A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Evaluation of dietary supplementation of a Meju, a fermented soybean meal, and *Aspergillus oryzae* for juvenile parrot fish (*Oplegnathus fasciatus*)

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GRADUATE SCHOOL

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CONTENTS

국문초록

본 연구는 돌돔 치어의 사료 내에 한국의 전통적인 메주에 사용되어지는 유용한 유포자효모균류를 이용한 발효 대두박 첨가가 어류의 성장, 면역 그리고, 인의 소화율에 어떠한 영향을 미치는 지를 알아보기 위해 수행되어졌다. 실험 사료 내 유포자효모균류의 첨가는 다음과 같은 3가지 형태인 일반적인 메주, 발효미생물인 *Asperguillus oryzae*를 이용하여 발효시킨 대두박과 *Asperguillus oryzae*의 단독첨가로 이루어 졌으며, 실험사료의 급여는 총 8주 동안 수행되어졌다.

총 4개의 실험사료는 8% 대두박, 4% 메주 (50% 대두박을 메주로 대체), 4% 대두박 메주(50% 대두박을 대두박 메주로 대체), 0.08% *Asperguillus oryzae*가 포함되게 각각 조성되어졌다. 또한 모든 실험사료는 동일한 에너지와 칼로리를 갖도록 조성되었으며, 4개의 실험사료는 어류에게 3반복으로 공급되어졌다. 실험 어류들은 4개의 실험사료에 빠르게 적응하였으며, 8주 동안의 사료공급 실험기간동안 사망한 개체는 없었다.

8주의 사료공급실험 후 성장률(Weight gain), 일간성장률(Specific growth rate), 먹이섭취율(Feed intake), 사료전환효율(Food conversion ratio), 단백질 전환효율(protein ehhiciency ratio), White blood cell count, Hematocrit과 NBT(Nitrobluetetrazolium)분석 결과 모든 실험구에서 유의적인 차이를 나타내지 않았다. 그러나 유의적인 차이를 보이지는 않았으나 4% 메주, 4% 대두박 메주, 0.08% *Asperguillus oryzae*를 함유한 사료를

섭취한 어류의 그룹은 8% 대두박 사료를 섭취한 어류의 그룹에 비하여 높은 먹이섭식과 인의 흡수를 나타내었고, 그리고 혈청 항산화능력 분석 결과에서도 높은 경향을 나타내었다.

본 연구의 결과를 종합하여 볼 때, 돌돔 치어의 사료 내 한국의 전통적인 메주에 사용되어지는 유용한 유포자효모균류인 ***Asperguillus oryzae***를 이용하여 발효한 대두박의 첨가는 돌돔 치어의 성장과 먹이 효율은 증가시키지 못하였으나, 인의 흡수와 어체 내 항산화력을 증가시킬 수 있을 것으로 보여진다.

ABSTRACT

In this study, dietary supplementations of a traditional Korean Meju, a fermented soybean meal (SBM-Meju) by *Asperguillus oryzae* and *Asperguillus oryzae* itself, a useful ascomycetous fungus used for Meju, were evaluated on growth performance, feed utilization, immune responses along with phosphorus availability in juvenile parrot fish (*Oplegnathus fasciatus*) for 8 weeks. Four experimental diets were formulated to contain 8% soybean meal, 4% Meju (50% soybean meal was replaced by Meju), 4% SBM-Meju (50% soybean meal was replaced by SBM-Meju), and 0.08% *Asperguillus oryzae* (designated as diet 1, 2, 3 and 4 respectively). The experimental diets were formulated to be isonitrogenous and isocaloric. One of four experimental diets was fed in triplicate groups of fish. After 8 weeks of feeding trial, there were no significant differences in growth parameters, feed utilization, antioxidant capacity, and immune responses. Fish fed the test diets (diets2, 3 and 4) showed a higher feed intake, phosphorus absorption and serum antioxidant activity compared to the fish fed the control diet, even though they were not significantly different. The survivals of fish fed all the experimental diets were 100%. Therefore, fermentation process of soybean meal was not able to increase growth performances and feed utilization in diets for parrot fish. However, the fermentation process of soybean meal could enhance the absorption of phosphorus and antioxidant capacity of juvenile parrot fish.

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I. REFERENCE REVIEW

Parrot fish (*Oplegnathus fasciatus*) is one of the emerging aquaculture species in Korea. Its high commercial value makes it a promising aquaculture species in the future. Parrotfish is a carnivore fish therefore requires high level of protein in diet. Optimum dietary protein and lipid level in diets for 7.0 g parrot fish (*Oplegnathus fasciatus*) was 46% and 16% respectively. Fish fed diets containing 16% lipid level showed significantly ($P < 0.05$) higher weight gain, feed efficiency and protein efficiency ratio than did fish fed diets containing 8% lipid level when diets contained 40 or 50% dietary protein (Kang, 1998).

At present, fish meal (FM) constitutes the major portion of the protein used in most commercial feeds for high value carnivorous species, such as parrot fish, sea bream, sea bass and salmon (New, 1991). Fish meal use will likely increase in pet and livestock feeds along with fish feeds (New, 1991; Rumsey, 1993). Therefore, future fish meal prices and the costs of fish production will be higher than at present unless suitable inexpensive alternative sources of protein, which are of constantly high quality, are found and/or developed (Higgs et al., 1995). Soybean meal which constitutes about 75% of all plant feedstuffs will likely be one of the key

ingredients for aquaculture feeds of the future (Crowder, 1990). Also fish, specially carnivorous species, such as sea bass and sea bream, use fats more efficiently than carbohydrates as an energy source. Thus soybean oil could also play an important role in fish feed feeding (Lovell, 1991).

Soybean meals (SBM) are widely used as the most cost-effective alternative for high-quality FM in feeds for many aquaculture animals (Storebakken et al., 2000) because of high protein content, relatively well-balanced amino acid profile, reasonable price and steady supply of soybeans. Different soybean products, such as soy protein concentrate, extracted and toasted (defatted) soybean meal, full-fat soybean meal, or low oligosaccharides soybean meal (Refstie et al., 1998), tended to produce contrasting fish growth because of the varied quantities of antinutrient compounds and/or lowly digestible carbohydrates present in these products such as, protease inhibitors, non- digestible carbohydrates, lectins, saponins, phytates, and possibly allergenic storage proteins (Liu, 1997). Among several soybean compounds which have been implicated in hindering digestion in fish (Spinelli et al., 1983; Olli and Krogdahl, 1994; Bureau et al., 1998; Storebakken et al., 1998; Refstie et al., 1999), non-starch polysaccharides may play a leading role. Refstie et al. (1999) have demonstrated a negative effect of soybean non-starch polysaccharides on

digestion and absorption of lipid in Atlantic salmon. Non-starch polysaccharides are probably also responsible for a slower rate of gastro-intestinal passage in fish fed diets with SBM when compared to FM (Storebakken et al., 1999). Inclusion of non-starch polysaccharides (guar galactomannans and alginates) in the diets for salmonid fish reduces the availability of nutrients when compared to non-starch polysaccharides free diets (Storebakken, 1985; Storebakken and Austreng, 1987). In Atlantic salmon the digestibility of dry matter coincided with the content of non-digestible soybean non-starch polysaccharides and a similar trend was also seen in Atlantic salmon for digestibility of organic matter (Refstie et al., 1999). In addition, this non-digestible fraction of carbohydrates results in a relatively low digestible energy content in SBM. Phytate in SBM may reduce bioavailability of protein and minerals (Spinelli et al., 1983; NRC, 1993; Storebakken et al., 1998). Phytate-phosphorus is unavailable or poorly available to monogastric animals, including fish (NRC, 1993). Improved absorption and retention of phosphorus from diets containing SBM with phytase supplementation has been demonstrated in rainbow trout (Riche and Brown, 1996; Lanari et al., 1998) and common carp (Kim et al., 1996). Moreover, Phytic acid affects the activation of trypsinogen and stability of trypsin (Caldwell, 1992). Different fishes show varied sensitivity

to soy antinutrients. In Atlantic salmon and rainbow trout, soybean meal has been found to cause distinct morphological alterations in the intestine, in addition to impaired growth and protein utilization and the effects escalate with increasing dietary level (Krogdahl and Bakke-McKellep, 2001).

Many studies have shown considerable success in partial or total replacement of FM with SBM and other soybean products in diets for various fish species (Reinitz, 1980; Mohsen and Lovell, 1990; Vivyakarn et al**.,** 1992; Webster et al., 1992a,b, 1995; Olli et al., 1995; Boonyaratpalin et al**.,** 1998; Quartararo et al., 1998; Arndt et al., 1999). The discrepancy among researchers regarding the use of SBM as a protein source for fish may be related to the quality and processing of SBM, variation in diet formulation and differences in fish species, size and culture system.

The main limitations in the use of SBM are attributed to the low level of methionine and the presence of antinutritional factors (Wilson and Poe, 1985; Olli et al., 1994a). On the other hand, heat treatment of soybeans improved growth performance and feed utilization in trout (Sandholm et al., 1976), common carp (Nour et al., 1989), and coho salmon (Arndt et al., 1999). Viola et al. (1983) reported that heating SBM at 105°C for 30-90 min. destroyed most of the protease inhibitors present. However, heating may cause loss of essential amino acids (Plakas et al., 1985).

Dietary soybean meal also appears to stimulate immune responses because of inflammation in the distance intestine (Krogdahl et al., 2000). Other fishes are not as sensitive to soybean antinutrients as the salmonids. Red drum (Reigh and Ellis, 1992) and Japanese flounder (Kikuchi, 1999) can effectively grow on diets containing almost equal amounts of FM and SBM without adversary effects.

SBM has a poorer balance of essential amino acids (EAA) than FM and is somewhat deficient in some certain EAAs such as methionine. Consequently, some researchers have tried to utilize alternative proteins by complementing them with other protein sources so as to obtain the required EAA profile for fish. The combination of SBM and corn gluten meal has been employed with succes to replace up to 63% of FM in the diet for rainbow trout (Watanabe, 1993).

In trial conducted with parrotfish (*Oplegnathus fasciatus*), to evaluate dietary protein sources for replacing FM, was found that weigth gain of fish fed diet containing 31% SBM was not different from that of fish fed the control diet (white fish meal), but feed efficiency of fish fed the diet was lower than that of fish fed the control diet (Kang, 1999).

Recently, special interest is drawn to Meju because of several functional effects of Korean soybean fermented food including antioxidant

effect (Cheigh et al., 1993), antimutagenesis, immuno-modulating and fibrinolytic activities (Kim et al., 1996). Moreover, the functional activity of such Korean food is higher than that of Japan and the reason is probably due to the difference of Meju and Koji (Kim et al., 1998). Fermented foods derived from soybean have been placed as an important portion of the diet in Korea and Japan.

Koji is made from cooked whole soybean and wheat-derived materials. After inoculation of pure culture of starter (*Aspergillus oryzae* or *A. sojae*), the mixture is spread on trays and incubated at 25-30 °C for 2-3 days. Contrary to Koji making, Meju is made from only cooked, chopped and molded soybean. The lump (0.5-2kg, usually brick shaped) is dried and fermented for 15-30 days, usually outdoor conditions in winter, without adding pure starter culture. The main fermentative microorganisms in Meju are fungi (surface of the lump) and *Bacillus subtilis* (inner space of the lump) originated from environment (Kim et al., 2000).

Soyproteins have been reported to benefit human health by lowering blood cholesterol, reducing the risks of breast cancer and atherosclerosis**.** (Carroll, 1991; Lu, 1996). However, consuming improperly processed or uncooked soybeans can be harmful because of anti-nutritional compounds in soybeans including trypsin inhibitors, lectins, flatulence-producing

compounds, etc. (Baker, 2000; Kim, 2003). Some of the antinutritional compounds can be destroyed or eliminated by proper processing or cooking (Clarke, 1999; Kaankuka, 1996). Use of soybean in infant foods may be even more problematic because of the high susceptibility of infants to these anti-nutritional compounds.

Fermentation processes have been used to prepare traditional soybeans foods in Far East Asia that are commonly known as “Dou-Bian-Jiang” in China, “Miso” in Japan, and “Duen-Jang” in Korea. These fermented soy foods are highly digestible and nutritious, contributing important nutrients including calcium and vitamins A and B, as well as functional properties, such as laxative effect, and anti-cancer properties (Lee, 1998; Kim, 1999).

Soybean meal is the most commonly used protein source in the animal feed industry (Easter, 1999). High protein content and wide availability make SBM a good source of protein in animal diets (Easter, 1999). However, use of soybean meal is mostly limited to adult animals because of inefficient digestibility of soy proteins by young animals (Dunsford, 1989**;** Li, 1990). However, use of SBM is mostly limited to adult animals because of inefficient digestibility of soy proteins by young animals. However, a recent European Union ban on the use of animal-

derived ingredients in feeds for food animals has accelerated the urgency for nutritionists to find alternative plant protein sources. Extensive use of SBM in adult animal feed is also undesirable, because of the detrimental effects of anti-nutritional compounds that commonly occur in soybean meals. Therefore, the development processing techniques that reduce the harmful and anti-nutritional properties of soy meal would make possible a high-quality and inexpensive protein with valuable functional benefits available for both humans and young animals.

Fermentation increased protein content, eliminated trypsin inhibitors and reduced peptide size in soybeans and soybean meals. These effects of fermentation might make soy foods more useful in human diets as a functional food and benefit it livestock as a novel feed ingredient by using *Aspergillus oryzae* GB-107 (Hong, 2004).

Therefore, this study was conducted to examine the effects of dietary supplementation of fermented soybean products on growth performances and feed utilization of juvenile parrot fish. Also, antioxidant capacity and immune responses of fish to the fermented soybean meal were studied.

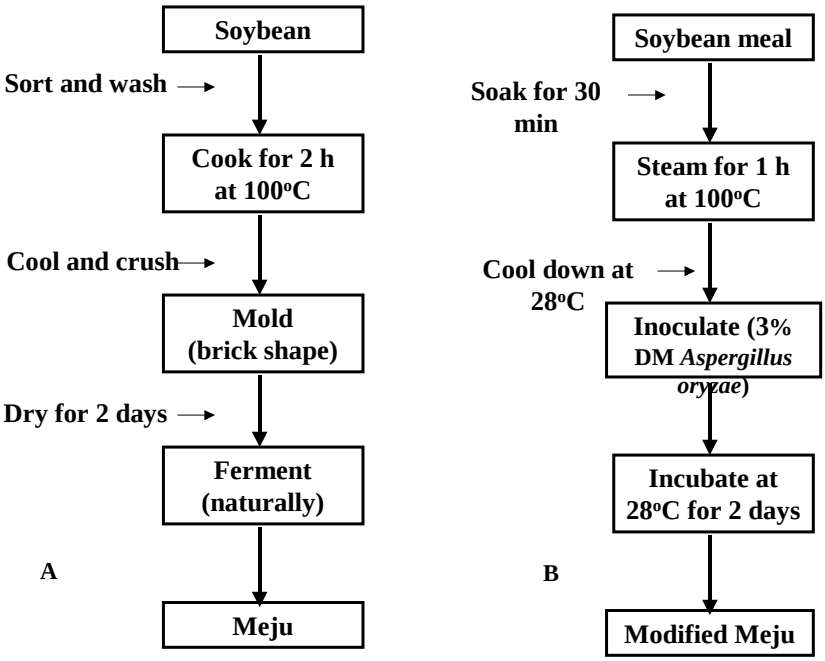

Fig 1. Preparation of Meju (A) and for Modified SBM Meju (B)

II. MATERIALS AND METHODS

1. Experimental diets

Four experimental diets were formulated to be isonitrogenous and isocaloric in term of crude protein (48.9%) and lipid (17.1%). The diet formulation and proximate compositions are presented in Table1. Four experimental diets were formulated to contain 8% soybean meal, 4% Meju (50% soybean meal was replaced by Meju), 4% SBM-Meju (50% soybean meal was replaced by SBM-Meju), and 0.08% *Aspergillus oryzae* (designated as diet 1, 2, 3 and 4, respectively). A commercial Meju was purchased from Munwha Meju (Daegu, Korea) and ground into fine powder using grinding machine (MF10 basis, Germany). SBM-Meju (soybean meal used Meju) was prepared as process in making meju, a traditional Korean fermented soy paste, with some modification (Fig. 1). Soybean meal was used instead of whole soybean in meju. Soybean meal was finely ground and soaked with water at a ratio of 1:3 for 30 min and steamed in a rice cook for 1 h. Then the mixture was cooled down to 28 °C in an incubator. *Asperguillus oryzae* of 3% dry matter was inoculated into the mixture and incubated in an incubator at 28 °C for 48 h. The fermented mixture was

directly used in dietary formulation. *Asperguillus oryzae* was kindly provided from Jeil BioTech. All dry ingredients were thoroughly blended with 30% distilled water to become dough. Pellets were extruded through the meat chopper machine (SMC-12, Korea) in 3.0 mm diameter size, dried at room temperature, crushed into desirable particles sizes (0.4-2.0 mm), and stored at –20 ºC until use.

Table 1. Formulation of experimental diets (% dry matter, DM)

Ingredients	Diets 1 (Control)	Diet 2 (Meju 4%)	Diet 3 (SBM Meju 4%)	Diet 4 (*Aspergillus oryzae*)
White fish meal	48.00	48.00	48.00	48.00
Soybean meal	8.00	4.00	4.00	8.00
Meju	0.00	4.00	0.00	0.00
SBM Meju	0.00	0.00	4.00	0.00
A. oryzae	0.00	0.00	0.00	0.08
Cottonseed meal [1]	8.00	8.00	8.00	8.00
Corn gluten meal	8.00	8.00	8.00	8.00
Potato Starch	13.00	13.00	13.00	13.00
Mineral Mix [2]	1.00	1.00	1.00	1.00
Vitamin Mix [3]	0.50	0.50	0.50	0.50
Vitamin C & E	0.50	0.50	0.50	0.50
Squid liver oil	12.00	12.00	12.00	12.00
CMC	1.00	1.00	1.00	0.92

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

[2] *$MgSO_4.7H_2O$, 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, 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] *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 2. Proximate composition of the experimental diets (% DM)

Ingredients	Diets 1 (Control)	Diet 2 (Meju 4%)	Diet 3 (SBM - Meju 4%)	Diet 4 (*Aspergillus oryzae*)
Dry matter, %	94.13	94.32	93.53	94.86
Protein, % DM	50.50	50.64	51.83	50.32
Lipid, % DM	12.95	17.23	16.55	16.85
Ash, % DM	9.57	9.56	9.71	9.44
Gross energy, MJ/kg DM	18.09	18.17	18.04	18.10

Table 3. The proximate composition of major ingredients used in experimental diets (% DM)

Ingredients	Moisture	Protein	Lipid	FNE [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 [3]	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.*
[3] *Meju was purchased from Munwha Meju (Daegu, Korea).*

2. Fish, facilities and feeding trial

Parrot fish juveniles were obtained from a private hatchery (Chang-Hae Fisheries, Jeju) in Jeju Island, transported to the Marine and Environmental Research Institute, Cheju National University, Korea, and conditioned on a commercial formulated diet to the experimental condition until the start of feeding trial. Two hundred and sixteen fish (initial body weight 22.0 g) were randomly distributed into twelve 100 L PVC tanks (18 fish per tank) in a flow through system supplied with sand filtered sea water at flow rate of 3 l/min. After 2 weeks of acclimation period, the fish were fed at a rate of 4% body weight, twice a day (8:00 and 18:00), 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 weighing.

3. Sample collection and analysis

3.1 Whole body composition

At the beginning and the end of the feeding trial, all fish were weighed and counted to calculate percent body weight gain (WG), feed conversion ratio (FC), protein efficiency ratio (PER), specific growth rate (SGR). Three fish from each tank (9 fish per diet) were sampled and stored at –20 °C for whole body proximate analysis. Proximate analysis of whole body was performed by the standard procedures (AOAC, 1995).

3.2 Serological assay

During the experiment three fish per tank (9 fish per diet) were anesthetized using tricaine methane sulfonate (MS222) solution (100 mg l^{-1}) and blood was taken from caudal veins by medical syringes with heparin as anticoagulant. Red blood cell (RBC) and white blood cell (WBC) were determined as described by Kumar et al. (2005). NBT (Nitro blue tetrazolium) assay was conducted by the Anderson et al (1992) method. Hematocrit (Ht) was determined using microhematocrit technique. Blood was drawn into plastic capillary tubes and centrifuges at 12,000 x g for 10 min in a micro-hematocrit (VS-12000, Korea). The hemoglobin was

determined using a slightly modified method as the following description. Twenty five ul blood sample (without heparin) was diluted into 5 ml modified hemoglobin solution (composed of 0.7g $K_3Fe(CN)_6$ and 0.1g KCN in 11 double distilled water). Absorbance of mixture was measured using spectrophotometer (Genesys 10 UV, Rochester, NY, USA) at a wave length of 540 nm. The hemoglobin was calculated using the formula; Hb = 0.146 x F x OD, where Hb: hemoglobin; F: dilution factor (201) and OD: optical density.

3.3 Antioxidant capacity assay (DPPH)

One hundred μl serum from 3 fish per tank (9 fish per diet) was pipetted into a 1.5 ml cuvette, then 900 ul of DPPH methanolic solution (100 μM) was added to obtain a final volume of 1 ml. The absorbance of the mixture was observed at wavelength of 517nm with 1 min intervals for 10 min by a spectrophotometer (Genesys 10 UV, Rochester, NY, USA). The antioxidant capacity 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, A_S are the absorbance of sample at 0 and s min, respectively.

4. 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 ± standard deviations (SD). The percentage data of weight gain and specific growth rate were arcsine transformed before the ANOVA analysis. Differences were considered significant at $P < 0.05$.

III. RESULTS

1. Growth and feed utilization of juvenile parrot fish

The growth and feed utilization of fish fed experimental diets are presented in Table 4. After 8 weeks of feeding trial, the final body weight (Fig 2), weight gain (Fig 3), specific growth rate (Fig 4), feed conversion ratio (Fig 5) and feed intake (Fig 6) of fish fed all the experimental diets were not significantly different compared to that of fish fed diet 1 (control). Fish fed the test diets (diets2, 3 and 4) showed a higher feed intake (Fig 6) compared to the fish fed the control diet (diet 1), even though it was not significantly different. The experimental fish did not show any resistance to the experimental diets and consumed well during the feeding trial. The survivals of fish fed all the experimental diets were 100%.

Table 4. Growth performance of juvenile parrot fish (IBW, 22 g) fed different experimental diets for 8 weeks

Diets	**Diet 1 (CONTROL)**	**Diet 2 (MEJU 4%)**	**Diet 3 (SBM-MEJU4%)**	**Diet 4 (*Aspergillus oryzae*)**
Initial body weight, g	22.0±0.04	21.9±0.31	22.1±0.03	22.0 ±0.01
Final body weight, g	81.7±2.32	80.6 ± 2.88	78.4±2.34	79.7 ± 2.54
Weight gain (WG)[1]	372 ± 11	368 ± 13	355±11	362 ± 11
Specific growth rate (SGR)[2]	1.0±0.02	1.0 ±0.03	1.0±0.02	1.0 ±0.03
Protein efficiency ratio (PER)[3]	1.8±0.06	1.7±0.04	1.7±0.05	1.7±0.04
Feed conversion ratio (FCR)[4]	1.1±0.04	1.2±0.02	1.2±0.03	1.2± 0.02
Feed intake (FI, g/g, BW)[5]	0.8±0.02	0.8 ±0.01	0.8±0.01	0.8±0.01
Survival (%)	100	100	100	100

Values are presented as mean ± std. Value in the same row having different superscript letters is significantly different (P < 0.05)

[1] *WG (%) = 100 x (final mean body weight - initial mean body weight)/initial mean body weight*

[2] *SGR (%) = [(loge final body weight - loge initial body weight)/days] x 100*

[3] *PER = wet weight gain/ total protein given5 FCR = dry feed fed/wet weight gain*

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

[5] *FI (g/g body weight) = dry feed consumed (g)/ body weight (g)*

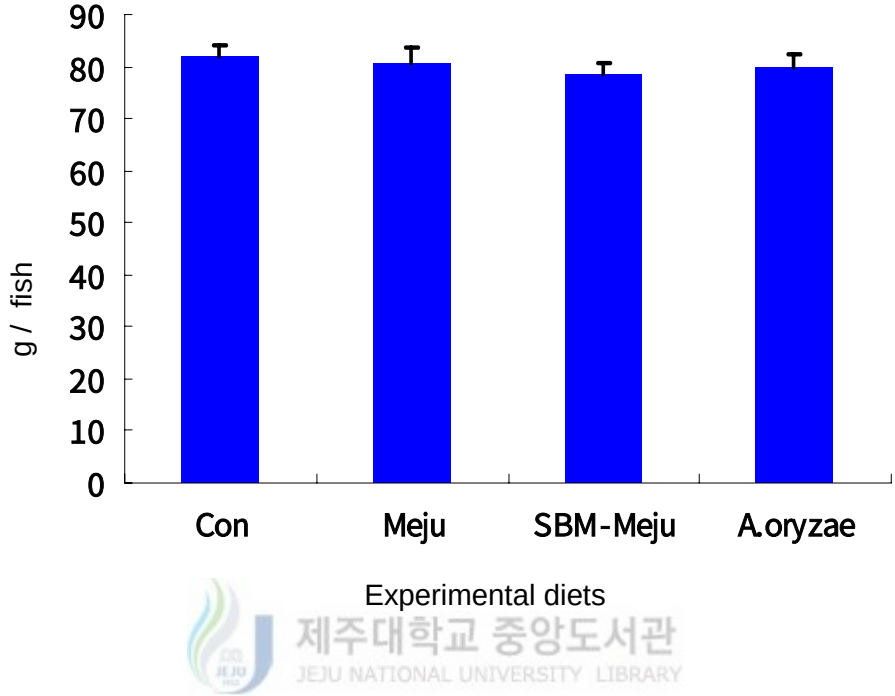

Figure 2. Final body weight (g/fish) of fish fed the experimental diets for 8 weeks.

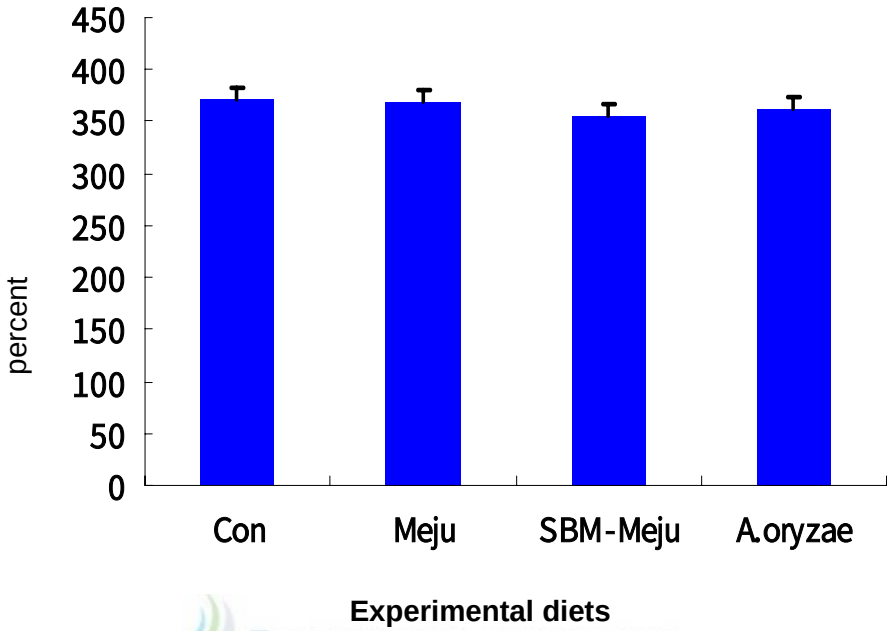

Figure 3. Weight gain (%) of fish fed the experimental diets for 8 weeks.

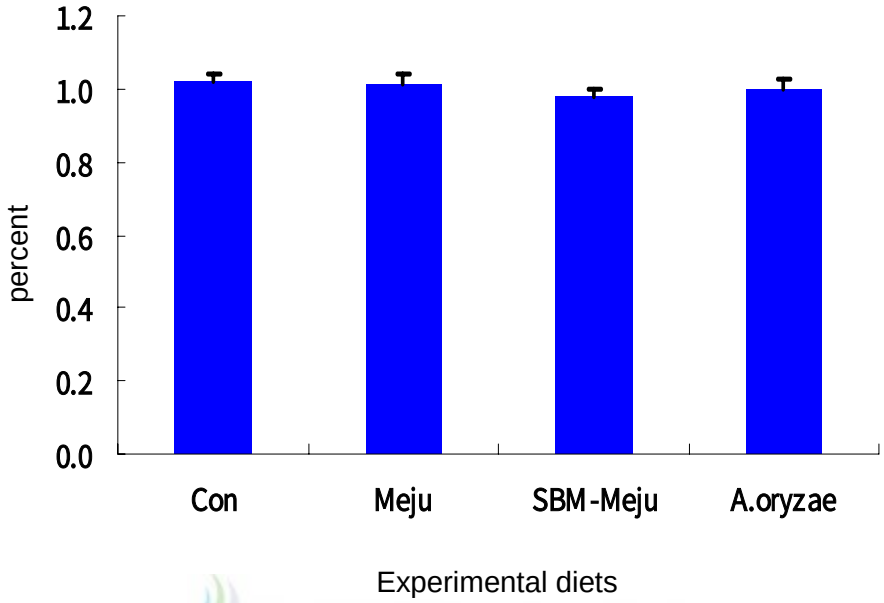

Figure 4. Specific growth rate (%), of fish fed the experimental diets for 8 weeks.

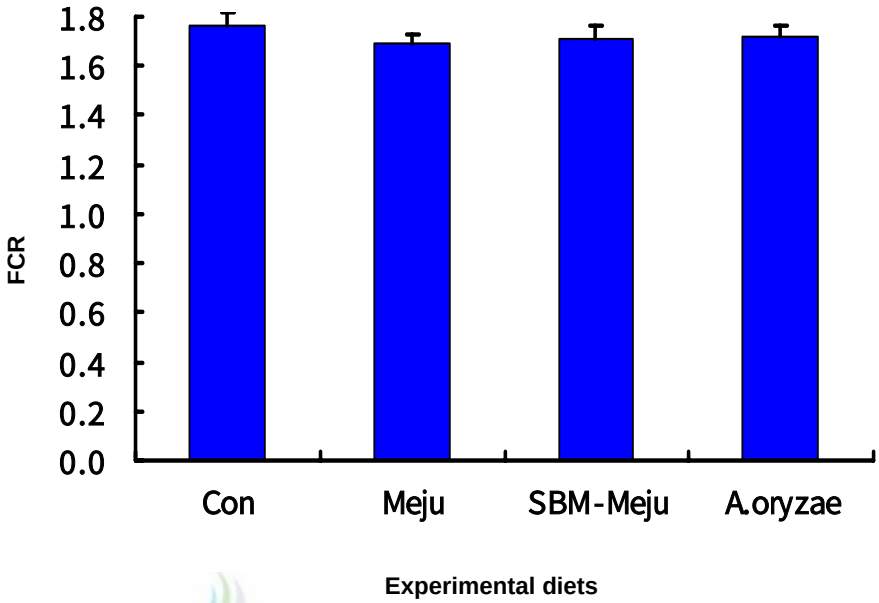

Figure 5. Feed conversion ratio of fish fed the experimental diets for 8 weeks.

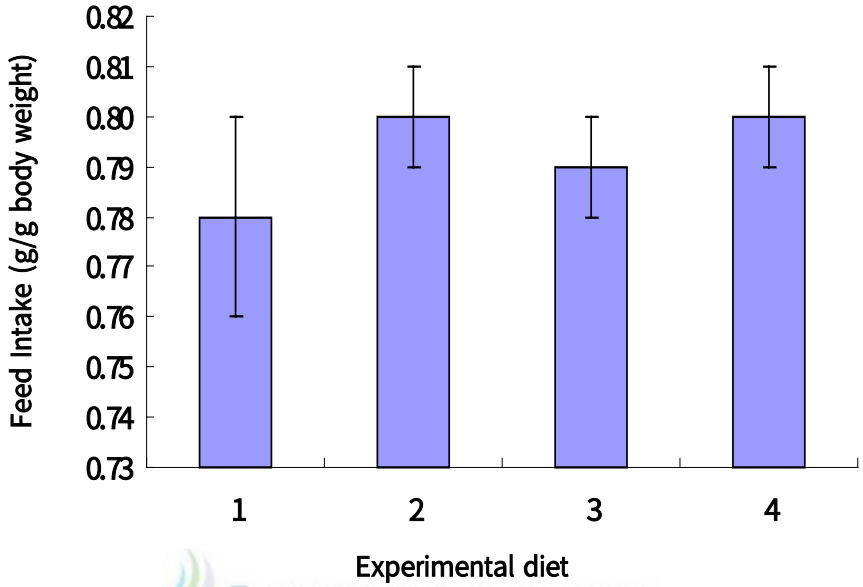

Figure 6. Feed intake of fish fed the experimental diets for 8 weeks.

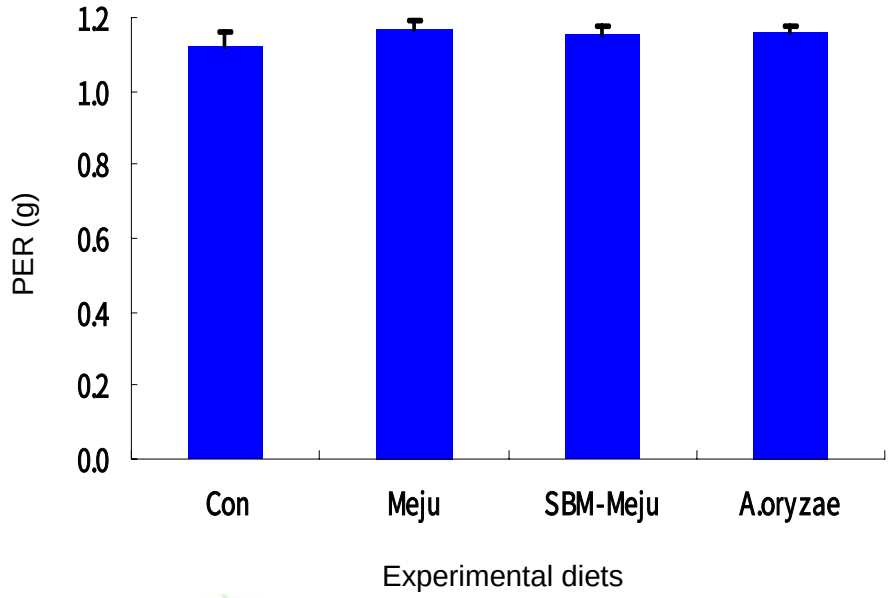

Figure 7. Protein efficiency ratio of fish fed the experimental diets for 8 weeks.

2. Blood Parameters

Blood was taken from caudal veins of fish fed the experimental diets for 8 weeks. Hematocrits (Ht%), hemoglobin (Hb, g/dL), nitro blue tetrazolium (NBT), white blood cell (WBC), red blood cell (RBC) were evaluated to verify the supplemental effects of fermented soybean meal and/or *Aspergillus oryzae* (Table 5).

Hematocrit (Fig 8) of fish fed diets 3 (SBM-Meju) and 4 (*A. oryzae*) showed a higher value than that of fish fed the control diet, even though there was no significant difference. Hemoglobin (Fig. 9) was not significantly different among all the dietary groups. Interestingly, phagocytic activity (Fig 10) of fish (measured by NBT) fed diets 3 and 4 was numerically elevated compared to that of fish fed the control diet. However, it was not significant. For white blood cell count (Fig 11), fish fed diets 2 and 3 exhibited slightly increased ($P > 0.05$) values than that of fish fed the control diet. Red blood cell (Fig. 12) of fish fed diet 4 (*A. oryzae*) was significantly increased compared to that of fish fed the control and other diets ($P < 0.05$).

Table 5. Blood parameters of juvenile parrot fish (IBW, 22 g) fed the experimental diets for 8 weeks.

Diets	**Diet 1 (CONTROL)**	**Diet 2 (Meju4%)**	**Diet 3 (SBM - Meju4%)**	**Diet 4 (*Aspergillus oryzae*)**
Hematocrits (Ht, %)	34.96 ±2.37	34.67±2.70	35.17±2.62	36.83±3.72
Hemoglobin (Hb, g/dL)	7.78 ± 7.48	7.69 ± 8.14	7.36±8.10	7.85±7.06
Nitro Blue Tetrazolium	50.3±7.7	50.0 ±20.0	55.5±28.1	57.3±24.3
White blood cell (million cell/mm^3)	0.125	0.143	0.131	0.116
Red blood cell (million cell/mm^3)	3.7 ± 0.38	3.9 ± 0.39	4.4 ± 0.48	5.1 ± 0.42

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

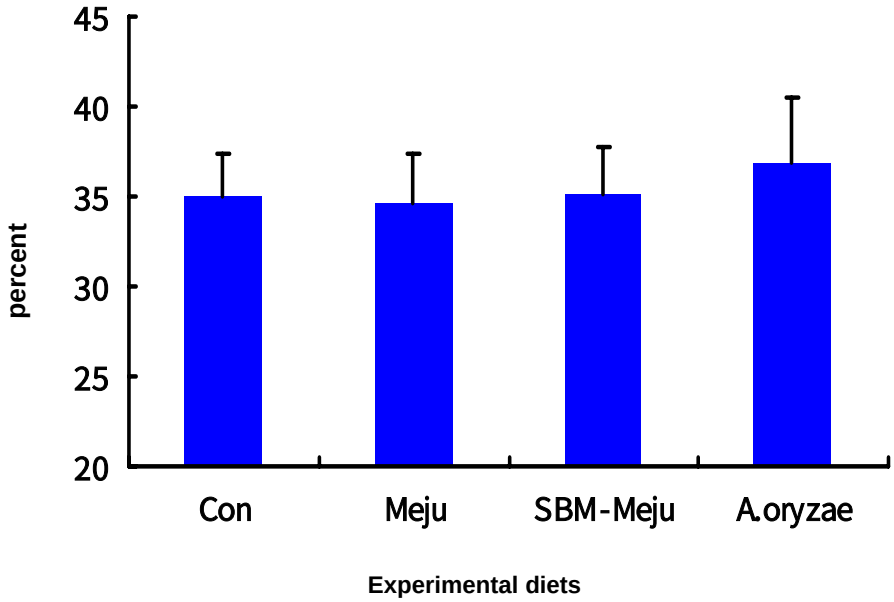

Figure 8. Hematocrits of fish fed the experimental diets for 8 weeks.

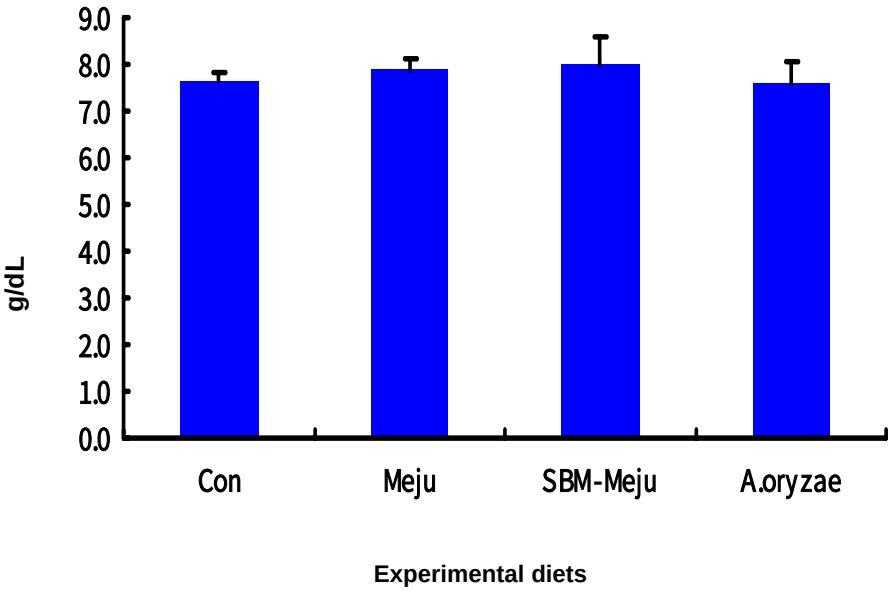

Figure 9. Hemoglobin of fish fed the experimental diets for 8 weeks.

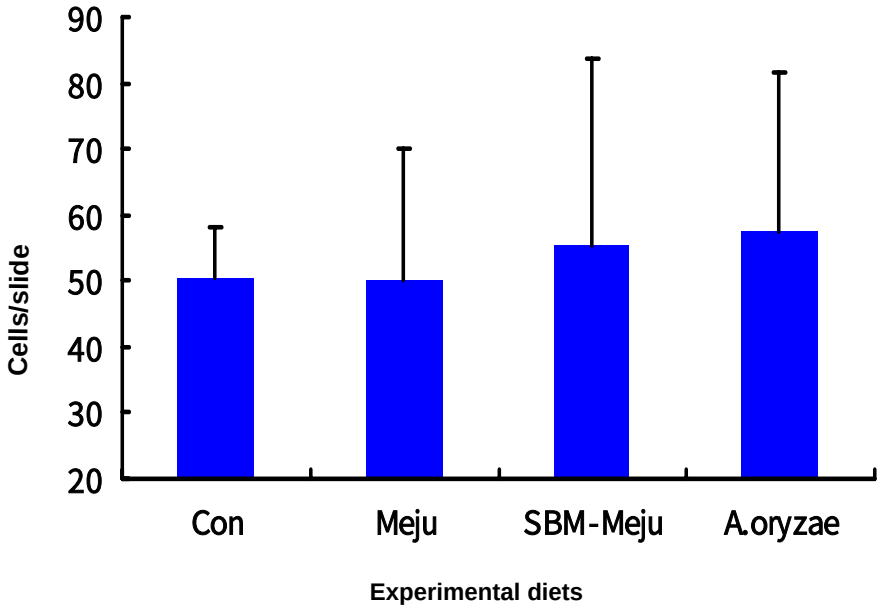

Figure 10. Nitro blue tetrazolium (NBT) of fish fed the experimental diets for 8 weeks.

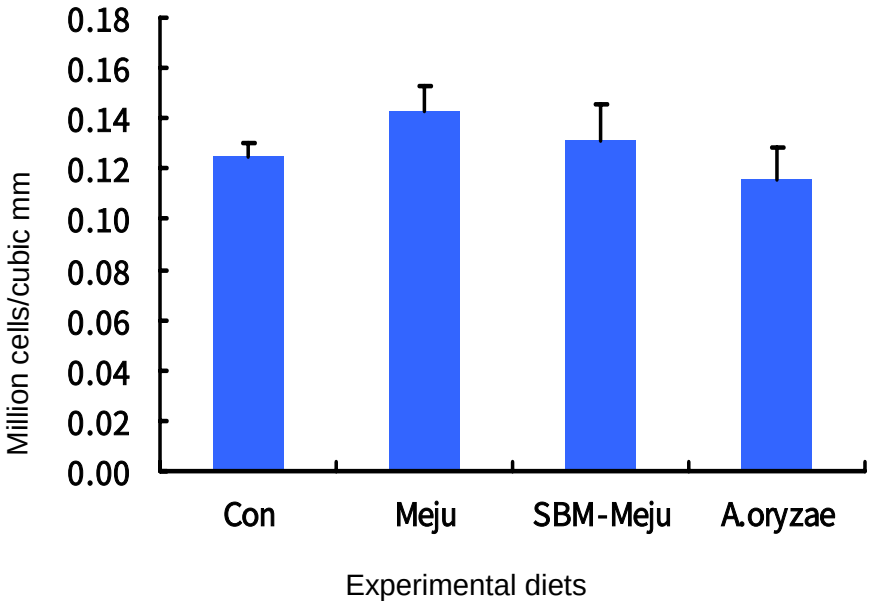

Figure 11. White blood cell (million cell/cubic mm), of fish fed the experimental diets for 8 weeks.

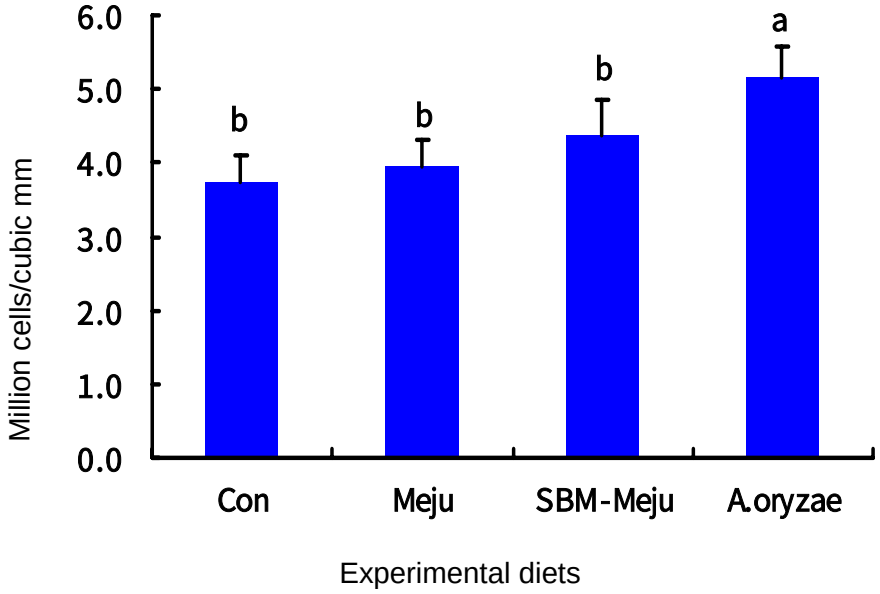

Figure 12. Red blood cell (million cell/cubic mm) of fish fed the experimental diets for 8 weeks. Significant differences ($P<0.05$) was indicated by different letter above the bars.

3. Whole body composition

Whole body composition of juvenile (initial body weight 22.0g) parrot fish fed the experimental diets for 8 weeks are given in Table 6. No significant differences were observed in protein, lipid and ash contents in fish fed all the experimental diets.

Table 6. Whole body composition of juvenile parrot fish (IBW, 22 g) fed the experimental diets for 8 weeks

Diets	**Diet 1 (Control)**	**Diet 2 (Meju 4%)**	**Diet 3 (SBM - MEJU4%)**	**Diet 4 (*Aspergillus oryzae*)**
Moisture content, %	67.1 ±0.26	66.7 ±0.23	66.3 ± 0.60	67.5±0.40
Protein, % DM	51.4±0.28	51.0 ± 2.02	52.7 ± 3.66	52.2 ±0.94
Lipid, % DM	33.6±3.44	33.6±1.72	32.3±2.18	35.4±1.35
Ash, % DM	5.49 ±0.48	5.60 ±1.46	5.36 ± 1.05	5.36 ± 0.68

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

4. Antioxidant activity

DPPH free radical scavenging activity (%) in serum of fish fed the experimental diets is expressed in Fig. 13. The serum antioxidant capacity was higher in fish fed the test diets (diets 2, 3 and 4) than that of fish fed the control diet. The highest oxidation inhibitory activity was exhibited by diet 2(Meju), followed by diet 3 (SBM-Meju) and 4 (*A. oryzae*), however, these values were not significantly different compared to that of the control diet.

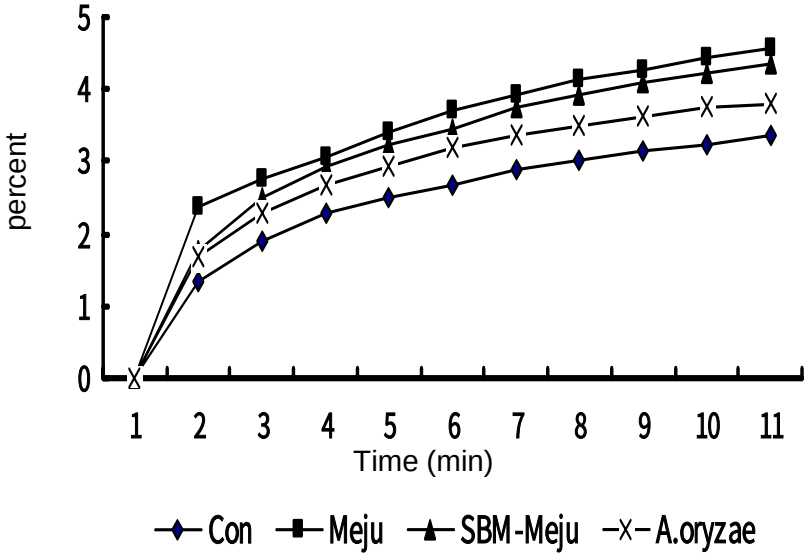

Figure 13. The antioxidant capacity of serum of parrot fish fed the experimental diets for 8 weeks. The absorbance of 50μL serum and 950μl was measured at 517nm for 10min with 1min interval.

Digestibility test

In the present study, digestibility of protein and phosphorus was examined to verify the supplemental effects of fermented soybean meal and/or *A. oryzae* on their digestibility. The apparent digestibility coefficients of protein and phosphorus in fish fed the experimental diets were provided in Table 7. The apparent digestibility coefficients of protein (Fig 14) of fish fed all the diets were not significantly different. Interestingly, however, phosphorus absorption was numerically increased in fish fed diets 3 (SBM-Meju) and 4 (*A. oryzae*) compared to that of fish fed the control diet, even though it was not significantly different.

Table 7. Digestibility parameters of juvenile parrot fish (IBW, 22 g) fed experimental diets for 8 weeks

Diets	**Diet 1 (Control)**	**Diet 2 (Meju4%)**	**Diet 3 (SBM - MEJU4%)**	**Diet 4 (*Aspergillus oryzae*)**
Protein digestibility %	73.7 ±13.6	66.4 ±10.9	72.7 ± 9.7	66.8±10.8
Phosphorus, %	16.2 ±3.1	16.0 ± 2.9	27.0± 10.7	26.1 ±5.5

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

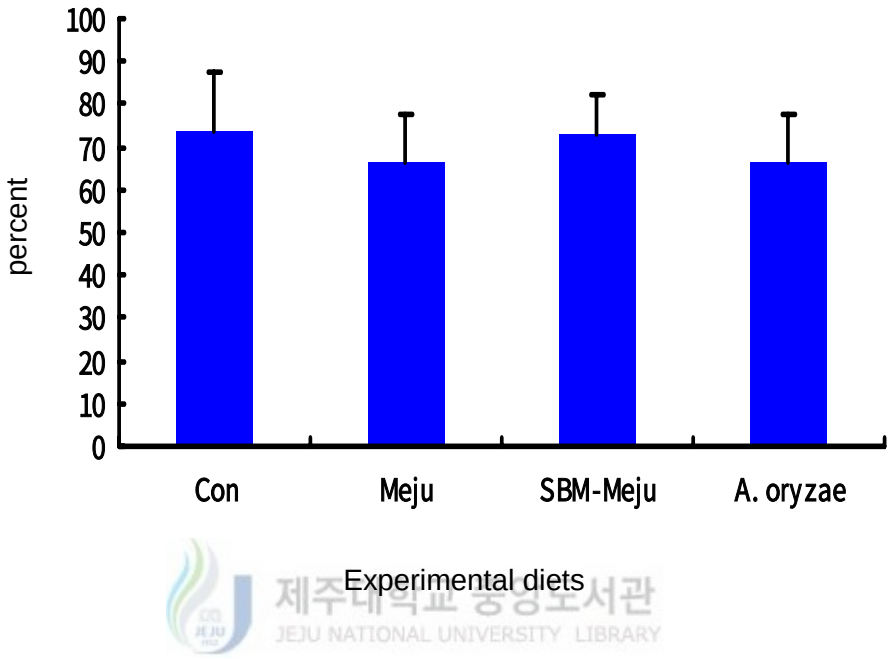

Figure 14. Protein digestibility (%), of fish fed the experimental diets for 8 weeks.

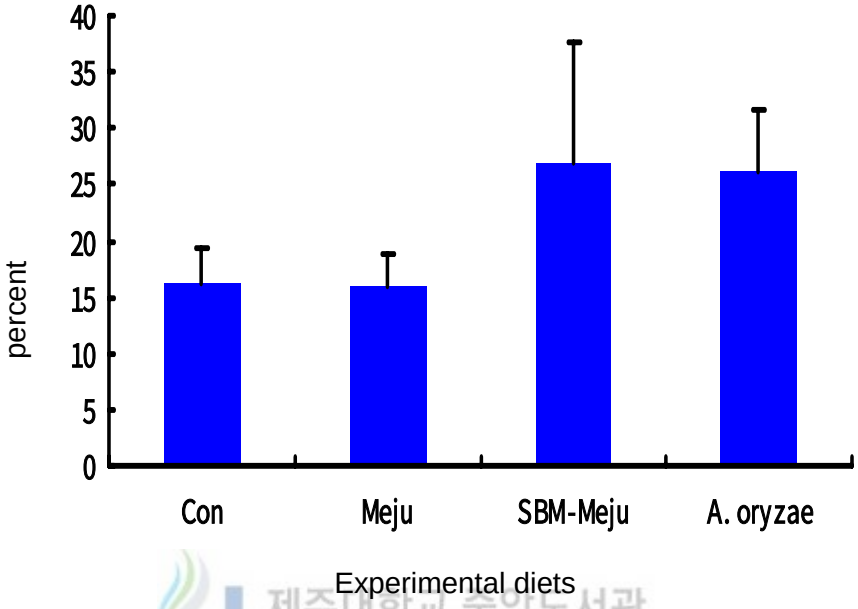

Figure 15. Phosphorus digestibility of fish fed the experimental diets for 8 weeks.

IV. DISCUSSION

In this experiment, the crude protein (48.9% DM) and lipid content (17.1%) of diets were formulated based on the protein and lipid requirement of parrot fish juveniles suggested by Kang et al. (1998) and Wang et al. (2003). The growth and feed utilization of fish fed test diets (diets 2, 3 and 4) were not significantly different compared to that of fish fed the control diet. Meanwhile, there were no significant differences in specific growth rate, food conversion ratio, feed intake and protein efficiency ratio of fish fed all the experimental diets. The survivals of fish fed all the experimental diets were 100% showing that the experimental fish grew well on the experimental diets during the feeding trial. The results in this study suggested that dietary incorporation of soybean meal, cottonseed meal and corngluten meal do not affect the dietary palatability and thereby do not impair the growth of juvenile parrot fish.

The results in the present study are in agreement with other studies on a mixture of cottonseed and soybean meal, with animal by-products as fish meal substitutes (Lee et al., 2002; Chou et al., 2004). Some antinutritional factors, such as gossypol, protease inhibitors and phytic acid exist in cottonseed meal and soybean meal. Gossypol, a yellow pigment

found in the gland of cottonseed, has been demonstrated to be toxic for many fish species (Dorsa et al., 1982; Dabrowski et al., 2000; Lee et al., 2002; Garcia-Abiado et al., 2004). The toxicity of gossypol depends on several factors including the form of gossypol (free or bound), the amount of consumption and varieties of the cottonseed.

Gossypol molecule can easily combine with lysine resulting the deficiency of lysine. Phytic acid (6-inositol hexaphosphate) exits in both cottonseed and soybean meal, and it also has been reported as a major antinutrient factor that limits the utilization of these ingredients in fish diets (Bransden and Carter, 1999; Lee et al., 2002; Barual et al., 2004; Riche and Garling JR, 2004). Owing to its unique chemical structure, phytic acid is able to combine with other minerals, such as calcium, magnesium and zinc, which reduce biological availability of the minerals in mono-gastric animals including fish (NRC, 1993; Storebakken et al., 2000). However, the dietary inclusion level of cottonseed meal in the present study was lower than that reported by Mbahinzireki et al. (2001). In the 16 weeks of feeding trial, Mbahinzireki et al. (2001) showed that up to 50% fish meal protein could be replaced by cottonseed meal in tilapia diets without any adverse effect on fish growth performance. Whereas, Cheng and Hardy (2002) reported that only 5% to 10% of fish meal protein can be replaced by cottonseed meal in

diet for rainbow trout fingerlings (initial body weight 11.2 g). It is apparent that level of cottonseed meal inclusion in fish diets widely varies among fish species. In addition, the incorporation level of cottonseed meal in fish diet also depends on the developmental stage of the fish. Therefore, it was demonstrated from the present study that 8% cottonseed meal cannot be a problem in diets for juvenile parrot fish.

Recently, the use of mixture of plant protein meals has been reported to be superior to single one. The essential amino acid profile in multiple plant protein might be able to meet their requirements in many cultured fish species. In addition, the level of anti-nutritional substances in individual plant protein source can be reduced during feed processing (Riche et al., 2001; Kaushik et al., 2004). Pongmaneerat and Watanabe (1993) reported that mixture of soybean meal and corn gluten meal can replace up to 63% fish meal protein in rainbow trout diet. Tilapia fingerlings (initial body weight 3.7 g) were fed diets containing 100% protein from plant meals (El-Saidy and Gaber, 2003). In the present experiment, it was demonstrated that the use of different types of fermented soybean meal (Meju and SBM-Meju) with cottonseed meal and corn gluten meal was possible for juvenile parrot fish.

Whole body compositions of fish fed the experimental diets are given in Table 6. There were no significant differences in moisture, crude protein, crude lipid and ash contents of whole body of fish fed all the experimental diets after feeding trials. The same results have been indicated in a study conducted by Cheng and Hardy (2002). The authors reported that whole body composition of juvenile rainbow trout fed 10% CSM inclusion diets were not significantly different among fish groups. The results in our study indicated that the inclusion of cottonseed meal 8% DM, soybean meal 8% DM and corn gluten meal 8% DM, did not affect the whole body composition of parrot fish at the juvenile stage.

Red blood cell (RBC) in fish fed diet 4 (0.08% *Asperguillus oryzae*) was significantly higher than that of fish fed the control diet containing 8% soybean meal. All the other parameters were not significantly different from that of control diet. In another work with juvenile hybrid catfish, fed Jackbean (*Canavalia ensiformis*), the haematocrit, red blood cell count, white blood cell count and haemoglobin concentration decreased significantly ($P < 0.05$) with increasing dietary Jackbean seed meal (JBSM) (Osouigwe et al., 2005). Variables such as age, sex, dietary state and stress have been known to alter blood values (Barnhart, 1969; McCarthy et al., 1973). Stress factors due to capture, handling and sampling procedures are

factors which can cause intra-species haematological variations (Bouck and Ball, 1966). It has also been shown that haemaglobin concentration and haematocrit of fish blood decreases after the stress of capture and transportation (Hatting and Van Pletzer, 1974). Soybean hemagglutinin is readily inactivated by pepsin in the stomach (Mickelsen and Yang, 1966), and therefore would not appear to cause any significant problems for fish with true stomachs. Some of these antinutritional factors have been known to adversely affect some haematological parameters. Concanavalin A causes agglutination of red blood cells in monogastric (Liener, 1979) while saponins are known to cause erythrocyte haemolysis and reduction of blood (Cheeke, 1971). The progressive reduction in the values of the haematological parameters of hybrid catfish was caused by the increasing presence of antinutritional factors with increasing dietary JBSM level. This is in agreement with the finding of Dick et al. (1976) that nutritional toxicity is associated with anaemia. Herman (1970) also observed that gossypol an antinutritional factor found in some legumes severely reduced blood haematocrit and haemoglobin concentration in rainbow trout.

In terms of the processing type, it was observed that juvenile hybrid catfish fed the control diet had haematocrit, red blood cell count, white blood cell count and haemoglobin concentration that were higher ($P < 0.05$)

than the values of those fed boiled JBSM diets which were in turn higher and significantly different from those fed raw JBSM diets (Osuigwe et al., 2005). In the present study, the high values of red blood cell in diet 4 (*Asperguillus oryzae*), can be explained by the lower level of anti-nutritional factors in relation to the control group.

The apparent digestibility coefficients of protein and phosphorus in fish fed the experimental diets were provided in Table 7. The apparent digestibility coefficients of protein (Fig 14) of fish fed all the diets were not significantly different. Interestingly, however, phosphorus absorption was numerically increased in fish fed diets 3 (SBM-Meju) and 4 (*A. oryzae*) compared to that of fish fed the control diet, even though it was not significantly different.

In other experiments phytase initiates the release of phosphorus from phytate (myo-inositol hexakisphosphate), the storage form of phosphorus present in various seeds and grains. Phytase has been used as feed supplement to improve phosphorus nutrition and reduce phosphorus in excretory products of animal (Ravindran et al., 2001). Furthermore, hydrolysis of phytate also prevents protein-phytate complex formation, leaving more free protein available to be digested and absorbed for animal growth. Most of the studies on phytase production have been carried out in

fungi, and especially those in the genus *Aspergillus sp*. due to their high production yields and their low-pH tolerance. (Wodzinski et al., 1996). In another study, phytase production was studied in solid substrate fermentation with a fungal strain (*Aspergillus oryzae* AK9) normally used to produce soy sauce. For soy sauce koji preparation, AK9 is utilized for its protein digestibility with cooked soybean. It was reasoned that this fungus would also exhibit phytase activity since phytate is a normal constituent of soybean.

The increased DPPH radical scavenging activity in serum of fish fed Meju and SBM-Meju was not clear. However, it might be assumed that the increased antioxidant activity was due to fermentation of soybean. Even though the results of digestibility were not significant, fermentation process of soybean meal could increase the phosphorus absorption in parrot fish.

In conclusion, fermentation process of soybean meal was not able to increase growth performances and feed utilization in diets for parrot fish. However, the fermentation process of soybean meal could enhance the absorption of phosphorus and antioxidant capacity of juvenile parrot fish. The processing method of soybean meal is needed to investigate and a longer feeding period of the products should be adopted in the further studies. And, studies on different supplementation levels of SBM-Meju in

diets for parrot fish and other fish species would be helpful to determine its optimum supplementation for commercial use.

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VI. SUMMARY

This study was conducted to examine the effects of dietary supplementation of fermented soybean products on growth performances, feed utilization, antioxidant capacity and immune responses of juvenile parrot fish. Dietary dehulled soybean meal was replaced by traditional Meju and fermented soybean (SBM-Meju) by *Aspergillus oryzae*. The experimental diets were as follows; diet 1 = control, diet 2 = 4% Meju, diet 3 = 4% SBM-Meju, and diet 4 = 0.08% *Asperguillus oryzae* supplementation into the control. After 8 weeks of feeding trial, there were no significant differences in growth parameters, feed utilization, antioxidant capacity, and immune responses. Fish fed the test diets (diets2, 3 and 4) showed a higher feed intake, phosphorus absorption and serum antioxidant activity compared to the fish fed the control diet, even though they were not significantly different. The survivals of fish fed all the experimental diets were 100%. In conclusion, fermentation process of soybean meal was not able to increase growth performances and feed utilization in diets for parrot fish. However, the fermentation process of soybean meal could enhance the absorption of phosphorus and antioxidant capacity of juvenile parrot fish.

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