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FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Nutritional and Immunological Studies on Fermented Soybean Products in Diets for Marine Fishes

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GRADUATE SCHOOL

CHEJU NATIONAL UNIVERSITY

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CONTENTS

요 약 문

최근 양식산업에 있어서 양식어류의 건강과 질병에 대한 면역력을 증진시키기 위한 많은 연구가 수행되고 있다. 따라서 이 연구는 발효시킨 콩, 대두박 그리고 면실박의 사료 내 첨가가 돌돔 (*Oplegnathus fasciatus*)과 넙치 (*Paralichthys olivaceus*)의 성장과 선척적 면역반응에 미치는 영향에 대해 알아보기 위해 수행되었다.

이 연구에서는 청국장 (Chapter I), 메주 (Chapter II), 발효 대두박 (Chapter III), 그리고 발효 대두박-면실박 혼합물 (Chapter IV)을 각각 이용하여 총 4 번의 사양실험이 수행되었다. 돌돔과 넙치 사료 내 각각 청국장과 메주를 첨가하여 어류의 성장과 인 이용률 그리고 면역에 미치는 영향을 알아보고자 수행한 연구결과 (Part □,□)에서는 어류사료 내 25% 청국장 (돌돔)과 6% 메주 (넙치) 첨가가 간 내 SOD 활성(superoxide dismutase activity)과 인 이용률에서 대조구에 비해 유의적으로 높은 값을 나타내었으며, 성장과 생존율에 아무런 영향을 미치지 않는 것으로 조사되었다. 이러한 어체 내 항산화 활성과 인 이용률의 증가는 청국장과 메주에 존재하는 micro-flora 와 몇몇 효소들에 의한 것으로 사료된다.

사양실험 □과 □를 바탕으로 한 3 번째 사양실험(Chapter III)에서는 4 가지의 유용균주 *Aspergillus oryzae* (AO), *Sacchromyces cerevisiae* (SC), *Pediococcus pentosaceus* (PP) 및 *Bacillus subtilis* (BS)를 이용하여 발효시킨 각각의 대두박을 돌돔사료 내 첨가하여 어류의 면역력에 미치는 영향을 조사하였다. 사양실험 결과, AO-발효대두박을 섭취한 어류는 다른

유용균주로 발효시킨 대두박을 섭취한 어류에 비해 간 내 높은 catalase (CAT)와 antioxidant (DPPH) 활성을 나타내었다. 이러한 연구결과는 *Aspergillus oryzae* 를 이용한 대두박의 발효는 돌돔에 있어 높은 면역능력을 야기시킬 수 있을 것으로 판단된다. 발효대두박을 섭취한 어류에 있어서의 낮은 성장률은 몇몇 필수 아미노산의 결핍과 발효 과정에서 생산되는 항영양소적 요소에 의한 것으로 사료된다.

4 번째 사양실험(Chapter IV)은 돌돔을 대상으로 하여 AO 를 이용하여 발효시킨 대두박-면실박 혼합물(F-CS)을 사료 내 첨가하여 어류의 성장과 혈액학적 요소에 미치는 영향에 대해 알아보고자 수행되었다. 어류의 혈장 내 콜레스톨과 중성지방함량에 있어서 F-CS 사료를 섭취한 어류는 어분을 기초로 한 사료와 일반 대두박-면실박 혼합물(CS) 사료를 섭취한 어류에 비해 유의적으로 낮은 함량을 나타내었다. 그러나 F-CS 와 CS40 사료를 섭취한 어류에 있어서의 성장률은 어분을 기초로 한 사료를 섭취한 어류에 비해 낮은 것으로 나타났다. 이러한 성장결과는 과도한 식물성 단백질원의 사료 내 첨가(40%)와 몇몇 필수 아미노산의 결핍 및 발효 과정에서 생산되는 항영양소적 요소에 의한 것으로 사료된다.

결론적으로, 사료 내 25% 청국장 또는 6% 메주의 첨가는 돌돔과 넙치에 있어 비특이적 면역반응과 인 이용률을 증가시킬 수 있을 것으로 판단된다. *Aspergillus oryzae* 로 발효시킨 대두박은 다른 유용균주를 이용하여 발효시킨 대두박에 비해 돌돔에 있어서 비특이적 면역반응을 효과적으로 증가시킬 수 있을 것으로 여겨진다. 현재의 연구 결과를

바탕으로 하였을 때, 양식산업에 있어서 발효시킨 대두박은 어류의 면역 증강제로서 양어사료에 효과적으로 사용될 수 있을 것이며, 향후 연구에서는 발효 부산물의 양어사료 내 이용을 위한 최적의 발효 공정과 발효 과정 중 발생하는 아미노산의 손실과 낮은 단백질 용해로 인한 낮은 성장에 대한 연구가 수행되어야 할 것이라고 판단된다.

SUMMARY

Many studies recently have been being conducted to minimize the feed costs and to enhance immune responses and diseases resistances of farmed fish. In line of the current direction, this study was conducted to investigate the effects of fermented soybean and cottonseed meal with different bacteria and fungi on growth performance and non-specific immune response of marine fish species, including parrot fish, *Oplegnathus fasciatus* and olive flounder, *Paralichthys olivaceus*.

To pursue the purposes of the study, four feeding experiments were conducted with different Korean traditional fermented soybean products including Cheongkukjang (CKJ-Chapter I), Meju (MEJU-Chapter II) and fermented soybean and cottonseed meal with MEJU microorganisms (Chapter III and IV).

In the first two feeding experiments, effects of dietary CKJ and MEJU on growth performance, phosphorus utilization and immune response in juveniles of parrot fish and olive flounder were examined. Higher liver superoxide dismutase (SOD) activity and phosphorus retention were observed in both parrot fish and olive flounder fed the diets contained either 25% CKJ or 6% MEJU, compared to those of fish fed the fish meal based diets. The diets supplemented with 25% CKJ or 6% MEJU did not affect the growth performance and survival of the fishes. The findings from Chapter I and II suggested that increase of liver SOD activity and phosphorus retention might be contributed by presence of some enzymes and higher antioxidant activities produced by micro-flora in both CKJ and MEJU.

Based on the findings in the previous two feeding experiments, a six week feeding experiment was conducted to investigate the effects of fermented soybean

meal by four probiotics including *Aspergillus oryzae* (SBM-AO), *Sacchromyces cerevisiae* (SBM-SC), *Pediococcus pentosaceus* (SBM-PP), and *Bacillus subtilis* (SBM-BS) on immune response of juvenile parrot fish. At the end of the feeding trial, fish fed the diet contained SBM-AO produced higher liver catalase (CAT) and antioxidant activities than did the fish fed the SBM diet. The findings suggest that *Aspergillus oryzae* was superior to the other examined probiotics. Fermented SBM with *A. oryzae* might enhance immune response of parrot fish. *A. oryzae* could be a suitable fermentation microorganism for other plant protein sources such as cottonseed meal. The lower growth performance in the fish fed the fermented soybean meal diets might be associate with deficiency of some essential amino acids and antinutitional factors produced in fermentation process.

Finally, a six week feeding experiment was conducted to investigate the effects of fermented cottonseed and soybean meal (CS) with *A. oryzae* on growth performance, hematological parameters of parrot fish. Fermentation process produced higher antioxidant activities in CS. Lower plasma cholesterol and trygliceride levels were found in fish fed the diets containing fermented cottonseed and soybean meal (F-CS), compared to fish fed the fish meal and CS containing diets. However, fish fed F-CS and CS40 diets grew slower compared to fish fed the control and the other diets. The findings indicate that CS could replace up to 30% dietary fish meal for parrot fish without adverse effects on growth performance. Deficiency of some essential amino acids and antinutitional factors produced in fermentation process might have affected growth and utilization of nutrients in the fish.

In conclusion, dietary inclusion level of 25% CKJ or 6% MEJU could enhance innate immune response and increase phosphorus utilization of parrot fish and olive flounder. Fermented soybean meal with *Aspergillus oryzae* could increase immune response in juvenile parrot fish. For effective use of fermented cottonseed and soybean meal as immune-stimulants in aquaculture, further studies are necessary to optimize the fermentation condition and to address the growth impairment due to the loss of amino acids and lower protein solubility during fermentation process.

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REFERENCE REVIEW

Aquaculture is one of the most rapidly extended food animal producing industries. The extension of aquaculture industry has attempted to fill the gap between supply and demand of fish. Delgado et al. (2003) estimated that the global consumption of fish has doubled from 1973 and continued to increase due to the world population growth. Currently, aquaculture production accounted for 30 percent of total food fish production. And incoming decades, aquaculture will likely become the greatest of source of increased fish production due to the extension of water areas for cultivation and the increase of productivity. However, aquaculture has been reported to be constrained by some major challenges including disease infections and the shortage of fish meal and fish oil. It was estimated that nearly one-third of the fisheries productions are produced for fish meal and fish oil which are used in feeds for livestock and aquaculture feed industries. The stagnated fisheries and the faster extension of aquaculture could lead to a significant soar of price of fish meal and fish oil. On the other hand, outbreak of some diseases has been reported to lead to mass mortality in some intensive aquaculture systems, resulted in loss of millions of dollars and cut-down thousands of jobs. For instance, the infection of a virus in Chile salmon farms currently has cut down thousand of jobs in salmon aquaculture and one-quarter of the operation was closed (New York Times, March 27th, 2008). Use of antibiotics, and drugs for preventions and treatments of diseases in aquaculture currently can lead to pollution of surround environments. Moreover, the residues of antibiotics and chemicals in aquaculture products can cause serious health problems of consumers. Recently, many interests have been being paid on some natural

compounds which are potential to replace for antibiotics and chemicals in aquaculture. Use of natural bioactive compounds or probiotics to enhance non-specific immune responses and disease resistances of farmed fish has been promoted environment friendly alternative solutions.

To reduce production costs, many studies have been conducted to replace expensive fish meal by plant-original protein sources, such as soybean, cotton seed and rapeseed meal. Hardy (1995) reported that dietary replacement of fish meal protein by plant origin by-products, such as soybean meal, cottonseed meal and rapeseed meal, has been increasing in aquaculture industry, due to their low price, highly market availabilities and sufficient protein contents. Among plant protein sources, soybean meal has been used predominantly in diets for many fish species because of its high protein content and well balanced amino acid profiles (Degani et al., 1997; Refstie et al., 2000; Catacutan and Pagador, 2004; Chou et al., 2004; Zhou et al., 2005; Tomas et al., 2005; Pham et al., 2007). Studies have demonstrated that soybean meal alone or in combination with other protein sources can replace from 20% up to 90% fish meal protein in diets for some fish species such as yellowtail (Shimeno et al., 1993), red drum (McMoogan and Gatlin III, 1997), Asian seabass (Boonyaratpalin et al., 1998; Tantikitti et al., 2005), European seabass (Kaushik et al., 2004; Tibaldi et al., 2006), rainbow trout (Gomes et al., 1995), Australian snapper (Quantararo et al., 1998), cobia (Chou et al., 2004; Zhou et al., 2005; Romarheim et al., 2008), Korean rockfish (Lim et al., 2004) and olive flounder (Kikuchi et al., 1994; Kikuchi, 1999; Saitoh et al., 2003; Pham et al., 2005, 2007). Shimeno et al. (1993) reported up to 20% of fish meal was able to be substituted by commercial defatted

soybean meal in the diets for fingerling yellowtail. For Asian seabass, Boonyaratpalin et al. (1998) conducted a ten-week feeding trial with different soybean products, including solvent extracted, extruded full-fat, steamed full-fat and soaked raw full-fat soybean meal. At the end of feeding trial, the authors found that fish fed the diets contained 37.5% solvent extracted soybean meal as fish meal alternative had similar growth and feed efficiency compared to the fish fed the fish meal-based diet. Latter on, Tantikitti at al. (2005) reported that defatted soybean meal can replace only 10% of fish meal protein in the diets for Asian seabass with good growth and feed utilization relative to the control diet. It is apparent that different fish species and fish at developmental stages response differently with diets containing soybean meal and soybean meal in combination with other protein sources. For juvenile olive flounder, Kikuchi (1999) reported that 45% fish meal protein can be replaced by soybean meal in combination with other animal protein sources. He suggested that the inclusion of soybean meal in diets for fish could be increased by the supplementation of some deficient components such as methionine, lysine, and phosphate. Meanwhile, dietary inclusion level of soybean meal for red drum was much higher compared to the other fish species. McMoogan and Gatlin III (1997) reported that soybean meal could be incorporated up to 95% with supplementation of 2% glycine in the diets for red drum. The inclusion of soybean meal remarkably reduced feeding costs in shrapsnout sea bream (Hernandez et al., 2007). However, use of soybean meal in fish feeds, particularly for marine carnivorous fish species is still limited because of the presence of some antinutritional factors (ANFs) such as protease inhibitors, phytates, lectins, saponins, non-starch polysaccharide, high fiber content (NRC, 1993; Storebakken et al., 2000;

Francis et al., 2001; Hendricks, 2002; Deng et al., 2006), and deficiency of essential amino acids, such as methionine in soybean meal (NRC, 1993). The ANFs also can induce severe enteritis in digestive tracts, reduce its absorbability of nutrients; consequently impair the growth of fish. Recently, many techniques have been being applied to eliminate the ANFs and increase the inclusion of soybean meal in aqua-feeds, including enzyme and heat treatment, and fermentation. Fermentation has been reported to be able to degrade ANFs and improve the nutritive quality of plant protein sources for animals including fish (Compbell-Platt, 1994; Matsui et al., 1996; Hirabayashi et al., 1998; Hong et al., 2004; Mukhopadhyay and Ray, 2005; Feng et al., 2007a, b; Zhang et al., 2007). Matsui et al. (1996) reported that *Aspergillus usami* fermentation could improve phosphorus availability of soybean meal in chicks and the supplementation of exogenous inorganic phosphorus was not necessary in the diets contained the fermented soybean meal. In the followed experiment, Hirabayashi et al. (1998) revealed the lower phosphorus excretion in chicks fed fermented soybean with *A. usami,* compared to the control diet. Mukhopadhyay and Ray (2005) recommended that fermentation of oilseed meals which result in the reduction of ANFs may be applied as an efficient tool in formulation of feeds for rohu fingerlings. On the other hand, fermentation could also increase the antioxidant capacity of plant derived protein sources (Yang et al., 2000; Feng et al., 2007a; Zhang et al., 2007; Choi et al., 2008; Kim et al., 2008; Lee et al., 2008; Radha et al., 2008; Wang et al., 2008), and enhance non-specific immune responses and diseases resistances of farmed animals including fish (Ashida et al., 2002; Ashida and Okimasu, 2005; Ashida et al., 2006; Feng et al., 2007a; Yamonto et al., 2007). Yang et al. (2000) concluded that fermented soybean was superior to soybean and might be able to be

applied in functional feed ingredients.

Therefore, the present study with four different feeding experiments was conducted to investigate the effects of Korean traditional fermented soybean products and its application in the diets containing soybean and cottonseed meal on growth performance, feed utilization, phosphorus retention, and non-specific immune response in two common marine cultured fish species in Korea, including olive flounder, *Paralichthys olivaceus* and parrot fish, *Oplegnathus fasciatus*.

CHAPTER I

DIETARY CHEONGKUKJANG INCREASED LIVER SUPEROXIDE DISMUTASE ACTIVITY IN PARROT FISH, *Oplegnathus fasciatus*

ABSTRACT

A four-week feeding trial was conducted to investigate the effects of dietary Cheongkukjang (CKJ) on non-specific immune response of parrot fish, *Oplegnathus fasciatus*. Three isonitrogenous (42 % crude protein) and isocaloric (17.1 MJ/kg) diets were formulated to replace fish meal by 0, 25% SBM or 25% CKJ (designated as FM, 25SBM and 25CKJ, respectively). Ninety fish (initial body weight of 122 g/fish) were randomly allotted into nine 150 L tanks. One of the three experimental diets was fed to triplicate groups of fish for 4 weeks. After the feeding trial, no differences were observed in growth performance and feed utilization among the fish groups. Liver superoxide dismutase activity of the fish fed CKJ containing diet was significantly higher than that of the control groups. DPPH radical scavenging and Fe^{2+}-chelating activities of the experimental diets containing SBM or CKJ were significantly higher than that of the control diet. The results of the present feeding trial suggest that dietary inclusion of 25% CKJ significantly increased liver superoxide dismutase activity and antioxidant activities in muscle and liver of parrot fish and did not affect the growth performance, feed utilization, morphological parameters, as well as hematological values in the fish. Fermentation process in CKJ might increase the availability of phosphorus in diets containing plant protein sources.

1.1. INTRODUCTION

Cheongkukjang (CKJ), a traditional Korean fermented soybean with rice straw, has been reported to have unique flavor, antimicrobial, anticarcinogenic, and antioxidant activities, and high nutritional compositions (Kim et al., 1998; Kim et al., 1999; Kim et al., 2004; Lee et al., 2005; Mine et al., 2005; Youn et al., 2001). CKJ is the most popular seasoning food in Korea. Kim et al. (1998) reported that CKJ is an important source of essential amino acids and fatty acids, supplementing with rice and barley protein. Conventionally, CKJ is made of fermented soybeans and salt with red pepper powder and garlic as additives. Lee et al. (2005) has discovered that CKJ produced using *Bacillus licheniformis* increases the antimutagenic properties and improves the sweet and savory taste by increasing glycine, glutamic acid, aspartic acid and arginine concentration.

Use of dietary fermented vegetable products, recently was reported to be able to enhance non-specific immune responses and disease resistances in fish (Ashida et al., 2002; Ashida and Okimasu, 2005; Ashida et al., 2006). And the use of natural fermented products has become more attractive since consumers are seriously paying concerns on quality and safety of aquaculture products. However, no information is available on the use of CKJ in diets for fish.

Parrot fish, *Oplegnathus fasciatus* is one of the most important aquaculture fish species in Korea. However, outbreak of diseases has been reported as a cause mass mortality in captive parrot fish and decelerated the aquaculture development of this species. Jung and Oh (2000) reported that high mortality of net-caged parrot fish were observed in southern coastal areas of Korea peninsula due to the outbreak of a

disease in all developmental stages of fish including 3-year-old marketable fish.

Therefore, the aim of the present feeding trial was to evaluate the effect of dietary CKJ on growth performance and immune response of parrot fish, *Oplegnathus fasciatus*.

1.2. MATERIALS AND METHODS

1.2.1. Experimental diets

Three experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (42 %) and gross energy (17.1 MJ/kg). The energy value of the experimental diets was estimated on the basis of mammalian physiological fuel value, i.e., 16.7 KJ/g protein or carbohydrate and 37.7 KJ/g lipid (Lee and Putman, 1973). The dietary formulation and proximate compositions are presented Table 1.1. For the control diet (FM), white fish meal was used as major dietary protein source. For diet 25SBM and 25CKJ, fish meal in the control diet was replaced by 25% soybean meal (SBM) and 25% Cheongkukjang (CKJ), respectively. In diets containing SBM and CKJ, methionine and lysine were supplemented to meet their requirement of fish. CKJ, made of traditional fermented soybean, was purchased from a local market. The proximate compositions of the experimental diets and major protein sources used in this study are given in Table 1.2, 1.3. All dry ingredients were thoroughly mixed with 30% distilled water. Pellets were extruded through a meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size and dried with a freeze drier (Operon FDT-8605, Korea) for 24 h. The pellets were crushed into desirable particle sizes (0.4 – 2.0 mm) and stored at –20^{o}C until use.

Table 1.1. Formulation of the experimental diets (% DM)

	Diets		
Ingredients	**FM**	**25SBM**	**25CKJ**
White fish meal	52.0	39.0	39.0
Soybean meal	0.0	20.0	0.0
Cheongkukjang[1]	0.0	0.0	21.8
Corn gluten meal	8.2	7.4	8.6
Starch	24.8	17.4	19.4
Yeast	2.0	2.0	2.0
Mineral premix[2]	1.0	1.0	1.0
Vitamin premix[3]	1.0	1.0	1.0
Squid liver oil	8.0	8.7	4.2
Lysine	0.0	0.4	0.4
Methionine	0.0	0.2	0.2
Monocalcium phosphate	0.0	0.6	0.6
Cellulose	3.0	2.3	1.8

[1] Cheongkukjang was purchased from a local market.

[2] Mineral premix (g/kg): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4.7H_2O$, 20.0; Ca-lactate, 356.5; $CuCl_2$, 0.2; $AlCl_3.6H_2O$, 0.15; $Na_2Se_2O_3$, 0.01; $MnSO_4.H_2O$, 2.0; $CoCl_2.6H_2O$, 1.0.

[3] Vitamin premix (g/kg): L-ascorbic acid, 121.2; DL-α tocopheryl acetate, 18.8; thiamin hydrochloride, 2.7; riboflavin, 9.1; pyridoxine hydrochloride, 1.8; niacin, 36.4; Ca-$_D$-pantothenate, 12.7; myo-inositol, 181.8; $_D$-biotin, 0.27; folic acid, 0.68; p-aminobezoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalficerol, 0.003; cyanocobalamin, 0.003.

Table 1.2. Proximate compositions of the experimental diets

Diets	**FM**	**25SBM**	**25CKJ**
Proximate composition			
Dry matter, %	96.3	96.9	96.2
Protein, % DM	42.1	43.6	42.9
Lipid, % DM	10.5	11.3	11.1
Ash, % DM	8.7	8.7	8.3
Gross energy, MJ/kg DM	17.1	17.1	17.1

DM: dry matter.

Table 1.3. Proximate compositions of ingredients in the experimental diets (% DM)

Ingredients	Moisture	Protein	Lipid	NFE[1]	Ash
White fish meal	8.72	68.33	8.56	9.04	14.07
Soybean meal	11.68	46.91	2.52	32.35	6.54
Corn gluten meal	9.50	61.70	1.03	26.59	1.18
Cheongkukjang	6.51	41.26	23.13	24.31	4.79

[1] Nitrogen Free Extracts = 100 - (%Moisture + %Protein + %Lipid + %Ash).

DM: dry matter.

1.2.2. Fish and feeding trial

Parrot fish were transported from a private hatchery in Jeju Island to Marine and Environmental Research Institute, Cheju National University. Fish were fed with a commercial diet for 4 weeks in a 1000 L tank. Ninety fish (initial body weight 122 g/fish) were randomly distributed into nine 150 L tanks (10 fish per tank) in a flow through system supplied with sand filtered seawater at a flow rate of 3 L/min. One of the three experimental diets was fed to three groups of fish at a feeding rate of 3.5% body weight per day, twice a day, 7 days a week, for 4 weeks. Growth of fish was measured at the end of feeding trial. Feeding was stopped 24 h prior to weighing.

1.2.3. Growth performance and feed utilization

At the beginning and the end of feeding trial, all fish were weighed and counted for weight gain, feed conversion ratio, protein efficiency ratio, specific growth rate and survival calculation. Three fish from each tank (9 fish per diet) were randomly sampled and stored at –20 ^{o}C for muscle proximate compositions analysis. Analysis of crude protein, moisture, and ash were performed using the standard procedures (AOAC 1995). Lipid was determined using Soxhlet System (SH6, Korea).

1.2.4. Morphological parameters

The total length, body weight, liver weight and gonad weight of 9 fish per diet (3 fish per tank) were individually measured. Condition factors (CF), hepato-somatic index (HSI) and gonad somatic index (GSI) were calculated.

1.2.5. Hematological parameters

At the end of feeding trial, 3 fish per tank (9 fish per diet) were anesthetized in tricaine methanesulfonate (MS-222) solution (100 ppm). Blood was taken from caudal veins using non-heparinsed syringes. Hematocrit was measured using microhematocrit technique (Micro-hematocrit VS-12000, Vision Scientific Co. Ltd., Korea). The remained blood samples were used for nitro blue tetrazolium (NBT) assay, serum cholesterol and triglyceride. Serum cholesterol and triglyceride were measured using a Photometer CH100 Plus (Calenzano, Firenze, Italy).

Superoxide anion radical production by neutrophils during respiratory burst was measured by the nitroblue tetrazolium (NBT) assay as a method described by Anderson and Siwicki (1995) with some modification (Kumari and Sahoo, 2005). Briefly, blood and 0.2% NBT solution were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 µL was taken out and dispensed in glass tubes. Then, 1 mL of dimethyl formamide (Sigma) was added and centrifuged at 2000 x g for 5 min. Finally, the optical density (OD) of supernatant was measured at 540 nm. Dimethyl formamide was used as the blank.

1.2.6. Ferric reducing activity of plasma (FRAP)

Antioxidant activity in plasma measured by FRAP assay with tripyridyltriazine (Benzie and Strain 1996), with a confirmation of Benzie et al. (1999). The blue colored Fe^{2}- tripyridyltriazine compound formed in reaction solution was monitored by a spectrophotometer at 593 nm (Genesys 10 UV, Rochester, NY, USA). Ferrous solution was used to make the standard curve.

1.2.7. Serum lysozyme activity

A turbidometric method described by Swain et al. (2007) was used to measure serum lysozyme activity in fish fed the experimental diets. *Micrococcus lysodeikticus* concentration of 0.2 mg/mL (in 0.02 M sodium citrate buffer, pH 5.5) was added to serum sample at 10:1 ratio. Absorbance was measured at 450 nm immediately after adding *M. lysodeikticus* suspension. Final absorbance was measured after incubating for 1 h at 25 ^{o}C. Lyophilized hen egg white lysozyme (HEWL) was used as standard. Serum lysozyme values are expressed as µg/mL equivalent of HEWL activity.

1.2.8. Antioxidant activity

Antioxidant activity of the experimental diets and fish liver was measured using 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay described by Brand-William et al. (1995) with some modifications. Two g of diets (3 replicates per diet) were homogenized in 20 mL aqueous methanol (80%) and kept at room temperature for 10 min. The homogenates were centrifuged at 5000 rpm, 4 ^{o}C for 10 min and filtered through 0.45 µm syringe filters (Whatman Inc., Clifton, NJ) prior to the assay. Whole liver of 3 bled fish per tank (9 fish per treatment) were homogenized in the aqueous methanol (80%) at a ratio of 1:4 for 1 min using a homogenizer (X-120, Germany). The homogenate was centrifuged at 5000 rpm, 4 ^{o}C for 10 min. The supernatant was filtered through a 0.45 µm syringe filter. One hundred µL of filtered extract was pipetted into a 1.5 mL cuvette, then 900 µL of DPPH methanolic solution (100 µM) was added to obtain a final volume of 1 mL. The absorbance of the mixture was measured at 517 nm with 1 min intervals for 10

min using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The antioxidant activity of the extracts against the DPPH radicals was calculated as follow: Percent inhibition = $[(A_0 - A_s)/A_0] \times 100$, where A_0 and A_s are the absorbance of sample at 0 and s min, respectively.

1.2.9. Measurement of total polyphenol compounds

Total polyphenol compounds in the experimental diets were measured using a colorimetric method described by Skerget et al. (2005). Briefly, 1 g of diets was extracted with 250 mL methanol for 2 h at 40 ^{o}C. The solution was cooled and filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ). To 0.5 mL filtered extract, 2.5 mL of Folin-Ciocalteu reagent (0.2 N, Sigma) was added and kept for 5 min at room temperature, then 2 mL of Na_2CO_3 solution (75g/L) was added. The mixture was incubated for 5 min at 50 ^{o}C and cooled. The absorbance was measured at 760 nm using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The results were expressed in gram of gallic acid per kilogram of dry diet.

1.2.10. Measurement of total flavonoids

Total flavonoids content in the experimental diets was measured using colorimetric method (Moreno et al., 2000). One gram of the experimental diet or 0.5 g of liver was extracted in 10 mL of aqueous ethanol (80%) using a shaker (Wise Cube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 24 h. The solution was centrifuged at 27000 rpm for 20 min. The supernatant was used for flavonoids assay. Total flavonoids content was expressed in mg of quercetin per g dry diet.

1.2.11. Measurement of reducing activity

Dietary sample (2 g) was finely ground and extracted in 20 mL aqueous methanol (80%) for 12 h with three replicates. The extract was filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ) prior to assay.

Reducing activity of the experimental diets was measured using a method described by Oyaizu (1986). Filtered extract (0.3 mL) was mixed with 0.3 mL of 1.0% potassium ferricyanide and 0.3 mL sodium phosphate buffer (0.2 M, pH 6.6). The mixture was incubated at 50 ^{o}C for 20 min. After cooling, 10% trichloroacetic acid (0.3 mL) was added and centrifuged at 6000 rpm for 10 min at 4 ^{o}C. The supernatant (0.6 mL) was mixed with 0.1% ferric chloride solution (0.12 mL) and deionized water (0.6 mL) and incubated at room temperature for 10 min. The absorbance was measured at 700 nm using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA).

1.2.12. Ferrous chelating assay

Fe^{2+}-chelating activity of the experimental diets was measured according to Decker and Welch (1990). The reaction mixture containing dietary extract (1.0 mL), methanol (3.7 mL), 2 mM $FeCl_2$ (0.1 mL), and 5 mM ferrozine (0.2 mL) was incubated at room temperature for 10 min. The absorbance of mixture was measured at 562 nm. The chelating effect of dietary extract was calculated as follows: Chelating effect (%) = 100 x (1- absorbance sample/absorbance control).

1.2.13. Superoxide anion radical scavenging assay

Superoxide anion radical scavenging activity of the experimental diets was evaluated using a method described by Nagai et al. (2001). The system contained 1.2 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.1 mL of 3 mM xanthine, 0.1 mL of 3 mM ethylenediamine tetraacetic acid disodium salt (EDTA), 0.1 mL of 0.15% bovine serum albumin, 0.1 mL of 0.75 mM NBT, and 0.1 mL dietary extract. After incubation at 25 ^{o}C for 10 min, the reaction was initiated by adding 0.1 mL of xanthine oxidase (6 mU) and kept at 25 ^{o}C for 20 min. The reaction was stopped by adding 0.1 mL of 6 mM CuCl. The absorbance of the mixture was read at 560 nm.

1.2.14. Measurement of liver thiobarbituric acid reactive substances (TBARS)

Liver TBARS in fish fed the experimental diets was measured using a method of Burk et al. (1980) and modified by Tocher et al. (2002). Liver (30 mg) was homogenized in 1.5 mL of 20% (w/v) trichloroacetic acid containing 0.05 mL of 1% butylated hydrotoluene in ethanol. To the homogenate, 2.95 mL of freshly prepared 10 mM thiobarbituric acid was added. The mixture was vortexed in a glass tube and heated at 100 ^{o}C for 10 min. Protein was removed by centrifugation of 12000 x g. Absorbance of supernatant was measured at 532 nm. The concentration of TBARS, expressed as μM TBARS/g liver, was calculated using the extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$.

1.2.15. Measurement of liver superoxide dismutase activity

Fish liver was homogenized in 9 volumes of 20 mM phosphate buffer pH 7.4,

1mM EDTA and 0.1% Triton X-100. The homogenate was centrifuged at 10000 rpm (4 ^{o}C, 10 min) to remove debris. The supernatants were used for superoxide dismutase assay as method of Ukeda et al. (1999). Into 2.4 mL of a 50 mM sodium carbonate buffer (pH 10.2), 0.1 mL of 3 mM xanthine, 3mM EDTA, 0.75 mM NBT, 15% bovine serum albumin and 0.1 mL supernatant was added. The reaction was initiated by adding 0.1 mL of 100 mU/mL xanthine oxidase. The absorbance of mixture was measured at 560 nm after incubation at 25 ^{o}C for 25 min.

1.2.16. Measurement of phosphorus

The experimental diets and fish tissues were digested with 10 mL of a mixture of concentrated H_2SO_4 and HNO_3 (1:1; v: v) in Kjeldahl flasks. The digested mixture was volume up to 100 mL with distilled water and used for total phosphorus measurement. Inorganic phosphorus of the experimental diets was extracted with 12.3% trichloroacetic acid solution using a shaker (Wise Cube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 12 h at room temperature. After centrifugation at 2000 x g for 20 min, the supernatant was used for measurement of inorganic phosphorus. Phosphorus concentration was measured using spectrophotometrically method described by Nahapetian and Bassiri (1975).

1.2.17. Measurement of vitamin C

Concentrations of total ascorbic acid (AA) and dehydroascorbic acid (DAA) in diets, liver, muscle and plasma were measured using the 2, 4-dinitrophenylhydrazine colorimetric method (Dabrowski and Hinterleitner, 1989). Samples of the

experimental diets, liver and muscle were homogenized in 5% trichloroacetic acid solution containing 250 mM $HClO_4$ and 0.08% ethylenediaminetetraacetic acid using homogenizer (X-120, Germany). The homogenates were centrifuged at 15000 x g for 30 min at 4 ^{o}C. Plasma extracts were prepared as the same manner with 10% of TCA solution at a ratio of 1:1 (plasma: extract solution).

1.2.18. Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple test. Data presented are means ± SD. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significantly at $P < 0.05$.

1.3. RESULTS

Growth performance, feed utilization and survival of fish fed the experimental diets are presented in Table 1.4. After 4 weeks of feeding trial, no significant differences were observed in final body weight, weight gain, specific growth rate, protein efficiency ratio, feed conversion ratio and feed intake among fish groups fed all the experimental diets. No mortality occurred during feeding trial. The growth performance was not affected by the supplementation of SBM and CKJ in the present feeding trial, although the period of feeding was short. Muscle proximate compositions did not differ among fish groups fed the experimental diets, including the control diet (Table 1.5).

Condition factor, visceral somatic index, hepato somatic index and gonad somatic index were not different in all fish groups fed the experimental diets (Table 1.6). Hematocrit (%), blood respiratory burst (NBT assay) activity (OD, 540 nm), serum cholesterol, serum triglyceride, and liver DPPH radical scavenging activity were not influenced by the treatments (Table 1.7). Dietary Fe^{2+}-chelating and superoxide radical scavenging activities were increased by the supplementation of SBM and CKJ (Table 1.8). The reducing activity of the diets was only increased by SBM supplementation. Interestingly, liver superoxide dismutase activity was significantly increased by dietary CKJ after four weeks of feeding trial (Fig. 1.1). The liver of fish fed the diets containing SBM or CKJ also showed a decreasing trend in lipid peroxidation, even though it was not significant (Fig. 1.2).

There were no significant differences in dietary total phosphorus concentration, but diet 25CKJ had higher concentration of inorganic phosphorus (Table 1.9). Higher

serum phosphorus concentration was obtained in fish fed the diet 25CKJ and lightly related to the dietary inorganic concentration (Fig. 1.3). Serum protein did not differ among fish groups fed the all experimental diets. Bone and muscle phosphorus contents were not altered by the dietary phosphorus concentration (Table 1.10).

Dietary total ascorbic acid (AA), dehydroascorbic acid (DAA) and ascorbic phosphate (AAP) concentrations are showed in Table 1.11. The diets contained CKJ and SBM had higher concentration of total AA and DAA compared to the control diet. The 25CKJ diet had higher ascorbic phosphate concentration than the control and SBM diets. Concentrations of AA, DAA and AAP in muscle and liver of fish fed the experimental diets are given in Table 1.12. Liver AA concentration showed a positively relationship with the dietary concentrations of polyphenol compounds, flavonoids and AA (Fig. 1.4, 1.5, 1.6). No differences were found in AA, DAA and AAP concentration in the fish tissues of fish fed the experimental diets. Concentrations of AA, DAA and AAP in plasma did not differ among the fish groups fed the experimental diets (Table 1.13).

Concentration of polyphenol compounds and flavonoids in muscle and liver of fish fed the experimental diets are given in Table 1.14. Polyphenol and flavonoids concentrations in both muscle and liver of fish fed the 25CKJ and 25SBM diets were significantly higher than those of fish fed the control diet. Higher DPPH radical scavenging activity in muscle and liver was also observed in fish fed the 25CKJ and 25SBM diets. Muscle DPPH radical scavenging activity positively associated to the muscle polyphenol concentration (Fig. 1.7, 1.8).

Table 1.4. Growth performance, feed utilization and survival of parrot fish fed the experimental diets for 4 weeks*

Diets	FM	25SBM	25CKJ
Initial body weight, g	122.4 ± 0.5	123.0 ± 0.6	122.5 ± 0.9
Final body weight, g	141.0 ± 0.2	143.8 ± 1.2	142.0 ± 1.3
Weight gain (WG)[1]	15.2 ± 0.6	17.0 ± 1.3	15.9 ± 0.6
Specific growth rate (SGR)[2]	0.51 ± 0.0	0.56 ± 0.0	0.53 ± 0.0
Protein efficiency ratio (PER)[3]	1.17 ± 0.04	1.26 ± 0.09	1.20 ± 0.04
Feed conversion ratio (FCR)[4]	2.03 ± 0.07	1.89 ± 0.09	1.95 ± 0.07
Feed intake (g/g BW)[5]	0.27 ± 0.0	0.26 ± 0.0	0.27 ± 0.0
Survival (%)	100	100	100

*Values presented are means ± SD. Value in the same row having different superscripts is significantly different ($P < 0.05$).

[1] WG (%) = 100 x (FBW - IBW)/IBW.

[2] SGR (%) = [(ln FBW - ln IBW)/days] x 100.

[3] PER = wet weight gain/ total protein given.

[4] FCR = dry feed fed/wet weight gain.

[5] FI (g/g BW) = dry feed consumed/BW.

BW: body weight.

IBW: initial mean body weight.

FBW: final mean body weight.

Table 1.5. Muscle proximate compositions of parrot fish fed the experimental diets for 4 weeks*

Diets	**FM**	**25SBM**	**25CKJ**
Moisture content, %	73.8 ± 0.2	74.7 ± 0.0	73.8 ± 0.0
Protein	20.5 ± 0.7	19.6 ± 1.2	20.6 ± 0.4
Lipid, % DM	4.4 ± 0.2	4.5 ± 0.4	4.3 ± 0.1
Ash, % DM	1.3 ± 0.0	1.2 ± 0.1	1.3 ± 0.2

* Values presented are means ± SD of triplicates. Value in the same row having different superscripts is significantly different ($P < 0.05$).

DM: dry matter.

Table 1.6. Condition factor and viscera, gonad and hepato somatic index of parrot fish fed the experimental diets for 4 weeks*

Diets	**FM**	**25SBM**	**25CKJ**
Condition factor (%)	2.6 ± 0.0	2.6 ± 0.2	2.6 ± 0.0
Viscera somatic index (%)	13.4 ± 0.8	13.2 ± 1.3	12.0 ± 0.8
Gonad somatic index (%)	0.08 ± 0.01	0.08 ± 0.02	0.09 ± 0.01
Hepatic somatic index (%)	3.4 ± 0.2	3.3 ± 0.4	3.2 ± 0.9

* Values presented are means ± SD of triplicates. Value in the same row having different superscripts is significantly different ($P < 0.05$).

Table 1.7. Hematological parameters and liver DPPH radical scavenging activity in parrot fish fed the experimental diets for 4 weeks*

Diets	**FM**	**25SBM**	**25CKJ**
Hematocrit (%)	39.9 ± 2.4	38.9 ± 4.8	40.5 ± 1.9
Blood NBT ($OD_{540\ nm}$)	1.01 ± 0.11	1.14 ± 0.11	1.02 ± 0.05
Serum lysozyme (μg/mL)	1.87 ± 0.21	1.46 ± 1.14	2.00 ± 0.14
Serum cholesterol (mg/dL)	185 ± 27	150 ± 13	179 ± 4
Serum triglyceride (mg/dL)	454 ± 120	508 ± 208	622 ± 250
Liver DPPHRS (%)	10.7 ± 1.7	11.5 ± 0.61	10.6 ± 1.9

*Values presented are means ± SD of triplicates. Value in the same row having different superscripts is significantly different ($P < 0.05$).

OD: optical density.

DPPHRS: DPPH radical scavenging activity (% inhibition).

Table 1.8. Concentration of polyphenol compounds, reducing, Fe^{2+} chelating and superoxide radical scavenging (SRS) activities in the experimental diets*

Diets	**FM**	**25SBM**	**25CKJ**
Polyphenol (g/kg)	0.018 ± 0.006	0.018 ± 0.002	0.025 ± 0.014
Fe^{2+} chelating activity (%)	6.77 ± 0.38^{b}	9.42 ± 0.32^{a}	8.47 ± 0.63^{b}
Reducing activity (OD_{700nm})	1.014 ± 0.02^{b}	1.071 ± 0.01^{a}	1.014 ± 0.01^{b}
SRS ($OD_{560\ nm}$)	0.042 ± 0.002^{a}	0.052 ± 0.002^{b}	0.055 ± 0.003^{b}

*Values presented are as means ± SD of triplicates. Value in the same row having different superscripts is significantly different ($P < 0.05$).

OD: optical density.

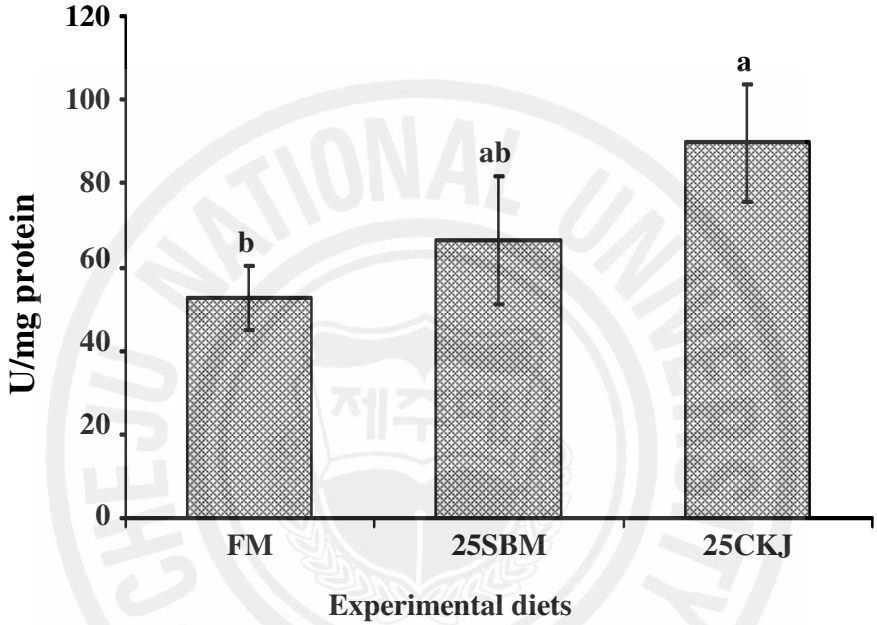

Figure 1.1. Superoxide dismutase activity in liver of parrot fish fed the experimental diets for 4 weeks. Bars having different letters are significantly different ($P<0.05$).

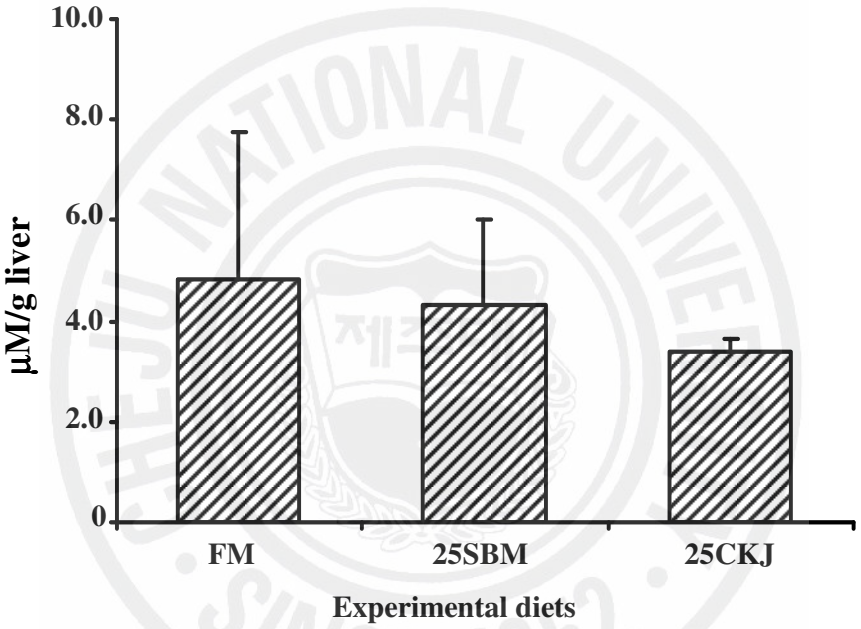

Figure 1.2. Concentration of thiobarbituric acid reactive substances in liver of parrot fish fed the experimental diets for 4 weeks

Table1.9. Concentrations of different phosphorus fractions in the experimental diets*

Diets	Total phosphorus (mg/g)	Inorganic phosphorus (mg/g)	Acid extractable phosphorus (mg/g)
FM	4.85 ± 0.02	2.08 ± 0.01[b]	2.54 ± 0.03[a]
25SBM	3.83 ± 0.04	2.05 ± 0.02[c]	1.60 ± 0.02[c]
25CKJ	3.77 ± 0.03	2.12 ± 0.01[a]	1.75 ± 0.01[b]

*Values presented are means ± SD of triplicates. Value in the same column having different superscripts is significantly different ($P < 0.05$).

Table 1.10. Phosphorus concentrations in serum, muscle and bone of parrot fish fed the experimental diets for 4 weeks*

Diets	Serum protein (g/dL)	Serum phosphorus (mg/dL)	Muscle phosphorus (mg/g)	Bone phosphorus (mg/g)
FM	4.51 ± 0.39	9.29 ± 0.97[b]	0.49 ± 0.05	13.64 ± 2.51
25SBM	4.01 ± 0.48	10.27 ± 0.23[b]	0.45 ± 0.04	13.81 ± 1.08
25CKJ	4.29 ± 0.35	12.64 ± 1.65[a]	0.42 ± 0.03	13.62 ± 1.36

*Values presented are means ± SD of triplicates. Value in the same column having different superscripts is significantly different ($P < 0.05$).

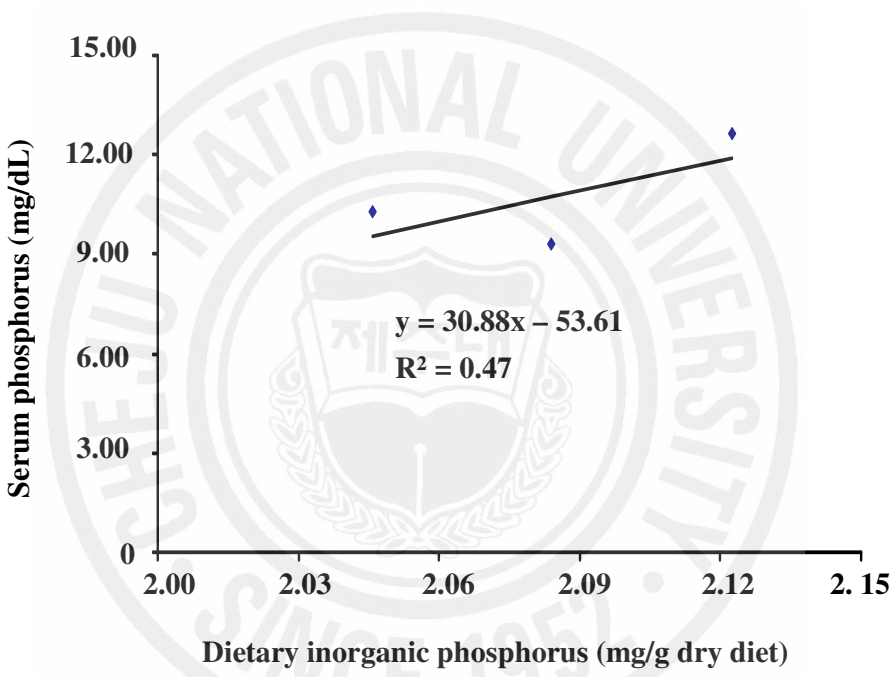

Figure 1.3. Relationship between serum phosphorus and dietary inorganic phosphorus concentrations in parrot fish fed the experimental diets for 4 weeks

Table 1.11. Dietary concentrations of ascorbic acid (AA), dehydro-ascorbic acid (DAA) and ascorbic phosphate (AAP) in the experimental diets*

Diets	AA (ng/g diet)	DAA (ng/g diet)	AAP (ng/g diet)
FM	87.8 ± 4.4^{c}	94.3 ± 5.7^{c}	475.8 ± 13.7^{b}
25SBM	127.6 ± 6.1^{a}	126.6 ± 6.7^{a}	482.4 ± 30.4^{b}
25CKJ	107.5 ± 0.8^{b}	113.2 ± 4.8^{b}	541.2 ± 17.0^{a}

*Values presented are means ± SD of triplicates. Value in the same column having different letters is significantly different ($P < 0.05$).

Table 1.12. Concentrations of ascorbic acid (AA), dehydro-ascorbic acid (DAA) and ascorbic phosphate (AAP) in muscle and liver of parrot fish fed the experimental diets for 4 weeks*

Diets	AA (ng/g tissue)	DAA (ng/g tissue)	AAP (ng/g tissue)
Muscle			
FM	61.9 ± 2.02	36.9 ± 4.65	108.6 ± 7.3
25SBM	56.5 ± 2.42	33.8 ± 3.68	106.6 ± 16.8
25CKJ	59.9 ± 2.77	35.2 ± 5.21	115.9 ± 15.7
Liver			
FM	85.1 ± 6.7^{a}	54.2 ± 2.3	203.6 ± 20.7^{b}
25SBM	109.5 ± 14.6^{b}	66.9 ± 9.9	251.7 ± 26.9^{a}
25CKJ	94.6 ± 8.3^{a}	59.7 ± 9.5	229.9 ± 10.9^{a}

*Values presented are means ± SD of triplicates. Value in the same column having different letters is significantly different ($P < 0.05$).

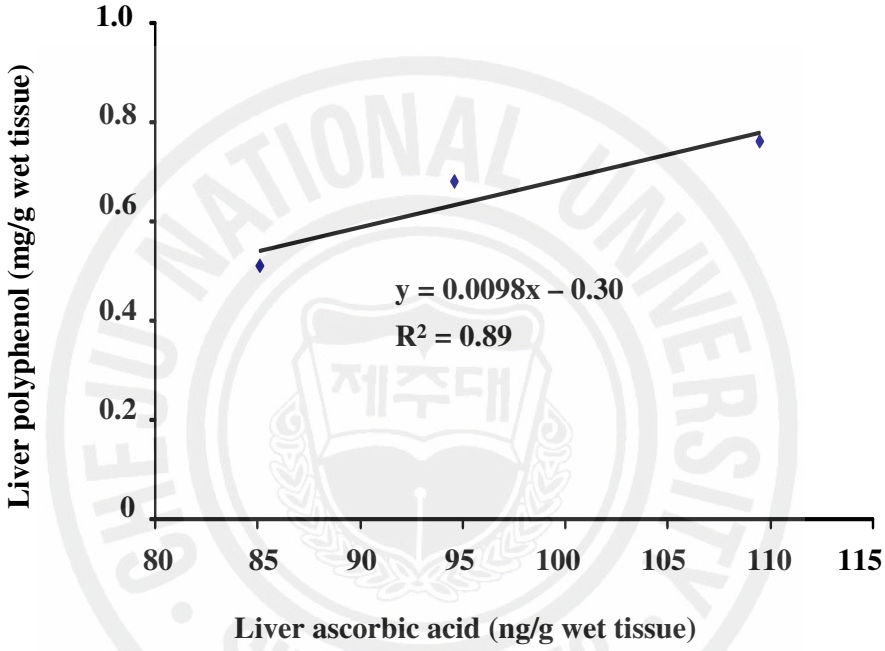

Figure 1.4. Relationship between liver AA concentration and concentration of liver polyphenol compounds in parrot fish fed the experimental diets for 4 weeks

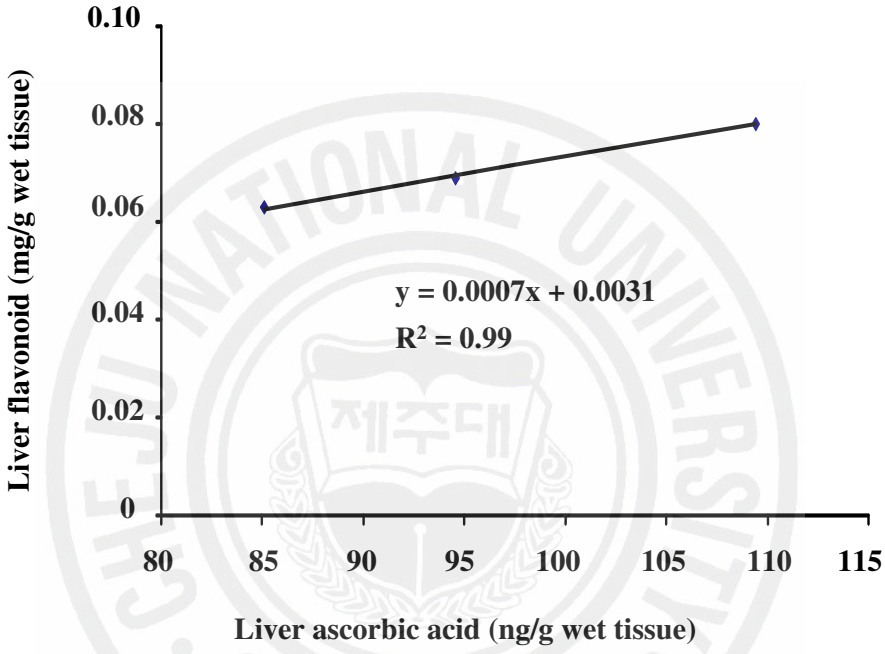

Figure 1.5. Relationship between liver total AA concentration and liver flavonoids concentration of parrot fish fed the experimental diets for 4 weeks

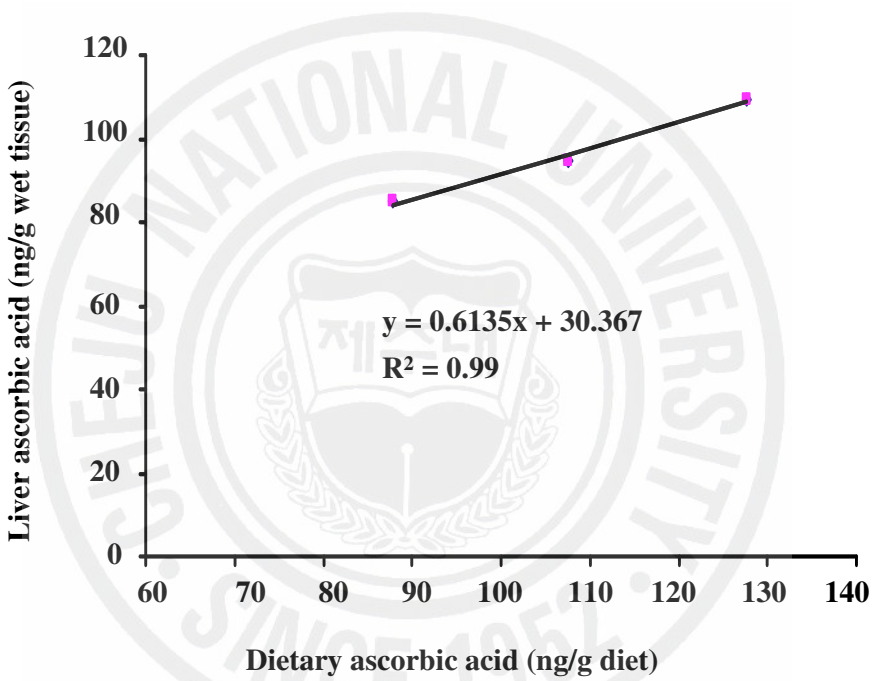

Figure 1.6. Relationship between dietary total AA concentration and liver total AA concentration of parrot fish fed the experimental diets for 4 weeks

Table 1.13. Concentrations of ascorbic acid (AA), dehydro-ascorbic acid (DAA), ascorbic phosphate (AAP) and ferric reducing activity of plasma (FRAP) of parrot fish fed the experimental diets for 4 weeks*

Diets	AA (ng/mL)	DAA (ng/mL)	AAP (ng/mL)	FRAP (μM/L)
FM	172.8 ± 6.0	146.1 ± 5.0	653.3 ± 33.5	188.0 ± 37.0
25SBM	164.9 ± 4.2	136.6 ± 4.8	627.0 ± 23.5	207.0 ± 27.7
25CKJ	175.2 ± 5.2	142.2 ± 5.9	654.5 ± 7.8	228.5 ± 59.8

*Values presented are as means ± SD of triplicates. Value in the same column having different letters is significantly different ($P < 0.05$).

Table 1.14. Concentration of polyphenol compounds and flavonoids and DPPH radical scavenging activity (DPPHRS) in muscle and liver of parrot fish fed the experimental diets for 4 weeks*

Diets	Polyphenol (ng/g tissue)	Flavonoids (ng/g tissue)	DPPHRS (%)
Muscle			
FM	164.5 ± 1.9^{b}	2.09 ± 0.00^{b}	8.64 ± 0.75^{c}
25SBM	203.5 ± 27.3^{a}	2.63 ± 0.93^{b}	10.17 ± 0.78^{a}
25CKJ	191.8 ± 10.4^{ab}	5.31 ± 1.61^{a}	10.67 ± 0.46^{a}
Liver			
FM	513.4 ± 2.1^{b}	63.2 ± 4.9^{b}	12.36 ± 1.28^{c}
25SBM	759.9 ± 12.6^{a}	80.4 ± 9.3^{a}	32.20 ± 4.55^{a}
25CKJ	680.4 ± 10.7^{ab}	69.9 ± 1.7^{ab}	21.08 ± 0.18^{b}

*Values presented are as means ± SD of triplicates. Value in the same column having different letters is significantly different ($P < 0.05$).

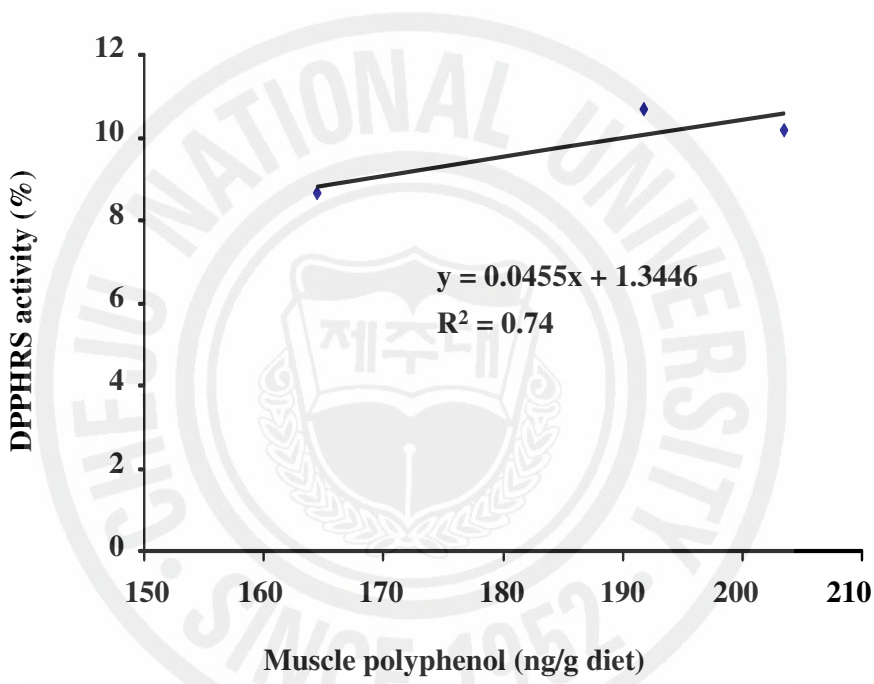

Figure 1.7. Relationship between concentration of muscle polyphenol compounds and muscle DPPH radical scavenging activity in parrot fish fed the experimental diets for 4 weeks

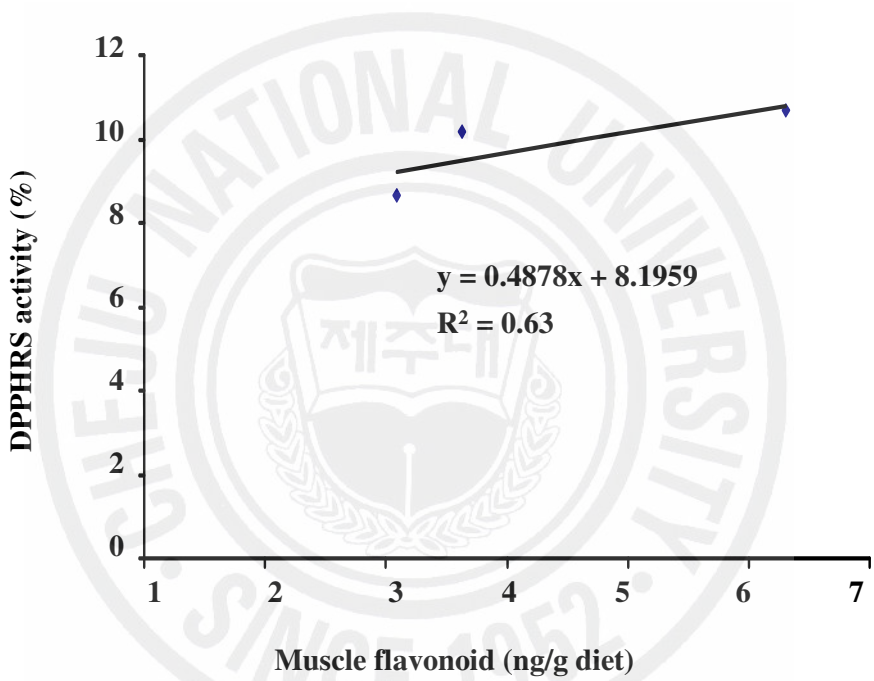

Figure 1.8. Relationship between concentration of muscle flavonoids and muscle DPPH radical scavenging activity in parrot fish fed the experimental diets for 4 weeks

1.4. DISCUSSION

Growth performance of parrot fish fed the diets containing 25% CKJ or 25% SBM was comparable to that of the control diet (Table 1.4). Low growth rate was observed in this feeding trial because of low water temperature (~15 ^{o}C). No significant differences were found in growth performance, muscle compositions, morphological parameters, hematocrit and serum cholesterol and triglyceride (Table 1.4~1.7). It suggests that 25% dietary fish meal protein can be replaced with SBM or CKJ with supplementation of methionine and lysine in parrot fish.

Parrot fish has been considered as a potential candidate for marine intensive aquaculture in Korea. However, there have been few works on this species to date (An et al., 2006; Cho et al., 2006; Choi et al., 2006; Jung and Oh, 2000; Lee et al., 2004; Makino et al., 2006; Nam et al., 2005; Oh et al., 2006; Tachibana et al., 1997; Wang et al., 2003). Recently, the outbreak of diseases has been announced to result in severe economic loss. Therefore, finding a method to prevent the infection of diseases is prioritized for aquaculture development of parrot fish. Unlike terrestrial animals, innate immune response plays more crucial important role in preventing diseases in fish (Ellis 2001; Kollner et al., 2002; Magnadottir 2006). Recently many studies have been reported that the innate immune response can be enhanced by dietary supplementation of some immunostimulants, such as vitamin C (Ai et al., 2004; Lin and Shiau, 2005a; Xie et al., 2006), vitamin E (Lin and Shiau, 2005b; Puang et al., 2004), β-glucan (Kumari and Sahoo, 2006) and other components. Use of natural products, such as fermented materials as immunostimulants, has been promoted in aquaculture because it is cheap, easy to treat, and rich in bioactive

compounds (Ashida et al., 2002). CKJ has been reported to have many beneficial effects on antimicrobial, anticarcinogenic, and antioxidant activities (Kim et al., 1998; Kim et al., 1999; Kim et al., 2004; Lee et al., 2005; Mine et al., 2005; Shon et al., 2007; Youn et al., 2001). However, in the present feeding trial, there were no clear effects of dietary supplementation of CKJ on serum lysozyme, respiratory burst and liver DPPH radical scavenging activities in parrot fish after 4 weeks of feeding trial. Contrastingly, several studies demonstrated that fermented vegetable products reduced oxidative stress, suppressed the lipid peroxidation, enhanced the antioxidant, and increased the phagocytic and lysozyme activities in Japanese flounder, *Paralichthys olivaceus* (Ashida et al., 2002; Ashida and Okimasu, 2005; Ashida et al., 2006). It was notable that there were some differences between the present feeding trial and the others. Firstly, different method in administration of the fermented products was used in the present feeding trial. In the others, only extractants of fermented products were used instead of whole fermented products. The condensed bioactive compounds in the extractant could increase their absorption, and thereby it could be more effective in enhancing the immune response of the fish. Secondly, a highly fluctuation of water temperature (from 27 ^{o}C at the beginning to 15 ^{o}C at the end of the present feeding trial) during feeding period might have also affected the immune response of fish. Kumari et al. (2006) reported that the seasonal variation of temperature remarkably had fluctuated innate immune parameters including serum myeloperoxidase, lysozyme, haemagglutination and alternative complement activities in Asian catfish.

Interestingly, the present feeding trial demonstrated that 25% dietary CKJ

significantly increased superoxide dismutase (SOD) activity in liver of fish (Fig. 1.1). The effects of dietary CKJ on liver antioxidant defense enzymes, such as SOD, could result in increased oxidant scavenging activities. Higher superoxide anion radical scavenging (SRS) activities in the diet supplemented 25% CKJ (Table 1.8) might have increased the SOD activity in the liver of fish fed the CKJ containing diet. The elevation of liver SOD activity consequently inhibits the generation of thiobarbituric acid reactive substances (TBARS, Fig. 1.2). It is well demonstrated that liver SOD and lipid peroxidation are influenced by dietary antioxidants. Ashida et al. (2002) reported that administration of fermented vegetable products significantly inhibited the lipid peroxidation of erythrocytes induced by tert-butyl hydroperoxide *in vivo*.

Dietary inclusion level up to 25% fish meal by either SBM or CKJ with supplementation of monocalcium phosphate did not alter the dietary total phosphorus concentration. However, concentration of inorganic phosphorus in the diet containing 25% CKJ was significantly higher than that of the control and 25SBM diets (Table 1.9). It is apparent that some natural microorganisms in CKJ could synthesize phytase that can liberate orthophosphate from soybean phytate, and consequently result in higher concentration of phosphorus in serum of fish fed the 25CKJ diet. Matsui et al. (1996) and Hirabayashi et al. (1998) concluded that *Aspergillus usami* fermentation could improve phosphorus availability of soya bean meal in chicks and the supplementation of inorganic phosphorus was not necessary. Boilers fed the diets supplemented fermented soybean meal with *Aspergillus oryzae* had higher concentration of plasma phosphorus and IgM, compared to those of animals fed the SBM-based diet (Feng et al., 2007a). In addition, the increment of some digestive

enzymes, such as total proteases and trypsin activity in the animals fed the diets supplemented with fermented soybean meal also could enhance the absorption of nutrients and result in their higher contents in plasma (Feng et al., 2007b).

Ascorbic acid (vitamin C) is an essential vitamin, response for normal physiological functions in animals and it also acts as an antioxidant vitamin which can enhance non-specific immune responses and diseases resistant in farmed fishes (Chew 1996; Mulero et al., 1998; Ortuno et al., 2001; Guesta et al., 2002; Ai et al., 2004). Ai et al. (2004) reported that the ascorbic acid concentrations in muscle and liver of Japanese seabass correlated positively with dietary AA contents. In the present feeding trial, the high concentration of other antioxidants compounds in the experimental diets and tissues, such as polyphenol compounds and flavonoids could have some sparing effects of AA concentrations. It suggests that the increase of some immune responses in parrot fish could be consequences of higher AA concentration.

Fermentations have been reported to improve the antioxidant capacity of plant protein sources (Doblado et al., 2005). In the present feeding trial, higher concentration of the antioxidant compounds, such as polyphenol compounds and flavonoids in the diet containing CKJ could result in the increase of radicals scavenging activity in the tissues of fish. Fang et al. (2002) elucidated that antioxidants and antioxidant enzymes exert synergistic action in scavenging radicals.

1.5. CONCLUSION

The present results suggest that dietary CKJ significantly increased liver superoxide dismutase and antioxidant activities in muscle and liver of growing parrot fish after four week feeding trial. Fermentation process in CKJ might increase the availability of phosphorus in parrot fish. To evaluate immunostimulatory effects of dietary CKJ in parrot fish, a long term feeding trial with dietary CKJ extract is recommended for further study.

CHAPTER II

EFFECTS OF DIETARY MEJU ON GROWTH PERFORMANCE, PHOSPHORUS RETENTION AND IMMUNE RESPONSE IN JUVENILE OLIVE FLOUNDER, *Paralichthys olivaceus*

ABSTRACT

An eight-week feeding trial was conducted to investigate the effects of dietary Meju (Korean traditional fermented soybean) on growth performance, feed utilization, phosphorus retention, and immune response in juvenile (mean body weight of 10 g/fish) olive flounder, *Paralichthys olivaceus*. Three isonitrogenous and isocaloric experimental diets supplemented with 0, 3 and 6% Meju (designated as Meju 0, Meju 3, and Meju 6) were formulated. After eight weeks of feeding trial, growth performance, feed utilization and survival were not different among all the fish groups fed all the experimental diets. Higher dietary inorganic phosphorus content was observed in the diet containing 6% Meju compared to the control diet. Phosphorus retention in juvenile olive flounder fed the Meju 6 diet was significantly higher than that of fish fed the control diet. Liver superoxide dismutase activity in the fish fed the experimental diets linearly increased with increment of dietary Meju supplementation level. In conclusion, dietary supplementation of Meju at 6% could increase phosphorus retention and enhance liver SOD activity in juvenile olive flounder.

2.1. INTRODUCTION

Olive flounder, *Paralichthys olivaceus,* is the most important marine cultured species in Korea, Japan and China. Its aquaculture production increased from 1,037mt in 1990 to 34,533mt in 2004 (Ministry of Maritime Affairs and Fisheries 2004) and contributed over 90% finfish aquaculture production in Korea. However, one of the current problems in olive flounder aquaculture is the fact that farmers use a large quantity of antibiotics to prevent the species from bacterial diseases. Moreover, residual of antibiotics in aquaculture products are being seriously concerned by consumers. Administration of natural bioactive compounds which can enhance immune responses and disease resistances of cultured fish might be a promising solution to avoid the use of antibiotics in olive flounder culture. Fermented products are very popular in oriental countries and have been reported to be rich in bioactive compounds. Recently, several studies reported that the use of fermented vegetable products could enhance non-specific immune response and disease resistances in fish (Ashida et al., 2002; Ashida and Okimasu, 2005; Ashida et al., 2006; Pham and Lee, 2007). The liver superoxide dismutase activity and DPPH radical scavenging activity in liver and muscle of parrot fish fed the diet containing 25% CKJ were significantly higher than those of fish fed the fish meal based diet (Chapter I). The diet 25CKJ had higher concentration of inorganic phosphorus, compared to the control and 25SBM diets (Pham and Lee, 2007).

Additionally, fermentations have been mentioned as the most effective techniques to improve palatability and flavor of soybean and reduce its anti-nutritional factors. The soy protein conversion ratio into amino acids in traditional

Korean soy-sauce fermentation is over 75%, approximately 15 times higher than feed protein conversion ratio in beef production and 6 times higher than that in pork production (Lee and Jul, 1982). Meju, a Korean traditional fermented soybean, is prepared by spontaneous fermentation (Fig. 2.1). The outer layer of Meju balls is covered with fungi such as *Aspergillus* sp., while on the inside bacteria, mainly *Bacillus subtilis* grow (Lee and Jul, 1982). It is recently commercialized and prepared using *Aspergillus oryzae* as a culture starter. Meju has been reported to have high antioxidant activity (Park and Jung, 2005). It possesses a beneficial mold *Aspergillus oryzae* that has been reported to be capable to produce extra-cellular enzymes, such as proteinases (Kundu et al., 1968; Kundu and Manna, 1975), alpha-amylase (Kundu and Das, 1970; Yabuki et al., 1977) and carboxypeptidase (Blinkovshi et al., 1999), cellulose-degrading enzymes (Yamane et al., 2002), and phytases (Fujita et al., 2000; 2003a, b). Esaki et al. (1999) reported that *A. oryzae* can synthesize high antioxidant compounds such as 6-hydroxyldaidzein, 8-hydroxydaidzein and 8-hydroxygenistein from soybean isoflavones. On the other hand, phytase, an enzyme synthesized during fermentation, can release orthophosphate from phytic acid that is composed of 70% phosphorus in plant protein sources. It was proposed that dietary inclusion of Meju could have beneficial effects on immune response and phosphorus utilization in animals, including fish. However, no information is available on the use of Meju in diets for olive flounder.

Therefore, the aim of the present experiment was to investigate the effects of Meju on growth performance, feed utilization, phosphorus retention and immune response in olive flounder fed the diets containing cottonseed and soybean meal.

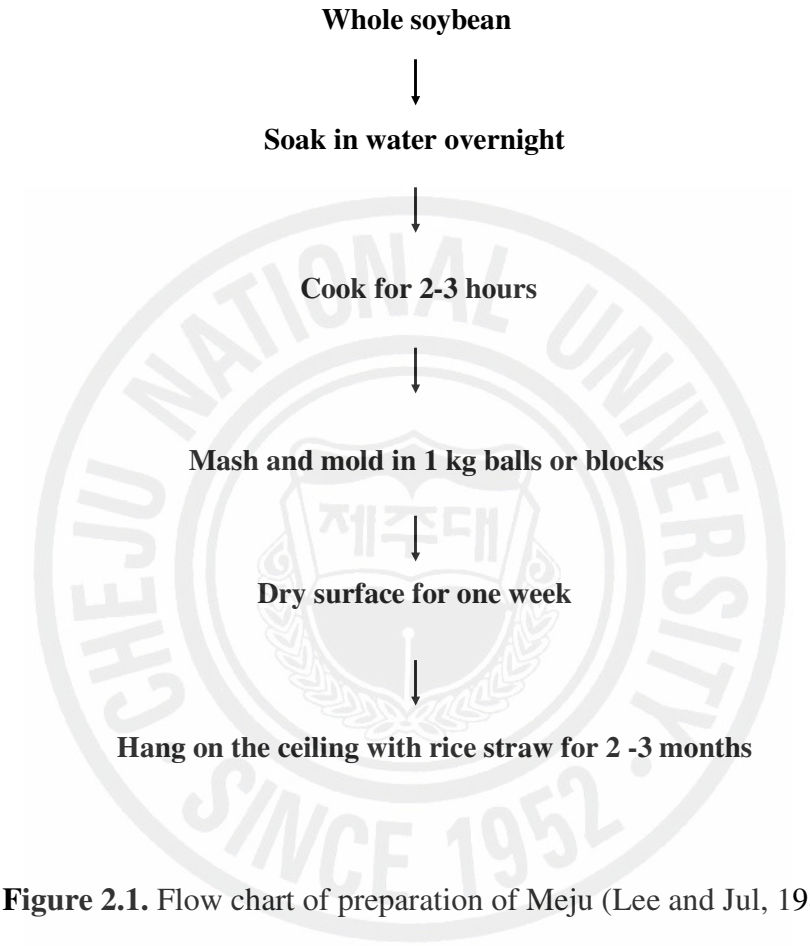

Figure 2.1. Flow chart of preparation of Meju (Lee and Jul, 1982)

Figure 2.2. Meju was purchased from Meju Munhwa Co. Ltd., Korea

2.2. MATERIALS AND METHODS

2.2.1. Experimental diets

Three experimental diets were formulated to be isonitrogenous and isocaloric in term of crude protein (54%) and gross energy (17.2 MJ/kg). The dietary formulation and proximate compositions are presented in Table 2.1, 2.2. Meju was supplemented in the experimental diets by 0, 3 and 6% (designated as Meju 0, Meju 3 and Meju 6, respectively) at the expense of soybean meal. Prior to its inclusion into diets, a dried Meju (purchased from Munwha-Meju Co., Daegu, Korea) was finely ground using grinding machine (MF 10 Basic, Germany). The proximate compositions of major protein sources used in the present feeding trial are given in Table 2.3. All dry ingredients were thoroughly mixed with 30% distilled water. Pellets were extruded through the meat chopper (SMC-12, Kuposlice, Busan, Korea) in 3.0 mm diameter and dried by an electric fan at room temperature for 24 h. The pellets were crushed into desirable particle sizes (0.4 – 2.0 mm) and stored at –20 ^{o}C until use.

2.2.2. Fish, facility and feeding trial

Olive flounder juveniles were transported from a hatchery in Jeju Island to Marine and Environmental Research Institute, Cheju National University. One hundred and eighty fish (initial body weight of 10 g/fish) were randomly distributed into nine 35 L plastic tanks (20 fish per tank) in a flow through system supplied with sand filtered seawater at a flow rate of 3 L/min. One of three experimental diets was fed to triplicate groups of fish at feeding rate of 3.5% BW per day, twice a day, 7

days a week, for 8 weeks. The growth of fish was measured every two weeks and feeding rate was adjusted accordingly. Feeding was stopped 24 h prior to weighing.

Table 2.1. Formulation of the experimental diets (% DM)

	Diets		
Ingredients	**Meju 0**	**Meju 3**	**Meju 6**
White fish meal	48.0	48.0	48.0
Soybean meal	9.0	6.0	3.0
Meju	0.0	3.0	6.0
Cottonseed meal [1]	8.0	8.0	8.0
Corn gluten meal	8.0	8.0	8.0
Wheat flour	13.4	13.8	14.2
Yeast	2.0	2.0	2.0
Mineral premix [2]	0.5	0.5	0.5
Vitamin premix [3]	0.5	0.5	0.5
Vitamin C and E	0.5	0.5	0.5
Choline chloride	0.1	0.1	0.1
Squid liver oil	9.0	8.6	8.2
CMC	1.0	1.0	1.0

[1]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.

[2] Mineral premix was mentioned in Chapter I.

[3] Vitamin premix was mentioned in Chapter I.

CMC: carboxymethyl cellulose.

DM: dry matter.

Table 2.2. Proximate compositions and energy of the experimental diets (% DM)

	Diets		
Proximate composition	**Meju 0**	**Meju 3**	**Meju 6**
Dry matter, %	94.8	93.2	94.0
Protein, % DM	52.7	52.8	52.0
Lipid, % DM	14.1	13.4	14.7
Ash, % DM	7.9	8.3	8.3
Total phosphorus, mg/g DM	6.4	5.7	5.5
Gross energy, MJ/kg DM[1]	17.2	17.2	17.2

[1]Energy in experimental diets was measured using a bomb calorimeter (Parr Instrument Company, Moline, IL, USA).

DM: dry matter.

Table 2.3. Proximate compositions of the ingredients used in the diets (% DM)

Ingredients	Moisture	Protein	Lipid	NFE[1]	Ash
White fish meal	8.72	68.33	8.56	0.32	14.07
Soybean meal	11.68	46.91	2.52	36.44	6.54
Cottonseed meal [2]	11.40	43.54	3.18	34.52	7.36
Corn gluten meal	9.50	61.70	1.03	26.59	1.18
Meju	10.85	41.42	17.93	24.54	5.62

[1] Nitrogen Free Extracts = 100 - (%Moisture + %CP + %Lipid + %Ash).

[2]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.

DM: dry matter.

2.2.3. Growth performance and feed utilization

At the beginning and the end of feeding trial, all fish were weighed and counted for weight gain, feed conversion ratio, protein efficiency ratio, specific growth rate, and survival calculation. Three fish per tank (nine fish per diet) were sampled and stored at –20 ^{o}C for whole body proximate compositions analysis. Analysis of crude protein, moisture, and ash were performed using the standard procedures (AOAC, 1995). Lipid was determined according to the method described by Folch et al. (1957).

2.2.4. Hematological parameters

At the end of feeding trial, three fish per tank (nine fish per diet) were randomly selected and anesthetized using tricaine methane sulfonate (MS-222) solution (100 mg/L). Blood was taken from caudal veins by heparinsed syringes. Hematocrit was determined using a VS-12000 (Vision Scientific Co. Ltd., Korea). Hemoglobin was determined by the method described in Pham et al. (2007).

2.2.5. Respiratory burst activity assay

Respiratory burst was measured by the nitro-blue-tetrazolium (NBT) assay described by Anderson and Siwicki (1995) with modificantion by Kumari and Sahoo (2005). Blood and 0.2% NBT were mixed in equal proportion (1:1), incubated for 30 min at room temperature, then 50 µL was taken out and dispensed in glass tubes. Then, 1 mL dimethyl formamide (DMF) was added and centrifuged at 2000 x g for 5 min. The OD of supernatant was measured at 540 nm. DMF was used as the blank.

2.2.6. Phosphorus measurements

The experimental diets and fish tissues were digested with 10 mL of a mixture of concentrated H_2SO_4 and HNO_3 (1:1; v:v) in Kjeldahl flasks. The digested mixture was volumed up to 100 mL with distilled water and used for total phosphorus measurement. Inorganic phosphorus of the experimental diets was extracted with 12.3% trichloroacetic acid solution using a shaker (WiseCube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 12 h at room temperature. After centrifugation at 2000 x g for 20 min, the supernatant was used for measurement of inorganic phosphorus. Phosphorus concentration was measured using spectrophotometrically method described by Nahapetian and Bassiri (1975). Phosphorus retention (PtR, %) was calculated by the following equation described by Nordrum et al. (1997): PtR=100 x (final body phosphorus - initial body phosphorus)/dietary phosphorus consumed.

2.2.7. Liver superoxide dismutase assay

Fish liver was homogenized in 9 volumes of 20 mM phosphate buffer pH 7.4 containing 1 mM EDTA and 0.1% Triton X-100. The homogenate was centrifuged at 10000 rpm to remove debris. The supernatants were used for superoxide dismutase assay according to method of Ukeda et al. (1999). Protein content in the supernatants was measured using method of Bradford (1976).

2.2.8. Measurement of total polyphenol compounds

Concentration of total polyphenol compounds in the experimental diets was measured using a method described by Skerget et al. (2005). The final value was

expressed in equivalent of gram of gallic acid per kilogram of dry diet.

2.2.9. Measurement of total flavonoids

Total flavonoids concentration in the experimental diets was measured using colorimetric method (Moreno et al., 2000). One gram of the experimental diet was extracted in 50 mL of 80% ethanol in a shaker (Wise Cube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 24 h at room temperature. The solution was centrifuged at 27,000 rpm for 20 min. The supernatant was used for total flavonoids assay. Total flavonoids content was expressed in mg of quercetin/g DM.

2.2.10. DPPH radical scavenging activity

Antioxidant activity of the experimental diets was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay described by Brand-Williams et al. (1995) with some modifications. Two g of diets (3 replicates per diet) were homogenized in 20 mL aqueous methanol (80%) and kept at room temperature for 10 min. The homogenates were centrifuged at 5000 rpm and 4 ^{o}C for 10 min. The supernatant was filtered through a 0.45 μm syringe filter (Whatman Inc., Clifton, NJ) and used for DPPH and superoxide anion radical scavenging activities. One hundred μL of filtered extract was pipetted into a 1.5 mL cuvette, then 900 μL of DPPH methanolic solution (100 μM) was added to obtain a final volume of 1 mL. The absorbance of the mixture was measured at 517 nm with 1 min intervals for 10 min using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The antioxidant activity of the extract against the DPPH radicals was calculated Percent inhibition:

$[(A_0 - A_s)/A_0] \times 100$, where A_0 and A_s are the absorbance at 0 and S min, respectively.

2.2.11. Superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity in the experimental diets was evaluated using a method described by Nagai et al. (2001). The reaction solution consisted of 1.2 mL of 0.05 M sodium carbonate buffer (pH 10.5), 0.1 mL of 3 mM xanthine, 0.1 mL of 3 mM ethylenediamine tetraacetic acid di-sodium salt (EDTA) 0.1 mL of 0.15% bovine serum albumin, 0.1 mL of 0.75 mM nitro-blue-tetrazolium and 0.1 mL dietary extract. After incubation at 25 ^{o}C for 10 min, the reaction was initiated by adding 0.1 mL 6 mU xanthine oxidase and kept at 25 ^{o}C for 20 min. The reaction was stopped by adding 0.1 mL of 6 mM CuCl. The absorbance of the mixture was measured at 560 nm.

2.12. Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. The significant differences between group means were compared using Duncan's multiple test. Data presented are means ± SD. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

2.3. RESULTS

At the end of feeding trial, up to 6% of dietary inclusion level of Meju did not affect the growth performance and feed utilization of juvenile olive flounder. Survival of the fish fed all the experimental diets was greater than 90 % (Table 2.4).

Whole body proximate compositions of juvenile olive flounder fed the experimental diets for 8 weeks are presented in Table 2.5. There were no significant differences in protein, lipid, and ash of fish fed the experimental diets supplemented with Meju for 8 weeks.

Blood parameters are given in Table 2.6. Hematocrit and hemoglobin showed an increasing trend related to the increment of dietary Meju. The nitro-blue-tetrazolium activity did not differ among fish groups fed the experimental diets.

Dietary inorganic phosphorus content was significantly increased with the increment of dietary Meju inclusion (Fig. 2.3). Significantly higher phosphorus retention was obtained in juvenile olive flounder fed the diet containing 6% of Meju (Fig. 2.4).

Liver superoxide dismutase (SOD) activity in juvenile olive flounder fed the experimental diets are presented in Figure 2.5. Liver SOD activity in the fish fed the diet containing 6% Meju was significantly higher than that of the fish fed the control diet. Concentrations of polyphenol compounds and flavonoids in the diets contained 6% Meju were significantly higher than those of the control diet (Fig. 2.6, 2.7). The antioxidant capacity of the experimental diets determined by 1,1-diphenyl-2-picryhydrazyl (DPPH) radical scavenging assay was significantly higher in the diet

containing 6% Meju (Fig. 2.8). Superoxide anion radical scavenging activity of the experimental diets is presented in Figure 2.9. It was gradually increased with the increment of dietary inclusion of Meju. Dietary superoxide anion radical scavenging activity showed a positive relationship with liver SOD activity ($R^2 = 0.79$).

Table 2.4. Growth performance and feed utilization of juvenile olive flounder fed the experimental diets for 8 weeks*

Diets	Meju 0	Meju 3	Meju 6
Initial body weight, g	9.90 ± 0.36	10.11 ± 0.36	9.78 ± 0.06
Final body weight, g	65.54 ± 1.82	67.63 ± 5.59	63.24 ± 1.08
Weight gain (WG)[1]	562.8 ± 36.6	569.4 ± 64.3	546.5 ± 14.6
Specific growth rate (SGR)[2]	1.17 ± 0.03	1.18 ± 0.06	1.16 ± 0.01
Nitrogen retention (NR)[3]	43.76 ± 2.02	41.07 ± 4.01	42.39 ± 1.39
Protein efficiency ratio (PER)[4]	2.26 ± 0.11	2.12 ± 0.22	2.22 ± 0.07
Feed conversion ratio (FCR)[5]	0.86 ± 0.04	0.94 ± 0.09	0.88 ± 0.03
Feed intake (FI)[6]	0.70 ± 0.04	0.75 ± 0.08	0.70 ± 0.02
Survival, %	93.2 ± 5.9	90.4 ± 5.1	95.0 ± 0.0

*Values presented are means ± SD. Value in the same row having different superscripts is significantly different ($P < 0.05$).

[1] WG (%) = 100 x (FBW - IBW)/IBW.

[2] SGR (%) = [(ln FBW - ln IBW)/days] x 100.

[3] NR (%) = 100 x (FBW x final CP – IBW x initial CP)/CP intake.

[4] PER = wet weight gain/ total protein given.

[5] FCR = dry feed fed/wet weight gain.

[6] FI (g/g BW) = dry feed consumed/BW.

IBW and FBW: initial and final mean body weight, respectively.

BW: body weight.

Table 2.5. Whole body compositions of juvenile olive flounder fed the experimental diets for 8 weeks*

Diets	**Initial**	**Meju 0**	**Meju 3**	**Meju 6**
Moisture content, %	78.7 ± 0.1	73.7 ± 0.5	73.4 ± 0.7	72.8 ± 0.6
Protein, % DM	74.3 ± 1.1	72.4 ± 0.4	70.7 ± 0.8	68.1 ± 2.3
Lipid, % DM	8.3 ± 0.1	14.5 ± 1.1	14.5 ± 1.8	13.7 ± 1.1
Ash, % DM	17.4 ± 0.2	13.3 ± 0.6	13.2 ± 0.6	13.5 ± 0.1

*Values presented are means ± SD of triplicates. Value in the same row having different superscripts is significantly different ($P < 0.05$).

DM: dry matter.

Table 2.6. Blood parameters of juvenile olive flounder fed the experimental diets for 8 weeks*

Diets	**Meju 0**	**Meju 3**	**Meju 6**
Hematocrit, %	23.7 ± 3.2	26.0 ± 1.0	27.2 ± 2.9
Hemoglobin, g/dL	2.8 ± 0.5	2.9 ± 0.3	3.1 ± 0.4
Blood NBT, $OD_{540\ nm}$	1.05 ± 0.03	1.25 ± 0.23	1.21 ± 0.05

*Values presented are means ± SD. Value in the same row having different superscripts is significantly different ($P < 0.05$).

OD: optical density.

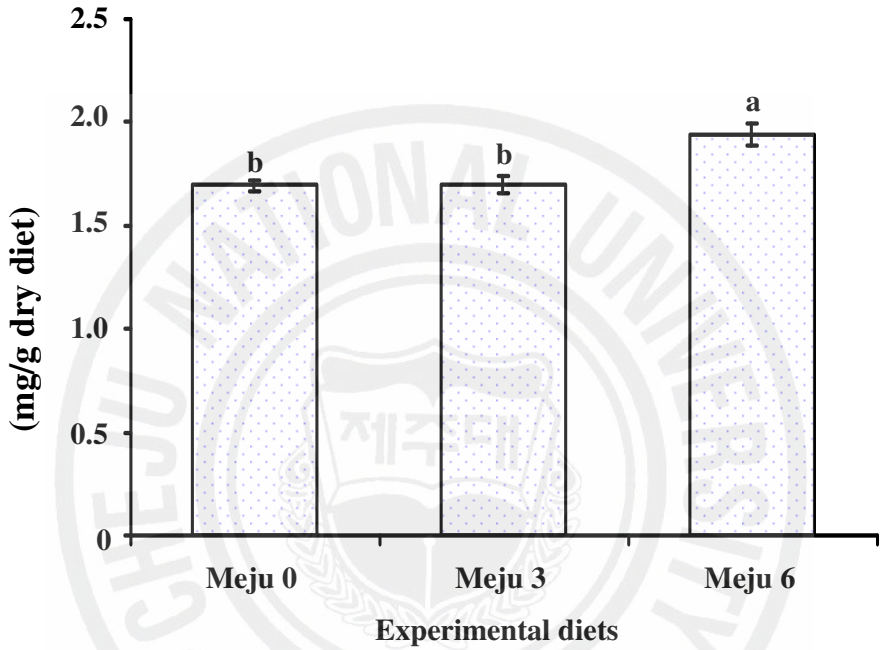

Figure 2.3. Concentration of inorganic phosphorus in the experimental diets containing Meju. Bar having different letters are significantly different ($P<0.05$).

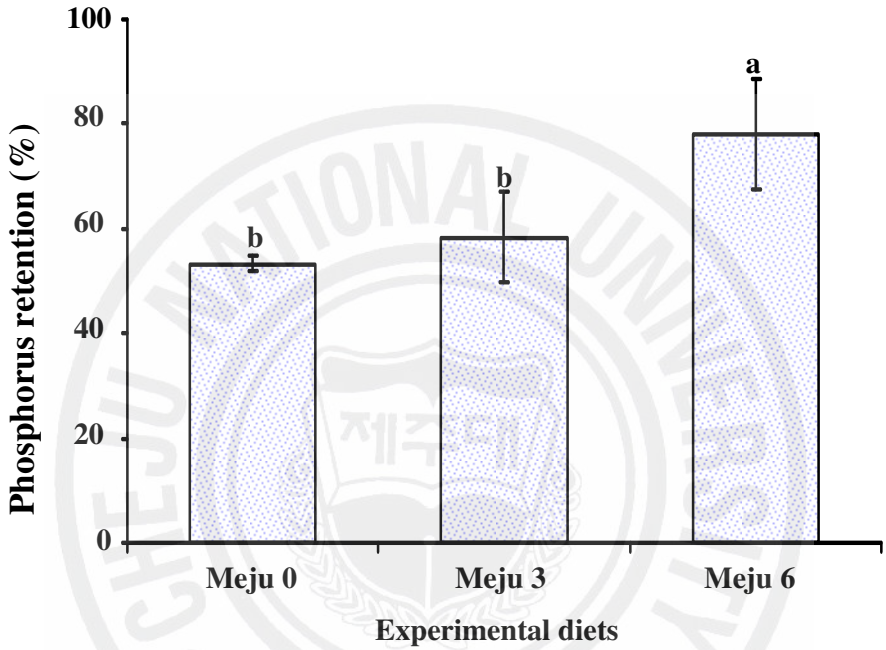

Figure 2.4. Phosphorus retention in juvenile olive flounder fed the experimental diets for 8 weeks. Bars having different letters are significantly different ($P < 0.05$).

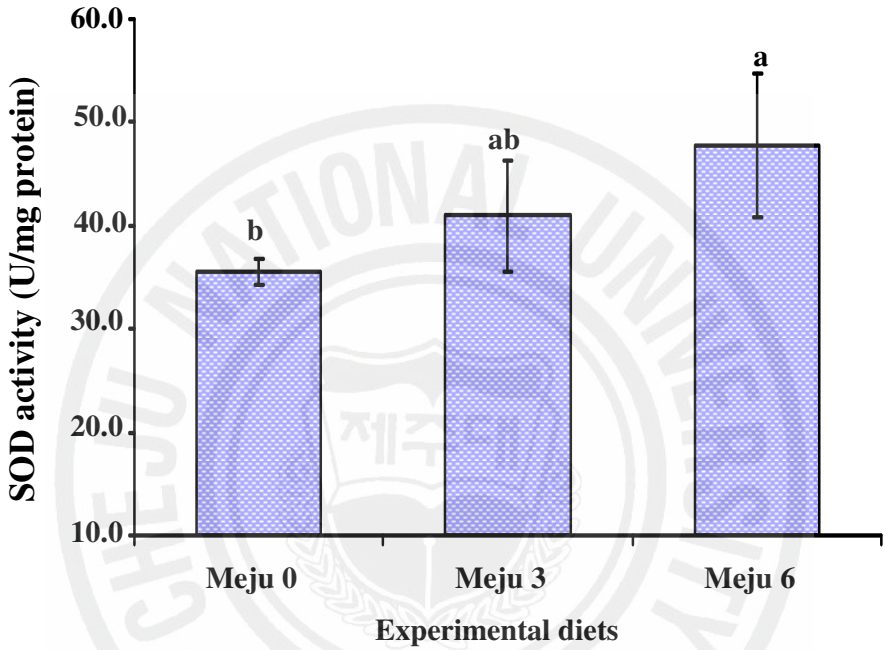

Figure 2.5. Liver superoxide dismutase activity in juvenile olive flounder fed the experimental diets for 8 weeks. Bars having different letters are significantly different ($P < 0.05$).

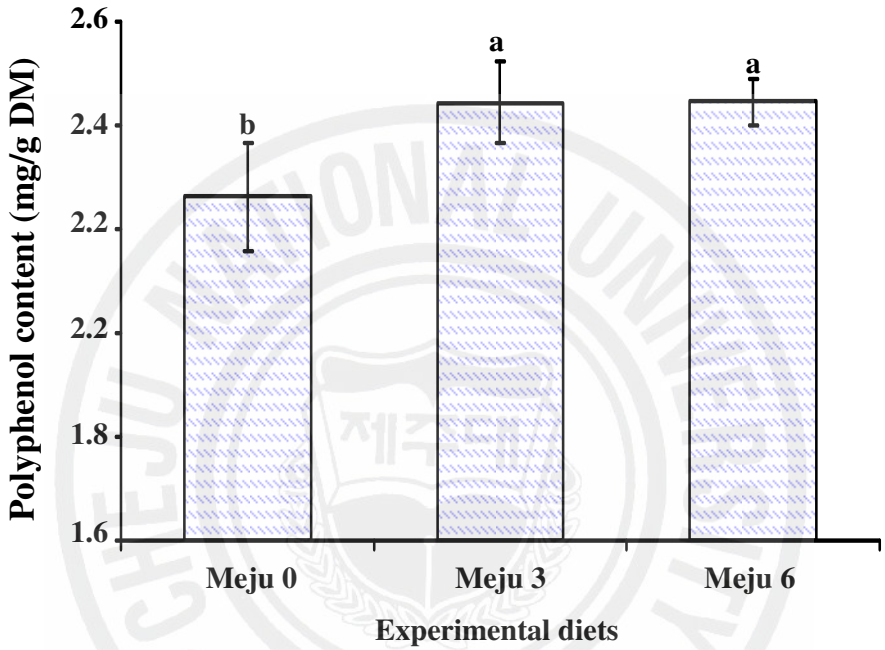

Figure 2.6. Concentration of polyphenol compounds in the experimental diets containing Meju. Bars having different letters are significantly different ($P<0.05$).

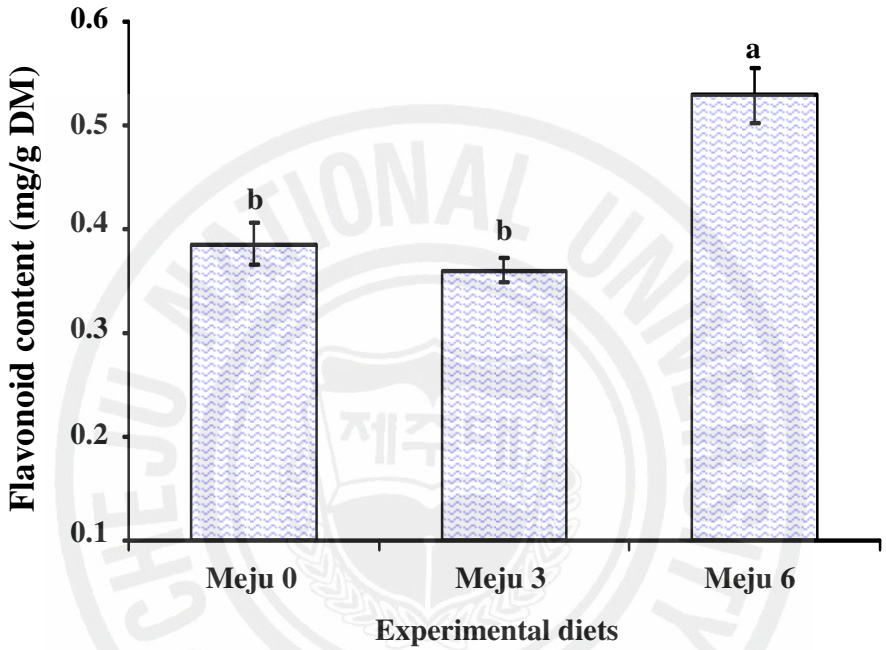

Figure 2.7. Concentration of flavonoids in the experimental diets containing Meju. Bars having different letters are significantly different ($P < 0.05$).

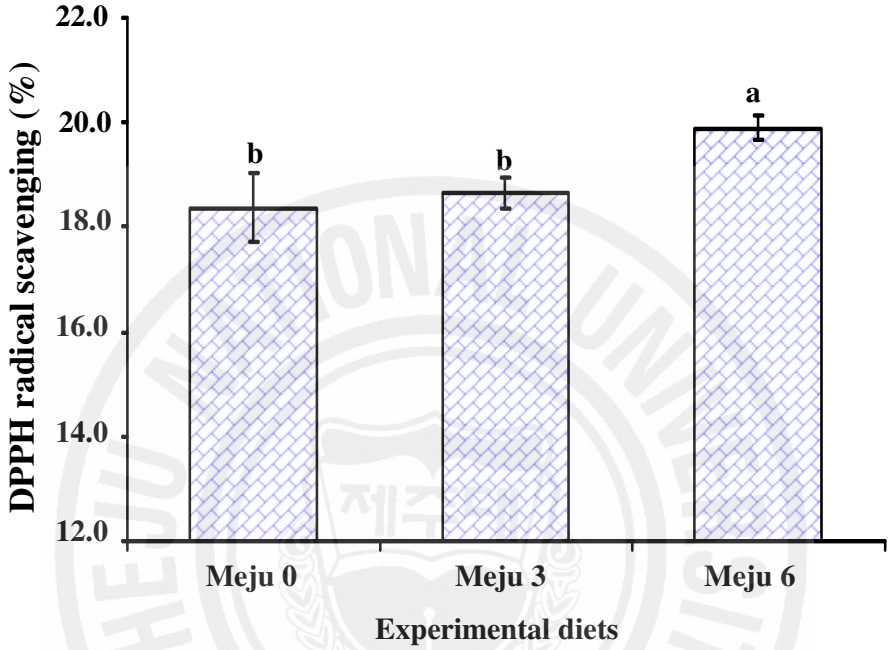

Figure 2.8. DPPH radical scavenging activity in the experimental diets containing Meju. Bars having different letters are significantly different ($P < 0.05$).

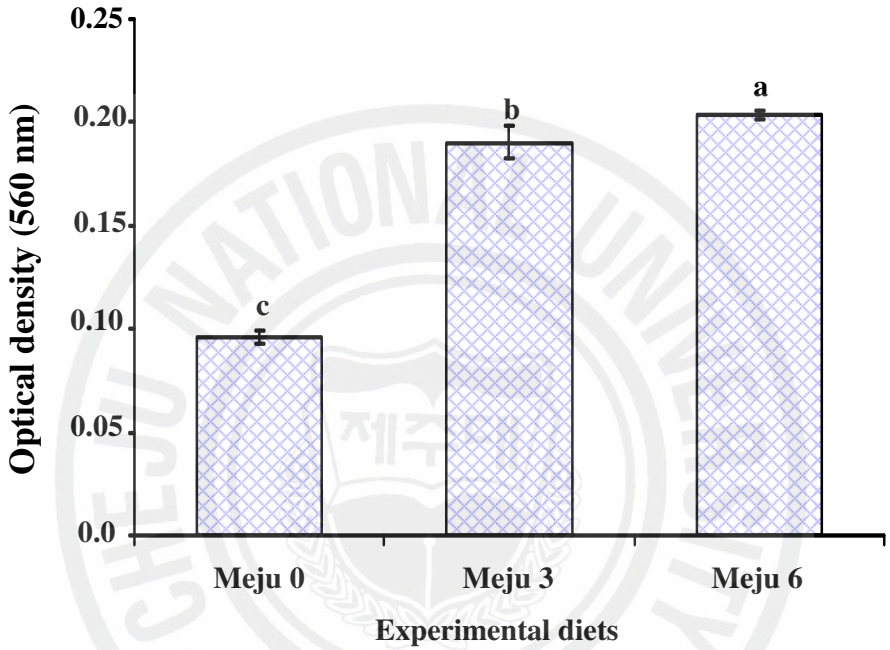

Figure 2.9. Superoxide anion radical scavenging activity in the experimental diets containing Meju. Bars having different letters are significantly different ($P<0.05$).

2.4. DISCUSSION

No significant differences were observed in final body weight, weight gain, specific growth rate, nitrogen retention, protein efficiency ratio, feed conversion ratio and whole body compositions among fish groups fed all the experimental diets for 8 weeks. It suggests that supplementation up to 6% of Meju do not affect the palatability and acceptability of the diets containing cottonseed and soybean meal; thereby it does not impair the growth performance, feed utilization and whole body compositions of olive flounder. Survival of fish fed the experimental diets was comparable to that of fish fed the control diet. It indicates that dietary Meju inclusion up to 6% does not possess any mycotoxic compounds to juvenile olive flounder. Strong antimutagenicity of Meju against mycotoxic compounds including aflatoxin B1 was reported by Jung et al. (2000).

Hematocrit and hemoglobin showed an increasing trend related to the increment of dietary Meju inclusion levels. There were no significant differences in blood respiratory burst activity among fish groups fed the experimental diets. Up to date, there has been no information on the effects of dietary Meju on hematocrit and hemoglobin of animals. Further studies on the effects of Meju on hematological parameters in fish are recommended.

Dietary inorganic phosphorus content significantly increased with increment of Meju in the experimental diets. Higher phosphorus retention was obtained in juvenile olive flounder fed the diet Meju 6. Elevation of inorganic phosphorus in the experimental diets might be related to phytase enzymes produced by microorganisms in Meju. Park and Jung (2005) reported that the primary microorganisms involved in

Meju fermentation are *Bacillus subtillis* and other filamentous fungi, such as Rizopus, Mucor, and Aspergillus sp. Particularly, *Aspergillus oryzae* has been reported to produce varieties of enzymes, such as phytases, cellulose-degrading enzymes, proteinases, alpha-amylase and carboxypaptidase (Kundu et al., 1968; Kundu and Das, 1970; Kundu and Manna, 1975; Yabuki et al., 1977; Blinkovshi et al., 1999; Yamane et al., 2002; Fujita et al., 2000, 2003a, b). In the present study, phytase produced by Meju microorganisms might have increased the dietary inorganic phosphorus and phosphorus retention in juvenile olive flounder fed the diet containing 6% Meju for 8 weeks. Masumoto et al. (2001) reported that a higher absorption of phosphorus was observed in juvenile olive flounder fed a diet containing soybean meal protein with phytase compared to fish fed the fish meal based diet. Moreover, Meju microorganisms, including *A. oryzae* and *B. subtilis*, could stimulate digestive enzymes and indirectly enhance absorbability of nutrients in fish. Yanbo and Zirong (2006) demonstrated that digestive enzymes of common carps significantly increased by feeding *Bacillus* sp. for 60 days and the effects of mix of probiotics were superior to a single one. There was a positive interaction between phytase, inorganic phosphorus on bone ash, bone phosphorus and whole body phosphorus concentration (Sajjadi and Carter, 2004). It is evident that the supplementation of soybean meal and its supplementation into diets containing cottonseed and soybean meal might improve phosphorus utilization in juvenile olive flounder.

Liver superoxide dismutase activity in juvenile olive flounder fed the experimental diets significantly increased with dietary Meju inclusion level as dose

dependent-manner. Higher liver SOD activity in fish fed diet containing 6% Meju might be related to higher concentration of total polyphenol compounds and antioxidant activities in the experimental diet. Total polyphenol compounds concentration, DPPH and superoxide anion radical scavenging activities were gradually increased with increment of dietary Meju. Concentration of flavonoids in the diet containing 6% Meju was significantly higher than that of the control diet. There was a positive regression between dietary superoxide anion radical scavenging activity and liver SOD activity of fish fed the experimental diets. *Aspergillus oryzae* is main functional microorganisms and being used as fermentation starter in producing commercial Meju (Jung et al., 2006). Higher antioxidative and anti-mutagenic activities have been reported in fermented soybean meal with *A. oryzae* by Lin et al. (2006). The present findings are well agreement with our previous one. Pham and Lee (2007) reported that significantly increased liver SOD activity was observed in parrot fish *Oplegnathus fasciatus* fed the diet contained 25% Cheongkukjang, a another Korean fermented soybean, for 4 weeks. Microorganisms including *A. oryzae* and *B. subtilis* in Meju might also have stimulatory effects on production of liver SOD in juvenile olive flounder. The Meju microorganisms could also play a role as probiotics, particularly *B. subtilis,* a Gram-positive, aerobic, endospore forming bacterium which has been reported to be able to enhance the innate immune response and disease resistances of many fish species. Kumar et al. (2006) and Kumar et al. (2008) reported that Rohu, an Indian major carp, produced maximum per cent survival, weight gain, total blood leukocyte cell counts, hemoglobin contents, and total protein and globulin contents by oral administration of *B. subtilis*. The authors elucidated that the colonization of *B. subtilis* in fish gut

epithelium could reduce the risk of pathogenic bacteria infection and hence develop the ability to protect them from various diseases. Newaj-Fyzul et al. (2007) demonstrated that *Bacillus subtilis* AB1 was an effective probiotic at controlling the *Aeromonas* sp infection in rainbow trout. They proposed that the bacteria in live and inactivated cells and their subcellular components could have ability to stimulate innate immune systems in fish.

2.5. CONCLUSION

The supplementation of Meju up to 6% did not affect the palatability and acceptability of diets for juvenile olive flounder thereby did not impair growth performance, feed utilization and survival of the fish. Phosphorus retention and liver superoxide dismutase activity significantly increased in juvenile olive flounder fed the diet containing 6% Meju. The findings of the present feeding trial could be related to the functional microorganisms in Meju including *Aspergillus sp.* and *Bacillus sp*.

CHAPTER III

EFFECTS OF DIETARY FERMENTED SOYBEAN MEAL ON GROWTH PERFORMANCE AND NON-SPECIFIC IMMUNE RESPONSE OF PARROT FISH, *Oplegnathus fasciatus*

ABSTRACT

A six week feeding trial was conducted to investigate the effects of fermented soybean meal (F-SBM) with four microorganisms on growth performance, feed utilization and non-specific immune response of juvenile parrot fish, *Oplegnathus fasciatus*. Based on our previous findings (Pham and Lee, 2007; Chapter II), five isonitrogenous and isocaloric experimental diets (designated as SBM, SBM-AO, SBM-SC, SBM-PP, SBM-BS) were formulated to contain 20% SBM or F-SBM with one of the four microorganisms including *Aspergillus oryzae*, *Sacchromyces cerevisiae, Pediococcus pentosaceus,* or *Bacillus subtilis,* respectively. Diet containing SBM was considered as the control diet. After six weeks of feeding trial, superoxide anion concentration produced by blood leucocytes (NBT) was significantly higher in fish groups fed the diet SBM-AO and SBM-PP compared to the control diet. Liver catalase activity of fish fed the F-SBM diets was significantly higher than that of the control. Liver DPPH radical scavenging activity of fish fed the diet SBM-AO was significantly higher compared to the control diet, and strongly related to concentration of liver polyphenol compounds. Higher weight gain was observed in the control group, and positively contributed to the higher protein solubility and phosphorus retention (R^2= 0.98 and 0.83, respectively). Survival did not differ among fish groups fed the experimental diets. Plasma cholesterol and triglycerides were altered by feeding of the diets containing F-SBM, and negatively related to the dietary flavonoids content (R^2 = 0.56 and 0.58, respectively). In conclusion, the diets containing F-SBM at inclusion level of 20% could enhance blood NBT and liver catalase activities of juvenile parrot fish after 6 week feeding

trial, regardless of microorganisms. Protein solubility is again proved as a useful indicator for quality control of heat treated soybean protein and affects the growth of juvenile parrot fish. An optimum temperature in treatment of soybean meal in aquafeed is necessary to be determined.

3.1. INTRODUCTION

Soybean meal (SBM) has long history use as protein sources for terrestrial and aquatic animals. It is well demonstrated that dietary inclusion of SBM at an optimum level could reduce feeds costs and did not affect growth performance of animals. However, fish species respond differently to the inclusion level of dietary SBM. Recently, many studies have been conducted to examine the use of SBM as alternative protein sources for fish meal in the diets for fish species including rainbow trout (Kaushik et al., 1995; Yamamoto et al., 1995; Refstie et al., 1997; Nordrum et al., 2000; Ogunkoya et al., 2006), Asia seabass (Boonyaratpalin et al., 1998; Tankikitti et al., 2005), European seabass (Kaushik et al., 2004), Atlantic salmon (Olli and Krogdahl, 1995; Refstie et al., 1998; Nordrum et al., 2000; Refstie et al., 2000), Atlantic cod (Olsen et al., 2007; Hansen et al., 2007), tin foil barb (Elangovan and Shim, 2000), cobia (Chou et al., 2004; Zhou et al., 2005), red snapper (Quartararo et al., 1998; Catacutan et al., 2004), gilthead sea bream (Robaina et al., 1995), sharpnout sea bream (Hernandez et al., 2007), Mediterranean yellowtail (Tomas et al., 2005), red sea bream (Biswas et al., 2007) and flounder (Kikuchi 1999; Saitoh et al., 2003). However, the presence of ANFs, such as trypsin inhibitors, phytic acid, saponins, non-digestible carbohydrates and lectins have been reported to restrict the use of SBM in aquaculture diets (NRC 1993; Francis et al., 2001). Therefore, a solution to increase SBM content in aqua-feeds is urgently needed.

On the other hand, SBM also contains large content of bioactive compounds which could enhance the immune responses of animals. Fermentation process has been reported to improve physical and nutritional quality of SBM, being considered

as a prospective technique in utilization of plant original proteins for animals (Arndt et al., 1999; Feng et al., 2007; Frias et al., 2008; Refstie et al., 2005) and also increase its antioxidant capacity (Choi et al., 2008; Gyorgy et al., 1964; Fernandex-Orozco et al., 2007; Frias et al., 2008; Kim et al., 1998; Kim et al., 1999; Kim et al., 2004; Lee et al., 2005; Lee et al., 2006; Lee et al., 2008; Machado et al., 2008; Mine et al., 2005; Yang et al., 2000; Youn et al., 2001). Refstie et al. (2005) reported that the lactic acid fermentation improved the nutritional value of SBM by partly eliminating soybean allergens and soy factors that affect the absorption of lipid in Atlantic salmon. Machado et al. (2008) demonstrated that heating was sufficient in the urease, trypsin inhibitor and lectin inactivation and improvement of the nutritional quality of SBM. Yang et al. (2000) concluded that fermented soybean was superior to soybean and might be applied as potential antioxidants in functional food.

Therefore, purpose of the present feeding trial was to select a suitable microorganism for fermentation process and to investigate the effects of fermented soybean meal with different microorganisms on growth performance and immune response of juvenile parrot fish.

3.2. MATERIALS AND METHODS

3.2.1 Fermentation of soybean meal

Aspergillus oryzae (AO) were purchased from Biotech Center, Korea. Three other bacteria strains including *Sacchromyces cerevisiae*-KCCM-53053 (SC), *Pediococcus pentosaceus*-KCCM-40820 (PP), and *Baciilus subtillis*-KCCM-40464 (BS) were obtained from Korean Culture Center of Microorganisms and grown on BHI broth at 25 ^{o}C (Difco Laboratories, Detroit, MI) and MRS broth (Difco Laboratories, Detroit, MI) for 24 h in Aquatic Pathological and Microbiological Laboratory, Department of Aquatic Medicine, Cheju National University. The harvested cells were washed three times with sterile saline solution and used as inocula. Dry SBM were soaked with distilled water at ratio of 1:3 (SBM: distilled water) for 1 h, and steamed for 1 h at approximately 100 ^{o}C. After cooling down at 40 ^{o}C in an incubator (Micro-253, Sanyo Electronic Co., Ltd., Japan), one of the four microorganisms including AO, SC, PP and BS were inoculated at 10^{7} CFU/g of dry SBM and incubated at 30 ^{o}C for 8 h, and followed at 28 ^{o}C for 40 h (Fig. 3.1). Fermented SBM (F-SBM) was freeze dried at -40 ^{o}C for 24 h using a freeze drier (Operon FDT-8605, Korea) and finely ground prior to supplementing in the experimental diets.

3.2.2. Experimental diets

Five isonitrogenous (48% crude protein) and isocaloric (17.1 MJ/kg DM) the diets were formulated to containing 20% SBM or F-SBM with four different microorganisms including AO, SC, PP, and BS (designated as SBM, SBM-AO,

SBM-SC, SBM-PP and SBM-BS, respectively). Formulation and proximate compositions of the experimental diets are presented in Table 3.1 and 3.2. Methionine and lysine were supplemented in the experimental diets to meet their requirements of fish. All dry ingredients were thoroughly mixed with 30% distilled water. Pellets were extruded through the meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size and dried using a freeze drier (Operon FDT-8605, Korea) for 24 h. The pellets were crushed into desirable particle sizes (0.4 – 2.0 mm) and stored at –20 ^{o}C until use.

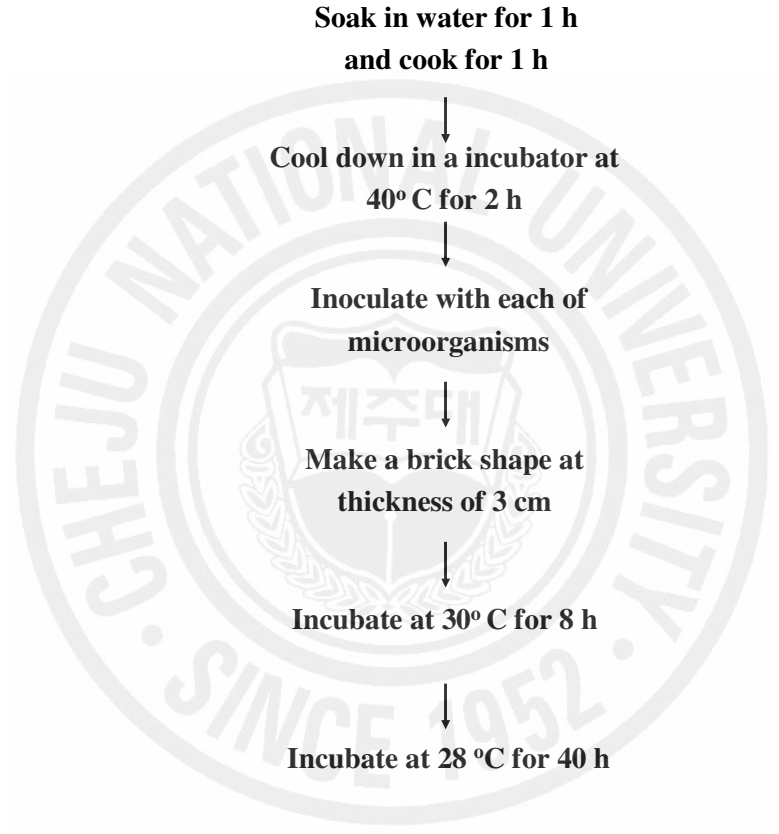

Fig. 3.1. Flow chart of making fermented SBM with different microorganisms

Fig. 3.2. Fermented soybean meal with different microorganisms

Table 3.1. Formulation of the experimental diets (% DM)

	Diets				
Ingredients	**SBM**	**SBM-AO[1]**	**SBM-SC[2]**	**SBM-PP[3]**	**SBM-BS[4]**
White fish meal	39.0	39.0	39.0	39.0	39.0
SBM	20.0	0.0	0.0	0.0	0.0
Fermented SBM	0.0	20.0	20.0	20.0	20.0
Corn gluten meal	7.4	7.4	7.4	7.4	7.4
Starch	17.4	17.4	17.4	17.4	17.4
Yeast	2.0	2.0	2.0	2.0	2.0
Mineral premix[5]	1.0	1.0	1.0	1.0	1.0
Vitamin premix[6]	1.0	1.0	1.0	1.0	1.0
Squid liver oil	8.7	8.7	8.7	8.7	8.7
Lysine	0.4	0.4	0.4	0.4	0.4
Methionine	0.4	0.4	0.4	0.4	0.4
MCP	0.6	0.6	0.6	0.6	0.6
Cellulose	2.1	2.1	2.1	2.1	2.1

[1] SBM-AO: SBM fermented with *Aspergillus oryzae*, EasyBio Co. Ltd., Korea.

[2] SBM-SC: soybean meal fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: soybean meal fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: soybean meal fermented with *Bacillus subtilis* - KCMM-40464.

[5]Mineral premix was mentioned in Chapter I.

[6] Vitamin premix was mentioned in Chapter I.

MCP: mono calcium phosphate.

DM: dry matter.

Table 3.2. Compositions, pH and protein solubility of the experimental diets*

	Diets				
	SBM	**SBM-AO[1]**	**SBM-SC[2]**	**SBM-PP[3]**	**SBM-BS[4]**
Dry matter, %	90.7	86.0	85.1	88.3	87.1
Protein, % DM	47.5	48.7	48.3	48.1	48.4
Lipid, % DM	12.1	12.4	12.8	12.7	12.5
Ash, % DM	9.3	9.5	9.2	9.2	9.3
pH	6.1	6.4	5.9	5.9	6.1
PS (%)	39.7[a]	38.4[b]	35.2[c]	34.3[d]	38.2[b]
GE, MJ/kg DM	17.1	17.1	17.1	17.1	17.1

*Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae*, EasyBio Co. Ltd., Korea

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

PS: protein solubility.

GE: Gross energy

3.2.3. Fish and feeding trial

Parrot fish juveniles were transported from a private hatchery in Jeju Island to Marine and Environmental Research Institute, Cheju National University. Fish were fed a commercial diet for 2 weeks in a 1000 L tank. Four hundred and fifty fish (initial body weight of 13.0 ± 0.2 g) were randomly distributed into fifteen 150 L tanks (30 fish per tank) in a flow through system supplied with sand filtered seawater at a flow rate of 3 L/min. One of the five experimental diets was fed to triplicate groups of fish at feeding rate of 3.5% body weight, twice a day (8:00 and 18:00), 7 days a week, for 6 weeks. The growth of fish was measured every three weeks and feeding rate was adjusted accordingly. Feeding was stopped 24 h prior to weighing.

3.2.4. Growth performance and feed utilization

At the beginning and the end of feeding trial, all fish were weighed and counted for weight gain, feed conversion ratio, protein efficiency ratio, specific growth rate and survival calculation. Three fish from each tank (9 fish per diet) were randomly sampled and stored at –20 ^{o}C for proximate compositions analysis. Analysis of crude protein, moisture and ash were performed using the standard procedures (AOAC 1995). Lipid was determined using Soxhlet System (SH6, Korea).

3.2.5. Morphological parameters

The total length, body weight, liver weight and gonad weight of 9 fish per diet (3 fish per tank) were individually measured. Condition factors (CF), hepato somatic index (HSI) and gonad somatic index (GSI) were calculated.

3.2.6. Hematological parameters

At the end of feeding trial, 3 fish per tank (9 fish per diet) were anesthetized in tricaine methanesulfonate (MS-222) solution (100 mg/L). Blood was taken from caudal veins using non-heparinsed syringes. Hematocrit was measured using a Microhematocrit VS-12000 (Vision Scientific Co., Ltd, Korea). The remained blood samples were centrifuged at 5000 rpm for 10 min at 4 ^{o}C and collected plasma was used for cholesterol and triglyceride analysis. Plasma cholesterol and triglyceride were measured using a Photometer CH100 Plus (Calenzano, Firenze, Italy).

3.2.7. Respiratory burst assay

Superoxide anion content produced by blood leukocytes during respiratory burst was measured using nitro-blue-tetrazolium (NBT) assay described by Anderson and Siwicki (1995) with some modified by Kumari and Sahoo (2005).

3.2.8. Lysozyme activity

Plasma lysozyme activity in parrot fish fed the diets containing F-SBM was measured using a colorimetrical method. Plasma sample was added in *Micrococcus lysodeikticus* suspension (0.2 mg/ml *M. lysodeikticus* in 0.02 M sodium citrate buffer) at 1: 10 ratio. Initial optical density was measured immediately after adding plasma. After incubating for 5 min at room temperature, final optical density was measured at 450 nm using an UV/VIS spectrophotometer (DU 730, Beckman, USA). Hen egg white lysozyme (HEWL, Sigma) was used as standard. Lysozyme values were expressed as μg/ml equivalent of HEWL activity.

3.2.9. Liver superoxide dismutase assay

Fish liver was homogenized in 9 volumes of 20 mM phosphate buffer pH 7.4 containing 1 mM EDTA and 0.1% Triton X-100. The homogenate was centrifuged at 10000 rpm to remove debris. The supernatants were used for superoxide dismutase assay according to method of Ukeda et al. (1999). Protein content in the supernatants was measured using a method of Bradford (1976).

3.2.10. Liver catalase assay

Catalase activity was measured based on the bleaching ability of H_2O_2 to potassium permanganate as method of Cohen et al. (1970). The reaction mixture consisted of 100 µL sample supernatant and 1 mL 6 mM H_2O_2 was incubated for 3 min on ice. After incubation on ice, 200 µL 6 N H_2SO_4 was added to stop the reactions, and 1.4 mL 2mM $KMnO_4$ was added to each tube. The mixture was vortexed and the optical density was measured within 60 seconds using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA) at 480 nm. The 0.25 M sucrose buffer and water were used as blank and standard, respectively. The optical densities were converted into units of catalase/mg protein based on following equation: 1 unit = k/0.00693.

3.2.11. Measurement of total polyphenol compounds

Total polyphenol compounds in the experimental diets and liver of fish were spectrophotometrically measured using a method described by Skerget et al. (2005). The results were expressed in equivalent of gram of gallic acid per kilogram of DM.

3.2.12. Measurement of total flavonoids

Total flavonoids concentration in the experimental diets was measured using colorimetric method (Moreno et al., 2000). One gram of the experimental diet or 0.5 g of liver was extracted in 10 mL of 80% ethanol using a shaker (Wise Cube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 24 h at room temperature. The solution was centrifuged at 27000 rpm for 20 min. The supernatant was used for total flavonoids assay. Flavonoids concentration was expressed in equivalent of mg of quercetin/g DM.

3.2.13. DPPH radical scavenging activity

Antioxidant activity of liver was measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay described by Brand-Williams et al. (1995) with some modifications. One g of diets (3 replicates per diet) or 0.1 g of liver were homogenized in 1 mL aqueous methanol (80%) and kept at room temperature for 10 min. The homogenates were centrifuged at 5.000 rpm for 10 min at 4 ^{o}C. The supernatant was filtered through a 0.45 µm syringe filter (Whatman Inc., Clifton, NJ) and used for DPPH assay. One hundred µL of filtered extract was pipetted into a 1.5 mL cuvette, then 900 µL of DPPH methanolic solution (100 µM) was added to obtain a final volume of 1 mL. The absorbance of the mixture was measured at 517 nm with 1 min intervals for 10 min using a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The antioxidant activity of the extract against the DPPH radicals was calculated as percent inhibition. Percent inhibition = $[(A_0 - A_s)/A_0]$ x 100, where A_0 and A_s are the absorbance of sample at 0 and S min, respectively.

3.2.14. Dietary pH

One gram of the experimental diets was homogenized in 10 mL distilled water for 1 min. The mixture was kept for 15 min at room temperature. pH of mixture was measured by a pH meter (Seveneasy pH meter, Mettler Toledo GmbH, Switzerland).

3.2.15. Protein solubility

Protein solubility in the experimental diets containing F-SBM was determined using 0.2% KOH solution (Araba and Dale, 1990). After extraction, solid particles were removed by centrifugation at 1250 x g for 10 min (VS-550, Vision Scientific Co., Ltd. Korea) and soluble protein in the supernatants was measured using Kjeltec 2300 (Foss Korea Ltd., Korea).

3.2.16. Dietary amino acids analysis

Concentrations of amino acids of the experimental diets containing F-SBM with different microorganisms were measured by Woo Sung Feed Co. Ltd., Korea.

3.2.17. Phosphorus measurements

The experimental diets and fish tissues were digested with 10 mL of a mixture of concentrated H_2SO_4 and HNO_3 (1:1; v:v) in Kjeldahl flasks. The digested mixture was volumed up to 100 mL with distilled water and used for total phosphorus measurement. Inorganic phosphorus of the experimental diets was extracted with 12.3% trichloroacetic acid solution using a shaker (WiseCube, DAIHAN Scientific, Co., Ltd., Seoul, Korea) for 12 h at room temperature. After centrifugation at 2000 x

g for 20 min, the supernatant was used for measurement of inorganic phosphorus. Phosphorus concentration was measured using spectrophotometrically method described by Nahapetian and Bassiri (1975). Phosphorus retention was calculated as the equation described by Nordrum et al. (1997): Phosphorus retention (%) = 100 x (final body phosphorus - initial body phosphorus)/dietary phosphorus consumed.

3.2.18. Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0 for Windows. The significant differences between group means were compared using Duncan's multiple test. Data presented are means ± SD. The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

3.3. RESULTS

In the present feeding trial, dietary protein, lipid, ash contents and pH did not differ among the experimental diets. However, protein solubility of the control diet was significantly higher than that of the diets contained the F-SBM (Table 3.2). The contents of amino acids in the experimental diets are given in Table 3.3. The total essential amino acids significantly decreased in the F-SBM diets.

Growth performance and feed utilization of fish fed the experimental diets are presented in Table 3.4. Highest weight gain, protein efficiency ratio and nitrogen retention were observed in the fish group fed the diet SBM, followed by SBM-AO, SBM-BS, SBM-PP and SBM-SC, respectively. However, feed intake showed highest value in the fish group fed the SBM-SC diet, followed by SBM-PP, SBM-AO, SBM-BS, and lowest in fish group fed the control diet. Positively relationship was observed between growth performance of fish and dietary protein solubility (R^2 = 0.83). There were no significant differences in survival of among all the fish groups fed all the experimental diets.

Whole body compositions of the fish fed the experimental diets are given in Table 3.5. Whole body moisture, protein and ash contents did not differ within the fish groups fed the experimental diets. However, whole body lipid content of fish fed the control diet was significantly higher than that of fish fed SBM-SC, SBM-PP and SBM-BS diets. There were no significant differences in whole body lipid content among the fish groups fed the diets containing F-SBM.

No significant differences were observed in morphological parameters of juvenile parrot fish fed all the experimental diets for 6 weeks (Table 3.6).

F-SBM did not influence dietary total phosphorus, inorganic phosphorus. No differences were observed in plasma and whole body phosphorus contents among fish groups fed the experimental diets. However, highest phosphorus retention was observed in the fish fed the SBM diet, followed by SBM-BS, SBM-AO, SBM-SC and SBM-PP, respectively (Table 3.7). Phosphorus retention also had positively relationship to growth performance of the fish fed the experimental diets.

Blood parameters of the fish fed the experimental diets are given in Table 3.8. No significant differences were observed in hematocrit (Ht), hemoglobin (Hb), plasma protein and glucose contents among fish groups fed all the experimental diets. Plasma triglyceride and cholesterol in the fish fed SBM-SC, SBM-PP and SBM-BS diets were significantly higher than those of the fish fed the control diet. There were no significant differences in plasma triglyceride and cholesterol among fish fed the F-SBM. Plasma lysozyme and superoxide dismutase did not influenced by F-SBM. Respiratory burst activity of blood leukocytes was measured by nitro blue tetrazolium assay. The fish fed the diets SBM-AO and SBM-PP showed higher NBT activity than that of fish fed the control diet.

F-SBM significantly enhanced liver catalase activity, regardless of different microorganisms. No significant differences in liver SOD and lipid peroxidation were observed among fish groups fed all the experimental diets (Table 3.9).

Liver DPPH activity in fish fed the SBM-AO diet was significantly higher than that of fish fed the control diet. Liver antioxidant activity seemed to be attributed by the dietary total polyphenol compounds and positively associated with liver concentration of polyphenol compounds (Table 3.10).

Table 3.3. Concentrations of amino acids in the experimental diets (%)

Diets	**SBM**	**SBM-AO[1]**	**SBM-SC[2]**	**SBM-PP[3]**	**SBM-BS[4]**
Arginine	2.62	2.61	2.54	2.23	2.28
Histidine	1.19	1.21	1.21	1.20	1.17
Isoleucine	1.62	1.16	1.48	1.58	1.54
Leucine	2.97	2.31	2.65	2.92	2.66
Lysine	3.44	3.91	3.69	3.01	3.13
Methionine	1.00	1.20	0.93	0.94	0.89
Cysteine	0.37	0.49	0.34	0.35	0.33
Phenylalanine	1.77	0.90	1.56	1.66	1.74
Threonine	2.54	3.66	1.50	1.57	1.63
Valine	2.06	1.21	0.80	1.50	1.64
Alanine	4.68	3.61	6.82	2.11	1.87
Aspartic acid	4.77	6.83	2.91	3.49	3.49
Glutamic acid	6.10	6.49	6.05	5.67	5.39
Glycine	2.18	1.50	2.09	2.00	2.04
Serine	2.79	4.31	3.77	1.45	1.53
EAAs	19.21	18.17	16.36	16.61	16.68
N-EAAs	20.89	23.23	21.98	15.07	14.65
Total	40.10	41.40	38.34	31.68	31.33

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* - KCMM-40464.

EAAs: Essential amino acids. **N-EAAs:** Non essential amino acids.

Table 3.4. Growth performance, feed utilization and survival of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
FBW (g)	25.8 ± 1.0^{a}	23.5 ± 0.2^{b}	20.4 ± 1.0^{d}	21.8 ± 0.4^{c}	23.3 ± 0.3^{b}
WG (%)[5]	94.8 ± 5.5^{a}	80.7 ± 2.5^{b}	56.0 ± 6.9^{d}	65.5 ± 2.3^{c}	79.1 ± 3.7^{b}
SGR (%)[6]	1.59 ± 0.07^{a}	1.41 ± 0.03^{b}	1.14 ± 0.0^{c}	1.20 ± 0.03^{c}	1.39 ± 0.1^{b}
NR (%)[7]	33.9 ± 1.5^{a}	29.8 ± 0.8^{b}	22.2 ± 0.3^{e}	25.5 ± 0.7^{d}	27.9 ± 0.9^{c}
PER[8]	1.97 ± 0.1^{a}	1.73 ± 0.1^{b}	1.37 ± 0.0^{d}	1.48 ± 0.0^{c}	1.70 ± 0.1^{b}
FCR[9]	1.07 ± 0.1^{d}	1.19 ± 0.0^{c}	1.51 ± 0.0^{a}	1.41 ± 0.0^{b}	1.22 ± 0.0^{c}
FI[10]	0.51 ± 0.0^{c}	0.53 ± 0.0^{bc}	0.57 ± 0.0^{a}	0.56 ± 0.0^{a}	0.53 ± 0.0^{b}
Survival	98.9 ± 1.1	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

[5] Weight gain (WG) = 100 x (FBW - IBW)/IBW.

[6] Specific growth rate (SGR) = 100 x [(ln FBW - ln IBW)/days].

[7] Nitrogen retention (NR) = 100 x (FBW x final CP - IBW x initial CP)/CP intake.

[8] Protein efficiency ratio (PER) = wet weight gain/total protein given.

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

[10] Feed intake (g/g body weight) = dry feed consumed (g)/body weight (g).

Table 3.5. Whole body proximate compositions of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
Moisture, %	71.5 ± 1.0	71.8 ± 0.8	72.5 ± 0.3	72.4 ± 0.3	72.7 ± 0.4
Protein, % DM	58.1 ± 0.3	58.5 ± 0.6	58.9 ± 1.0	59.3 ± 0.4	58.9 ± 0.7
Lipid, % DM	16.1 ± 1.9[a]	13.3 ± 2.3[ab]	12.4 ± 0.6[b]	13.0 ± 1.2[b]	12.5 ± 1.4[b]
Ash, % DM	16.9 ± 1.2	17.1 ± 0.7	17.0 ± 0.5	17.5 ± 0.4	17.8 ± 0.7

* Values are presented as mean ± SD of triplicates. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* - KCMM-40464.

DM: dry matter.

Table 3.6. Morphological parameters of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
CF (%)[5]	2.27 ± 0.2	2.35 ± 0.2	2.35 ± 0.1	2.31 ± 0.1	2.32 ± 0.2
HSI (%)[6]	1.91 ± 0.2	1.92 ± 0.2	2.03 ± 0.3	1.96 ± 0.1	1.89 ± 0.2
VSI (%)[7]	8.32 ± 0.9	7.58 ± 0.8	7.98 ± 0.9	7.58 ± 0.3	7.44 ± 0.8

* Values are presented as mean ± SD of triplicates. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

[5] Condition factor (CF) = fish weight (g) x 100/fish length $(cm)^3$.

[6] Hepato somatic index (HSI) = 100 x (liver weight/body weight).

[7] Viscera somatic index (VSI) = 100 x (viscera weight/body weight).

Table 3.7. Blood parameters of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
Ht	36.2 ± 1.4	35.4 ± 2.2	36.2 ± 2.7	38.0 ± 5.0	37.5 ± 5.7
Hb	6.7 ± 0.3	6.8 ± 0.6	6.2 ± 0.3	6.4 ± 0.8	6.7 ± 0.2
P-Trigly.	171 ± 17.9^{b}	176 ± 16.5^{b}	240 ± 7.6^{a}	243 ± 40.3^{a}	240 ± 47.5^{a}
P-Chol.	125 ± 7.6^{b}	125 ± 24.4^{b}	149 ± 6.9^{ab}	163 ± 19.4^{a}	150 ± 21.1^{ab}
P-protein	2.7 ± 0.1	2.9 ± 0.4	3.4 ± 1.0	3.1 ± 0.1	2.7 ± 0.1
P-glucose	93 ± 36.7	82 ± 27.9	105 ± 42.3	102 ± 17.6	83 ± 10.0
P-SOD	8.3 ± 1.0	9.0 ± 0.5	8.4 ± 1.1	7.8 ± 0.2	72 ± 0.21
P-Lyso.	1.47 ± 0.24	1.76 ± 0.44	1.26 ± 0.17	1.75 ± 0.29	1.80 ± 0.58
NBT	1.24 ± 0.10^{b}	1.46 ± 0.13^{a}	1.12 ± 0.12^{b}	1.46 ± 0.04^{a}	1.26 ± 0.10^{b}

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *A. oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

Ht: Hematocrit (%). Hb: Hemoglobin (g/dL).

P-Trigly.: Plasma triglyceride (mg/dL). P-Chol.: Plasma cholesterol (mg/dL).

P-protein: Plasma protein (g/dL). P-glucose: Plasma glucose (mg/dL).

P-SOD: Plasma superoxide dismutase (U/mg protein).

P-lyso.: Plasma lysozyme (μg Hen egg white lysozyme /ml).

NBT: nitro blue tetrazolium ($OD_{540\ nm}$).

Table 3.8. Liver superoxide dismutase, catalase activities and thiobarbituric acid reactive substances of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
L-SOD	27.7 ± 7.4	27.0 ± 3.7	24.6 ± 3.7	25.9 ± 1.6	28.7 ± 4.5
L-CAT	46.1 ± 24.1[b]	107.5 ± 31.9[a]	96.2 ± 2.1[a]	109.0 ± 19.4[a]	108.2 ± 18.8[a]
L-TBARS	7.94 ± 2.25	7.51 ± 1.83	5.78 ± 0.75	6.39 ± 1.56	6.57 ± 1.24

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

L-SOD: liver superoxide dismutase activity (U/mg protein).

L-CAT: liver catalase activity (U/mg protein).

L-TBARS: liver thiobarbituric acid reactive substances (μM/g tissue).

Table 3.9. Concentrations of dietary inorganic, total and whole body phosphorus and phosphorus retention of parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
D-Pi	8.9 ± 0.17	9.4 ± 0.38	9.1 ± 0.21	9.1 ± 0.37	8.9 ± 0.34
D-Pt	10.4 ± 0.15	11.2 ± 0.68	10.8 ± 0.35	11.5 ± 0.96	10.9 ± 0.60
P-Pi	9.34 ± 2.17	8.99 ± 1.24	9.65 ± 1.80	9.93 ± 1.41	8.53 ± 0.80
WBP	21.2 ± 2.35	21.2 ± 1.02	22.4 ± 1.06	22.2 ± 1.53	20.9 ± 0.60
PR	61.4 ± 2.44^{a}	51.1 ± 1.16^{c}	47.5 ± 1.91^{d}	46.0 ± 0.96^{c}	54.7 ± 1.46^{b}

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

D-Pt: Dietary total phosphorus (mg/g DM).

D-Pi: Dietary inorganic phosphorus (mg/g DM).

P-P: Plasma phosphorus (mg/dL).

WBP: whole body phosphorus (mg/g DM).

PR: Total phosphorus retention (%).

Table 3.10. Concentrations of dietary polyphenol compounds, flavonoids and DPPH radical scavenging activity of the diets, and liver of juvenile parrot fish fed the experimental diets for 6 weeks*

Diets	SBM	SBM-AO[1]	SBM-SC[2]	SBM-PP[3]	SBM-BS[4]
D-poly.	1602 ± 31^{b}	1770 ± 86^{a}	1673 ± 41^{ab}	1617 ± 38.5^{b}	1749 ± 96^{a}
D-flav.	303 ± 26.5	208 ± 28.5	196 ± 14.0	161 ± 13.5	205 ± 28.0
L-poly.	1969 ± 11^{b}	2619 ± 17^{a}	2298 ± 43^{ab}	2540 ± 14^{ab}	2206 ± 17^{ab}
L-flav.	980 ± 26.2	1367 ± 50.9	1000 ± 28.2	1130 ± 8.5	1139 ± 37.7
LDPPHRS	11.5 ± 1.2^{b}	22.4 ± 8.9^{a}	14.8 ± 1.1^{ab}	17.2 ± 2.8^{ab}	17.8 ± 4.2^{ab}

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

[1] SBM-AO: SBM fermented with *Aspergillus oryzae,* EasyBio Co. Ltd., Korea.

[2] SBM-SC: SBM fermented with *Sacchromyces cerevisiae* KCMM-35053.

[3] SBM-PP: SBM fermented with *Pediococcus pentosaceus* KCMM-40820.

[4] SBM-BS: SBM fermented with *Bacillus subtilis* KCMM-40464.

D-poly.: Dietary polyphenol compounds (μg/g).

D-flav.: Dietary flavonoids (μg/g).

L-poly.: Liver polyphenol compounds (μg/g).

L-flav.: Liver flavonoids (μg/g).

LDPPHRS: Liver DPPH radical scavenging activity.

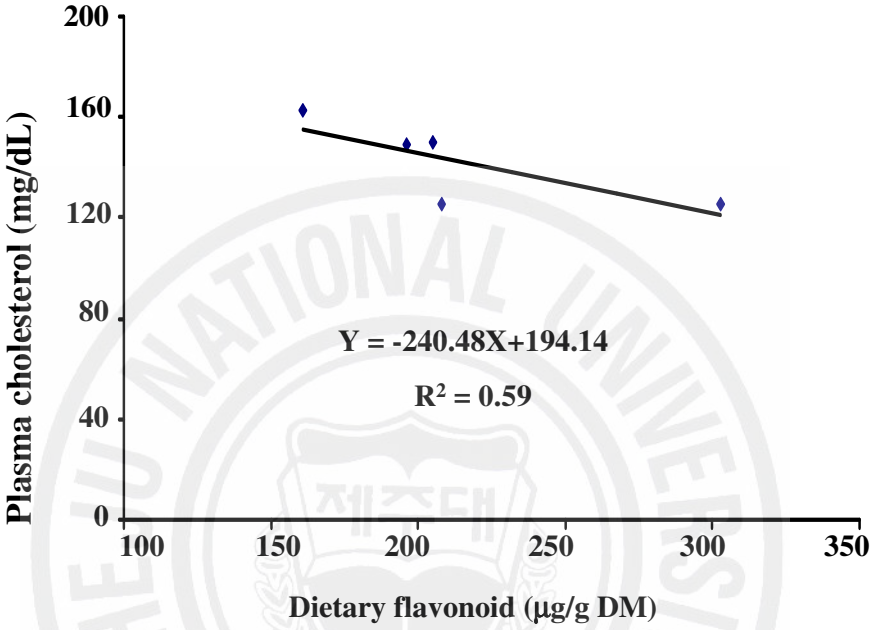

Figure 3.3. Relationship between concentration of dietary flavonoids and plasma cholesterol concentration in juvenile parrot fish fed the diets containing fermented soybean meal with microorganisms for 6 weeks

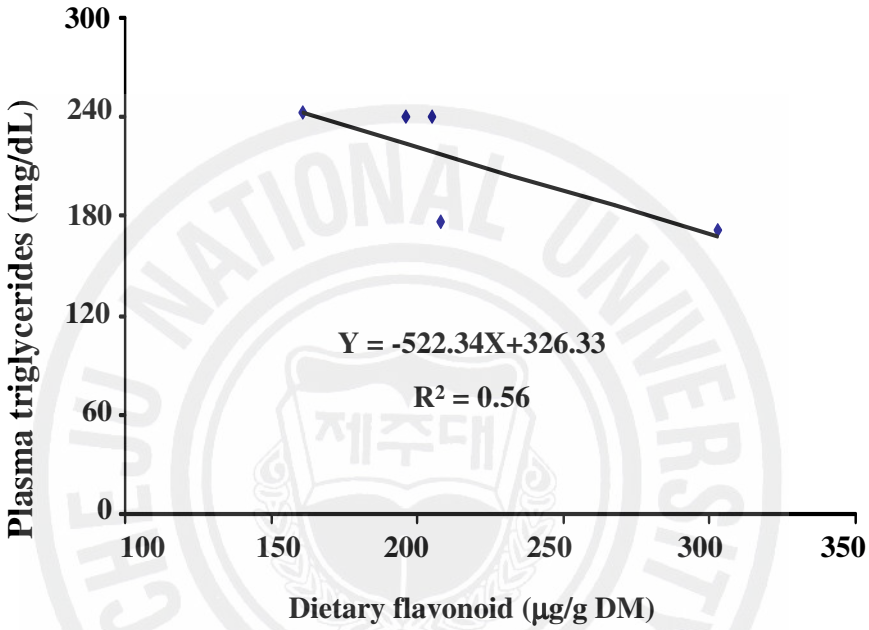

Figure 3.4. Relationship between concentration of dietary flavonoids and plasma triglyceride concentration in juvenile parrot fish fed the diets containing fermented soybean meal with microorganisms for 6 weeks

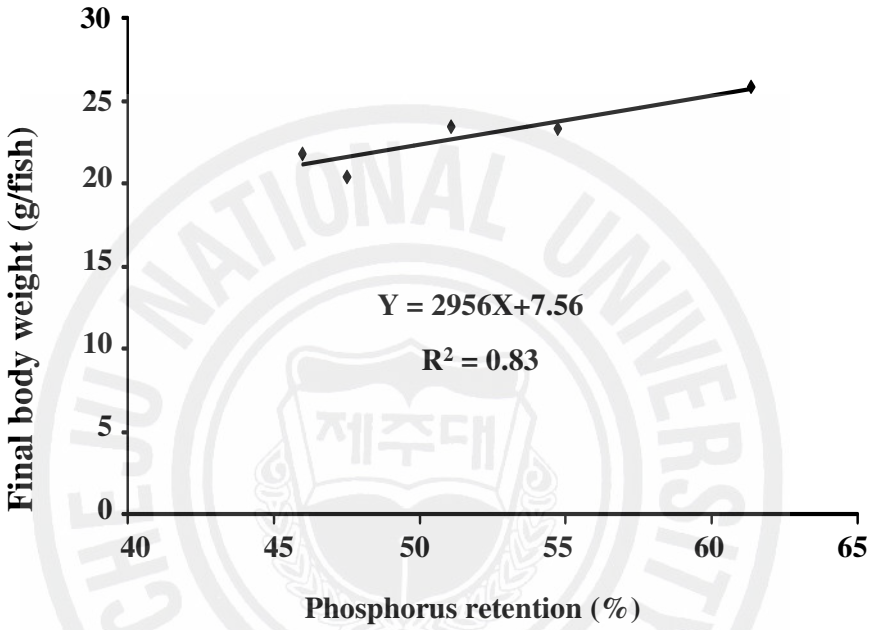

Figure 3.5. Relationship between dietary phosphorus retention and final body weight gain in juvenile parrot fish fed the diets containing fermented soybean meal with microorganisms for 6 weeks

3.4. DISCUSSION

Protein solubility, nitrogen and phosphorus retention showed significant differences among the F-SBM diets. Frias et al. (2008) elucidated that reduction of protein solubility in fermented soybean could be attributed by microorganisms and enzymes by easily hydrolyzing of soluble protein. However, the lower protein solubility of the F-SBM in the present feeding trial could be more associated to the inconsistent in temperature rather than the effects of different microorganisms. Similar finding also was well demonstrated by Lee et al. (2007).

Fish species respond differently with different processed SBM and SBM products (Boonyaratpalin et al., 1998; Kasper et al., 2007; Kaushik et al., 1995; Refstie et al., 1999; Refstie et al., 2000). In the present feeding trial, growth performance of fish fed the experimental diets containing F-SBM was significantly lower than that of the control diet meanwhile feed intake was significantly higher in the F-SBM groups. It suggests that extremely high temperature (100 ^{o}C for 60 min) significantly decreased protein solubility of the F-SBM and resulted in lower growth performance of fish. Lower nitrogen retention in the fish fed the diets contained F-SBM could be a consequence of reduced protein solubility. Protein solubility was crucial index for evaluating heat-treated soybean protein quality *in vivo* and significantly decreased with increase of heat-treatment (Lee et al., 2007; Parsons et al., 1991; Parsons et al., 1992; Radha et al., 2008). Parson et al., (1991) concluded that protein solubility of autoclaved SBM quadratically decreased with increase of autoclaving period and significantly influenced feed efficiency ratio of pigs. Excessive heat treatment also can cause the decomposition of amino acids, activate

oxidation of cysteine and methionine and facilitate the Maillard reaction between the lysine and aldehyde groups of carbohydrates, resulting in growth retardation of animals and decreased feed efficiency (Parsons et al., 1992). Lee et al. (2007) reported that extremely heat-treated soyflakes significantly decreased digestibility in both nitrogen and energy and growth performance of pigs. Radha et al. (2008) interpreted that lower solubility in the heat-treated SBM was consequence of the formation of soy protein aggregates. The aggregates might have caused an allergic reaction and induce abnormality in digestive tract of the fish. Therefore, it was recommended that investigations the histology of digestive tract of fish fed diets containing F-SBM should be included (Baeverford and Krogdahl, 1996; Boonnyaratpalin et al., 1998; Evans et al., 2005; Storebakken et al., 2000). Growth performance of parrot fish fed the experimental diets, in the present feeding trial, also showed a highly positively relationship with retention of dietary phosphorus (R^2 = 0.82), however, there were no significant differences in dietary total and inorganic phosphorus. In our best knowledge, there have been no evidences on the effects of the tested microorganisms on growth performances of fish. The finding suggests that over heat-treatment might reduce availability of phosphorus and cause amino acid loss and consequently decrease growth performance of juvenile parrot fish.

Whole body moisture, protein and ash contents did not differ among fish groups fed all the experimental diets. However, whole body lipid content in fish fed the control was significantly higher than that of fish fed diet SBM-SC, SBM-PP and SBM-BS. However, dietary crude protein, lipid and ash contents are the same among the experimental diet. Hossain et al. (2001) reported that whole body lipid content of

common carp fed untreated Sesbania meal was significantly higher than that of fish groups fed autoclaved one. It is possible that the production of aggregates in extremely heated SBM might have reduced the nutrients digestibility including lipid and resulted in reduction of whole body lipid concentration.

In the present feeding trial, SBM or F-SBM with different microorganisms did not influence morphological parameters of juvenile parrot fish. Hossain et al. (2001) found that no significant differences in hepato somatic index of common carp fed the diets contained different inclusion levels of untreated, soaked and soaked and autoclaved Sesbania meal. Tibaldi et al. (2006) reported that different processed SBM did not affect morphological parameters in European sea bass.

Triglycerides and cholesterol lowering effects of SBM have been reported in many studies and it was suggested that triglycerides and cholesterol lowering effects of SBM consumption could be attributed by many dietary components including nutrients, non-nutrients and endocrines (Ali et al., 2004; Anderson and Wolf, 1995; Chisholm et al., 2005; Dias et al., 2005; Golgberg et al., 1982; Hossain et al., 2001; Kaushik et al., 1995). In the present feeding trial, no significant differences were observed in plasma triglycerides and cholesterol concentrations among fish groups fed the experimental diets. Plasma triglycerides and cholesterol concentration of fish fed diet SBM-SC, SBM-PP and SBM-BS were significantly higher than that of fish fed the control diet and negatively related to dietary flavonoids concentrations (R^2 = 0.59, 0.56, respectively). The present results are well in agreement with findings of Ali et al. (2004) and Manzoni et al. (2005). The authors reported that plasma cholesterol concentration in rats was decreased by soy isoflavones consumption.

Jenkins et al. (2008) demonstrated that plant sterols contributed over one third of LDL-cholesterol reduction. Meanwhile, Kaushik et al. (1995) demonstrated that cholesterol lowering effects of dietary SBM consumption in rainbow trout mainly associated to soy protein concentrate. Hossain et al. (2001) concluded that the hypocholesterolemic response of common carp in different dietary groups contained untreated and treated Sesbania meal might be related to the dietary content of non-starch polysaccharides which results in highly viscosity in the digestive tract of fish. Up to now, metabolism and regulation mechanism of cholesterol in fish is still ambiguous.

Dietary supplementation of fermented products has been extensively reported to be able to enhanced immune response and disease resistances in some fish species (Ashida et al., 2002; Ashida and Okimasu, 2005; Ashida et al., 2005; Pham and Lee, 2007). In the present feeding trial, fish fed the SBM-AO and SBM-PP diets had higher blood nitroblue tetrazolium (NBT) activity compared to that of the control and there were no significant differences in blood NBT activity among fish groups fed the F-SBM with different microorganisms. No significant differences were observed in liver superoxide dismutase and lipid peroxidation in fish fed all the experimental diets. However, significantly higher liver catalase activity was found in the fish fed the F-SBM compared to that in the control diet. Liver DPPH radical scavenging activity was significantly enhanced by the consumption of the diets containing F-SBM. Concentrations of polyphenol compounds in the diets containing the F-SBM were significantly higher than that in the control diet. It suggests that the increase of blood cells NBT and liver catalase activities in fish fed the F-SBM could associate to

the dietary antioxidant capacity. Yang et al. (2000) reported that antioxidant properties of the F-SBM were superior to those of SBM and F-SBM could be potential antioxidant sources in functional foods. Ashida and Okimasu (2005) revealed that superoxide generation of intraperitoneal leukocytes in olive flounder was significantly increased by the administration of the fermented plant products and probably related to its antioxidant capacity. Although, the enhancement of immune response in the fish fed the F-SBM could be attributed by the micro-organisms. Toaka et al. (2006a) found that immune responses of tilapia fed the diet supplemented with the commercial probiotics contained *B. subtilis, L. acidophilus, Clostridium butyricum* and *S. cerevisiae* were significantly higher compared to the control. Toaka et al. (2006b) re-confirmed that the administration of the commercial probiotics also enhanced the stress tolerance and non-specific systems of Japanese flounder. The present results suggest that non-specific immune response of parrot fish could be affected by several dietary factors including microorganisms and antioxidants and their interactions.

3.5. CONCLUSION

The present results indicate that the diet containing the fermented soybean meal with microorganisms could enhance the nonspecific immune responses juvenile parrot fish after 6 week-feeding trial. *Aspergillus oryzae* showed superior to the other strains. However, proper steaming temperature for soybean meal without adverse effects on its nutrient value is necessary to investigate.

CHAPTER IV

EFFECTS OF FERMENTED COTTONSEED AND SOYBEAN MEAL WITH *Aspergillus oryzae* ON HEMATOLOGICAL VALUES OF JUVENILE PARROT FISH, *Oplegnathus fasciatus*

ABSTRACT

A six week feeding trial was conducted to investigate the effects of cottonseed and soybean meal (CS) and fermented cottonseed and soybean meal (F-CS) with *Aspergillus oryzae* on growth performance, feed utilization, hematological parameters and immune response of juvenile parrot fish, *Oplegnathus fasciatus*. Based on the findings in our previous experiments, seven isonitrogenous (45% crude protein) and isocaloric (17.7 MJ/kg) experimental diets (designated as CS0, CS20, CS30, CS40, F-CS20, F-CS30 and F-CS40, respectively) were formulated to replace 20%, 30%, 40% of dietary fish meal by CS and F-CS. The CS0 diet without replacement of fish meal protein was considered as the control diet. Triplicate groups (20 fish per group) of fish (initial body weight of 22.6 g/fish) were fed one of the experimental diets for 6 weeks. At the end of the feeding trial, there were no significant differences in growth performance and feed utilization between fish groups fed the diets CS0, CS20 and CS30. However, the fish groups fed the diets CS40, F-CS20, F-CS30 and F-CS40 showed significantly lower growth performance and feed utilization compared to the other fish groups and the control diets. F-CS did not affect the survival and muscle compositions of juvenile parrot fish. Hepato somatic index of fish was decreased along with the increase of dietary CS, regardless of the fermentation process. Serum protein concentration in fish groups fed the F-CS diets were significantly lower than that of the other fish groups. Blood nitro blue tetrazolium (NBT) activity showed increasing tendency along with the increase of dietary CS and F-CS. The fish groups fed the F-CS40 diet show highest NBT activity compared to the control. Plasma glucose concentration had a positively relation with

the dietary CS inclusion levels. Meanwhile, concentration of plasma cholesterol and triglyceride was negatively associated with dietary CS contents. Phosphorus retention in the fish groups fed the diets F-CS30 and F-CS40 was significantly lower than that of the other diets. No significant differences were observed in liver superoxide dismutase activity and lipid peroxidation. Liver antioxidant activity showed slightly increase in fish groups fed the diets containing CS and F-CS. Methanol extract yield, polyphenol compounds content and antioxidant activities of CS diets were dramatically increased during fermentation process. The present results suggest that (1) CS might replace up to 30% of fish meal in the diet for juvenile parrot fish; (2) fermentation process significantly enhances the antioxidant capacity of CS which could be used as immuno-stimulants for fish; (3) the decline in growth performance of fish fed the F-CS diets probably related to the anti-nutritional factors and the loss of some essential amino acids due to over-heat treatment. Therefore, a proper fermentation process including heating, incubation periods should be further investigated.

4.1. INTRODUCTION

Aquaculture is the most rapidly extending animal food producing sector and feed expenses contribute major production costs (Coyle et al., 2004), because of the use of the expensive fish meal protein with a large dietary proportion. The shortage of fish meal has resulted in the increase of fish meal price over 30% in 2006 compared to the last two year (personal communication). Many studies recently have been conducted to search for less expensive protein sources to replace fish meal protein (Kikuchi et al., 1994; Kikuchi 1999; Choi et al., 2004; Pham et al., 2007). Hardy (1995) reported that dietary replacement of fish meal protein by plant origin by-products, such as soybean meal, cottonseed meal and rapeseed meal, has been increasing in aquaculture industry, due to their low price, highly market availabilities and sufficient protein contents.

Among plant protein sources, soybean meal (SM) has been used predominantly in diets for number of fish species (Degani et al., 1997; Refstie et al., 2000; Catacutan and Pagador, 2004; Chou et al., 2004; Zhou et al., 2005; Tomas et al., 2005; Pham et al., 2007). Studies have demonstrated that soybean meal alone or in combination with other protein sources can replace from 20% to 90% fish meal protein in diets for many fish species, such as yellow tail (Shimeno et al., 1993), red drum (McMoogan and Gatlin, III, 1997), seabass (Boonyaratpalin et al., 1998), rainbow trout (Gomes et al., 1995), Australian snapper (Quantararo et al., 1998), cobia (Chou et al., 2004) and olive flounder (Kikuchi et al., 1994; Kikuchi 1999; Saitoh et al., 2003; Pham et al., 2005). Kikuchi (1999) reported that 45% fish meal protein can be replaced by soybean meal in combination with other animal protein

sources in diets for juvenile olive flounder. However, the use of soybean meal in fish feeds, particularly in carnivorous marine fish species is still limited because of the presence of anti-nutritional factors, such as protease inhibitors, phytates, lectins, saponins, non-starch polysaccharide and high fiber content (NRC 1993; Storebakken et al., 2000; Francis et al., 2001; Hendricks 2002). In addition, limiting essential amino acids in soybean meal, such as methionine and lysine, reduce its inclusion level in fish feeds (NRC 1993).

Cottonseed meal (CM), a by-product of cottonseed processing, has been examined in diets for many fish species, such as olive flounder (Pham et al., 2005; 2007), channel catfish (Dorsa et al., 1982; Robinson and Brent, 1989; Robinson and Li, 1994; Robinson and Tiersch, 1995), rainbow trout (Hendricks et al., 1980; Lee and Dabrowski, 2002; Rinchard et al., 2003) and tilapia (El-Sayed 1990; Robinson et al., 1984; Mbahinzireki et al., 2001; Rinchard et al., 2002). Despite its high nutritional value, cottonseed meal contains gossypol which is toxic to fish (Herman 1970), leading to restriction of its use. On the other hand, gossypol was also reported to have high antioxidant activity (Rhee et al., 2001) as well as anticarcinogenic activities (Benz et al., 1990; Shelley et al., 1999). Many studies recently have been reported that the fermentation process could degrade anti-nutritional factors in plant protein sources and improve their antioxidant capacity, particularly they could be apply in functional foods.

The findings in the previous experiments demonstrated that *Apsergillus oryzae* was superior to the other tested microorganisms and it might increase phosphorus utilization and enhance immune response of juvenile parrot fish fed the diets

contained plant protein sources.

Therefore, the aim of this feeding trial was to investigate the effects of fermented cottonseed and soybean meal with *A. oryzae* as partial replacer for dietary fish meal protein on growth performance, feed utilization, hematological parameters and immune response in juvenile parrot fish, *Oplegnathus fasciatus*.

4.2. MATERIALS AND METHODS

4.2.1. Fermentation of cottonseed and soybean meal

Process of making fermented cottonseed meal and soybean meal (CS) are described in Chapter III. CS were finely ground using a milling machine (M12, Seoul, Korea). One part of CS was soaking in three parts of distilled water for 1 h and steamed at 100 ^{o}C for another 1 h using a laboratory steaming system (Fig. 4.1). The steamed CS were cooled down in an incubator (MIR-253, Sanyo, Japan) at 40 ^{o}C for 2 h before *Aspergillus oryzae* was inoculated at 3.5% of CS in dry matter. The *A. oryzae* inoculated CS was made into a brick shape (3 cm x 15 cm x 10 cm) and incubated at 30 ^{o}C for 8 h and at 28 ^{o}C for 40 h since the yellow layer occurred. The fermented CS was dried at room temperature for 72 h and finely ground prior to supplementation into the experimental diets.

4.2.2. Experimental diets

Seven experimental diets were formulated to be isonitrogenous and isocaloric in terms of crude protein (45% crude protein) and gross energy (17.7 MJ/kg). The energy value of each diet was estimated on the basis of mammalian physiological fuel value, i.e., 16.7 KJ/g protein or carbohydrate and 37.7 KJ/g lipid (Lee and Putman, 1973). Proximate compositions of cottonseed meal (CM), soybean meal (SM), fermented cottonseed meal (F-CM) and fermented soybean meal (F-SM) are given in Table 4.1. Total gossypol concentration in CM was 1.65%. The dietary formulation and proximate compositions are provided in Table 4.2 and 4.3. The experimental diets were formulated to replace 0, 20, 30, and 40% of fish meal protein

by CS and fermented cottonseed mean (F-CS) and designated as CS0, CS20, CS30, CS40, F-CS20, F-CS30 and F-CS40, respectively. The CS and F-CS containing diets were supplemented with methionine and lysine to meet their requirements (NRC 1993). Experimental diets were pelleted through the meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size, freeze dried to approximately 5% moisture, crushed into desirable particle sizes (0.4 – 2.0 mm) and stored at –20 ^{o}C until use.

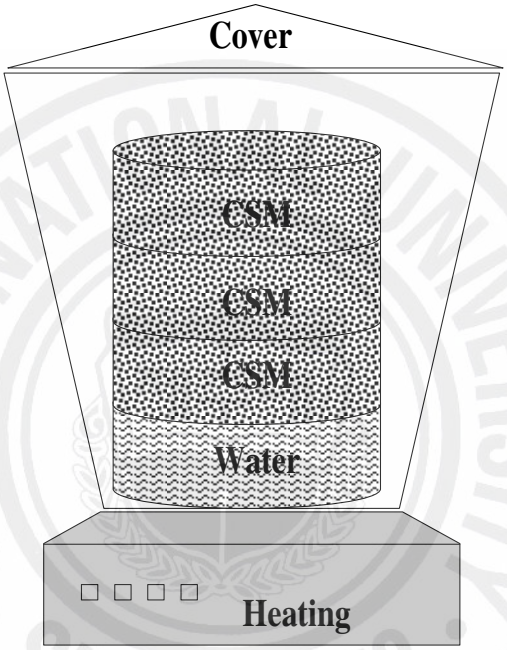

Fig. 4.1. Steaming system

Fig. 4.2. Fermented cottonseed and soybean meal with *Apergillus oryzae*

Table 4.1. Proximate compositions and pH of soybean meal (SM), cottonseed meal (CM), fermented soybean (F-SM) and fermented cottonseed meal (F-CM)

Diets	**SM**	**CM**	**F-SM**	**F-CM**
Moisture (%)	12.37	13.01	20.55	16.39
Protein (% DM)	50.54	48.44	58.33	49.27
Lipid (% DM)	1.33	5.11	1.89	5.85
Ash (% DM)	7.02	7.26	8.87	9.06
pH	-	-	6.55	6.98

DM: dry matter.

Table 4.2. Dietary formulation of the experimental diets (% in DM basis)

	Diets						
Ingredients	**CS0**	**CS20**	**CS30**	**CS40**	**F-CS20**	**F-CS30**	**F-CS40**
White fish meal	52.0	41.6	36.4	31.2	41.6	36.4	31.2
Soybean meal	0.0	8.0	12.0	16.0	8.0	12.0	16.0
Cottonseed meal[1]	0.0	8.2	12.3	16.4	8.2	12.3	16.4
Corn gluten meal	8.0	7.5	7.0	6.5	7.5	7.0	6.5
Starch	22.0	18.0	14.8	11.0	18.0	14.8	11.0
Yeast	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Mineral premix[2]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Vitamin premix[3]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Squid liver oil	11.0	11.0	11.6	12.2	11.0	11.6	12.2
Lysine	0.0	0.4	0.6	0.8	0.4	0.6	0.8
Methionine	0.0	0.2	0.3	0.4	0.2	0.3	0.4
Ferrous Sulfate-7H2O	0.0	0.1	0.2	0.3	0.1	0.2	0.3
Monocalciumphosphate	0.0	0.4	0.8	1.2	0.4	0.8	1.2
Cellulose	3.0	0.6	0.0	0.0	0.6	0.0	0.0

[1] Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.

[2] Mineral premix was mentioned in Chapter I.

[3] Vitamin premix was mentioned in Chapter I.

F-CS20, F-CS30, and F-CS40: CS fermented with *A. oryzae*.

Table 4.3. Proximate compositions of the experimental diets*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
Proximate composition							
Dry matter (%)	93.7	93.5	93.3	93.5	93.3	93.4	93.6
Protein (%, DM)	46.5	47.4	46.2	45.9	46.9	47.2	47.0
Lipid (%, DM)	14.7	15.2	15.0	15.4	14.8	15.0	14.9
Ash (%, DM)	8.5	7.8	8.1	7.7	8.6	8.5	9.1
Gross energy (MJ/kg)	17.7	17.8	17.8	17.7	17.8	17.8	17.7
Protein solubility (%)	75.1^{bc}	73.2^{c}	82.7^{a}	84.0^{a}	68.1^{d}	76.9^{b}	72.9^{c}

*Values in the same row having different letters are significantly different at $P < 0.05$.

DM: dry matter.

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

4.2.3. Fish and feeding trial

Parrot fish juveniles were transported from a private hatchery in Jeju Island to Marine and Environmental Research Institute, Cheju National University, Korea. The fish were fed with a commercial diet for 4 weeks to allow adaptation to experimental condition. Fish (mean body weight of 22.6 g/fish) were randomly distributed into twenty one 150 L polyvinyl tanks (20 fish per tank) in a flow through system supplied with sand filtered seawater at a flow rate of 3L/min. One of the experimental diets was fed triplicate groups of fish to apparent satiation (twice per day) for six weeks. Aeration was also provided to maintain dissolved oxygen levels near to the saturation. The growth of fish was measured every 3 weeks.

4.2.4. Muscle proximate compositions

At the end of feeding trial, all fish were weighed for the calculation of weight gain, feed conversion ratio, protein efficiency ratio and specific growth rate. Three fish per tank (9 fish per diet) were sampled and stored at –20 ^{o}C for muscle proximate analysis. Analysis of crude protein, moisture and ash were performed by the standard procedures (AOAC 1995). Lipid was measured using a Soxhlet System (SH6, Korea).

4.2.5. Morphological parameters

The total length, whole body weight, liver weight and gonad weight of 9 fish per diet were individually measured. Calculation of condition factor (CF), hepato somatic index (HSI), and gonad somatic index (GSI) was described in Chapter III.

4.2.6. Hematological parameters

At the end of feeding trial, 3 fish per tank (9 fish per diet) were randomly selected and anaesthetized in tricaine methane sulfonate (MS-222) solution (100 mg/L). Blood were taken from caudal veins with heparinised syringes. Hematocrit was determined using microhematocrit technique. Hemoglobin, plasma protein, cholesterol, triglyceride, and glucose concentrations were measured using a Photometer CH100 Plus (Calenzano, Firenze, Italy).

Plasma lysozyme activity in parrot fish fed the experimental diets was measured using a colorimetrical method. Plasma sample was added in *Micrococcus lysodeikticus* suspension (0.2 mg/ml *M. lysodeikticus* in 0.02 M sodium citrate buffer) at 1: 10 ratio. Initial optical density (OD) was measured immediately after adding plasma. After incubating for 5 min at room temperature, final OD was measured at 450 nm using a spectrophotometer (DU 730, Beckman, USA). Hen egg white lysozyme (HEWL, Sigma) was used as standard. Lysozyme values were expressed as µg/ml equivalent of HEWL activity.

Another portion of the heparinized blood was used for nitro tetrazolium assay according to Anderson and Siwicki (1995) and Kumari and Sahoo (2005).

4.2.7. Measurement of phosphorus

Phosphorus concentration was measured using a method described by Nahapetian and Bassiri (1975). Phosphorus retention was calculated as the equation described by Nordrum et al. (1997): Dietary phosphorus retention (%) = 100 x (final body phosphorus - initial body phosphorus)/dietary phosphorus consumed.

4.2.8. Iron and heme iron analysis

Total dietary and whole body iron concentrations were measured according to a colorical method as described by Turhan et al. (2004). Sample was combusted at 450 ^{o}C in a muffle furnace (Ceber, Daihan Scientific Co., Ltd., Korea) for 12 h. After cooling down, concentrated HNO_3 (1 mL) was added, heated at 80 ^{o}C for 12 h. The digested sample was again combusted at 450 ^{o}C for 2 h. Then concentrated HCl was added and kept at room temperature for 2 h. The reaction mixture contained diluted sample (2 mL), 3% hydroxylamine hydrochloride solution (1 mL) and total iron color reagent (1.5 mL) and left at room temperature for 10 min. Optical density was measured at 533 nm using an UV/VIS spectrophotometer (DU 730, Beckman, USA). Total iron content was estimated according to Gomez-Basauri and Regenstein (1992).

Heme iron concentration in the experimental diets was measured using the method established by Hornsey (1956) with modification of Clark et al. (1997). Dietary sample was homogenized using a blender (Polytron MR-2100, Switzerland) and extracted in acid- acetone mixture for 1 h. The extract was centrifuged at 2200 x g for 10 min. The supernatant was filter using a syringe filter (0.45 μm, Whatman Inc., Clifton, NJ) and the optical density was measured at 640 nm. The heme iron content was calculated with the factor of 0.0882 μg iron/μg hematin (Merc 1989).

4.2.9. Methanol extract yield

Five gram of diets was extracted in 100 ml methanol for 24 h in a shaking incubator at room temperature. The mixture was filtered through a Whatman filter paper (Whatman #4) and dried using a rotary vacuum drier to remove the methanol.

4.2.10. Antioxidant capacity assay

Antioxidant capacity of experimental diets, fish liver and muscle was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay as described by Brand-Williams et al. (1995).

4.2.11. Total dietary polyphenol compounds

Total polyphenol compounds in the experimental diets were measured by a colorimetric method described by Skerget et al. (2005).

4.2.12. Reducing activity

Reducing activity of the experimental diets was measured as a method described by Oyaizu (1986). Filtered extract (0.3 mL) was mixed with 0.3 mL of 1.0% potassium ferricyanide and 0.3 mL sodium phosphate buffer (0.2 M, pH 6.6). The mixture was incubated at 50 ^{o}C for 20 min. Then, 10% trichloroacetic acid (0.3 mL) was added and centrifuged at 6000 rpm for 10 min at 4 ^{o}C. The supernatant (0.6 mL) was mixed with 0.1% ferric chloride solution (0.12 mL) and deionized water (0.6 mL). The mixture was incubated at room temperature for 10 min and the OD was read at 700 nm by a spectrophotometer (Genesys 10 UV, Rochester, NY, USA).

4.2.13. Ferrous chelating activity

Reaction mixture containing dietary extract (1.0 mL), 3.7 mL methanol, 2 mM $FeCl_2$ (0.1 mL) and 5 mM ferrozine (0.2 mL) was incubated at room temperature for 10 min (Decker and Welch, 1990). Chelating activity was expressed as percent.

4.2.14. Measurement of liver thiobarbituric acid reactive substances (TBARS)

Liver TBARS in fish fed the experimental diets was measured according to method of Burk et al. (1980) and modified by Tocher et al. (2002). Liver (30 mg) was homogenized in 1.5 mL of 20% (w/v) trichloroacetic acid containing 0.05 ml of 1% butylated hydrotoluene in ethanol. To the homogenate, 2.95 mL of freshly prepared 10 mM thiobarbituric acid was added and heated at 100 ^{o}C for 10 min. Protein was removed by centrifugation of 12000 x g. OD was measured at 532 nm. The concentration of TBARS, expressed as μM TBARS/g liver, was calculated using the extinction coefficient of 0.156 $\mu M^{-1}cm^{-1}$.

4.2.15. Measurement of liver superoxide dismutase activity

Liver superoxide dismutase activity was measured using a Cayman kits (Cayman, MI, USA). The reaction mixture composing of radical detector solution (200 μL), dilute sample extract (10 μL) and xanthine oxidase (20 μL) was incubated in 96-microwell plate for 20 min. OD was read using a microwell reader (Multiskan Ex, Thermo Electron Corporation, Shanghai, China).

4.2.16. Statistical analysis

Data were subjected to one-way ANOVA in SPSS version 11.0. Significant differences between group means were compared using Duncan's multiple test. Data were presented as means ± standard deviations (SD). The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significantly at $P < 0.05$.

4.3. RESULTS

Proximate composition of SM, CM, F-SM and F-CM are given in Table 4.1. Crude protein, lipid and ash contents in F-SM and F-CM were numerically increased compared to the SM and CM.

Growth performance and feed utilization of juvenile parrot fish fed the CS20 and CS30 were comparable to that of fish fed the control diet. Fish groups fed the diets CS40, F-CS20, F-CS30 and F-CS40 showed lower final body weight, weight gain, feed conversion and protein conversion ratios than those of fish groups fed the control diet. However, the daily feed intake of fish groups fed the F-CS diets was significantly higher than that in fish groups fed the control and the CS diets. Survival did not differ among fish groups fed all the experimental diets and was greater than 95% at the end of feeding trial (Table 4.4).

Muscle body compositions of parrot fish juveniles fed the experimental diets were not affected by fermentation process, except for whole body ash content. Fish fed the diet CS40 showed significantly lower whole body ash content, compared to that of the control diet (Table 4.5).

Morphological parameters including condition factor, hepato somatic and viscera somatic indexes are presented in Table 4.6. There were no significant differences in condition factor and viscera somatic index of fish fed the experimental diets for 6 weeks. Hepato somatic index (HSI) was decreased with the increase of dietary cottonseed and soybean meal content. HSI of the fish groups fed the CS30, CS40, F-CS30 and F-CS40 diets was significantly lower than that of the control diet.

Hematocrit, hemoglobin and plasma lysozyme activity were not affected by the

dietary inclusion of the CS and F-CS. However, the blood nitro blue tetrazolium activity in fish groups fed the F-CS diets was significantly higher than that of fish fed the control diet (Table 4.7).

Plasma glucose concentration of fish fed the diets contained CS was significantly higher that that of the control diet, regardless of the different treatments. Meanwhile, concentration of plasma cholesterol and triglyceride were lower in the fish groups fed the CS and F-CS diets. Plasma protein concentration was not different among fish groups fed the CS and the control diets (Table 4.8).

Dietary total phosphorus concentration was significantly increased with the increment of dietary CS inclusion levels. The highest values were observed in the diets F-CS30 and F-CS40. The concentration of dietary inorganic phosphorus of the diets contained CS was lower than that in the control diet. The F-CS diets had higher concentration of inorganic phosphorus compared to the CS diets. However, phosphorus retention of fish groups fed the F-CS diets was significantly lower compared to the other dietary groups, except for F-CS20 diet (Table 4.9).

Dietary total iron significantly increased in the CS diets. Heme iron concentration the experimental diets significantly higher than that of the control diet. There were no differences in whole body iron content among the treatment, expect for diet F-CS40. Iron retention in fish groups fed the diets F-CS20 and F-CS30 were statistically lower than that of the other diets. Among the diets CS0, CS20, CS30, CS40 and F-CS40, no differences in iron retention were found (Table 4.10).

Methanol extract yield, polyphenol compounds concentration, and Fe-chelating, reducing and DPPH radical scavenging activities were dramatically improved during

fermentation process (Table 4.11). The F-CS diets had higher antioxidant activities than those in the CS diets and the control diet.

No significant differences were obtained in liver superoxide dismutase activity and lipid peroxidation in among the fish fed all the experimental diets (Fig. 4.3, 4.4). Liver DPPH radical scavenging activity was not affected by the treatments (Fig. 4.5).

Table 4.4. Growth performance and feed utilization of juvenile parrot fish fed the experimental diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
FBW (g/fish)	53.4^{a} ± 0.1	52.5^{a} ± 0.5	52.9^{a} ± 0.7	48.5^{b} ± 0.4	48.2^{b} ± 1.4	47.3^{b} ± 0.7	43.7^{b} ± 1.6
WG (%)[1]	136.3^{a} ± 1.1	132.1^{a} ± 2.4	133.5^{a} ± 3.6	114.3^{b} ± 1.5	114.4^{b} ± 4.3	109.3^{b} ± 3.1	93.1^{c} ± 7.7
SGR (%/day)[2]	0.89^{a} ± 0.0	0.87^{a} ± 0.0	0.88^{a} ± 0.0	0.79^{b} ± 0.0	0.79^{b} ± 0.0	0.76^{b} ± 0.0	0.68^{a} ± 0.0
FI (g/g BW)[3]	0.55^{c} ± 0.0	0.56^{c} ± 0.0	0.57^{c} ± 0.0	0.60^{bc} ± 0.0	0.61^{b} ± 0.0	0.61^{b} ± 0.0	0.66^{a} ± 0.0
FCR (g BW/g DM diet)[4]	1.05^{a} ± 0.0	1.01^{a} ± 0.0	0.98^{a} ± 0.1	0.89^{b} ± 0.0	0.85^{b} ± 0.1	0.85^{b} ± 0.0	0.72^{c} ± 0.1
PER (g BW/g protein)[5]	2.3^{a} ± 0.0	2.1^{a} ± 0.0	2.1^{ab} ± 0.1	1.9^{bc} ± 0.0	1.8^{c} ± 0.1	1.8^{c} ± 0.0	1.5^{d} ± 0.2
Survival (%)	100.0	100.0	96.7	100.0	96.7	100.0	100.0

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different (P<0.05).

F-CS20, F-CS30, and F-CS40: CS fermented with *A. oryzae*.

[1]Weight gain (WG) = 100 x (FBW - IBW)/IBW. [2] Specific growth rate (SGR) = 100 x [(ln FBW - ln IBW)/days].

[3] Protein efficiency ratio (PER) = wet weight gain/total protein given. [4] Feed conversion ratio (FCR) = dry feed fed/wet weight gain.

[5] Feed intake (FI, g/g BW) = dry feed consumed (g)/BW (g). FBW, IBW: Final and initial mean body weight (g/fish), respectively.

Table 4.5. Muscle proximate compositions of juvenile parrot fish fed the experimental diets for 6 weeks[*]

Diets	**CS0**	**CS20**	**CS30**	**CS40**	**F-CS20**	**F-CS30**	**F-CS40**
Moisture (%)	75.6 ± 0.4	75.7 ± 0.5	76.0 ± 0.2	75.9 ± 0.8	76.0 ± 0.3	75.9 ± 0.8	76.6 ± 0.4
Protein (% wet matter)	20.0 ± 0.3	19.9 ± 0.1	20.1 ± 0.4	19.6 ± 0.2	19.7 ± 0.4	20.1 ± 0.3	19.7 ± 0.3
Lipid (% wet matter)	2.82^{ab} ± 0.9	3.34^{a} ± 0.1	3.41^{a} ± 0.5	2.77^{ab} ± 0.2	2.86^{ab} ± 0.6	2.99^{ab} ± 0.5	2.21^{b} ± 0.2
Ash (% wet matter)	1.34^{a} ± 0.0	1.28^{ab} ± 0.0	1.30^{ab} ± 0.0	1.21^{b} ± 0.0	1.27^{ab} ± 0.0	1.30^{ab} ± 0.1	1.29^{a} ± 0.0

[*] Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

F-CS20, F-CS30, and F-CS40: CS fermented with *A. oryzae*.

Table 4.6. Morphological parameters of juvenile parrot fish fed the experimental diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
CF (%)[1]	2.08 ± 0.02	2.22 ± 0.21	2.16 ± 0.08	2.31 ± 0.06	2.18 ± 0.18	2.09 ± 0.11	2.47 ± 0.28
HSI (%)[2]	3.25^{a} ± 0.6	2.84^{ab} ± 0.2	2.46^{bc} ± 0.3	2.26^{c} ± 0.2	2.77^{abc} ± 0.2	2.51^{bc} ± 0.2	2.47^{bc} ± 0.25
VSI (%)[3]	9.87 ± 1.41	9.63 ± 0.49	9.10 ± 0.69	9.49 ± 0.88	9.96 ± 0.51	9.38 ± 0.23	9.21 ± 0.74

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different (P<0.05).

F-CS20, F-CS30, and F-CS40: CS fermented with *A. oryzae*.

[1] Condition factor = fish weight (g) x 100/fish length $(cm)^3$.

[2] Hepato somatic index = 100 x (liver weight/BW).

[3] Viscera somatic index = 100 x (viscera weight/BW).

BW: body weight (g)

Table 4.7. Hematocrit, hemoglobin, blood NBT and plasma lysozyme activity of parrot fish fed the diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
Hematocrit (%)	36.0± 2.2	38.0 ± 4.4	38.8 ± 2.6	35.8 ± 4.0	35.7 ± 0.6	38.8 ± 2.8	38.8 ± 2.1
Hemoglobin (mg/dL)	7.70 ± 0.4	8.94 ± 0.8	8.58 ± 0.7	8.50 ± 0.5	8.29 ± 0.9	9.22 ± 1.2	7.94 ± 0.7
Blood NBT (OD 540nm)	0.73^{b} ± 0.0	0.76^{ab} ± 0.0	0.76^{ab} ± 0.0	0.80^{ab} ± 0.0	0.78^{ab} ± 0.1	0.78^{ab} ± 0.1	0.81^{a} ± 0.0
Plasma lysozyme (μg/mL)	12.73 ± 3.7	13.76 ± 3.2	14.54 ± 3.4	11.06 ± 1.4	11.95 ± 2.0	13.18 ± 1.5	11.97 ± 0.4

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different (P<0.05).

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

Table 4.8. Plasma glucose, cholesterol, triglyceride and serum protein in parrot fish fed the experimental diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
Serum protein (g/dL)	$4.18^{abc} \pm 0.3$	$4.41^{ab} \pm 0.3$	$4.67^{a} \pm 0.5$	$4.36^{ab} \pm 0.4$	$3.92^{bc} \pm 0.2$	$3.97^{bc} \pm 0.1$	$3.67^{c} \pm 0.2$
Plasma glucose (mg/dL)	$85^{b} \pm 11.1$	$110^{ab} \pm 35.3$	$130^{ab} \pm 38.9$	$142^{a} \pm 28.1$	$139^{a} \pm 24.2$	$113^{ab} \pm 20.2$	$156^{a} \pm 20.9$
Plasma cholesterol (mg/dL)	$242^{a} \pm 37.5$	$197^{b} \pm 10.1$	$171^{bc} \pm 12.9$	$154^{cd} \pm 11.5$	$146^{cd} \pm 16.6$	$141^{cd} \pm 10.2$	$133.9^{d} \pm 4.3$
Plasma triglycerides (mg/dL)	$269^{a} \pm 36.0$	$223^{b} \pm 3.9$	$152^{c} \pm 21.2$	$131^{c} \pm 16.1$	$151^{c} \pm 13.9$	$158^{c} \pm 5.9$	$130^{c} \pm 28.6$

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

Table 4.9. Dietary total, inorganic phosphorus and phosphorus retention in parrot fish fed the experimental diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
Pt (mg/g DM diet)	$10.5^{d} \pm 0.3$	$10.9^{c} \pm 0.1$	$11.3^{b} \pm 0.1$	$11.5^{b} \pm 0.1$	$10.8^{c} \pm 0.1$	$12.9^{a} \pm 0.1$	$12.5^{a} \pm 0.1$
Pi (mg/g DM diet)	$10.1^{a} \pm 0.3$	$8.3^{c} \pm 0.2$	$8.0^{c} \pm 0.1$	$8.1^{c} \pm 0.3$	$9.2^{b} \pm 0.1$	$9.1^{b} \pm 0.4$	$8.9^{b} \pm 0.4$
FWBP (mg/DM tissue)	25.8 ± 1.3	26.0 ± 4.2	25.8 ± 0.8	28.9 ± 2.0	28.0 ± 1.8	26.3 ± 1.2	29.5 ± 1.3
Phosphorus retention (%)	$60.5^{a} \pm 0.3$	$60.6^{a} \pm 0.9$	$57.1^{a} \pm 3.6$	$61.2^{a} \pm 1.1$	$56.6^{a} \pm 4.6$	$42.1^{b} \pm 1.4$	$44.2^{b} \pm 6.1$

* Values are presented as mean ± SD. Values in the same row having different letters are significantly different ($P<0.05$).

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

Pt: Dietary total phosphorus

Pi: Dietary inorganic phosphorus

FWBP: Final whole body phosphorus

Table 4.10. Dietary total, heme iron and iron retention in parrot fish fed the experimental diets for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
Total iron (µg/g DM diet)	$83.0^{d} \pm 2.6$	$102.7^{c} \pm 2.0$	$111.5^{b} \pm 2.4$	$116.1^{a} \pm 2.2$	$102.3^{c} \pm 2.7$	$112.4^{a\,b} \pm 2.8$	$114.8^{ab} \pm 2.2$
Heme iron (µg/g DM diet)	$2.5^{b} \pm 0.2$	$2.7^{a\,b} \pm 0.6$	$3.4^{ab} \pm 0.2$	$3.4^{ab} \pm 0.6$	$3.3^{ab} \pm 0.6$	$3.6^{a} \pm 0.8$	$3.5^{a} \pm 0.4$
FWBI (µg /DM tissue)	$33.5^{b} \pm 3.9$	$35.0^{b} \pm 4.9$	$35.5^{b} \pm 2.4$	$40.6^{ab} \pm 7.3$	$36.6^{b} \pm 3.1$	$34.3^{b} \pm 1.3$	$44.5^{a} \pm 2.7$
Iron retention (%)	$6.1^{a} \pm 0.1$	$5.9^{a} \pm 0.1$	$6.0^{a} \pm 0.5$	$6.6^{a} \pm 0.2$	$4.1^{b} \pm 0.6$	$3.8^{b} \pm 0.2$	$5.9^{a} \pm 0.6$

* Values are presented as mean ± SD of triplicates. Values in the same row having different letters are significantly different ($P<0.05$).

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

FWBI: Final whole body iron

Table 4.11. Methanol extract yield, polyphenol compounds content and Fe-chelating, reducing and DPPH radical scavenging activities in the experimental diets fed parrot fish for 6 weeks*

Diets	CS0	CS20	CS30	CS40	F-CS20	F-CS30	F-CS40
ME (mg/g DM diet)	$4.25^{f} \pm 0.12$	$6.27^{de} \pm 1.5$	$5.86^{e} \pm 0.6$	$8.12^{d} \pm 1.6$	$12.39^{c} \pm 0.1$	$12.99^{b} \pm 0.0$	$13.67^{a} \pm 0.1$
Polyphenol (mg/g DM diet)	$2.77^{e} \pm 0.1$	$3.93^{d} \pm 0.0$	$4.01^{d} \pm 0.1$	$4.25^{c} \pm 0.1$	$7.59^{ab} \pm 0.4$	$7.71^{ab} \pm 0.1$	$6.79^{b} \pm 0.5$
Fe-chelating (%)	$0.0^{f} \pm 0.0$	$6.37^{e} \pm 1.4$	$17.59^{bc} \pm 0.7$	$22.27^{a} \pm 2.3$	$19.99^{ab} \pm 1.8$	$13.40^{d} \pm 0.7$	$16.52^{cd} \pm 1.1$
Reducing activity (OD 700nm)	$0.850^{e} \pm 0.0$	$0.960^{d} \pm 0.0$	$1.118^{c} \pm 0.0$	$1.231^{b} \pm 0.0$	$1.376^{a} \pm 0.0$	$1.371^{a} \pm 0.1$	$1.373^{a} \pm 0.0$
DPPHRS (% inhibition)	$10.3^{e} \pm 0.7$	$17.6^{d} \pm 0.2$	$20.2^{c} \pm 1.4$	$31.4^{b} \pm 0.4$	$47.1^{a} \pm 5.7$	$47.3^{a} \pm 2.5$	$48.8^{a} \pm 0.1$

* Values are presented as mean ± SD of triplicates. Values in the same row having different letters are significantly different ($P<0.05$).

F-CS20, F-CS30 and F-CS40: CS fermented with *A. oryzae*.

ME: Methanol extract yield.

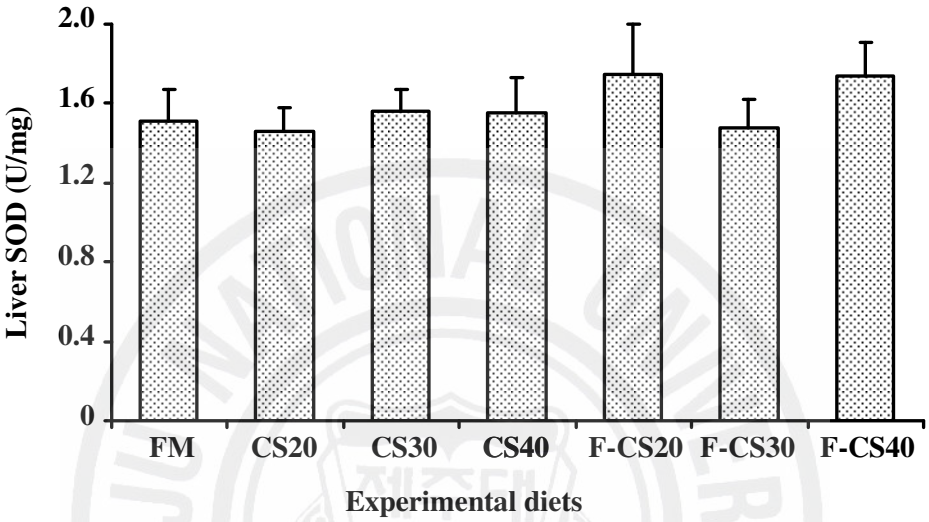

Fig. 4.3. Liver superoxide dismutase activity in parrot fish fed the diets for 6 weeks

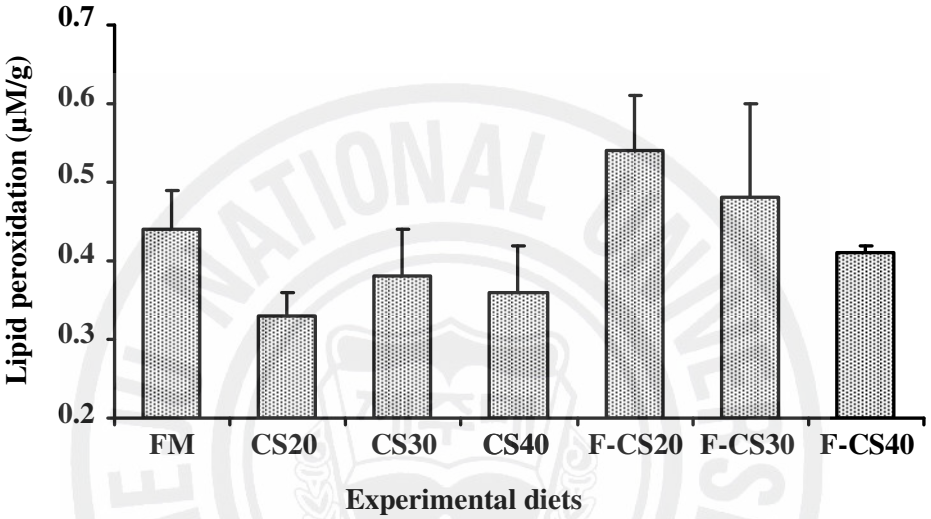

Fig. 4.4. Lipid peroxidation in liver of fish fed the experimental diets for 6 weeks

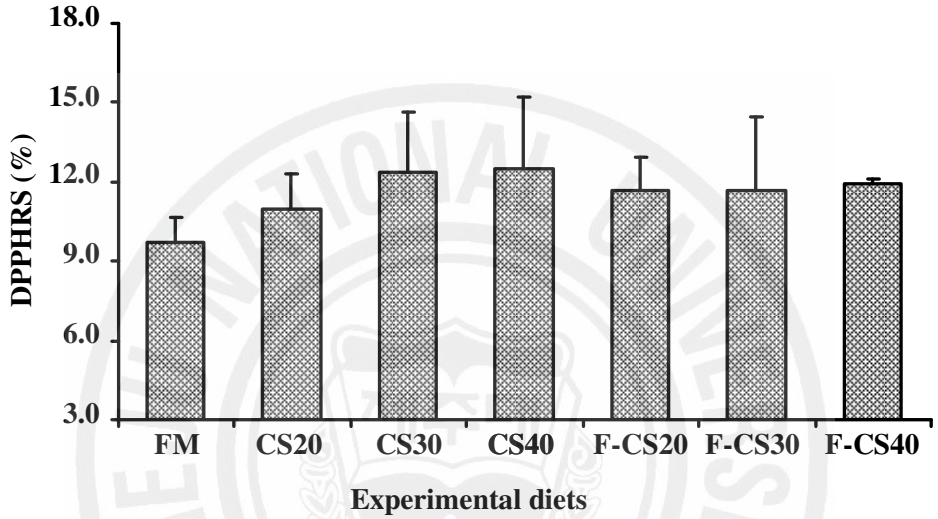

Fig. 4.5. DPPH radical scavenging activity in liver of fish fed the experimental diets for 6 weeks

4.4. DISCUSSION

Fermentation process has been used to improve nutritional quality and flavor of plant protein sources including legumes and cereals. In the present feeding trial, crude protein, lipid and ash contents in both F-SM and F-CM were numerically increased compared to SM and CM, respectively (Table 4.1). The finding was well in agreement with many studies. Zhang et al. (2007) reported that the fermentation significant affected the chemical composition of soybean. Yousif and El Tinay (2001) demonstrated that protein concentration in the fermented sorghum was slightly increased after 24 h incubation. They interpreted that the kirsa fermentation could attribute to the increment of sorghum protein concentration.

In the present feeding trial, growth performance and feed utilization of juvenile parrot fish fed the CS20 and CS30 were comparable to those of fish fed the control diet. The fish fed the diets F-CS20, F-CS30, F-CS40 and CS40 showed lower final body weight, weight gain, feed conversion and protein efficiency ratios, compared to those in fish groups fed the control diet. However, daily feed intake of the fish groups fed the F-CS diets was significantly higher than that in fish groups fed the control and CS diets. No differences were observed in survival among the fish groups fed all the experimental diets. Survival was greater than 95% in all fish groups at the end of feeding trial (Table 4.4). It suggests that fermentation of CS did not produced any toxic to the fish, however, there might be some anti-nutritional factors (ANFs) which caused the decrease of growth performance and feed utilization in parrot fish fed the CS40 and the F-CS diets. Firstly, higher ANFs, such as phytic acid and gossypol, could result in lower growth performance of the fish

groups fed the diet CS40 and F-CS40. Poor growth performance and feed utilization were also found in juvenile olive flounder fed the diets incorporated up to 40% of CS with or without supplementation of exogenous phytase (Pham et al., 2005; 2007 and Pham et al., 2008 accepted in Asian-Australia Journal of Animal Science). The present results indicate that the up to 30% fish meal protein could be replaced with CM (16.4% dry matter) and SM (16.0% dry matter) in the diet for juvenile parrot fish. The lower growth performance and feed utilization of the fish fed the F-CS, including F-CS20, F-CS30, and F-CS40 could be related to the other factors which probably were produced during fermentation process. Parson et al. (1992) reported that excessive heat treatment can cause the decomposition of amino acids, activate oxidation of cysteins and methionine and facilitate the Maillard reaction between lysine and aldehyde groups of carbohydrates and consequently resulting in growth retardation and lower feed efficiency in animals. The conclusion was re-confirmed by Lee et al. (2007) and our previous findings (Chapter III). Lee et al., (2007) demonstrated that extremely heat-treated soy-flakes dramatically decreased digestibility of both nitrogen and energy and retarded growth performance of pigs. Radha et al. (2008) suggested that the formation of soy protein aggregates in extremely heat-treated soybean might induce abnormality and reduce the nutrients absorbability in digestive tract. Therefore, it is necessary to investigate the histological changes of digestive tracts in fish fed the diets containing CS and F-CS.

No significant differences were observed in muscle proximate compositions among the fish groups fed all the experimental diets, in the present feeding trial, except for the diet CS40. Whole body ash content of the CS40 groups was

significantly lower than that of the control groups (Table 4.5). Pham et al. (2005, 2007) reported that substitution of fish meal protein with CS up to 40% did not alter the whole body compositions of juvenile olive flounder. Cheng and Hardy (2002) also did not observe any difference in whole body composition of rainbow trout fed the diet contained 10% cottonseed meal.

Hepato somatic index of the fish fed the diets CS30, CS40, F-CS30 and F-CS40 was significantly lower than that of fish fed the control diet and no significant differences were observed among the fish groups fed the control and CS20 and F-CS20 diets. Condition factor and viscera somatic index were not affected by dietary CS, regardless of different treatments (Table 4.6). It indicates that HSI might be altered by high concentration of dietary CS with the supplementation of monocalcium phosphate. However, we did not find any significant differences in HSI of olive flounder fed the diets contained graded concentration of cottonseed and soybean meal (Pham et al., 2007). Toko el al. (2008) demonstrated that HSI of African catfish was not affected by the dietary graded level of CM or SM. Meanwhile, Hansen et al. (2007) demonstrated that HSI was significantly decreased in fish fed the diet contained 100% plant protein in Atlantic cod. They suggested that the liver sizes of fish strongly relate to dietary concentration of macro-nutrients balances and energy density is of the most important factors to regulate the hepato lipid deposition. Albrektsen et al. (2006) found a significant reduction of liver sizes of Atlantic cod fed the diet containing larger content of plant protein sources. The present results suggest that liver sizes of the juvenile parrot fish might be affected by dietary CS inclusion levels.

Superoxide anion produced by blood leukocytes was measured using nitro blue tetrazolium assay (NBT). NBT activity slightly increased with the increase of dietary CS. Fish groups fed the diet F-CS40 produced highest NBT activity compared to the control diet (Table 4.8). It indicates that the higher anti-oxidant activities in the diets containing the F-CS might enhance the non-specific immune response in juvenile parrot fish. In the present feeding trial, anti-oxidant activities in the diets containing the F-CS was significantly higher than that of the control and the CS diets (Table 4.9). It clearly demonstrated that heat treatment in fermentation process could produce higher antioxidant activities in the F-CS diets. Anton et al. (2008) reported that total polyphenol content and DPPH radical scavenging activity was significantly increased with heat drying. Large amount of polyphenol compounds were consumed in fish groups fed the F-CS diets might directly enhance the NBT activity. Manach et al. (2004) found that the effects of Polyphenol depend on their amount consumed and on their bioavailability. It is therefore concluded that the higher NBT activity in fish groups fed the diets containing F-CS might be directly related to the larger amount of dietary polyphenol compounds consumed.

Plasma glucose concentration was significantly increased in fish groups fed the CS diets, regardless of the different treatments (Table 4.10). Higher sugar concentration including glucose in the CS diets might result in the increment of plasma glucose concentration. Further studies on the effects of fermented plant protein sources on plasma glucose of fish are recommended.

Plasma cholesterol and triglyceride were significantly lower in the fish groups fed the CS and F-CS diets. The fish fed the F-CS diets produced lower plasma

cholesterol and triglyceride compared to the CS diets. Cholesterol lowering effects of dietary soybean meal and other plant protein sources has been reported in many vertebrate animals including fish (Golgberg et al., 1982; Kaushik et al., 1995; Ali et al., 2004; Chisholm et al., 2005; Dias et al., 2005; Romarheim et al., 2008). It was suggested that there were various dietary factors including proteins, non-proteins in soybean and cottonseed meal might lower the plasma cholesterol and triglyceride. Goldberg et al. (1982) demonstrated that the decrement in plasma cholesterol concentration in hypercholesterolemia patients was attributed by soy protein components. Meanwhile, Anderson and Wolf (1995) reported that cholesterolemia was affected by various soybean non-protein components, such as trypsin inhibitors, saponins, phytoestrogen, fibers, phytosterols, phytic acids and minerals. Ali et al. (2004) also found that soy isoflavones could lower plasma cholesterol in rats. It is apparent that there are many factors including nutrients, non-nutrients and endocrines involve in the regulation of cholesterol synthesis and metabolism in vertebrates. In fish, Kaushik et al. (1995) revealed that plasma cholesterol concentration was reduced in rainbow trout fed the diets contained soybean protein in comparison to the fish fed the fish meal protein based diet. Similar results were observed in European sea bass fed the soybean diets (Dias et al., 2004). Moreover, cottonseed meal also could contribute in the reduction of plasma cholesterol concentration. Studies recently have demonstrated that cottonseed by-products, including cottonseed meal might affect the cholesterol synthesis and metabolism and lead to the reduction of plasma cholesterol level in animals (Nwoha and Aire, 1995; Edwarsd and Radcliffe, 1995; Radcliffe et al., 2001). The results in our previous feeding trial (Pham et al., 2008, accepted on Asian-Australia Journal of Animal Science) strongly support to

the present findings and indicate a negatively relationship between the dietary concentration of polyphenol compounds, particularly with some flavonoids and plasma cholesterol (R^2 = 0.68). The effect of dietary flavonoids on the decrement of plasma cholesterol concentration was well demonstrated by Sudheesh et al. (1997). The authors interpreted those higher dietary flavonoids levels significantly increased the concentration of hepatic and fecal bile acids and fecal neutral sterols and resulted in higher rate of degradation of cholesterol.

Fermented soybean has been considered as a potential source of antioxidant compounds which could alter the antioxidant enzymes in including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and inhibit lipid peroxidation (thiobarbituric acid reactive substances, TBARS) in some animals' tissues both *in vitro* and *in vivo* (Ashida et al., 2002; Ashida and Okimasu 2005; Ashida et al., 2005; Pham and Lee, 2007; Kwak et al., 2007; Wang et al., 2008). In the present feeding trial, the F-CS diets did not influence the liver antioxidant enzymes and liver DPPH activity in juvenile parrot fish after six weeks, even the antioxidant activity and polyphenol compounds in the F-CS diets were significantly higher than those of the control diet (Fig. 4.3, 4.4, 4.5). Contradictorily, Pham and Lee (2007) reported that higher liver SOD activity was obtained in juvenile parrot fish fed the diets containing Cheongkukjang. Kwak et al. (2007) also demonstrated that Chungkukjang (CKJ) diet elevated liver SOD activity compared to the soy and high fat control diet. Both Chungkukjang and soy diets showed the significant reduction in hepatic lipid peroxidation and slightly increase in CAT activity compared to the control diet. They postulated that the findings probably resulted in

much higher antioxidant activities of KCJ due to the increase of aglycone and malonylglycosode isoflavones produced during fermentation process. Therefore, further studies on the effects of fermented CS on immune response of parrot fish are necessary.

4.5. CONCLSUSION

The present findings suggest that (1) cottonseed and soybean meal (CS) could replace up to 30% of fish meal in the diet for juvenile parrot fish; (2) fermentation process significantly improves the antioxidant activity and increases content of polyphenol compounds in CS; (3) the decline in growth performance of fish fed the F-CS diets is probably associated to the anti-nutritional factors produced in over-heated CS. A proper fermentation process including heating period, starter microorganisms should be further determined.

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APPENDIX (June 2008)

CURRICULUM VITAE

PERSONNEL INFORMATION

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EDUCATIONAL BACKGROUND

2006.03 - 2008.08	**Ph.D.** Cheju National Univ., Faculty of Marine Life Science.
2004.03 - 2006.08	**M.Sc.** Cheju National Univ., Faculty of Marine Life Science.
1997.09 - 1999.09	**B.Sc.** Nhatrang Fisheries University, Nhatrang, Vietnam.
1995.09 - 1997.09	**Certificate.** Hanoi Agriculture Univ. Hanoi, Vietnam
1992.09 - 1995.09	**General Diploma**, Lienha High School, General Diploma
1983.09 - 1992.09	**Ordinary:** Viethung Secondary School, Hanoi, Vietnam

AWARDS

2006-current	Post Brain of Korea 21 Scholarship for Doctor Course
2006	Second Prize for Poster Presentation at International Conference on Oceanography and Sustainable Marine Aquaculture Confluence and Synergy held in Malaysia, from 2nd to 4th May, 2006.
2005-2006	New Regional University of Innovation Scholarship for Master Course in Cheju National University, Korea
2005	First Prize for Presentation at Student Conference organized by New Regional University of Innovation Scholarship Program at Cheju National University on 31st May, 2005.
2004-2005	Brain of Korea 21 Scholarship for Master Course
1997-1999	International scholarship from Asian Institute of Technology, Thailand at Research Institute for Aquaculture #1, Vietnam.
1995-1997	Government scholarship at Hanoi Agriculture University #1.

WORKING EXPERIENCES

2003.01- 2004.03	Aquaculture Expert, Support for Freshwater Aquaculture, SUFA, DANIDA-Vietnam Ministry of Fisheries.
2002.01- 2003.03	Researcher, Research Institute for Aquaculture No.1, Dinh Bang, Tu Son, Bac Ninh, Vietnam.
2000.01 - 2002.01	Research Assistant, Research Institute for Aquaculture No.1, Ding Bang, Tu Son, Bac Ninh, Vietnam.

TRAININGS

2000-03	Participant in Training Course on Sperm Cryoprservation of Aquatic Species, held at Research Institute for Aquaculture #1, Vietnam, from 21st - 26th March.
2000-04	Participant in Workshop on Aquaculture, held at Research Institute for Aquaculture #1, Vietnam, from15th - 16th April.

2000-12 Training course on Cage aquaculture, held in Bangladesh and Thailand, from 9 - 18 December.

2001-09 Participant in Training course on Sex Reverse of Freshwater Prawn (*Macrobrachium rosenbergii* de Man), held at Research Institute for Aquaculture #1, from 28th September to 1st October.

2002-03 Participant in Gardening for Food Around the World, held at EPCOT, Florida, United State, from 18th March - 10th June.

2003-03 Participant in training course on Sex Reverse of Freshwater Prawn (*Macrobrachium rosenbergii* de Man) in Ben Gurion University, Ben Shava, Israel, from 2nd March - 14th March.

PUBLICATIONS

1. **Pham, M.A.,** Lee, K.-J., Dang, T.M., Lim, S.J., Ko, G.Y., Eo, J.N., Oh, D.H., 2008. Effects of Microbial Phytase in Diets Containing Cottonseed and Soybean Meal for Juvenile Olive flounder (*Paralichthys olivaceus*): **It was accepted in Asian-Aust. J. Anim. Sci.**
2. **Pham, M.A.,** Lee, K.-J., Lim, S.-J., Park, K.-H., 2007. Evaluation of cottonseed and soybean meal as partial replacement for fishmeal in diets for juvenile Japanese flounder *Paralichthys olivaceus*. Fisheries Science 73, 760-769.
3. **Pham, M.A.,** Lee, K.-J., 2007. Effects of dietary Cheongkukjang on liver superoxide dismutase activity of parrotfish *Oplegnathus fasciatus*. J. Korean Aquaculture Society 20, 132-139.
4. **Pham, M.A.,** Lee, K.-J., Lee, B.-J., Lim, S.-J., Kim, S.-S., Lee, Y.-D., Heo, M.-S., Lee, K.-W., 2006. Effects of dietary *Hizikia fusiformis* on growth and immune responses in juvenile olive flounder (*Paralichthys olivaceus*). Asian-Aust. J. Anim. Sci. 19, 1769-1775.
5. Dang, T. T. M., **Pham, M. A**., Pham, A. T., Lee, K.-J., 2006. Effects of dimethyl sulfoxide concentration on quality of cryopreserved sperm of grass carp (*Ctenopharygodon idellus*). J. Korean Aquacult. Soc., 19. 52-56.
6. Lee, B.-J., Lee, K.-J., **Pham, M.A**., Lee, S.-M., 2006. Myo-inositol requirement

in diets for juvenile olive flounder (*Paralichthys olivaceus*). J. Korean Aquacult. Soc., 19, 225-230.

7. Pham, N. S., Pham A. T., **Pham M. A.**, Nguyen, D. T., 2005. "All –male freshwater prawn (*Macrobabrachium rosenbergii* de Man) production by bilateral ablation of androgenic gland from normal male". Annual research reports, 153-159.

8. Pham, M. A., Lee, K.-J., Lim, S.-J., Lee, B.-J., Kim, S.-S., Park, Y.-J. and Lee, S.-M., 2005. Fish meal replacement by cottonseed and soybean meal in diets for juvenile Olive flounder, *Paralichthys olivaceus*. J. Korean Aquacult. Soc., 18, 215-221.

9. Edwards, P., Hiep, D. D., **Pham, M. A.**, Mair, G. C., 2000. Traditional culture of indigenous common carp in rice fields in northern Vietnam: does it have a future role in poverty reduction? World Aquaculture, 31 (4), 34-40.

ORAL PRESENTATION

1. Pham, M.A., Lee, K.J., 2006. Prospects of fermented and phytase treated cottonseed and soybean meal in marine aquafeeds. XIIth AAAP Animal Science Congress 2006, 18th – 22th September, 2006 BEXCO, BUSAN, KOREA.

2. Pham, M.A., Dang, T.T.M., Lee, K.-J., 2007. Dietary Choengkukjang increased liver superoxide dismutase in parrot fish *Oplegnathus fasciatus*. World Aquaculture Society Conference, August 5-8, Hanoi, Vietnam.

3. Pham, M.A., Eo, J.-Y., Kim, S.-S., Lee, K.-J., 2007. Replacement of fish meal by spirulina, *Spirulina pacifica*, in diets for parrot fish *Oplegnathus fasciatus.* World Aquaculture Society Conference, August 5-8, Hanoi, Vietnam.

POSTER PRESENTATION

1. Pham, M.A., Lee, K.J., Lim, S.J., Lee, B.J., Kim, S.S. Park, Y.J., Lee, Y.D., Lee, S.M., 2004. Dietary supplementation of cottonseed and soybean meal containing high levels of natural antioxidants as plant protein sources for

olive flounder (*Paralichthys olivaceus*). Korean Aquaculture Society Meeting 2004.

2. Pham, M.A., Lee, K.J., Kim, K.Y., 2005. Preliminary study on supplementation of Meju in diet containing cottonseed and soybean meal for juvenile olive flounder (*Paralichthys olivaceus*). Korean Aquaculture Society Meeting 2005.

3. Pham, M.A., Lee, K.J., Lim, S.J., Kim, S.S., Lee, B.J., Jeoung, Y.W., Hur, M.S., 2005. Immune response of juvenile olive flounder (*Paralichthys olivaceus*) fed diet containing graded level of *Hizikia fusiformes*. Korean Aquaculture Society Meeting 2005.

4. Pham, M.A., Dang, T.M., Pham, A.T., Lee, K.-J., 2005. Effect of dimethyl sulfoxide on cryopreserved sperm of Grass carp (*Ctenopharygodon idellus*). Korean Aquaculture Society Meeting 2005.

5 Dang, T.M., **Pham, M.A.,** Lee, K.-J. 2006. Improved apparent digestibility coefficient of phosphorus by supplementation of phytase in diets containing cottonseed and soybean meal for juvenile olive flounder (*Paralichthys olivaceus).* International Conference on Oceanography and Sustainable Marine Aquaculture Confluence and Synergy held in Malaysia, from 2nd to 4th May, 2006.

6. Pham, M.A., Lee, K.-J., Lim, S.-J., Kim, S.-S., Lee, B.-J., Jeoung, Y.-U., Heo, M.-S., 2006. Replacement of antibiotic by dietary supplementation of *Hizikia fusiformis* and *Ecklonia cava* for juvenile olive flounder (*Paralichthys olivaceus*). International Conference on Marine Biotechnology. November 17, 2006. Pukyong National University, Busan, Korea.

7. Pham, M.A., Dang, T.T.M., Lee, K.-J., 2007. Fermentation and phytase supplementation treatment improved digestibility of phosphorus and protein in diets containing cottonseed and soybean meal for olive flounder *Paralichthys olivaceus.* World Aquaculture Society Conference, February 26-March 2, Texas, US.

8. Pham, M.A., Lee, B.-L., Lee, K.-J., Kim, G.-U., Heo, M.-S., Moon, D.-H., 2007.

Effects of citrus by-products fermented with *Bacillus subtilis* in diets for juvenile olive flounder *Paralichthys olivaceus.* World Aquaculture Society Conference, February 26-March 2, Texas, US.

MANUSCRIPTS ON REVISION AND PREPARATION

1. Fermentation and phytase treatment improved digestibility of protein and phosphorus in diets containing cottonseed and soybean meal for olive flounder *Paralichthys olivaceus.*
2. Preliminary study on effects of Meju on growth performance and immune responses of juvenile olive flounder (*Paralichthys olivaceus*). **Revising and submitting on Aquaculture**
3. Effects of probiotics and fermentation on immune responses of juvenile parrot fish *Oplegnathus fasciatus*. **Revising**
4. Effect of fermentation of CSM with AO on growth performances of Parrotfish.

PROJECT EXPERIENCES

Project name:	"Semen Cryoprservation of Common Cultured Species"
Location:	Research Institure for Aquaculture No1. Vietnam
Year:	2000
Position:	Research Assistant
Project name:	"Quality Improvement of YY male Tilapia *Oreochromis niloticus and O.aureus"*
Location:	Research Institure for Aquaculture No1. Vietnam
Year:	2001
Position:	Research Assistant
Project name:	"Sex control of Freshwater Prawn (*Macrobrachium*
Location:	*rosenbergii* de Man)*"*
Year:	Research Institure for Aquaculture No1. Vietnam
Position:	2002
	Researcher

Project name:	"Support for Freshwater Aquaculture, SUFA"
Location:	Vietnam Ministry of Fisheries
Year:	2003
Position:	Aquaculture Expert

LANGUAGES

	Read	**Write**	**Speak**
Vietnamese (mother tongue)	Excellent	Excellent	Excellent
English (second language)	Good	Good	Good
Korean (third language)	Fair	Fair	Good