA were supplied as lipid sources, and wheat flour as the carbohydrate or nitrogen free extract sources. Diet preparation was described in the previous chapters.

5.1.3.1.3 *Feeding protocol*

After being acclimatized to the laboratory environment, homogenous sized juveniles were sorted out. Triplicate groups of fish were assigned to each dietary treatment. Fifteen fish, having a mean initial body weight of 1.35 ± 0.05 g (mean ± SD) were randomly allocated to previously prepared fifteen tanks. Fish were fed the experimental diets by hand twice a day to visual satiation at 9.00 and 16.00 h. The daily feed supplied was recorded, and the uneaten feed was collected 30 min after feeding, followed by drying, weighing and finally subtracted from the total amount of supplied test diets to calculate the actual feed intake. All fish were weighted in bulk at every two weeks interval to determine growth and visually check their health condition. The monitored water quality parameters (mean ± S.D.) were: water temperature 22.3 ± 1.9°C; pH 8.0 ± 0.7 and salinity 33.9 ± 0.5 during the feeding trial. These ranges are considered within optimal values for juvenile red sea bream.

Table 5.1.1: Composition of experimental diet (% dry matter basis)

Ingredients	Diet group				
	FM100	FM40	FM20	FM0	COM*
Fishmeal[1]	60.00	24.00	12.00	0.00	
FSSq[2]	-	8.26	11.92	12.84	
FSSc[3]	-	8.26	11.92	12.84	
FS[4]	-	16.51	23.85	25.68	
KM[5]	-	3.48	3.48	6.97	
SM[6]	-	2.59	2.59	5.17	
DSM[7]	-	4.24	4.24	8.49	
Pollack liver oil[8]	2.00	4.50	5.50	6.00	
Soybean lecithin[8]	2.00	2.00	2.00	2.00	
HUFA[9]	0.50	0.50	0.50	0.50	
Wheat flour	10.00	7.00	5.00	3.00	
Vitamin mixture[10]	3.00	3.00	3.00	3.00	
Mineral mixture[11]	3.00	3.00	3.00	3.00	
Vitamin C ester[12]	0.30	0.30	0.30	0.30	
Activated gluten	5.00	5.00	5.00	5.00	
Carboxymethyl cellulose	1.00	1.00	1.00	1.00	
Chromium oxide	0.50	0.50	0.50	0.50	
α-cellulose	12.70	5.87	3.62	3.71	

Commercial diet

*The composition is unknown.

[1]Nippon Suisan Co. Ltd., Tokyo, Japan: proximate composition (% dry matter): moisture, 8.2; crude protein, 72.1; total lipid, 15.6 and ash 12.0.

[2]FSSq: fermented soybean meal and squid by-product mixture; AFCEP, Japan; proximate composition (% dry matter): moisture, 11.6; crude protein, 47.8; total lipid, 5.9 and ash, 6.3.

[3]FSSc: fermented soybean meal and scallop by-product mixture; AFCEP, Japan; proximate composition (% dry matter): moisture 17.24, crude protein 51.91, total lipid 7.16 and ash 6.44.

[4]FS: fish soluble; Makurazaki Fish Processors Cooperatives, Kagoshima, Japan; proximate composition (% dry matter): moisture, 50.2; crude protein, 73.4; total lipid, 4.0 and ash, 19.3.

[5]KM: krill meal; Nippon Suisan Co. Ltd., Tokyo, Japan; proximate composition (% dry matter): moistre, 6.9; crude protein, 62.1; total lipid, 23.4 and ash 9.7.

[6]SM: squid meal; Nippon Suisan Co. Ltd. Tokyo, Japan; proximate composition (% dry matter): moisture, 9.3; crude protein, 83.6; total lipid, 9.2 and ash 6.4.

[7]DSM: dehulled soybean meal; J. Oil Mills, Japan; proximate composition (% dry matter): moisture, 7.2; crude protein, 50.1; total lipid, 4.4 and ash 4.2.

[8]Riken Vitamin, Tokyo, Japan.

[9]Poweash A, Oriental Yeast Co, Ltd., Tokyo, Japan.

[10] Vitamin mixture (g kg^{-1} diet): ß-carotene 0.10; Vitamin D_3 0.01; Menadione $NaHSO_3.3H_2O$ (K_3) 0.05; DL-α-Tochopherol Acetate (E) 0.38; Thiamine-Nitrate (B_1) 0.06; Riboflavin (B_2) 0.19; Pyridoxine-HCl (B_6) 0.05; Cyanocobalamin (B_{12}) 0.0001; Biotin 0.01; Inositol 3.85; Niacine (Nicotic acid) 0.77; Ca Panthothenate 0.27; Folic acid 0.01; Choline choloride 7.87; ρ-Aminobenzoic acid 0.38; Cellulose 1.92.

[11]Mineral mixture (g kg^{-1} diet): $MgSO_4$ 5.07; Na_2HPO_4 3.23; K_2HPO_4 8.87; Fe Citrate 1.10; Ca Lactate12.09; Al $(OH)_3$ 0.01; $ZnSO_4$ 0.13; $CuSO_4$ 0.004; $MnSO_4$ 0.03; Ca $(IO_3)_2$ 0.01; $CoSO_4$ 0.04.

[12]L-Ascorbyl-2-phosphate-Mg.

Table 5.1.2: Chemical analysis of the experimental diets

Ingredients	Diet group				
	FM100	FM40	FM20	FM0	COM
Proximate composition (% dry matter basis)					
Crude protein	50.7	50.2	50.7	50.5	53.7
Crude lipid	14.9	14.5	15.2	15.2	12.5
Ash	11.4	11.3	11.3	11.3	14.3
Gross energy (KJ g^{-1})[1]	20.06	20.10	20.31	20.16	19.30
Amino acid (AA g $100g^{-1}$ dry sample)					
Arginine	4.12	3.49	3.05	2.57	2.96
Histidine	1.78	1.60	1.51	1.37	1.94
Isoleucine	1.69	1.34	1.18	1.16	1.53
Leucine	3.17	2.67	2.36	2.63	3.57
Lysine	3.42	3.61	3.01	3.48	4.18
Methionine	1.48	1.13	1.06	0.85	1.63
Phenylalanine	2.43	2.89	2.56	2.57	2.96
Threonine	1.73	1.55	1.46	1.48	1.84
Tryptophan	0.00	0.00	0.00	0.00	0.00
Valine	2.18	1.85	1.64	1.48	1.84

[1]Calculated using combustion values for protein, lipid and carbohydrate of 23.6, 39.5 and 17.2 KJ g^{-1}, respectively. Carbohydrate was calculated by the difference: 100 - (protein + lipid + ash + moisture)

5.1.3.1.4 *Sample collection and biochemical analysis*

The initial sample of 20 fish for whole body analysis was stored at −20°C. At the end of the feeding trial, fish were starved for 24 h prior to final sampling. All the fish were anaesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg l^{-1}. Then the total number, individual body weight and length of fish from each tank were measured. Five fish from each replicate tank were randomly collected and stored at −20°C for final whole body analysis. Using heparinized syringes, blood was collected from the caudal vein of three fish in each replicate tank and pooled. A small fraction of the heparinized blood was used to analyze the haematocrit level. Plasma samples were obtained by centrifugation at 3000 × *g* for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80°C. Liver was dissected out from three fish in each replicate tank, weighted individually to get hepatosomatic index (HSI), and finally pooled together and kept at −80°C.

All the chemical analysis was performed based on the methods described in earlier experiments.

5.1.3.2 Part II: <u>Effects on digestibility of fish</u>

5.1.3.2.1 *Digestibility assessment*

After finished the growth trial, the remaining fish from the triplicate groups of each treatment were distributed randomly into duplicate tanks. Test diets were prepared with the addition of 0.05% chromium oxide (Cr_2O_3) as inert marker to the previous diet formulation and fed to the fish under the same condition maintained for the feeding trial. After one week acclimatization with new diet, faeces collection started at 3-h interval between the morning and afternoon feeds by using a siphon. Sufficient amount of faeces were collected, freeze dried and dry matter, crude protein and lipid contents were determined as described above.

Chromic oxide content in faeces and diets was determined according to Furukawa and Tsukahara (1966).

5.1.3.3 Part III: Effects on organoleptic characteristics and element compositions in fillet of fish

5.1.3.3.1 *Assessment of organoleptic characteristics and element composition of fish fillet*

To evaluate the dietary effects on the organoleptic characteristics and heavy metal accumulation in fish fillets, a long term independent feeding trial was conducted. Duplicate groups of fish (5.50 g) were stocked in 100-l polycarbonate tanks at a rate of 8 fish per tank. The fish were fed with each of three diets, such as FM100, FM20 and FM0 that were used in the growth trial (Table 5.1.1). The FM40 diet was not used in this trial. Fish were supplied the respective test diets at the rate of 3-4% of their body weight for 120 days. Ten days before the end of the feeding trial, a group of similar size of red sea bream was collected from a commercial farm, Nobeoka, Miyazaki, Japan. This group of fish was fed by the similar commercial diet (COM) that supplied in the growth trial. At the end of the trial, ten fish from each group were randomly taken, slaughtered by dipping in ice cold water, and placed on ice. Following rigor mortis, the fillet from each fish was removed and sliced to obtain uniform sized sashimi of about 5-10 mm thickness, wrapped in aluminum foil and kept at 4^{o}C. The sensory panel consisted of ten non-trained personnel comprising teachers and students from Faculty of Fisheries, Kagoshima University. All the panelists were evaluated the sashimi according to freshness, odour, texture and taste for all dietary fish groups based on a 0 to 10 scale (Amerine *et al*., 1965).

Fillet samples were also collected and kept at −80°C for heavy metal analysis. The analyses of copper (Cu), zinc (Zn), cadmium (Cd) and lead (Pb) in diets and fish fillets were performed by atomic absorption spectrophotometer (AAS; Hitachi A-2300, Tokyo, Japan) after acid digestion.

5.1.3.4 *Evaluation of performance parameters*

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR %, day^{-1}) = {Ln (final weight) – Ln (initial weight) / duration} × 100

Survival (%) = 100 × (final no of fish / initial no of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed efficiency ratio (FER) = live weight gain (g) / dry feed intake (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Protein gain (PG, g kg weight $gain^{-1}$) = {(final weight (g) × final whole body protein content (%) / 100) – (initial weight (g) × initial whole body protein content (%) / 100)} / (weight gain (g)) × 1000

Protein retention (PR, % of intake) = (protein gain (g kg weight $gain^{-1}$) × 100) / protein intake (g kg weight $gain^{-1}$)

Condition factor (CF, %) = weight of fish / $(\text{length of fish})^3$ × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Apparent digestibility coefficient (ADC, %) = 100 – (100 × (% Cr_2O_3 in diet/% Cr_2O_3 in faeces) × (% nutrient in faeces/% nutrient in diet))

5.1.3.7 *Statistical analysis*

All data were subjected to statistical verification using Package Super ANOVA 1.11, Abacus Concepts, Berkeley, California, USA. Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

5.1.4 Results

Data on growth performance and feed utilization of the fish are presented in Table 5.1.3. At the end of the feeding trial, replacement of fishmeal had no negative ($P > 0.05$) effects on the survival of fish. There was no significant ($P > 0.05$) difference in final weight of fish fed FM100, FM40 and FM20 diets. Commercial diet (COM) also supported comparable growth performance with the control group. However, final weight was significantly ($P < 0.05$) decreased in fish fed fishmeal free diet (FM0). Other growth parameters (% weight gain and SGR, % day) followed the similar pattern as final weight. Similarly, FER, PER, PG and PR were also lowest in FM0 group while these parameters were not significantly different among the rests. In general, all the diets were well accepted by fish with no observed rejection. Although, fishmeal was successively replaced from the diets, feed intake was not significantly ($P > 0.05$) affected by the dietary treatments at the end of the feeding trial. Numerically highest feed intake was found in fish fed FM40 diet. Fig. 5.1.1 illustrates the relationship between SGR and feed intake of fish in different experiments. Fig. 5.1.2 shows the relative value (%) of protein cost in different experimental diets compared to control diet. Costs of protein were progressively decreased with fishmeal replacement levels and fishmeal free diet (FM0) represents 56% reduced cost compared to the control.

The proximate composition of whole body of juvenile red sea bream is shown in Table 5.1.4. In comparison with the control, dietary treatments had no significant influence on the whole body moisture, crude protein and ash contents at the end of the feeding trial. However, lipid content was significantly ($P < 0.05$) decreased in fish fed COM diet. No difference was also detected in CF and HSI of fish among treatments (Table 5.1.4).

Table 5.1.3: Growth parameters and nutrient utilization in red sea bream fed test diets for 56 days*

Parameters	Diet group				
	FM100	FM40	FM20	FM0	COM
In wt[1]	1.36 ± 0.01	1.35 ± 0.01	1.36 ± 0.01	1.35 ± 0.02	1.35 ± 0.01
Fn wt[2]	27.73 ± 0.48^{b}	27.88 ± 0.67^{b}	26.10 ± 0.94^{ab}	24.11 ± 0.85^{a}	27.42 ± 0.83^{ab}
WG[3]	1948 ± 38^{b}	1969 ± 47^{b}	1826 ± 72^{ab}	1690 ± 57^{a}	1931 ± 63^{ab}
SGR[4]	5.39 ± 0.03^{b}	5.41 ± 0.04^{b}	5.28 ± 0.07^{ab}	5.15 ± 0.06^{a}	5.37 ± 0.06^{ab}
FI[5]	24.88 ± 0.26	27.02 ± 1.11	25.22 ± 0.45	25.33 ± 0.59	24.16 ± 0.72
FER[6]	1.06 ± 0.03^{b}	0.98 ± 0.04^{ab}	0.98 ± 0.02^{ab}	0.90 ± 0.02^{a}	1.08 ± 0.04^{b}
PER[7]	2.09 ± 0.06^{b}	1.96 ± 0.07^{ab}	1.93 ± 0.04^{ab}	1.78 ± 0.04^{a}	2.01 ± 0.08^{ab}
PG[8]	161 ± 2.9^{b}	160 ± 2.2^{b}	162 ± 1.4^{b}	144 ± 1.2^{a}	162 ± 2.6^{b}
PR[9]	32.84 ± 0.36^{b}	31.43 ± 1.54^{b}	31.27 ± 0.45^{ab}	27.50 ± 0.63^{a}	32.43 ± 0.65^{b}
Sur[10]	95.00 ± 5.00	97.67 ± 2.33	97.67 ± 2.33	95.00 ± 3.14	97.67 ± 2.33

Abbreviations used: [1]In wt: initial weight (g), [2]Fn wt: final weight (g), [3]WG: percent weight gain (%), [4]SGR: specific growth rate (% day^{-1}), [5]FI: feed intake (g dry diet fish^{-1}56 days^{-1}), [6]FER: feed efficiency ratio, [7]PER: protein efficiency ratio, [8]PG: protein gain (g kg body weight gain^{-1}), [9]PR: protein retention (% of intake), [10]Sur: survival (%).

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

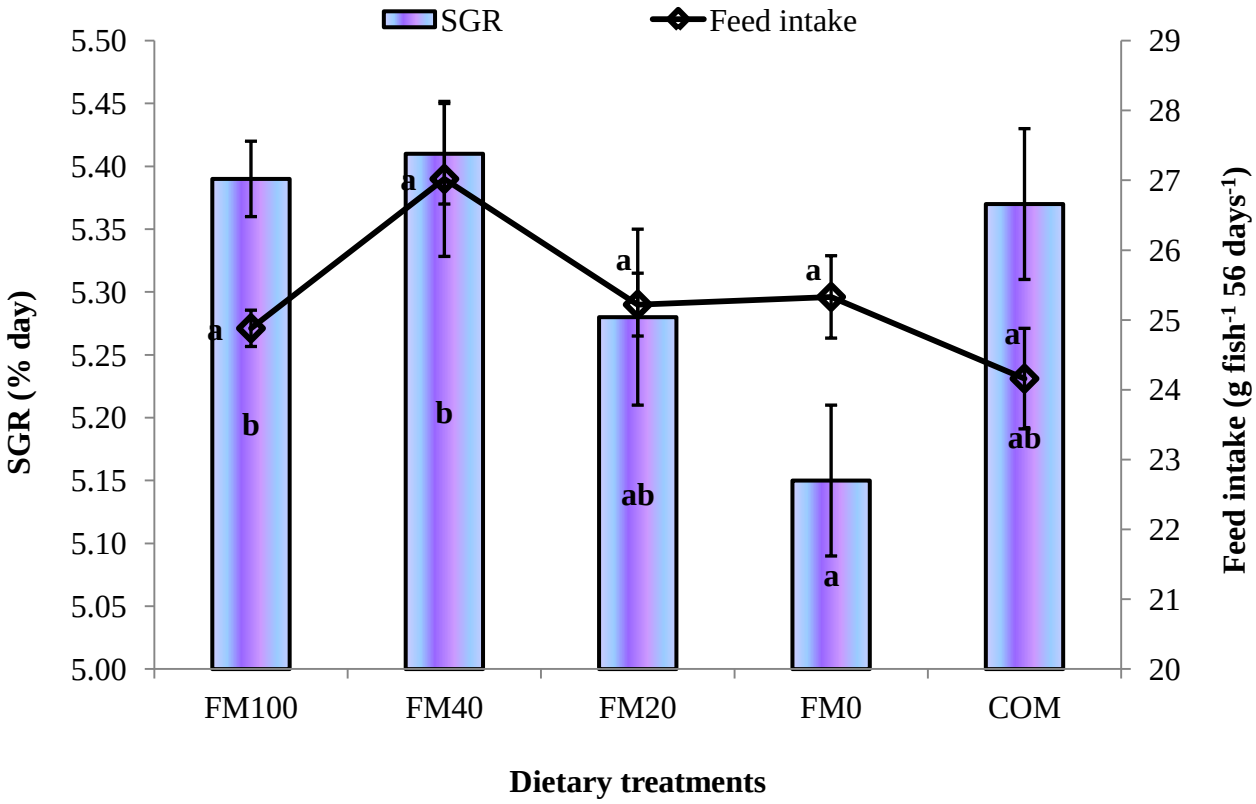

Fig. 5.1.1: Relationship between specific growth rate (SGR) and feed intake of red sea bream after 56 days feeding trial

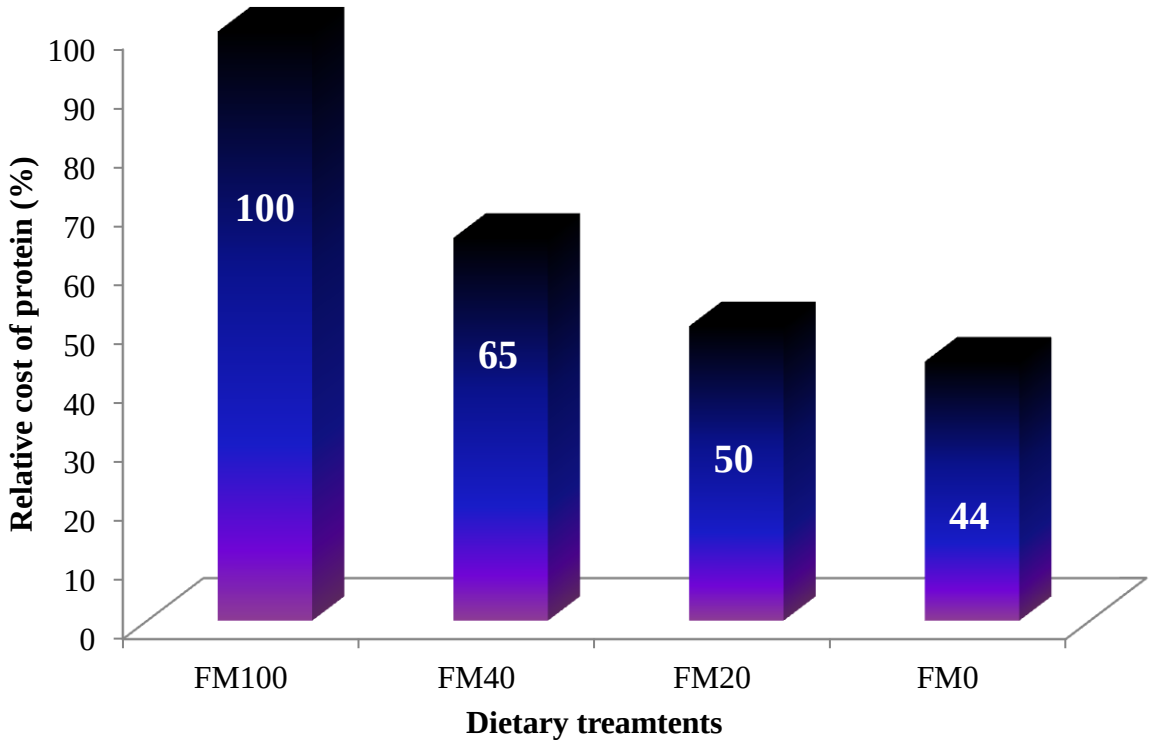

Fig. 5.1.2: Relative cost of protein (%) in the test diets compared to the cost of protein in fishmeal based control diet

Table 5.1.4: Whole body proximate analysis (%) and somatic parameters in juvenile red sea bream fed test diets for 56 days*

Parameters	Initial[1]	Diet group				
		FM100	FM40	FM20	FM0	COM
Moisture	76.85	72.20 ± 0.18	71.02 ± 0.92	70.58 ± 0.39	73.29 ± 0.56	73.04 ± 1.09
Crude protein	14.22	15.47 ± 0.57^{ab}	15.92 ± 0.21^{ab}	15.96 ± 0.11^{ab}	14.42 ± 0.12^{a}	16.06 ± 0.24^{b}
Total lipid	4.55	8.07 ± 0.60^{b}	8.74 ± 0.13^{b}	8.63 ± 0.10^{b}	8.43 ± 0.39^{b}	6.01 ± 0.36^{a}
Crude ash	4.30	4.21 ± 0.06	4.15 ± 0.10	4.09 ± 0.11	3.92 ± 0.39	4.40 ± 0.11
CF[2]		1.98 ± 0.04	1.86 ± 0.01	1.82 ± 0.07	1.76 ± 0.11	1.93 ± 0.03
HSI[3]		1.39 ± 0.09	1.46 ± 0.18	1.65 ± 0.03	1.64 ± 0.09	1.54 ± 0.07

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%), [3]HSI: hepatosomatic index (%).

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Crude protein, crude lipid and ash are expressed on a wet weight basis.

Table 5.1.5 represents the blood parameters of red sea bream after 8 weeks feeding trial. Overall, dietary treatments had no effect in hematocrit and blood chemical parameters of fish among different treatments except for those of plasma glucose and total cholesterol (T-Cho). Plasma glucose level was significantly increased in fish of all the replacement groups compared to FM100 and COM. Conversely, plasma T-Cho level had a decreasing ($P < 0.05$) trend with the increasing fishmeal replacement levels and the values were also significant between FM100 and COM. Oxidative status of fish was analyzed from plasma (Table 5.1.5). Although wide variations were found on oxidative parameters of fish, no significant change or recognizable tendency was indentified among treatments.

The ADC of dry matter, crude protein and lipid is presented in Table 5.1.6, and Fig. 5.1.3 and 5.1.4. The digestibility of dry matter and lipid were not significantly different from those of fish fed the control diet containing 100% fishmeal. However, both the values were lowest in fish fed the commercial diet (COM). The ADC of crude protein showed slightly decreasing trend with the increasing fishmeal replacement levels. The highest value was observed from the group fed control diet (FM100) while the lowest value was obtained in fish fed with 100% replacement (FM0) and there was significant difference between them.

No significant differences were found in organoleptic characteristics of fish fed the different diets (Fig. 5.1.5). Concentration of Cu, Zn, Cd and Pb in the diets and fish fillet are shown in Table 5.1.6 and 5.1.7 respectively. Dietary Cu and Cd were significantly increased in FM20, FM0 and COM diets compared to the control (FM100), while similar levels of Zn and very low levels of Pb were found among all diets. However, dietary concentration of heavy metals had no significant effects in the element contents of red sea bream fillets after 120 days feeding trial.

Table 5.1.5: Blood parameters in juvenile red sea bream fed test diets for 56 days*

Parameters	Diet group				
	FM100	FM40	FM20	FM0	COM
Hematocrit (%)	35.0 ± 0.58	37.0 ± 1.00	36.0 ± 1.53	34.0 ± 2.08	36.0 ± 2.52
Total protein (g/dl)	4.47 ± 0.12	4.37 ± 0.03	4.97 ± 0.19	4.47 ± 0.19	4.40 ± 0.25
Total albumin (g/dl)	< 1.00	< 1.00	< 1.00	< 1.00	< 1.00
Total bilirubin (mg/dl)	0.50 ± 0.15	0.33 ± 0.09	0.43 ± 0.09	0.37 ± 0.03	0.67 ± 0.18
Glucose (mg/dl)	61.0 ± 0.6^{a}	74.3 ± 0.9^{b}	79.0 ± 2.0^{b}	70.3 ± 2.9^{b}	57.7 ± 1.3^{a}
GOT (IU/l)[1]	95 ± 15	86 ± 25	93 ± 13	71 ± 10	123 ± 21
GPT (IU/l)[2]	18.7 ± 2.33	11.0 ± 1.00	17.0 ± 7.00	10.0 ± 0.00	14.0 ± 4.00
BUN (mg/dl)[3]	5.33 ± 0.33	5.50 ± 0.50	5.50 ± 0.50	5.33 ± 0.33	6.67 ± 0.67
Triglycerides (mg/dl)	168 ± 18	210 ± 23	213 ± 8	220 ± 10	196 ± 26
T-Cho (mg/dl)[4]	426 ± 22^{c}	358 ± 11^{bc}	308 ± 19^{ab}	305 ± 16^{ab}	267 ± 23^{a}
HDL-c (mg/dl)[5]	322 ± 30	351 ± 13	307 ± 22	308 ± 16	254 ± 23
Amylase (IU/l)	< 30.00	< 30.00	< 30.00	< 30.00	< 30.00
Oxidative stress parameters					
d-ROMs (U.Carr)[6]	161 ± 21.7	133 ± 36.5	138 ± 19.6	111 ± 9.9	102 ± 16.6
BAP (μ Mol l^{-1})[7]	4186 ± 449	4668 ± 92	4352 ± 138	3524 ± 256	4009 ± 186

Abbreviation used: [1]GOT: glutamyl oxaloacetic transaminase, [2]GPT: glutamic-pyruvate transaminase, [3]BUN: blood urea nitrogen, [4]T-Cho: total cholesterol, [5]HDL-c: high density lipoprotein cholesterol, [6]d-ROMs: reactive oxygen metabolites, [7]BAP: biological antioxidant potential.

*Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

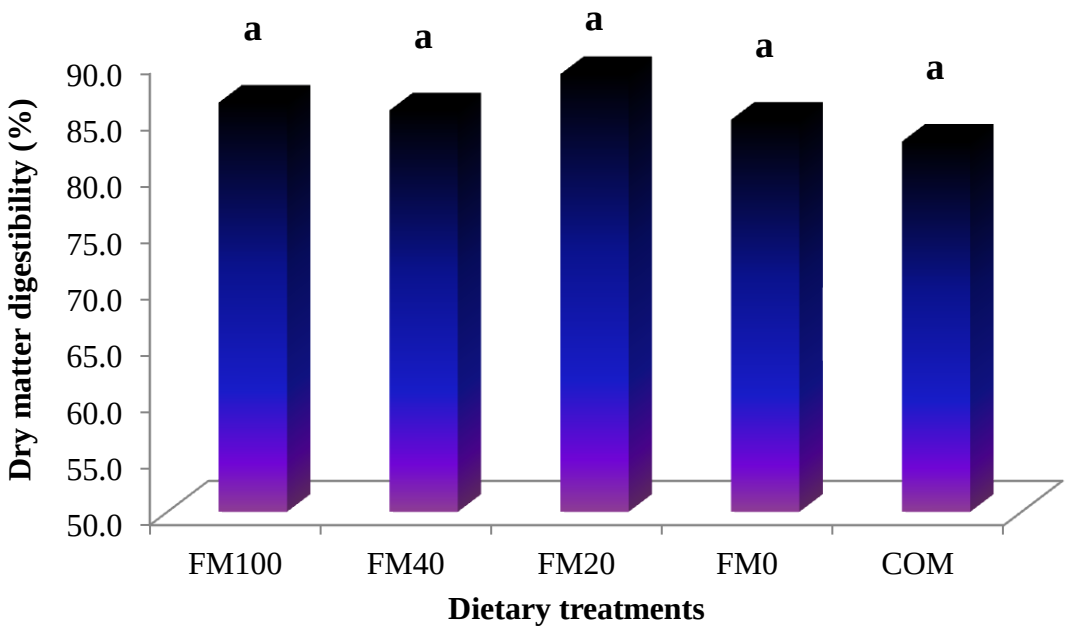

Fig. 5.1.3: Apparent digestibility coefficients (ADC) for dry matter of the test diets fed to juvenile red sea bream for 56 days

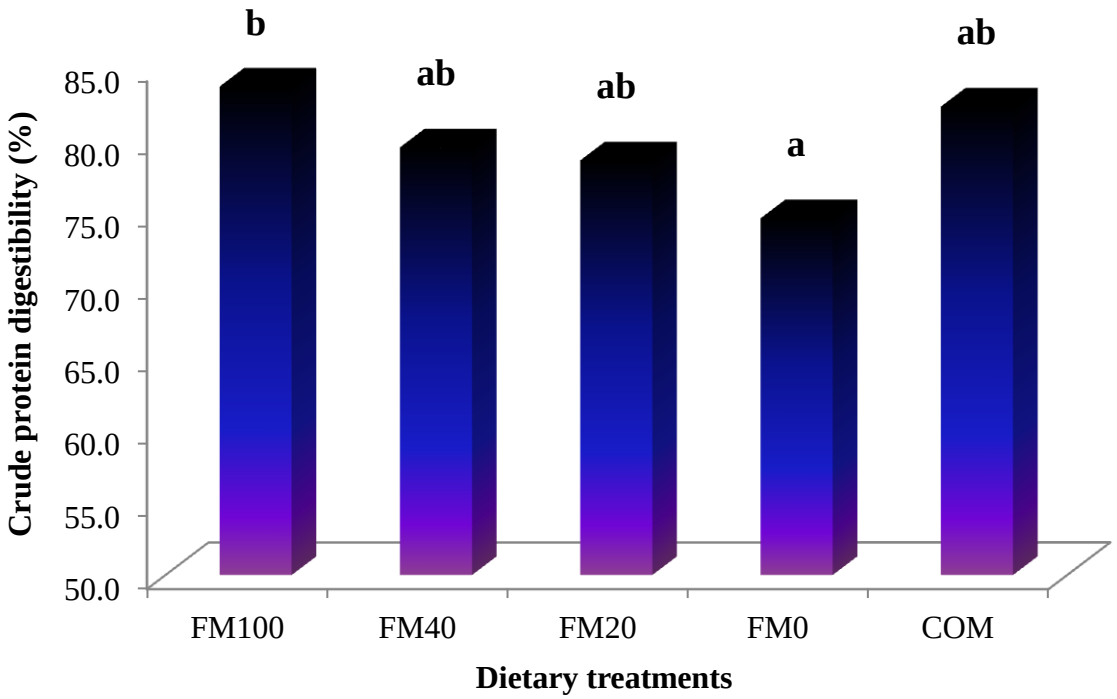

Fig. 5.1.4: Apparent digestibility coefficients (ADC) for crude protein of the test diets fed to juvenile red sea bream for 56 days

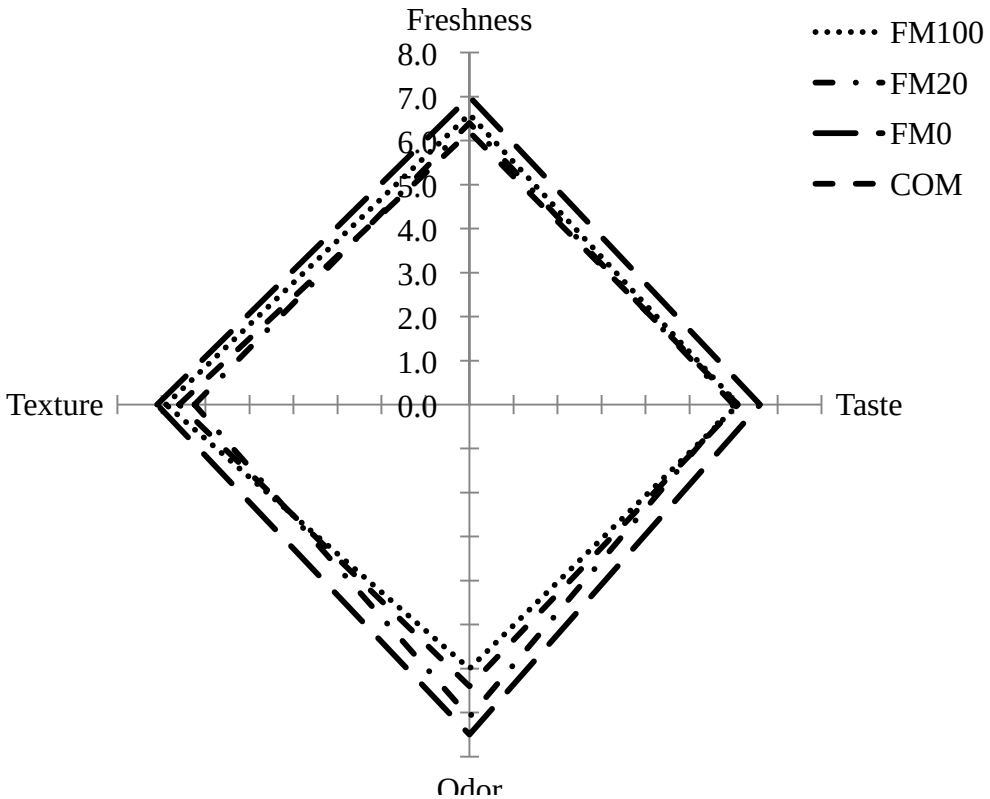

Fig. 5.1.5: Sensory analysis of red sea bream fillets fed different test diets for 120 days

Table 5.1.6 Element concentrations (μg g^{-1}) of the experimental diets

Element	Test diets			
	FM100	FM20	FM0	COM
Copper (Cu)	7.86 ± 0.59[a]	16.76 ± 0.48[b]	19.13 ± 0.99[b]	16.44 ± 0.67[b]
Zinc (Zn)	87.55 ± 0.89	85.05 ± 2.41	92.14 ± 4.59	82.19 ± 2.13
Cadmium (Cd)	0.58 ± 0.01[a]	2.00 ± 0.06[c]	2.34 ± 0.05[d]	0.96 ± 0.05[b]
Lead (Pb)	0.12	0.05	ND[*]	0.05

Values are means ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$).

[*]Not detectable (Pb, < 0.02 μg g^{-1}).

Table 5.1.7 Element concentrations (μg g^{-1}) in juvenile red sea bream fillet fed test diets for 120 days

Element	Test diets			
	FM100	FM20	FM0	COM
Copper (Cu)	0.43 ± 0.01	0.45 ± 0.05	0.40 ± 0.01	0.40 ± 0.08
Zinc (Zn)	5.24 ± 0.85	4.94 ± 0.39	5.02 ± 0.19	4.34 ± 0.14
Cadmium (Cd)	ND[*]	ND	ND	ND
Lead (Pb)	ND	ND	ND	ND

Values are means ± S.E.M.. Within a row, means with the same letters are not significantly different ($P > 0.05$).

[*]Not detectable (Cd, < 0.008 μg g^{-1}; Pb, < 0.02 μg g^{-1}).

5.1.5 Discussion

The most expansive components in compound aquafeed are the protein source. Therefore, to minimize the diet related cost, it is necessary to manipulate the dietary protein ingredients. By-products from seafood industries and plant proteins are potential candidate for this purpose. The present study clearly demonstrated that 80% fishmeal protein could be successfully replaced by a mixture of seafood by-products and soybean meal without any adverse effects on growth, feed utilization, body composition, health or welfare, digestibility and fillet quality of juvenile red sea bream. Growth performances were significantly decreased in fish fed diet from where 100% fishmeal was replaced. This suggests that certain level of fishmeal is necessary during formulation of diets with by-products or comparatively low valued protein sources in marine carnivorous fish such as red sea bream.

In the context of research of the substitution of fishmeal for different fish species, mostly single protein sources were evaluated rather combine use of different protein sources (Zhang *et al*., 2008). Previous studies have shown that it is possible to replace up to 30% fishmeal protein by SBM, malt protein flour, corn gluten meal and FSSc in the diets of juvenile red sea bream (Ukawa *et al*., 1994; Yamamoto *et al*., 1996; Takagi *et al*., 2000a; Kader *et al*., 2011). Solvent extracted plant protein such as SPC or seafood by-products such as tuna muscle by-product could replace 50% fishmeal protein in diets for juvenile fish (Takagi *et al*., 1999; Uyan *et al*., 2007). Fishmeal replacement levels could be accelerated to 50-60% in juvenile and 70-90% in yearling red sea bream by combining several protein sources (Aoki *et al*., 1998; Takagi *et al*., 2000b). Kader *et al*. (2010) also reported that 60% fishmeal protein could be replaced by SPC with the supplementation of crude ingredients such as FS, KM or SM and supplementation of all these three ingredients with SPC significantly improved the performances of juvenile red sea bream compared to control. In contrast, higher fishmeal replacement was reported in the present study, indicated that combination of seafood by-

products and soybean proteins could replace as high as 80% of the fishmeal protein in diets from juvenile red sea bream. Similar results were also found in other species, reported that 80-90% fishmeal protein could be replaced by combining different protein sources in the diets of cuneate drum (*Nibea miichthioides*), grouper (*Epinephelus coioides*) and rainbow trout (Guo *et al*., 2007; Millamena, 2002; Watanabe and Pongmaneerat, 1991). It has been suggested that appropriate combination of different protein sources would have several important advantages, these being to complement nutritional composition and to mask the unpalatable substances present in feed ingredients (Shimeno *et al*., 1993; Yamamoto *et al*., 1995; Tidwell *et al*., 2005; Guo *et al*., 2007; Kader *et al*., 2010).

The growth parameters (weight gain and SGR) were not significantly different among FM100, FM40, FM20 and COM groups. This growth promotion effect of fish, even with very low fishmeal levels (12%) can be attributed to the similar feed intake, FER and PER among those treatments. In general, feed intake has an inverse relationship to higher fishmeal replacement levels with by-products or plant proteins in marine carnivorous fish (Uyan *et al*., 2006; Pratoomyot *et al*., 2010). In the present experiment, feed intake was similar or slightly higher in all the replacement groups, even in FM0, compared to control and commercial diets. This might be explained by improved nutritional composition in FM40, FM20 and FM0 diets where a mixture of FSSc, FSSq and FS was used as protein source. This phenomenon was also confirmed by the analyzed values of TAA in test diets (Table 5.1.2). Although, most of the amino acids were decreased in FM40, FM20 and FM0 diets compared to fishmeal based diet (FM100), these values were comparable with the commercial diet (COM) except for methionine in FM0 diet and appear to have been adequate or sub-optimal for good growth and survival of juvenile red sea bream (Uyan *et al*., 2007; Kader *et al*., 2010). Feed intake of fish was also influenced greatly by the attractability or palatability of diets. Addition of palatability enhancers is an effective approach in order to maintain feed attractiveness and

induce adequate feed consumption rate by fish (Papatryphon and Soares Jr., 2000; Kissil *et al*., 2000; Kader *et al*., 2010). In the present experiment, FS, KM and SM were included to the diets which known as natural feeding stimulants. Improved feed intake was reported with the supplementation of FS in scorpion fish, *Sebastiscus marmoratus* (Kader, 2008) and red sea bream (Kader *et al*., 2010), KM in tilapia (Gaber, 2005;), krill hydrolysate in yellow perch (Kolkovski *et al*., 2000), squid viscera in Japanese seabass, *Lateolabrax japonicus* (Mai *et al*., 2006) and squid extract in Atlantic salmon, *Salmo salar* L. (Toften *et al*., 2003). The most promising feed stimulating feature was found in FS which is a water soluble compound and rich in soluble protein, minerals and vitamins; free amino acids, peptides, nucleotides and low molecular weight components such as taurine, creatinine, carnosine etc (Kousoulaki *et al*., 2009; Kader *et al*., 2010). In addition, FS have a good fishy smell which acted as a strong olfactory stimulant. It was suggested that high level of taurine might be one of the major characteristics as a feeding stimulant in FS, and other FAA help to enhance the stimulating effect (Kader *et al*., 2010). Taurine has been reported to be an effective feeding stimulant in high soybean protein based diets for common dentex (Chatzifotis *et al*., 2008) and an essential element for normal feeding behavior and growth of red sea bream (Matsunari *et al*., 2008) and Japanese flounder (Park *et al*., 2002). Therefore, similar feed intake among treatments in the present study might be attributed to the balanced dietary composition with amino acids and attractants. The lowest growth performances were found in fish fed FM0 di*et al*though feed intake was not significantly different. Significantly lower FER, PER, PG and PR in this group of fish might be a reason for the lower performances of fish. These results suggested that fish fed FM0 diet could not utilize the protein sources efficiently for body growth.

Moisture content of whole body of red sea bream was not influenced by dietary treatments. Whole body protein content was lowest in FM0 group which might be related to

the decreased protein utilization (PER, PG and PR) of fish in this group. There was no significant difference in whole body lipid and ash content among fish fed FM100, FM40, FM20 and FM0 diets. However, whole body lipid was significantly decreased in fish fed COM diet which was reflected by the dietary nutritional composition. CF and HSI were not influenced by the dietary treatments.

Blood parameters serve as reliable indicators for the physiological condition as well as welfare of fish. In the present experiment, no significant differences was detected in most of the hemochemical parameters and were found to be within values in the previous studies of juvenile red sea beam (Takagi *et al*., 1999, 2001; Kader *et al*., 2010, 2011). Plasma glucose was significantly increased in all the replacement groups compared to FM100 and COM. However, the values for glucose (57.7-79.0) in the present experiment was comparable to the values (61.7-78) reported previously for juvenile red sea bream (Takagi *et al*., 1999; Kader *et al*., 2010). Significantly higher T-Cho was found in fish fed FM100 diet which was gradually decreased in other diets. Takagi *et al*. (2001) also found a similar trend of higher T-Cho in fishmeal based diets, while it was decreased in fish fed high SPC based diets. In general, blood parameters suggests that physiological condition of fish were not affected by the dietary treatments and low or non fishmeal diets based on by-products and soybean proteins also supported similar health status as obtained with high quality fishmeal based diet. Further, health status was also confirmed by measuring oxidative stress condition of fish. Oxidative stress is an emerging health risk factor involved in many diseases of animals, and it can generate high level of reactive oxygen species (ROS) and/or decrease efficacy of antioxidant system (Pasquini *et al*., 2008). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani *et al*., 2001; Ballerini *et al*., 2003; Pasquini *et al*., 2008). Present study showed no significant difference in d-ROMs and BAP values and these values are comparable to those obtained in our laboratory in juvenile red sea bream (Kader *et al*., 2010, 2011).

In fish, protein digestibility is generally high ranging from 75 to 95% (NRC, 1993). In the present study protein digestibility varied between 74.6 to 83.7%. Comparatively low digestibility values recorded here could be due to the quality of raw material or to the method of faeces collection (Regost *et al*., 1999). There was no significant difference in protein digestibility of fish fed FM100, FM40, FM20 and COM diets while significantly lower value was found in FM0 group. Therefore, lowest growth performances in FM0 group might partly be due to the lowest protein digestibility.

Organoleptic characteristics or sensory evaluation of the cultured fish will provide valuable information regarding the consumer's acceptability of the finished product. In the present study, no differences were observed in the analyzed parameters among the fillets from fish fed fishmeal based control (FM100), low fishmeal (FM20), non fishmeal (FM0) or commercial diets. The high scores for freshness, odor, texture and taste of the sashimi indicated well acceptance by the panelists (Komilus, 2008). Long term exposure of diets with heavy metals may have various toxic effects in cultured fish and may lead to biomagnifications along the food chain, ultimately reaching the human system, which may pose a serious health hazard. Although some metals and their compounds like Cu and Zn are essential for fish metabolism, they are potentially harmful if consumed at higher levels. The other metals like Cd and Pb have no known role in biological systems and are harmful for the growth performances and health of fish (Canli and Atli, 2003; Moren *et al*., 2006). Even when humans consume fish with high levels of accumulated Cd, 3-7% of the ingested Cd is absorbed and transported to different parts of the body (Krajnc *et al*., 1987; Mai *et al*., 2006). Therefore, proper assessment and control of any food safety concerns in aquaculture products should be strictly maintained for the sustainability of the reliance on fish and shrimp products. In the present experiment, although some variation was found in some of the heavy metals such as Cu and Cd contents among different diets, it has no significant effects on the fillet composition. It is particularly mentioned that all the analyzed metals were very low ($P >$

0.05) or less than detectable limits in red sea bream fillet of different treatmetns after 120 days feeding trial. The values were also much lower than the values reported in the muscles of seabrean from the Mediterranean Sea (Canli and Atli, 2003). Moren *et al*. (2006) reported that dietary composition of heavy metals had no significant effect on the bioaccumulation in fish muscle even at very high levels of Cu and Cd (52 and 4.4 mg/kg). This might be due to the fact that accumulation of these metals mostly occurs in fish liver and kidney rather than muscle (Canli and Atli, 2003). Therefore, it is evident from the present study that replacement of fishmeal with composite mixture of seafood by-products and soybean proteins would not alter the sensory and fillet quality as well as consumer's acceptability of the cultured fish.

In summary, the present experiment shows that a composite mixture of seafood by-products and soybean proteins may be used in replacement of maximum fishmeal in a marine finfish such as red sea bream, having high dietary protein requirements. The results indicate that performances of fish fed 60, 24 and 12% fishmeal based diets were comparable; however the complete replacement of fishmeal (0% fishmeal) had detrimental effects. A typical commercial diet for red sea bream also performed similarly as with control group. Therefore, it is concluded that 80% of the fishmeal protein in a typical commercial diet could be replaced with a combination of sea food by-products and soybean proteins. In this recommended replacement level, dietary fishmeal inclusion level and cost of protein were reduced to about 12% and 50% respectively. Nutritional composition and availability of by-products are always variable which is a further consideration during the utilization of by-products.

CHAPTER V

Experiment II

Effect of complete replacement of fishmeal by dehulled soybean meal with crude attractants supplementation in diets for juvenile red sea bream, *Pagrus major*

(In Contribution: Aquaculture Nutrition)

5.2.1 Abstract

A feeding trial was carried out to develop non fishmeal practical diet for red sea bream, *Pagrus major* by gradually replacing fishmeal protein with dehulled soybean meal (DSM). Five isocaloric (22 KJ g^{-1}) diets were prepared by replacing 0 (FM100), 70 (FM30), 80 (FM20), 90 (FM10) and 100% (FM0) of the fishmeal protein with DSM. Based on recent findings (Kader *et al*., 2010), all the replacement diets were supplemented with 10% fish soluble (FS), 5% krill meal (KM) and 5% squid meal (SM) to act as attractant and complement amino acids. Triplicate groups of fish (7.3 g) were stocked in 100-l polycarbonate circulate tanks at a rate of 15 fish per tank. Fish were fed the respective test diets to satiation twice daily for 8 weeks. At the end of the feeding trial, there was no significant difference ($P > 0.05$) on final weight, weight gain and specific growth rate of fish fed FM100 and FM0 diets. The growth parameters were significantly ($P < 0.05$) increased in fish fed FM30 and FM20 diets compared to FM100 and FM0 diets. Numerically higher feed intake was found in all the replacement groups which might be due to the balanced nutritional composition of diets by supplementing FS, KM and SM. There was no significant difference in feed efficiency ratio and protein efficiency ratio among dietary treatments. Whole body proximate compositions were not influenced by the dietary treatments. Although wide variations in some of the blood parameters were observed, no significant alteration was identified among the treatments except for those of plasma total protein and triglyceride which were significantly increased in fish fed FM0 diet. Dietary treatments had no significant effects on serum cortisol and oxidative stress parameters of fish compared to the control. Based on present experimental condition, 30-40% protein cost can be reduced by gradually replacing fishmeal. Therefore, it is concluded that DSM supplemented with FS, KM and SM could completely replace fishmeal in diets for juvenile red sea bream without any adverse effects on fish performance.

Key words: Complete replacement, Fishmeal, Soybean meal, Growth, Health, Red sea bream

5.2.2 Introduction

Soybean proteins have been recognized as one of the most appropriate alternative protein sources for fishmeal in aquafeed because of their consistent nutritional composition, comparatively balanced amino acid profile, availability and reasonable price (Storebakken *et al*., 2000). However, the negative effects of soybeans such as anti-nutritional factors, imbalance of amino acid and less palatability should be considered in the process of soybean based diet formulation. The advance food processing technologies have enabled the improvement of the plant proteins through neutralizing antinutritional factors and improving digestibility. The amino acid and palatability issue could also be overcome with amino acid supplementation or appropriate feed formulation (Kader *et al*., 2010). Several studies have been conducted to utilize soybean proteins in replacement for fishmeal and it was found that 20-50% of the fishmeal protein could be replaced in the diets of red sea bream, *Pagrus major* (Takagi *et al*., 1999, 2001); gilthead sea bream, *Sparus aurata* L. (Kissil *et al*., 2000); Japanese flounder, *Paralichthys olivaceus* (Choi *et al*., 2004; Deng *et al*., 2006); turbot, *Scophthalmus masimus* L. (Day and González, 2000) and Korean rockfish, *Sebastes schlegeli* (Lim *et al*., 2004). In contrast, comparatively higher replacements (75-94%) of fishmeal with soybean proteins were found for Senegalese sole, *Solea senegalensis* post larvae (Aragão *et al*., 2003) and cobia, *Rachycentron canadum* juvenile (Salze *et al*., 2010). The complete replacement of fishmeal by soybean proteins was achieved by Kaushik *et al*. (1995) in rainbow trout, *Oncorhynchus mykiss* and Salze *et al*. (2010) in cobia. In most of the cases, higher inclusion levels of soybean proteins resulted in lower feed intake of fish because of imbalanced amino acids and decreased palatability of diets which had detrimental effects on the performances of fish. Therefore, crystalline amino acid (CAA) particularly methionine and/or lysine were usually supplemented in diets containing higher levels of soybean proteins. Takagi *et al*. (2008), Gaylord *et al*. (2006) and Lunger *et al*. (2007) reported that

supplementation of taurine in high plant protein based diets is beneficial to improve the production characteristics of carnivorous fish species. Non fishmeal diet was successfully developed for yellowtail, *Seriola quinqueradiata* with soy protein concentrate (SPC) by increasing 8-12% dietary protein level and supplementation of methionine, lysine and 4.5% taurine (Takagi *et al*., 2008). In a recent study, Salze *et al*. (2010) also indicated that supplementation of methionine and lysine in addition to taurine is imperative for the complete replacement of fishmeal with alternative protein sources from the diets of cobia. To summarize these research findings, soybean proteins are potential alternative protein source for the carnivorous fish and low or non fishmeal diet could be formulated with soybean proteins by meeting the deficient amino acids, increasing diet palatability and taurine supplementation.

Recently, Kader *et al*. (2010) investigated that supplementation of small amount (10%) of crude ingredients such as fish soluble (FS), squid meal (SM) and krill meal (KM) in high SPC based diet (without CAA) was effective to achieve similar performances of red sea bream as with fishmeal based control group. It was also reported that a mixer of FS, SM and KM each at 5% (total 15%) significantly improved the performances of red sea bream over those fed the control diet. Supplementation of FS, SM and KM acted as natural feeding attractants as well as being effective in balancing amino acids and masking the unpalatable substances present in SPC. Based on this recent finding, a feeding trial was conducted to develop fishmeal free diet for juvenile red sea bream by gradually replacing fishmeal with dehulled soybean meal (DSM) and crude attractants such as FS, KM and SM supplementation.

5.2.3 Materials and method

5.2.3.1 *Test fish and experimental system*

The feeding trial was carried out at the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University, Japan. The juvenile red sea bream were collected from a commercial hatchery located in Miyazaki prefecture, Japan. The fish were acclimatized for one week in the laboratory condition. During this period, a commercial diet (50% crude protein; Higashimaru, Japan) was supplied to the fish. The feeding trial was conducted in 100-l polycarbonate tanks (filled with 80l of water) in a flow through sea water system where each tank was equipped with an inlet, outlet, and continuous aeration. The tanks were maintained under natural light/dark regime. The seawater was pumped from the deep basin of Kagoshima bay, Japan; gravel filtered and supplied to the system. A flow rate of 1.5 l min^{-1} was maintained throughout the experimental period. During the experimental period, the monitored water quality parameters (mean ± S.D.) were: water temperature 26.1 ± 1.7°C; pH 8.1 ± 0.5 and salinity 33.1 ± 0.5. These ranges are considered within optimal values for juvenile red sea bream.

5.2.3.2 *Test diets*

The formulation and chemical composition of the experimental diets are shown in Table 5.2.1, 5.2.2 and 5.2.3. The dietary components and the basal diet were similar as those used in the recent publication (Kader *et al*., 2010). All the dietary components were obtained commercially, except for FS, which was provided by "Makurazaki Fish Processors Cooperatives, Kagoshima, Japan". High quality solvent extracted DSM (commercial name “soy pro”) was purchased from J. Oil Mills, Japan. The proximate composition of soy pro was closely related to SPC, used in our previous study (Kader *et al*., 2010).

Table 5.2.1: Composition of experimental diet (% dry matter basis)

Ingredients	Diet group				
	FM100	FM30	FM20	FM10	FM0
Fishmeal[1]	60.00	18.00	12.00	6.00	0.00
DSM[2]	-	30.72	39.21	47.70	56.19
FS[3]	-	10.00	10.00	10.00	10.00
KM[4]	-	5.00	5.00	5.00	5.00
SM[5]	-	5.00	5.00	5.00	5.00
Pollack liver oil[6]	2.00	5.50	6.00	6.50	7.00
Soybean lecithin[6]	2.00	2.00	2.00	2.00	2.00
HUFA[7]	0.50	0.50	0.50	0.50	0.50
Wheat flour	10.00	4.00	3.00	2.00	1.00
Vitamin mixture[8]	3.00	3.00	3.00	3.00	3.00
Mineral mixture[9]	3.00	3.00	3.00	3.00	3.00
Vitamin C ester[10]	0.30	0.30	0.30	0.30	0.30
Activated gluten	5.00	5.00	5.00	5.00	5.00
Carboxymethyl cellulose	1.00	1.00	1.00	1.00	1.00
α-cellulose	13.20	6.98	4.99	3.00	1.01
Total	100.00	100.00	100.00	100.00	100.00

[1]Nippon Suisan Co. Ltd., Tokyo, Japan: proximate composition (% dry matter): moisture, 8.2; crude protein, 72.1; total lipid, 15.6 and ash 12.0.

[2]DSM: dehulled soybean meal (soy pro); J. Oil Mills, Japan; proximate composition (% dry matter): moisture, 7.2; crude protein, 50.1; total lipid, 4.4 and ash 4.2.

[3]FS: fish soluble; Makurazaki Fish Processors Cooperatives, Kagoshima, Japan; proximate composition (% dry matter): moisture, 50.2; crude protein, 73.4; total lipid, 4.0 and ash, 19.3.

[4]KM: krill meal; Nippon Suisan Co. Ltd., Tokyo, Japan; proximate composition (% dry matter): moistre, 6.9; crude protein, 62.1; total lipid, 23.4 and ash 9.7.

[5]SM: squid meal; Nippon Suisan Co. Ltd. Tokyo, Japan; proximate composition (% dry matter): moisture, 9.3; crude protein, 83.6; total lipid, 9.2 and ash 6.4.

[6]Riken Vitamin, Tokyo, Japan.

[7]Poweash A, Oriental Yeast Co, Ltd., Tokyo, Japan.

[8]Vitamin mixture (g kg^{-1} diet): ß-carotene 0.10; Vitamin D_3 0.01; Menadione $NaHSO_3.3H_2O$ (K_3) 0.05; DL-α-Tochopherol Acetate (E) 0.38; Thiamine-Nitrate (B_1) 0.06; Riboflavin (B_2) 0.19; Pyridoxine-HCl (B_6) 0.05; Cyanocobalamin (B_{12}) 0.0001; Biotin 0.01; Inositol 3.85; Niacine (Nicotic acid) 0.77; Ca Panthothenate 0.27; Folic acid 0.01; Choline choloride 7.87; ρ-Aminobenzoic acid 0.38; Cellulose 1.92.

[9]Mineral mixture (g kg^{-1} diet): $MgSO_4$ 5.07; Na_2HPO_4 3.23; K_2HPO_4 8.87; Fe Citrate 1.10; Ca Lactate12.09; Al $(OH)_3$ 0.01; $ZnSO_4$ 0.13; $CuSO_4$ 0.004; $MnSO_4$ 0.03; Ca $(IO_3)_2$ 0.01; $CoSO_4$ 0.04.

[10]L-Ascorbyl-2-phosphate-Mg.

Table 5.2.2: Chemical analysis of the experimental diets

	Diet group				
Ingredients	FM100	FM30	FM20	FM10	FM0
Proximate composition (% dry matter basis)					
Crude protein	48.81	49.30	49.42	49.43	50.42
Total lipid	13.75	13.65	14.31	13.86	13.62
Ash	12.22	11.20	10.88	10.64	10.33
Gross energy (KJ g^{-1})	22.14	21.75	22.16	22.16	22.29
Amino acid (AA g $100g^{-1}$ dry sample)					
Arginine	4.12	3.57	3.61	3.81	3.83
Histidine	1.78	1.80	1.78	1.85	1.87
Isoleucine	1.69	1.27	1.21	1.32	1.22
Leucine	3.17	2.68	2.61	2.44	2.62
Lysine	3.42	2.88	2.82	2.85	2.71
Methionine	1.48	0.77	0.75	0.69	0.65
Phenylalanine	2.43	2.58	2.49	2.77	2.71
Threonine	1.73	1.56	1.46	1.54	1.50
Tryptophan	tr[1]	tr	tr	tr	tr
Valine	2.18	1.56	1.68	1.64	1.68
Taurine	0.40	0.49	0.47	0.41	0.37

[1]Trace amount

Table 5.2.3: Free amino acid content of experimental diets (FAA g $100g^{-1}$ dry sample)

Amino acids	Diet group				
	FM100	FM30	FM20	FM10	FM0
Indispensable					
Arginine	0.04	0.12	0.14	0.15	0.21
Histidine	0.09	0.30	0.28	0.32	0.32
Isoleucine	0.03	0.04	0.03	0.03	0.03
Leucine	0.08	0.09	0.08	0.08	0.06
Lysine	0.12	0.11	0.12	0.15	0.13
Methionine	0.04	0.03	0.00	0.01	0.01
Phenylalanine	0.12	0.28	0.30	0.34	0.30
Threonine	0.03	0.04	0.04	0.04	0.03
Tryptophan	0.03	0.03	0.03	0.02	0.00
Valine	0.08	0.04	0.03	0.04	0.04
Dispensable					
Taurine	0.34	0.42	0.42	0.42	0.35
Aspartic acid	0.01	0.04	0.04	0.05	0.04
Glutamic acid	0.07	0.14	0.14	0.15	0.14
Serine	0.01	0.03	0.03	0.03	0.02
Proline	0.04	0.07	0.07	0.08	0.07
Glycine	0.03	0.04	0.04	0.04	0.04
Alanine	0.11	0.11	0.10	0.10	0.08
Tyrosine	0.11	0.13	0.14	0.15	0.13
Total	1.39	2.05	2.05	2.21	1.99

Values are means of duplicate measurements.

Five experimental diets were formulated on the basis of nearly isonitrogenous (50% crude protein), isolipidic (14% total lipid) and isocaloric (22 KJ g^{-1} gross energy). The control diet was a 60% fishmeal based diet (FM100). Fishmeal protein was gradually replaced at 70, 80, 90 and 100% with DSM and designated as FM30, FM20, FM10 and FM0 respectively. All the replacement diets (except FM100) were supplemented with 10% FS, 5% KM and 5% SM to complement amino acid profile of the test diets and as attractants or palatability enhancer. Pollack liver oil, soybean lecithin and HUFA were supplied as lipid sources, and wheat flour as the carbohydrate or nitrogen free extract sources. Diet preparation was described in the previous chapters.

5.2.3.3 *Feeding protocol*

At the beginning of the feeding trial, juvenile fish, weighing 7.33 ± 0.06 g (mean ± SD) were stocked in previously prepared fifteen tanks with 15 fish per tank in triplicates per dietary treatment. All fish were fed the respective test diets to the satiation level by hand twice daily, 7 days per week for 8 weeks. Uneaten diet was removed one hour after feeding and dried using a freeze drier. The water quality was checked regularly. All fish were weighted in bulk at 2 weeks interval to determine growth and check their health condition.

5.2.3.4 *Sample collection and biochemical analysis*

A pooled sample of 10 fish at the beginning was stored at −20°C for whole body analysis. At the end of the feeding trial, all fish were fasted for 24 h prior to final sampling. All the fish were anaesthetized with Eugenol (4-allylmethoxyphenol, Wako Pure Chemical Ind., Osaka, Japan) at 50 mg l^{-1}. Then the total number, individual body weight and length of fish from each tank were measured. A pooled sample of five fish from each replicate tank were randomly collected and stored at −20°C for final whole body analysis. Blood was taken from

the caudal vein of three fish in each replicate tank using heparinized syringes. Hematocrit was determined using the microhematocrit technique. Plasma samples were obtained by centrifugation at 3000 × *g* for 15 min using a high-speed refrigerated microcentrifuge (MX-160; Tomy Tech USA Inc., Tokyo, Japan) and kept at −80°C. Blood samples collected with non-heparinized syringes from three fish per tank were kept at room temperature for 2 h and centrifuged at 3000 × *g* for 15 min to collect serum. Viscera with liver were dissected out from three fish in each replicate tank, weighted and then, only liver was weighted to get viscerasomatic index (VSI) and hepatosomatic index (HSI) respectively. Then, liver samples were pooled together and stored at −80°C.

Proximate composition, gross energy, amino acids (TAA and FAA), plasma chemical parameters and oxidative stress parameters were analyzed by the methods indicated in the previous chapters. For the analysis of serum cortisol, 100 µl serum was mixed with 1ml diethylether by using a vortex mixture and allow to separate the organic phase. The diethylether was evaporated under a gentle stream of nitrogen. The extract was then analyzed for cortisol concentration using an enzyme-linked immunosorbent assay (Cortisol EIA Kit, product number EA65, Oxford Biomedical Research Inc., Oxford, MI).

5.2.3.5 *Evaluation of growth performance parameters*

The following variables were evaluated:

Weight gain (%) = (final weight – initial weight) × 100 / initial weight

Specific growth rate (SGR %, day^{-1}) = {Ln (final weight) – Ln (initial weight) / duration} × 100

Survival (%) = 100 × (final no of fish / initial no of fish)

Feed intake (g $fish^{-1}$ 56 $days^{-1}$) = (dry diet given – dry remaining diet recovered) / no of fish

Feed efficiency ratio (FER) = live weight gain (g) / dry feed intake (g)

Protein efficiency ratio (PER) = live weight gain (g) / dry protein intake (g)

Protein gain (PG, g kg weight gain^{-1}) = {(final weight (g) × final whole body protein content (%) / 100) – (initial weight (g) × initial whole body protein content (%) / 100)} / (weight gain (g)) × 1000

Protein retention (PR, % of intake) = (protein gain (g kg weight gain^{-1}) × 100) / protein intake (g kg weight gain^{-1})

Condition factor (CF, %) = weight of fish / (length of fish)^3 × 100

Hepatosomatic index (HSI, %) = weight of liver / weight of fish × 100

Viscerotopic index (VSI, %) = weight of viscera / weight of fish × 100

5.2.3.6 *Statistical analysis*

Data were analyzed using one-way analysis of variance (Package Super-ANOVA 1.11, Abacus Concepts, Berkeley, California, USA) for significant differences among treatment means based on fishmeal protein replacement levels. Probabilities of $P < 0.05$ were considered significant. Significance differences between means were evaluated using the Tukey Kramer test.

5.2.4 Results

Growth performance, nutrient utilization and survival of fish are presented in Table 5.2.4. Survival (%) was not significantly ($P > 0.05$) different at the end of the feeding trial. Fishmeal free diet (FM0) supported growth equivalent to that of the 100% fishmeal based control diet (FM100) and there were no significant differences in final weight, weight gain and SGR of fish fed FM100, FM10 and FM0 diets. However, the growth parameters were significantly ($P < 0.05$) higher in fish fed FM30 and FM20 diets compared to FM100 and FM0 diets. Among the treatments, numerically the lowest growth performance was found in

fish fed fishmeal free diet (FM0). Similarly, FER, PER and PR were also lowest in FM0 groups while these parameters were not significantly different among treatments. No difference was also found in PG and PR among different treatments. The feed intake of fish was numerically higher in all the replacement groups compared to the fish fed control diet. Significantly higher feed intake was found in fish fed FM30 diet and then successively decreased in FM20, FM10 and FM0 groups and these were correlated to the growth of fish (Fig. 5.2.1 and 5.2.2). Fig 5.2.3 shows the relative value (%) for protein cost in different experimental diets compared to control diet. Costs of protein were progressively decreased with fishmeal replacement levels and fishmeal free diet (FM0) represents 40% reduced cost compared to the control.

The whole body proximate composition of fish at the start and end of the feeding trial is shown in Table 5.2.5. All the fish showed a change in the analyzed parameters compared to those of the initial values. However, there was no significant difference ($P > 0.05$) in the final whole body proximate composition between the groups of fish fed the different experimental diets. No difference was also detected in CF, HSI and VSI of fish among treatments (Table 5.2.5).

Table 5.2.6 represents the blood parameters of red sea bream after 8 weeks feeding trial. Although wide variations were observed on some of the parameters, no significant alteration was identified among the treatments except for those of total protein and triglycerides which were significantly increased in fish fed FM20 and FM0 diets, respectively. Oxidative status of fish was summarized in Table 5.2.6. Dietary treatments had no significant effect on the relative value (%) of serum cortisol levels compared to the control. No significant difference was found in d-ROMs and BAP values among fish fed different experimental diets. However, antioxidant levels (BAP) were slightly decreased in FM30 and FM0 groups while stress levels (d-ROMs) in those groups were comparable with control group.

Table 5.2.4: Growth parameters and nutrient utilization in red sea bream fed test diets for 56 days*

Parameters	Diet group				
	FM100	FM30	FM20	FM10	FM0
In wt[1]	7.39 ± 0.01	7.36 ± 0.03	7.30 ± 0.01	7.29 ± 0.01	7.31 ± 0.09
Fn wt[2]	45.91 ± 1.58^{a}	60.86 ± 2.82^{c}	56.73 ± 1.30^{bc}	50.34 ± 1.14^{ab}	45.10 ± 1.46^{a}
WG[3]	522 ± 21^{a}	727 ± 35^{c}	678 ± 19^{bc}	591 ± 17^{ab}	517 ± 12^{a}
SGR[4]	3.26 ± 0.06^{a}	3.78 ± 0.08^{c}	3.66 ± 0.04^{bc}	3.45 ± 0.04^{ab}	3.25 ± 0.04^{a}
FI[5]	45.81 ± 2.58^{a}	57.87 ± 0.98^{b}	55.65 ± 2.71^{ab}	50.82 ± 1.22^{ab}	47.56 ± 0.38^{ab}
FER[6]	0.84 ± 0.01	0.93 ± 0.04	0.89 ± 0.02	0.85 ± 0.04	0.80 ± 0.03
PER[7]	1.69 ± 0.03	1.88 ± 0.07	1.80 ± 0.04	1.72 ± 0.09	1.58 ± 0.05
PG[8]	169 ± 5.0	163 ± 6.0	165 ± 1.5	168 ± 2.5	169 ± 2.0
PR[9]	28.42 ± 0.33	30.55 ± 2.24	29.55 ± 0.37	28.78 ± 1.91	26.63 ± 1.02
Sur[10]	93	93	100	93	93

Abbreviations used: [1]In wt: initial weight (g), [2]Fn wt: final weight (g), [3]WG: percent weight gain (%), [4]SGR: specific growth rate (% day^{-1}), [5]FI: feed intake (g dry diet fish^{-1}56 days^{-1}), [6]FER: feed efficiency ratio, [7]PER: protein efficiency ratio, [8]PG: protein gain (g kg body weight gain^{-1}), [9]PR: protein retention (% of intake), [10]Sur: survival (%).

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

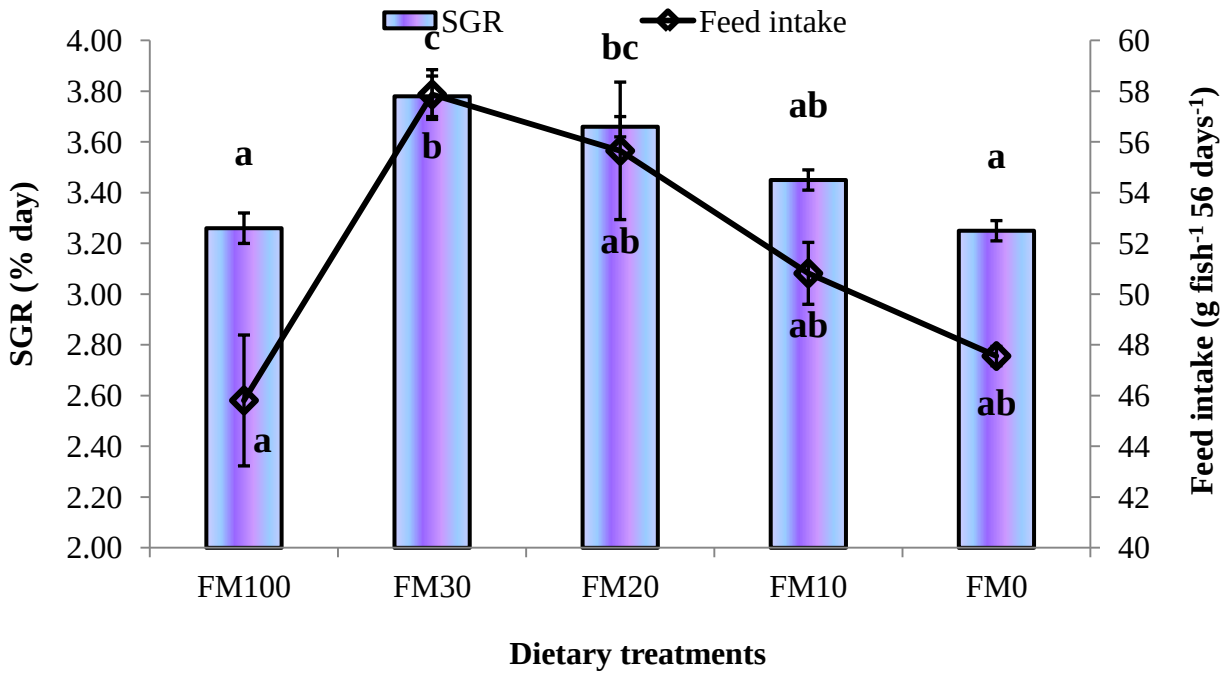

Fig. 5.2.1: Graphical presentation of specific growth rate (SGR) and feed intake of red sea bream after 56 days feeding trial

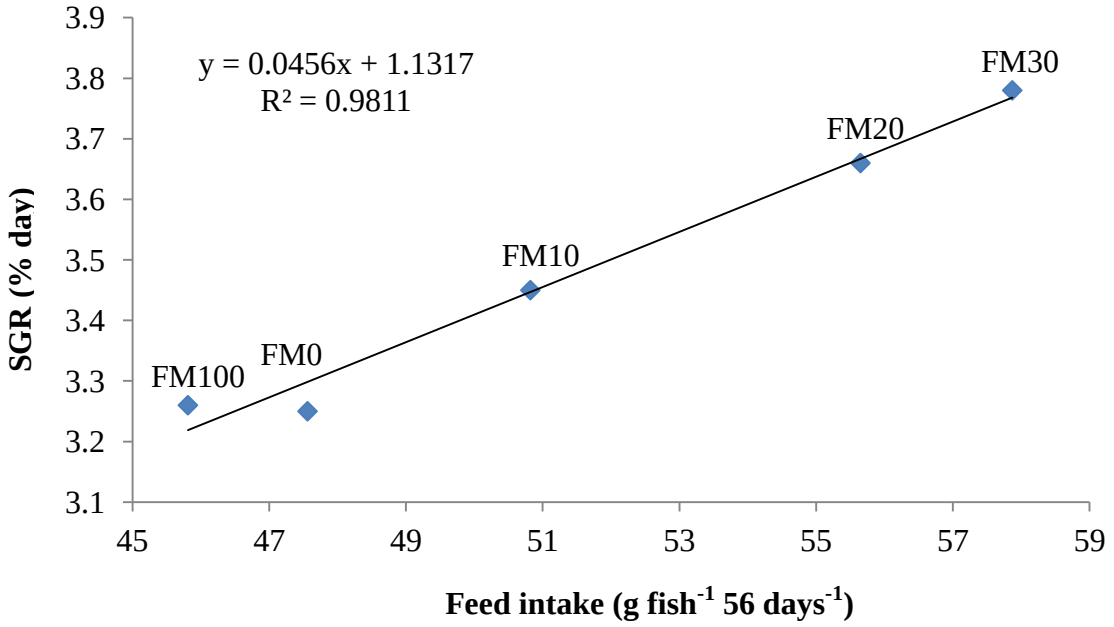

Fig. 5.2.2: The relationship between specific growth rate (SGR) and feed intake of red sea bream after 56 days feeding trial

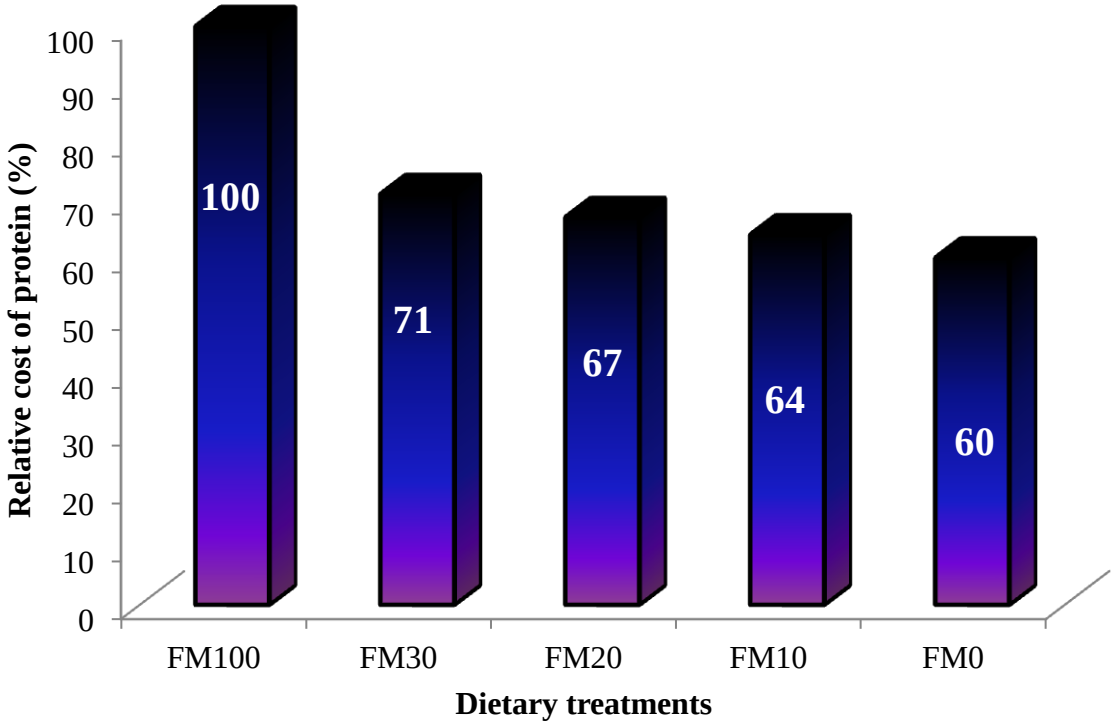

Fig. 5.2.3: Relative cost of protein (%) in the test diets compared to the cost of protein in fishmeal based control diet

Table 5.2.5: Whole body proximate analysis (%) and somatic parameters in juvenile red sea bream fed test diets for 56 days*

Parameters	Initial[1]	Diet group				
		FM100	FM30	FM20	FM10	FM0
Moisture	80.23	68.35 ± 0.25	66.68 ± 0.41	67.40 ± 0.34	68.13 ± 0.90	66.31 ± 0.50
Crude protein	13.94	16.47 ± 0.40	16.04 ± 0.56	16.15 ± 0.15	16.40 ± 0.24	16.48 ± 0.16
Total lipid	1.83	10.52 ± 0.59	12.03 ± 0.11	11.41 ± 0.26	10.96 ± 0.43	11.78 ± 1.15
Crude ash	4.60	4.48 ± 0.15	3.89 ± 0.20	4.15 ± 0.15	4.07 ± 0.18	4.25 ± 0.12
CF[2]		1.93 ± 0.03	1.95 ± 0.04	1.93 ± 0.01	1.98 ± 0.02	1.92 ± 0.06
HSI[3]		2.34 ± 0.11	2.38 ± 0.07	2.64± 0.06	2.19 ± 0.11	2.40 ± 0.11
VSI[4]		6.15 ± 0.39	6.05 ± 0.36	6.03 ± 0.17	6.04 ± 0.01	6.63 ± 0.29

*Values are means of triplicate groups ± S.E.M. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments. Crude protein, crude lipid and ash are expressed on a wet weight basis.

[1] Initial values are not included in the statistical analysis.

[2]CF: condition factor (%).

[3]HSI: hepatosomatic index (%).

[4]VSI: viscerotopic index.

Table 5.2.6: Blood parameters in juvenile red sea bream fed test diets for 56 days[1]

Parameters	Diet group				
	FM100	FM30	FM20	FM10	FM0
Hematocrit (%)	36.2 ± 2.0	44.4 ± 3.6	43.8 ± 1.2	41.6 ± 2.1	40.7 ± 0.7
Total protein (g/dl)	3.83 ± 0.03[a]	4.75 ± 0.25[ab]	5.40 ± 0.17[b]	5.10 ± 0.27[ab]	5.00 ± 0.50[ab]
Total albumin (g/dl)	1.00 ± 0.00	1.05 ± 0.05	1.17 ± 0.12	1.10 ± 0.06	1.07 ± 0.07
Total bilirubin (mg/dl)	0.27 ± 0.03	0.40 ± 0.10	0.40 ± 0.12	0.33 ± 0.03	0.37 ± 0.09
Glucose (mg/dl)	76.0 ± 5.0	81.5 ± 7.5	73.0 ± 3.0	73.0 ± 3.0	75.5 ± 6.5
GOT (IU/l)[2]	33.7 ± 3.8	38.5 ± 10.5	31.7 ± 8.7	32.0 ± 12.2	38.0 ± 7.4
GPT (IU/l)[3]	< 10.00	< 10.00	< 10.00	< 10.00	< 10.00
BUN (mg/dl)[5]	6.00 ± 0.00	5.00 ± 0.00	5.33 ± 0.33	5.33 ± 0.33	5.33 ± 0.33
Triglycerides (mg/dl)	328 ± 25[a]	301 ± 8[a]	410 ± 21[a]	455 ± 45[a]	684 ± 82[b]
T- Cho (mg/dl)[6]	367 ±23	382 ± 44	396 ± 76	386 ± 31	331 ± 32
HDL-c (mg/dl)[7]	343 ± 17	345 ± 36	368 ± 57	370 ± 33	307 ± 34
Amylase (IU/l)	25.7 ± 4.7	32.5 ± 7.5	28.0 ± 3.5	27.0 ± 0.6	27.0 ± 2.5
Oxidative status					
CORT (%)[8]	100 ± 2.1	100 ± 4.9	105 ± 2.5	97 ± 2.1	103 ± 1.1
d-ROMs (U.Carr)[9]	117 ± 2[ab]	54 ± 30[a]	165 ± 25[b]	186 ± 4[b]	139 ± 8[ab]
BAP (µ Mol l^{-1})[10]	3164 ± 108	2326 ± 51	3119 ± 343	2753 ± 538	2352 ± 618

Abbreviation used: [2]GOT: glutamyl oxaloacetic transaminase, [3]GPT: glutamic-pyruvate transaminase, [4]LDH: lactate dehydrogenase, [5]BUN: blood urea nitrogen, [6]T-Cho: total cholesterol, [7]HDL-c: high density lipoprotein cholesterol, [8]CORT: relative value of cortisol, [9]d-ROMs: reactive oxygen metabolites, [10]BAP: biological antioxidant potential.

[1]Values are means ± SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.

5.2.5 Discussion

There has been increasing interest to find out cost-effective and practical approaches to utilize alternative protein sources more efficiently in marine carnivorous species towards sustainable aquaculture practice. Our recent findings suggested that 60% fishmeal protein could be replaced by SPC with the supplementation of 10% FS, KM or SM in red sea bream diet (Chapter IV; Kader *et al*., 2010). In that study, it was also suggested that FS is the most promising supplement and FS alone or combining with KM and SM without any CAA, supported even significantly higher growth performances compared to those in control group. Based on these findings, it was hypothesized that higher or complete replacement of fishmeal could also be achieved by soybean protein with supplementation of proper ratio of FS, KM and SM. Results from the present experiment with juvenile red sea bream correlated to that hypothesis and it was found that growth performance, nutrient utilization, body composition and health parameters were not negatively affected by 70-100% fishmeal protein replacement and supported almost similar or even superior performances as with fishmeal based control group. Therefore, these results confirmed our previous study that plant proteins supplemented with a proper ratio of selected crude attractants is effective to formulate low or non fishmeal diet for marine carnivorous fish such as red sea bream.

Growth performances of fish in all the dietary treatments in the present experimental condition were satisfactory. The WG (%) ranged on 517-727%, which is comparatively higher than previous study with red sea bream, reported a WG (%) of 299-493 % for juvenile fish (6.60 g) fed diets replacing fishmeal with tuna muscle by-products for 50 days in a similar experimental condition as applied in the present experiment (Uyan *et al*., 2007). Sarker *et al*. (2007) also reported a WG (%) of 420-579% after 12 weeks feeding trial with juvenile red sea bream (12.60 g). Although several alternative protein sources were reported to partially replace fishmeal from the diets of many fish species, fewer studies were reported

for red sea bream. Takagi *et al*. (2000b) found that 50% fishmeal protein could be replaced with SBM in yearling red sea bream (260 g); while Biswas *et al*. (2007) reported a significant growth retardation by replacing 39% fishmeal with SBM in the diets of juvenile fish (19 g). Solvent extracted soybean products such as SPC have higher nutritional value and it could replace 50% fishmeal protein in juvenile red sea bream (Takagi *et al*., 1999). When SBM was combined with corn gluten meal and meat meal or corn gluten meal and poultry by-product meal, the replacement level of fishmeal protein could be accelerated to 50-60% in juvenile and 70-90% in yearling red sea bream, respectively (Aoki *et al*., 1998; Takagi *et al*., 2000a). In contrast, the present result suggested that 100% fishmeal can be replaced in diets of juvenile red sea bream. Therefore, it is clearly demonstrated that soybean protein could be utilized more efficiently with the supplementation of a mixture of FS, KM and SM which are in agreement with our previous findings (Kader *et al*., 2010). Comparatively higher fishmeal replacements (75-94%) with soybean proteins were also reported for other marine fish such as Senegalese sole post larvae (Aragão *et al*. ,2003) and cobia juvenile (Salze *et al*., 2010), and the complete replacement of fishmeal was achieved by Kaushik *et al*. (1995) in rainbow trout and Salze *et al*. (2010) in cobia. However, CAAs were supplemented to meet the dietary requirements in those studies.

Efficiency on the utilization of soybean proteins varies among different fish species, and this is related to the number of challenges associated with soybean products, such as imbalance amino acids, especially sulfur amino acids like lysine and methionine, less palatability, lower digestibility and the presence of antinutritional factors. Supplementation of lysine and methionine to compensate for the deficiency of essential amino acids and some other amino acids (e.g. glycine, alanine and taurine etc) as attractants, are beneficial in recovering amino acid balance and palatability in high soybean protein based diets (Fuke *et al*., 1981; Venou *et al*., 2006; Chatzifotis *et al*., 2008; Kader *et al*., 2010). In the present

experiment, CAA was not supplemented; rather some crude attractants were supplemented to compensate those deficiencies. The analyzed values showed that almost all the TAA contents were higher in FM100 while those values were comparable among other diets except for methionine which was decreased with the increasing fishmeal replacement levels. Dietary methionine content in FM10 and FM0 diets were comparatively low, however these diets supported similar growth performances of red sea bream as with control group (FM100). Therefore, it has been suggested that small amounts of FS, KM and SM are effective enough to improve the TAA of diets and the values obtained for TAA of different diets (Table 5.2.2) appears to have been adequate or sub-optimal for good growth and survival of juvenile red sea bream (Uyan *et al*., 2007; Kader *et al*., 2010; Kader *et al*., in press). It is also suggested that requirement of dietary amino acids might be altered depending on the diet formulation. Although methionine and lysine were supplemented in high soybean protein based diets to match the amino acid requirement of red sea bream, Takagi *et al*. (2001) failed to obtain similar performance of fish as that in the fishmeal based diet. In their study, it was also found that supplementation of CAA didn't improve feed intake which might partly be a reason for lower performances of fish. Likewise, Cheng *et al*. (2010) reported that supplemented CAA did not improve feed intake and growth performances of Japanese seabass fed graded levels of canola meal. Lower feed intake is directly proportional to the protein intake, which in turn affects growth rate (Phumee *et al*., 2010). Therefore, diet palatability should also be considered besides amino acid composition during formulating higher levels of soybean protein based diets to restore feed intake of fish.

Recovering depleted feed intake is one of the challenges for the utilization of higher levels of plant protein in aquafeeds. Kader *et al*. (2010) observed that supplementation of FS, KM and SM in high soybean protein based diets significantly improved the feed intake of fish, to the extent of being approximately equal to or higher than in fish fed fishmeal based diet.

Similarly, in the present study, numerically higher feed intake was found in all the replacement groups. This again suggests that small amount of FS, KM and SM are effective enough to restore feed intake in red sea bream even at 56% DSM inclusion level. It is well known that feed intake of fish will be affected by the amount as well as the kind of dietary FAA (Mackie and Mitchell, 1985). Since synthetic FAA was not added to the diets, however dietary total FAA was increased in all the replacement groups compared to FM100 diet (Table 3), it is attributed that inclusion of FS, KM and SM were acted as natural feeding stimulant and improved the diet palatability. Addition of palatability enhancers is an effective approach when developing diets containing high plant protein in order to maintain feed attractiveness and induce adequate feed consumption rate by fish (Papatryphon and Soares Jr., 2000; Kissil *et al*., 2000; Kader *et al*., 2010). Improved feed intake was reported with the supplementation of FS in scorpion fish, *Sebastiscus marmoratus* (Kader, 2008) and red sea bream (Kader *et al*., 2010), fish protein hydrolysate (FPH) in rainbow trout (Espe *et al*., 1999; Refsite *et al*., 2004), KM in tilapia (Gaber, 2005;), krill hydrolysate in yellow perch (Kolkovski *et al*., 2000), squid viscera in Japanese seabass, *Lateolabrax japonicus* (Mai *et al*., 2006) and squid extract in Atlantic salmon, *Salmo salar* L. (Toften *et al*., 2003). The most promising feed stimulating feature was found in FS which is a water soluble compound and rich in soluble protein, minerals and vitamins; free amino acids, peptides, nucleotides and low molecular weight components such as taurine, creatinine, carnosine etc (Kousoulaki *et al*., 2009). In addition, FS have a strong fishy smell which acted as a strong olfactory stimulant. It was suggested that high level of taurine might be one of the major characteristics as a feeding stimulant in FS, and other FAA help to enhance the stimulating effect. Taurine has been reported to be an effective feeding stimulant in high soybean protein based diets for common dentex (Chatzifotis *et al*., 2008) and an essential element for normal feeding behavior and growth of red sea bream (Matsunari *et al*., 2008) and Japanese flounder (Park *et al*., 2002).

Dietary taurine was markedly decreased when 60% fishmeal was replaced with SPC (Kader *et al*., 2010). Although fishmeal was gradually eliminated from diets in the present experiment, similar levels of taurine were found in all the diets and the levels also met the requirements for red sea bream (Matsunari *et al*., 2008). Therefore, comparable growth performances among FM100, FM10 and FM0 might partly be attributed with the similar levels of feed intake in those groups. The increased feed intake in fish fed FM30 and FM20 diets appears to explain the significantly better growth in those groups (Yamamoto *et al*., 1995). This might be due to the fact that higher feed intake would increase the amounts of protein and energy available for increasing fish growth (Phumee *et al*., 2010; Kader *et al*., in press). Feeding stimulatory effect of FS, KM and SM was more pronounced in FM30 and it was gradually decreased with the increasing levels of soybean protein in diets.

Blood parameters are important tools for the indication of the physiological stress response as well as the general health condition of fish. Blood parameters obtained in the present experiment are considered to be within the normal range for juvenile red sea beam, compared to the previous findings (Aoki *et al*., 1998; Uyan *et al*., 2007; Kader *et al*., 2010, 2011). Although total protein and triglyceride levels were increased in fish fed with low or non fishmeal diets, these values were comparable with those observed by Aoki *et al*. (1998; 2000) in juvenile red sea bream fed with low or non fishmeal diets. Stress is one of the emerging factors in aquaculture activities which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization (Li *et al*., 2009). Plasma or serum cortisol concentration is a reliable biological indicator of stress response in fish and terrestrial animals (Small and Davis, 2002; Li *et al*., 2009). In the present study, no differences were found in the relative values of serum cortisol concentrations among treatments compared to the control. Oxidative stress can be generated at high level of reactive oxygen species and/or decreased efficacy of antioxidant system, which is another health risk factor in human or

other mammals (Pasquini *et al*., 2008). The simultaneous analysis of d-ROMs and BAP provided valuable data on oxidative stress condition in humans, pig, rabbit and dog (Oriani *et al*., 2001; Ballerini *et al*., 2003; Pasquini *et al*., 2008). Recently, these tests have also been applied as a suitable tool for evaluating the oxidative stress in fish (Kader *et al*., 2010, 2011). In the present study, no significant differences nor any trend were detected in d-ROM and BAP values among dietary treatments and these values were comparable to the values obtained in earlier studies with juvenile red sea bream (Kader *et al*., 2010). Therefore, it has been suggested that no serious alteration in fish health was found in the present study and a normal physiological condition was maintained in all the fish among treatments.

Based on overall performances of fish, DSM is a suitable protein source as fishmeal replacement for red sea bream. Growth, nutrient utilization, body composition and blood parameters were either improved or were not significantly influenced by gradually replacing fishmeal with DSM and crude attractants supplementation, even in fishmeal free diet. Feed intake was significantly increased in fish fed FM30, while maintaining numerically higher feed intake in all the replacement groups compared to the control (FM100). Growth is directly correlated to the feed intake and it is appeared to be the major characteristics for the performances of fish. The similar or increased feed intake can be explained by improved nutritional composition, mainly amino acid concentration and increased palatability of the diets which were achieved by the supplementation of crude attractants such as FS, KM and SM. Unlike previous findings, the present results also confirmed that supplementation of FS, KM and SM with plant protein such as DSM could replace even 100% of fishmeal in diets for juvenile red sea bream. Interestingly it was also found that 30-40% protein cost would be reduced by gradually replacing fishmeal from control diet. Therefore, the findings of this study will encourage feed manufacturers to utilize plant proteins more efficiently in generating low-cost and sustainable aquafeed.

CHAPTER VI

General Discussion

6. General discussion

Aquaculture industry has grown very fast during last four decades and first time it reaches a landmark in 2008 when half of the fish consumed by human were cultured. Hence, aquaculture is increasing in importance as a means for food security to provide comparatively safe animal proteins. One of the next challenges for further expansion of aquaculture industry is to develop nutritionally balanced, cost-effective and sustainable aquafeeds.

Successful aquaculture venture mostly depends on appropriate feeds, as the feed cost constitutes majority (50-60%) of the total production cost. Fishmeal is still unparallel protein source for aquafeeds because of its unique nutritional specifications including indispensable amino acids, essential fatty acids, cholesterol, vitamins, minerals and attractants etc. Because of the increasing demand of fishmeal in different agro-industrial sectors together with decreasing wild stock of fishmeal producing fish, global warming and unpredictable raising costs of fishmeal, there has been considerable uncertainty on the future availability of this finite commodity.

In view of the need to reduce the dependence of the aquaculture industry upon the use of fishmeal, considerable researches have been carried on trying to find dietary replacements for fishmeal within compound aquafeeds. Fishmeal can be partially replaced with several plant and animal protein sources in the diets of many fish species, but in most cases, higher or complete replacements have detrimental effects, mainly due to imbalance amino acids, decrease bio-availability, and presence of toxins or anti-nutritional properties. Acceptability of the alternative diets by the fish is a further consideration, as feed intake is a major factor for growth performances of fish. In general, feed intake decreases as the level of alternative protein sources increases, especially plant proteins, due to decrease of diet palatability or acceptability. The present research emphasized on these two factors, firstly new alternative

protein sources, and secondly cost-effective approaches that can mitigate the negative effects of alternative proteins to formulate low or non fishmeal based aquafeeds.

Japanese are well known for their high consumption of fish and shellfish, which results in large amounts of by-products. These by-products are usually disposed according to the strict Japanese Government regulations for waste disposal. This is often seen as a threat to the industry as a considerable amount of money is spent on disposal of by-products, even though they are nutrient-rich resources which have the potential to be utilized in aquafeed. But there are problems associated with the utilization of by-products, especially by-product of seafood processing industry, including freshness, quality, availability, higher moisture, indigestible particle and contaminants or toxic metals. However, technological advances have made it possible to recycle fisheries waste products into acceptable protein supplements for the animal feed industry.

In the present research, Chapter III showed that the blend of co-drying wet fisheries by-product with another dry ingredient and subsequent fermentation is an effective approach for the utilization of fisheries by-products in aquafeed. Scallop by-product and squid by-product were mixed with soybean meal, and then the mixtures were fermented with combined bacteria to produce FSSc and FSSq respectively. These two fermented products could successfully replace 30 and 36% fishmeal protein in red sea bream and Japanese flounder diets, respectively without any negative effects on the performances of fish. Although soybean proteins have been recognized as one of the potential protein sources for cultured fish, it could replace only 20% or less fishmeal from the diets of Japanese flounder and red sea bream (Choi *et al*. 2004; Biswas *et al*. 2007). Thus it was suggested that fishmeal replacement levels were increased by blending fisheries by-products with SBM, and subsequent fermentation. One of the possible reasons for this improvement is the balanced nutritional composition in fermented products by complementing nutrients from two

products. Fermentation is also an efficient technique to decrease or eliminate anti-nutritional constituents from oilseeds and improve the overall nutritional quality. Therefore, FSSc and FSSq were assumed new dietary ingredients for marine cultured species.

A certain level of fishmeal could be replaced with different alternative protein sources in many fish species. However, higher replacement has generally resulted in a decrease in fish growth performance which is mainly attributed to the decrease feed intake. It is particularly noteworthy that feed intake is affected by the amount of fishmeal, amino acid composition or palatability of diets. Therefore, these factors should be considered when formulating low or non fishmeal based diets. Addition of CAA and feeding stimulants with alternative diets could recover the depleted feed intake of fish. Commonly recognized feeding stimulants are relatively small soluble molecules, such as certain amino acids (taurine, glycine, arginine, glutamic acid and alanine etc), betaine, nucleotides and organic acids (Grey *et al*., 2009), which are rich in marine organisms like fish, krill, squid, shrimp etc (Gaber, 2005; Smith *et al*., 2005; Mai *et al*., 2006). As CAA is expansive and not always available in developing countries, supplementation of marine by-products which are rich in feed stimulating substances might be a cost-effective approach. Chapter IV provides clear evidence that small amount of marine by-products such as FS, KM and SM in high soybean protein (60% fishmeal replacement) based diets is as effective as CAA to improve growth performance and welfare of red sea bream, *Pagrus major* (Kader *et al*., 2010). Supplementation of FS, KM and SM significantly increased feed intake to the extent of being approximately equal to or higher than in fish fed fishmeal or CAA supplemented diets. This suggests that only small amounts of FS, KM and SM are effective enough to improve the amino acid composition of diets and those acted as feeding stimulants. This was also confirmed by the analyzed values of TAA and FAA in test diets.

Chapter V dealt with the possibilities to formulate low or non-fishmeal based diets for red sea bream. Limited studies were reported for higher or complete replacement of fishmeal from the diets of marine carnivorous fish. It was reported that certain amounts of fishmeal and/or supplementation of CAA are imperative to formulate low or non-fishmeal based diets for cultured fish (Kaushik *et al*., 1995; Aragão *et al*., 2003; Wang *et al*., 2008; Salze *et al*., 2010). Combination of several protein sources is also effective for this purpose (Aoki *et al*., 1998; Guo *et al*., 2007, Kader *et al*., in press). Nevertheless, based on the encouraging finding from Chapter IV, two batches of non fishmeal diets were formulated for juvenile red sea bream by gradually replacing fishmeal with a blend of FS, FSSc and FSSq (2:1:1), and DSM respectively. Results showed that 80% of the fishmeal protein could be replaced by the blend and complete elimination of fishmeal was evident by DSM without compromising the performances of fish. The results obtained in these experiments were supported by previous studies showing that combination of different protein sources or supplementation of crude attractants is effective to achieve balanced nutritional composition, complementing amino acid profiles and masking the unpalatable substances present in feed ingredients (Yamamoto *et al*., 1995; Aoki *et al*., 1998; Tidwell *et al*., 2005; Guo *et al*., 2007; Kader *et al*., 2010). In Chapter IV, it was found that TAA, specially arginine, methionine and taurine, and the total amount of FAA fractions were markedly decreased by replacing 60% of the fishmeal protein with SPC. Thus, significantly lower growth performance of fish in that group was attributed to the lower feed intake of fish due to imbalanced amino acids and lower palatability of diet. Similarly, in both the experiments in Chapter V, it was found that TAA level in all the low or non-fishmeal diets were lower than that in fishmeal based diet, however these amounts were considered to have been adequate or sub-optimal for good growth and survival of juvenile red sea bream (Uyan *et al*., 2007; Kader *et al*., 2010). In contrary, total amounts of FAA were higher in all the diets of replacement groups compared to the fishmeal based diet in

experiment II. FAA were not analyzed in experiment I, however based on previous studies, it was assumed that total amount of FAA would be comparable or somewhat higher than fishmeal based diet. Therefore, imbalance amino acids and palatability issues related to by-products and soybean proteins might be minimized by blending different protein sources and supplementing crude attractants from marine by-products. Because of those complementing effects, a similar or even significantly higher feed intake was achieved in the replacement groups, which were correlated to the growth performances of fish. Thus, aside from having to increase feed consumption to achieve a similar growth, red sea bream tolerates a high level or even complete replacement of fishmeal protein with by-products and soybean protein without compromising performances of fish. The growths of fish obtained with the recommended fishmeal replacement levels were also comparable with fish fed a commercial diet.

Presently, both red sea bream and Japanese flounder aquaculture, farmers are using commercially manufactured feed, containing high amounts of fishmeal and this is the most expensive components in formulated feed. The use of economic alternative ingredients is the most important requisite to produce these fish cost-effectively. The present research tried to develop the formulation of cost-effective practical diets based on seafood by-products and soybean proteins for marine cultured species. These protein sources are considered as economical ones compared to fishmeal. From Chapter V, it was estimated that the newly formulated diets could reduce 40-50% of the protein cost compared to fishmeal based diet. Therefore, the recommended diets are surely cost-effective which would have a positive impact on the profitability of commercial fish production.

Nutritional imbalances might severely affect the general health condition and fillet quality of fish. Stress is another emerging factor in aquaculture activities which may affect hormonal secretion rates, intermediary metabolism, immunity and nutrient utilization. Thus, as aquaculture makes its transition to a major food-producing sector, proper assessment and

control of any food safety concerns are becoming increasingly important. Although, seafood by-products often contaminated with heavy metals, no mark differences were found in the Pb, Zn, Cu and Cd contents in dietary and whole body composition of red sea bream in the recommended inclusion level of FSSc (30%) during 45 days feeding trial (Chapter III, Experiment 1). In Chapter V (Part III), long term feeding trial showed that diets based on several seafood by-products had no significant effects on the element composition in red sea bream fillets after a 120-day feeding trial. In addition, sensory analysis also showed that no significant difference in the oragnoleptic characteristics of the fillets of fish fed fishmeal based diet, commercial diet and diets based on seafood by-products. Blood parameters are important tools for the indication of the physiological stress response as well as the general health condition of fish. Blood parameters in the present research showed no serious alteration in the health and stress condition of fish with similar growth performances. However, some of the parameters were severely affected when growth was significantly decreased by feeding SPC based diet replacing 60% fishmeal protein (Chapter IV). This confirms that health condition is closely related to the growth performances of fish. Oxidative stress is another emerging health risk factor involved in many diseases of animals (Pasquini *et al*., 2008). Simultaneous measurements of d-ROMs with BAP can provide a suitable tool for measuring the oxidative stress in humans, pig, rabbit and dog (Oriani *et al*., 2001; Ballerini *et al*., 2003; Pasquini *et al*., 2008). Recently, in our laboratory, these indices have been used for first time to determine the oxidative stress condition of fish species. The d-ROMs and BAP values could be interpreted as: animals with higher d-ROM values indicate that they are under more oxidative condition and animals with higher BAP values indicate they have more strong tolerance against oxidation. Although, some variations were found in d-ROMs and BAP values for juvenile red sea bream and Japanese flounder in the present

research, the values are considered as favorable for these species. Since this parameter is new for fish, more data will be needed to further understand the oxidative stress to fish.

Therefore, based on the overall findings the present research suggests that fish could maintain a normal growth performance, feed utilization, body composition, health condition and fillet quality by feeding diets based on by-products and soybean proteins in the recommended inclusion levels.

CHAPTER VII

Summary and Conclusion

7. Summary and Conclusion

The present research was conducted to evaluate the effects of several alternative proteins in the performances of marine fish and find out cost-effective approaches to maximize the utilization of alternative proteins in aquafeeds. To summarize the results from different experiments, it was found that-

1. Seafood by-products are potential dietary ingredients for marine finfish. Blend of scallop and squid by-product with soybean meal and subsequent fermentation is an efficient technology to produce new, cost-effective and comparatively balanced dietary ingredients for marine carnivorous fish.
2. Fermented soybean meal and scallop by-product blend (3:2) (FSSc) could replace at least 30% fishmeal protein in juvenile red sea bream diet while ensuring performances, health and carcass quality of fish.
3. Fermented soybean meal and squid by-product blend (1:1) (FSSq) could substitute 36% fishmeal protein in juvenile Japanese flounder diet without any negative effects on growth, feed utilization, nutrient retention and health/welfare of fish.
4. Inclusion of dietary fishmeal level could be reduced to less than 40% by incorporation of FSSc or FSSq individually in diets of marine carnivorous fish.
5. Fish soluble (FS), krill meal (KM) and squid meal (SM) are effective supplements with alternative proteins to complement amino acids and act as attractants.
6. Feed intake as well as growth was significantly decreased by replacing 60% fishmeal protein with soy protein concentrate in red sea bream diet. Supplementation of 10% FS, KM or SM, or a mixture (total 15%) of FS, KM, SM each at 5% in that diet could recover the depleted feed intake to the extent of being approximately equal to or higher than in fish fed fishmeal based or crystalline amino acid (CAA) supplemented diets.

7. Blend of FS, FSSc and FSSq (2:1:1) supplemented with KM and SM could replace 80% fishmeal protein in red sea bream diet by ensuring growth performances, digestibility, health/welfare and fillet quality of fish. In this recommended diet, fishmeal inclusion level was reduced to only 12%. Interestingly, it was also found that half of the protein cost could be reduced by using those marine by-products and soybean meal.
8. Dehulled soybean meal supplemented with FS, KM and SM could completely replace fishmeal in juvenile red sea bream diets without compromising the performances of fish. It was estimated that the newly formulated diet could reduce about 40% of the protein cost compared to fishmeal based diet.
9. Growth performance is directly correlated to the feed intake of fish and in general, higher feed intake supports better growth as higher feed intake would increase the amounts of protein and energy available for increasing fish growth. Therefore, it is recommended to emphasis on maintaining similar or higher degree of feed intake compared to control while formulating low or non fishmeal based diets for fish.
10. If feed intake could be assured, marine carnivore fish such as red sea bream could maintain normal growth performances with sub-optimal level of dietary amino acids. However, digestibility is another important factor which should be considered during formulation of low fishmeal or non-fishmeal based diets for marine carnivorous fish.

The overall findings of the research suggest that blend of seafood by-product and soybean meal together with subsequent fermentation is an efficient technology to produce new, cost-effective and comparatively balanced dietary ingredients which could partially replace fishmeal from the diets of marine fish. Dietary inclusion of fishmeal could be reduced to very low level in red sea bream diet by a mixture of these fermented products, FS, KM and SM.

Further, fishmeal could be completely eliminated by plant protein such as dehulled soybean meal with supplementation of small amount of FS, KM and SM in red sea bream diet. Therefore, it is recommended to supplement small amount of FS, KM and SM with alternative protiens during formulation of low or non fishmeal based diets for marine cultured species which might be effective to complement amino acids and act as attractants for maintaining normal feeding behavior and growth of fish. The increasing use of lower cost by-products and soybean proteins will allow for a significant reduction on the cost of the feeds as costly fishmeal are removed from the formulations, or at least used at more efficient levels.

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Appendices

Chapter II

Fig. 2.2a: Red sea bream

Fig. 2.2b: Japanese flounder

Fig. 2.2: Experimental fish

Fig. 2.3a: Test diet preparation

Fig. 2.3b: Test diet

Fig. 2.3: Experimental diet and preparation

Fig. 2.4a: Experimental system

Fig. 2.4b: Experimental tank

Fig. 2.4: Experimental system and tank

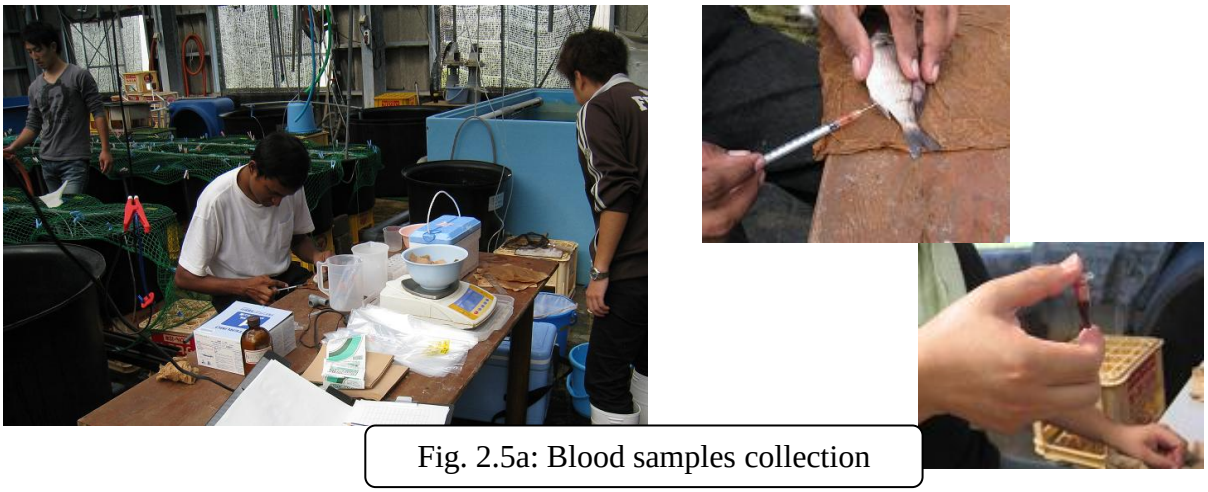

Fig. 2.5a: Blood samples collection

Fig. 2.5b: Organ samples collection

Fig. 2.5: Sample collection

Table 3.2.9: Nutrient retention (% of intake) in juvenile Japanese flounder after 8 weeks feeding trial

Nutrients	Test diets					*P value*
	FP0	FP12	FP24	FP36	FP48	
Protein	39.10 ± 1.36^{b}	38.42 ± 0.73^{b}	36.20 ± 0.24^{ab}	37.02 ± 0.83^{b}	32.13 ± 0.76^{a}	0.0031
Lipid	35.37 ± 0.99	31.95 ± 1.81	31.04 ± 1.61	34.87 ± 1.04	31.96 ± 1.25	0.3081

Values are means ± pooled S.E.M. from triplicate groups. Means in each row with different letters are significantly different ($P < 0.05$). Absence of letters indicates no significant difference between treatments.

Chapter IV

Table 4.8: Fatty acid composition (g $100g^{-1}$ dry diet) of the experimental diets.

Fatty acids	FM	SP	SPAA	SPFS	SPKM	SPSM	SPMX
Saturated	2.67	2.63	2.45	2.29	2.71	2.45	2.39
Monoenes	2.60	2.65	2.99	3.40	3.00	2.40	3.20
n-6 fatty acid	1.42	1.88	1.89	1.73	1.73	1.65	1.58
n-3 fatty acid	2.37	2.43	2.37	2.38	2.25	2.38	2.29
PUFA[1]	3.79	4.31	4.25	4.10	3.98	4.03	3.87
n-3 HUFA[2]	2.08	2.09	1.95	1.98	1.88	2.09	1.94
EPA+DHA[3]	1.92	1.94	1.86	1.84	1.77	1.95	1.81
n-3/n6 ratio	1.66	1.29	1.25	1.38	1.30	1.44	1.45

[1]PUFA, polyunsaturated fatty acids is expressed as sum of total n-3 and n-6 fatty acids.

[2]HUFA, highly unsaturated fatty acids is expressed as sum of n-3 fatty acids in carbons more than 20.

[3]Sum of eicosapentaenoic acid (C20: 5n-3) and docosahexanoic acid (C22: 6n-3).

Chapter V: Experiment I

Table 5.1.6: Apparent digestibility coefficients (ADC) for dry matter, crude protein and lipid of the test diets fed to juvenile red sea bream for 56 days[1].

Parameters	Diet Group				
	FM 100	FM 40	FM 20	FM 0	COM
Dry matter	86.3 ± 0.8	85.6 ± 4.3	88.8 ± 4.2	84.8 ± 4.0	82.8 ± 3.8
Crude protein	83.7 ± 2.6^{b}	79.5 ± 0.9^{ab}	78.6 ± 1.7^{ab}	74.6 ± 3.9^{a}	82.3 ± 0.2^{ab}
Lipid	71.2 ± 0.7ab	67.7 ± 0.3ab	72.9 ± 1.3b	70.3 ± 2.9ab	63.4 ± 0.2a

[1]Values are means±SE of triplicate groups. Within a row, means with the same letters are not significantly different ($P > 0.05$). Absence of letters indicates no significant difference between treatments.