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Effects of Fermented Cottonseed and Soybean Meal with Phytase Supplementation on Gossypol Degradation, Phosphorus Availability, and Growth Performance of Olive Flounder (*Paralichthys olivaceus*)

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To reduce anti-nutritional factors in plant protein sources for fish meal replacement in fish feeds, cottonseed and soybean meal (CS) were fermented with *Aspergillus oryzae*. A feeding trial was conducted to verify the effects of fermented CS (FCS) with phytase supplementation on gossypol detoxification, phosphorus digestibility, antioxidant activity, and growth performance of juvenile olive flounder over 10 weeks. Four diets were formulated to replace 0, 30, or 40% fish meal protein with CS or FCS (designated as CS0, CS30, FCS30P, and FCS40P). Phytase (1,000 FTU/kg) was added to FCS30P and FCS40P. The microbial fermentation significantly increased dietary total polyphenols and consequently led to higher DPPH radical-scavenging activities in fish feed and fish tissue. Dietary and liver gossypol concentrations were dramatically decreased by the fermentation process. Phosphorus digestibility was significantly increased in fish fed the FCS40P diet. However, growth performance decreased in fish fed FCS diets. This study demonstrates that the fermentation process and phytase supplementation can improve the phosphorus availability of plant protein sources in fish. The fermentation of CS by *A. oryzae* could increase antioxidant activities in feed and fish and effectively degrade toxic gossypol in cottonseed meal.

Key words: *Aspergillus oryzae*, Cottonseed meal, Fermentation, Olive flounder, Soybean meal

Introduction

To reduce feed costs, research on fish meal replacements with alternative plant protein sources have been widely investigated for many fish species (Kaushik, 1990; Gatlin et al., 2007). Cottonseed and soybean meal (CS) have been reported to be good protein sources for fish due to their high protein content, relatively well-balanced amino acid profiles, and reasonable prices (Pham et al., 2007; Lim and Lee, 2008). However, the presence of anti-nutritional factors in these plant meals limits their use in fish diets (Masumoto et al., 2001). Techniques including heat treatment (Arndt et al., 1999; Peres et al., 2003), phytase supplementation (Biswas et al., 2007; Dalsgaard et al., 2009), and microbial fermentation (Chelius and Wodzinski, 1994; Matsui et al., 1996; Liang et al., 2008) have been used to eliminate or reduce anti-nutritional factors.

Fermentation has long been used to prepare traditional soybean foods in Asia. Some examples are “Dou-Bian-Jiang” in China, “Miso and Natto” in Japan, and “Meju and Duen-Jang” in Korea. It is one of the most promising techniques to destroy or decrease anti-nutritional factors present in plant protein sources and improve their nutritive values. *Aspergillus oryzae* produces enzymes, such as phytases and alpha-amylase (Fujita et al., 2003; Hong et al., 2004; Rahardjo et al., 2005), and is the main functional microorganism being used as a fermentation starter in producing commercial Meju using whole soybeans (Jung et al., 2006). It also enhances the antioxidant activity of soybeans after fermentation (Lin et al., 2006). We recently reported that fermentation of soybean meal with *A. oryzae* increased

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antioxidant activities in diets and non-specific immune responses of parrot fish (Kim et al., 2009).

Gossypol ($C_{30}H_{30}O_8$) is a toxic polyphenolic pigment present in the cotton plant (*Gossipium* spp.) and is the main limiting factor in feeding cottonseed meal for most monogastric animals including fish. Gossypol toxicity depends on several factors including the form of gossypol ((+)- or (-)-enantiomer), the consumed amounts, and the cottonseed species. Furthermore, toxic effects of gossypol are reported to be much greater in non-ruminants including fish (Willard et al., 1995). To remove gossypol from cottonseed, solvent extraction (Canella and Sodini, 1977; Cherry and Gray, 1981), chemical treatment with ferrous sulfate (Lim and Lee, 2009; Barraza et al., 1991) or calcium hydroxide (Nagalakshmi et al., 2002; Nagalakshmi et al., 2003), and microbial fermentation (Zhang et al., 2006; Zhang et al., 2007) have been tested for their efficacy. Microbial fermentation can offer a practical solution to gossypol detoxification because fermented cottonseed meal usually contains certain types of exoenzymes such as cellulolytic enzyme, amylase, protease, and lipolytic enzymes as well as some vitamins (Brock et al., 1994). However, no information is available on using this technique for fish feeds.

Therefore, the aim of this study was to determine the effect of CS fermented by *A. oryzae* with supplementation of phytase on growth performance, phosphorus availability, antioxidant activity, and gossypol detoxification in juvenile olive flounder (*Paralichthys olivaceus*).

Materials and Methods

Fish rearing

A feeding trial was conducted at the Marine and Environmental Research Institute (Jeju National University, Jeju, South Korea) using juvenile olive flounder (*P. olivaceus*), with an initial body weight of 2.5±0.1 g. The fish were from a genetically homogenous stock obtained by a private hatchery (Jeju Island, South Korea), and adapted to a commercial diet (Suhyupfeed Co., Ltd., GyeongNam, South Korea).

Fermentation of cottonseed and soybean meal

Solvent-extracted cottonseed meal and soybean meal (CS) were fermented by a process similar to preparation of traditional Meju with a slight modification (Kim et al., 2009). Briefly, CS in equal proportion (v/v) was finely ground and hydrated with distilled water at a ratio of 1:1.5 (w/w) for 30 min. The materials were cooked for 30 min at 100°C, cooled, and dried at room temperature. Subsequently, the materials were in-oculated with 3% *A. oryzae* (Jeil Bio Tech. Co., Ltd., Hwaseong, South Korea) on a w/w basis. The in-oculated materials were then made into a brick shape (3×15×10 cm) and incubated at 28°C for 48 h until a yellow layer of fungus formed. The fermented cottonseed and soybean meal (FCS) were dried again at room temperature (25°C) for 48 h and ground prior to their supplementation into the experimental diets. The major difference between Meju and FCS is the inclusion of whole soybean versus defatted CS, respectively, and the duration of the fermentation.

Diet preparation

Four experimental diets (designated as CS0, CS30, FCS30P, and FCS40P) were formulated and fed to fish in triplicate (12 tanks) to investigate the effect of partial substitution of fish meal by unfermented CS or FCS with phytase supplementation (Table 1).

Diet CS0, a fish-meal-based diet, was considered as the control diet. In diet CS30, 30% fish-meal protein in the control diet was replaced by unfermented CS. In diets FCS30P and FCS40P, 30 and 40% fish-meal protein was replaced by FCS with phytase supplementation (1,000 FTU/kg diet). The CS- or FCS-containing diets were supplemented with L-methionine and L-lysine to give levels equal to the fish-meal-based control diet. The level of fish-meal replacement in the experimental diets was determined from earlier observations with olive flounder. Fish-meal protein has been successfully replaced by CS with iron and phosphorus in the presence of supplemental lysine and methionine (Lim and Lee, 2008).

All diets were prepared in the laboratory. Briefly, all of the dry ingredients were minced and mixed in a feed mixer (NVM-16, Gyeonggido, South Korea), and then squid liver oil was added and mixed for 5 min. Distilled water was added to the mixture (20-30 g/100 g of feed weight) and mixed until a pebble-like consistency was achieved. The mixture was then converted to pellets by a meat-chopper machine (SMC-12, Kuposlice, Busan, South Korea). Pellets were dried at room temperature for 48 h, crushed into desirable particle sizes (0.4-2.0 mm), and stored in a freezer at -20°C.

Experimental designs

Three hundred fish were randomly distributed into twelve 50 L tanks (three replicates per diet). The tanks were arranged in a line with a flow-through system supplied with sand-filtered seawater at a flow rate of 2.5 L/min and oxygenated to above 80% saturation by

Table 1. Formulation and proximate composition of the experimental diets (% DM)

Ingredient	Diets			
	CS0[f]	CS30[g]	FCS30P[h]	FCS40P[i]
White fish meal	54.0	37.8	37.8	32.4
Soybean meal	0.0	11.8	0.0	0.0
Cottonseed meal[a]	0.0	12.7	0.0	0.0
Fermented soybean meal	0.0	0.0	11.8	15.7
Fermented cottonseed meal	0.0	0.0	12.7	16.9
Corn gluten meal	6.6	7.2	7.2	7.4
Wheat flour	24.0	13.3	13.3	9.72
Mineral mix[b]	0.5	0.5	0.5	0.5
Vitamin mix[c]	0.5	0.5	0.5	0.5
Squid liver oil	12.0	13.0	13.0	13.3
CMC	1.0	1.0	1.0	1.0
Lysine	0.0	0.6	0.6	0.8
Methionine	0.0	0.3	0.3	0.4
Ferrous Sulfate-7H_2O	0.0	0.3	0.3	0.3
Phytase[d]	0.0	0.0	0.01	0.01
Cellulose	0.9	0.5	0.49	0.49
Chromic oxide	0.5	0.5	0.5	0.5
Proximate composition				
Dry matter, %	88.5	88.0	88.8	88.9
Protein, % DM	48.1	48.3	51.0	51.2
Lipid, % DM	17.1	17.0	17.1	17.5
Ash, % DM	8.8	8.2	9.0	8.9
Gross energy, MJ/kg DM	17.9	17.9	17.9	17.9
Phytase activity (FTU/ kg DM)[e]	0.0	0.0	1215	1275

[a]Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.
[b]Mineral premix (g/kg of mineral premix): $MgSO_4.7H_2O$, 80.0; $NaH_2PO_4.2H_2O$, 370.0; KCl, 130.0; Ferric citrate, 40.0; $ZnSO_4 \cdot 7H_2O$, 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.
[c]Vitamin premix (g/kg of vitamin premix): 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.
[d]Phytase (10,000 FTU/g) was purchased from Easy Bio System, Seoul, Korea.
[e]Phytase activity was analyzed according to the method described by Han et al. (1999).
[f]CS0=Fish-meal-based diet (control group).
[g]CS30=30% fish meal protein was replaced by unfermented cottonseed meal.
[h]FCS30P=30% fish meal protein was replaced by FCS with phytase supplementation.
[i]FCS40P=40% fish meal protein was replaced by FCS with phytase supplementation.

an air supply. The water temperature naturally ranged from 20 to 24°C, and the photoperiod was maintained at 12 h light/12 h dark during the feeding period. All tanks were cleaned as necessary.

Before starting the feeding trial, fish were acclimated for a week to the experimental conditions and fed the fish-meal-based control diets (CS0). Then, the fish were fed twice per day (8:00 h and 18:00 h) to apparent satiation, 7 days per week, for 10 weeks in accordance with normal flounder-culture practice. Uneaten food was collected 30 min after feeding, dried, and reweighed to determine feed intake and feed conversion ratio. Fish growth was measured every 2 weeks, and feeding was stopped 24 h prior to weighing. Experimental protocols followed the guidelines of the Animal Care and Use Committee of Jeju National University.

Feces collection and apparent digestibility test

When the feeding trial was completed, the remaining fish were transferred to a 150 L fecal collection tank. To measure apparent digestibility coefficient (ADC) of phosphorus in the experimental diets, the diets were reground, mixed with 0.5% chromic oxide (Cr_2O_3) as an inert marker, pelleted, and air-dried for 48 h. Feces were collected by a modified fecal collection system for olive flounder (Yamamoto et al., 1998) with a slight modification (Pham et al., 2008). To collect the feces, all the fish were fed their respective diets to apparent satiation at 18:00 h for 1 week. The

feces were collected the next morning and afternoon at 7:00 and 14:00 h, respectively. The collected feces were dried and analyzed. The ADC of phosphorus was calculated based on chromic oxide as a non-absorbable indicator (Storebakken et al., 1998) as follows:

ADC = 100 × [1 - (amount Cr_2O_3 in diets × amount phosphorus in feces) / (amount Cr_2O_3 in feces × amount phosphorus in diet)]

Chromic oxide in feces and diets was measured according to the method described by Furukawa and Tsukahara (1996). Total phosphorus in diets and feces was measured using an inductively coupled plasma emission spectrophotometer as described by Leske and Coon (1999).

Chemical analyses

At the end of the feeding trial, three fish per tank were sampled and stored at -70°C for whole-body proximate analysis. Crude protein was determined by the Kjeldahl method (N × 6.25), crude fat by extraction with diethyl ether using the soxhlet Tecator system, ash by burning samples in a muffle furnace at 550°C for 5 h, and moisture by drying at 110°C until constant weight.

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

Antioxidant activities in diets and livers were measured using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging assay described by Sandoval et al. (2002). Two grams of feed or whole livers were homogenized (PT 2100, Kinematica, Lucerne, Switzerland) in 80% aqueous methanol and centrifuged at 5000 rpm at 4°C for 10 min and filtered through 0.45 μm syringe filters prior to the assay. One hundred microliters 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 was measured at 517 nm after 10 min by a spectrophotometer (Genesys 10 UV). The antioxidant activity of the extract against the DPPH radicals was calculated as percent inhibition:

Percent inhibition = 100 × [(A_0 - A_{10})/A_0], where A_0 and A_{10} are the absorbance of the sample at 0 and 10 min, respectively.

Total gossypol concentration in diets and livers (9 fish per treatment) were determined with high-performance liquid chromatography (HPLC) according to the method described by Lee and Dabrowski (2002). The liver and dry feed were weighed, and 3-10 volumes of complexing reagent was added to obtain 2-amino-1-propanol derivatives of gossypol. The complexing reagent was composed of 2 mL 2-amino-1-propanol (Sigma-Aldrich), 10 mL glacial acetic acid (Sigma-Aldrich) and 88 mL N, N-dimethylformamide (Sigma-Aldrich). The samples were homogenized in complexing reagent for 30 sec, heated at 95°C for 30 min, cooled on ice and then centrifuged at 1,500 × *g* for 5 min. After centrifugation, an aliquot of the supernatant was diluted with mobile phase [800 mL acetonitrile with 10 mM KH_2PO_4 dissolved in 200 mL water (HPLC grade); adjusted to pH 3.0 with H_3PO_4] to obtain a desirable concentration, centrifuged again at 1,500 × *g* for 5 min, and filtered through a syringe filter (0.45 μm; Whatman Inc.) before injection. The mobile phase was delivered at a flow rate of 1.0 mL/min. The HPLC injection volume was 20 μL. The retention time for the (+)- and (-)-gossypol were 3.5 and 5.6 min, respectively.

Statistical analyses

All diets were assigned to tanks with a completely randomized design. Data were analyzed by one-way analysis of variance (ANOVA) in SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). When ANOVA identified differences among groups, the differences in mean values were evaluated with Duncan's multiple range test. Statistical significance was determined by setting the aggregate type I error at 5% ($P<0.05$) for each set of comparisons. Data are presented as mean ± SD. Percentage data were arcsine transformed before statistical analysis.

Results

Final body weight, specific growth rate, and protein efficiency ratio of fish fed the FCS30P and FCS40P diets were significantly lower than those of fish fed the control diet. No significant differences in growth performance were observed in fish fed the CS30 diet and the control diet. However, in contrast, fish fed FCS showed significantly higher feed intake than did

those fed the control diet, contrary to growth performance. Survival of fish fed all the experimental diets was over 90%, and no differences among diet groups were observed (Table 2).

There were no differences in whole-body proximate composition among fish groups fed the experimental diets, except for ash content. Whole-body ash contents of fish fed FCS30P and FCS40P were significantly higher than those of fish fed the control and CS30 diets (Table 3).

The fermentation process of CS increased dietary polyphenol concentration, and the highest concentration was observed in the FCS40P diet. Total polyphenol concentration in the FCS30P diet was also significantly higher than in the CS30 diet (Fig. 1).

Dietary supplementation of CS or FCS significantly increased antioxidant activities in diets and fish livers. DPPH radical-scavenging activity in diets increased with the addition of dietary CS or FCS, and this activity was significantly higher with the FCS30P diet than with the CS30 diet (Fig. 2a). Liver DPPH radical-scavenging activity was significantly higher in fish fed the diets containing CS or FCS compared with fish fed the control diet (Fig. 2b).

The ADC of phosphorus in fish fed FCS40 was significantly higher than that in fish fed the other diets. However, the ADC of phosphorus did not increase in fish fed FCS30 compared with fish fed CS30 or the control diet (Fig. 3).

Total gossypol concentrations in diets and livers were affected by the fermentation process of CS. In diets CS30 and FCS30P, the fermentation process decreased total, (+)-gossypol, and (-)-gossypol enantiomer concentrations in the diets by 17, 16, and 25%, respectively. Gossypol concentration was higher in the CS30 diet than in the FCS30P and FCS40P diets (Fig. 4a). Gossypol (both total and each enantiomer) in the liver was only detected in fish fed the CS30 diet, whereas it was not detected in fish fed the FCS-containing diets (Fig. 4b).

Discussion

The current study showed that diets containing 30 or 40% FCS negatively affected the growth performance of juvenile olive flounder, even though the fish fed the FCS diets had higher feed intake compared with those fed the control diet. Similar observations were made in our previous trial with parrot fish (*Oplegnathus fasciatus*), in which growth and feed utilization were decreased in fish fed fermented soybean meal or fermented whole soybeans, although the result was not significant (Kim et al., 2009). The lower growth performance of juvenile olive flounder fed the FCS-containing diets seemed to be a consequence of the presence of anti-nutritional factors or changes in

Table 2. Growth performance and feed utilization of juvenile olive flounder fed the experimental diets for 10 weeks[a]

Diets	CS0	CS30	FCS30P	FCS40P
Weight gain (WG)[b]	537 ± 19.1[a]	505 ± 48.1[a]	349 ± 23.2[b]	306 ± 31.7[b]
Specific growth rate (SGR)[c]	1.09 ± 0.02[a]	1.06 ± 0.05[a]	0.88 ± 0.03[b]	0.82 ± 0.02[b]
Feed intake (g/g BW)[d]	1.94 ± 0.13[a]	2.36 ± 0.13[a]	2.28 ± 0.02[a]	2.59 ± 0.43[b]
Feed conversion ratio (FCR)[e]	1.24 ± 0.01	1.30 ± 0.07	1.38 ± 0.04	1.49 ± 0.10
Protein efficiency ratio (PER)[f]	1.67 ± 0.01[a]	1.56 ± 0.04[a]	1.13 ± 0.09[b]	0.95 ± 0.07[c]
Survival (%)	100 ± 0.0	91.7 ± 7.8	98.3 ± 1.3	93.3 ± 8.9

[a]Values are presented as mean ± SD (n=3). Values in the same row having different superscripts are significantly different ($P<0.05$).
[b]WG = 100 × [FBW (g) – IBW (g)] / IBW.
[c]SGR = 100 × [(ln FBW (g) – ln IBW (g)) / days].
[d]FI = dry feed consumed (g)/ body weight (g).
[e]FCR = dry feed fed (g) /wet weight gain (g).
[f]PER = wet weight gain (g) / total protein given (g).

Table 3. Whole body proximate composition (wet weight) of juvenile olive flounder fed the experimental diets for 10 weeks[a]

Diets	CS0	CS30	FCS30P	FCS40P
Moisture content, %	74.6 ± 0.5	75.1 ± 0.9	75.1 ± 0.4	75.0 ± 0.3
Protein, % DM	16.6 ± 1.0	16.3 ± 0.8	16.4 ± 0.5	16.5 ± 0.2
Lipid, % DM	6.0 ± 0.6	5.3 ± 0.4	4.9 ± 0.5	5.1 ± 0.3
Ash, % DM	2.5 ± 0.2[a]	2.7 ± 0.3[a]	3.4 ± 0.2[b]	3.3 ± 0.2[b]

[a]Values are presented as mean ± SD (n=3). Values in the same row having different superscripts are significantly different ($P<0.05$).

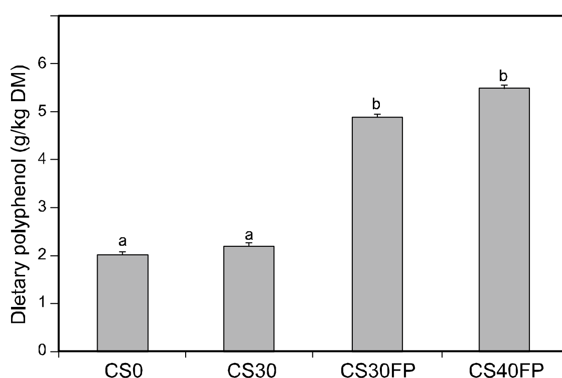

Fig. 1. Total polyphenol contents in the experimental diets (CS0=Fish-meal-based diet, CS30=30% fish meal protein was replaced by unfermented cottonseed meal, FCS30P and FCS40P=30 and 40% fish meal protein were replaced by the FCS with phytase supplementation (1,000 FTU/kg diet), respectively). Bars represent mean±standard errors of three fish from each of triplicate groups. The bars with different letters are significantly different (P<0.05).

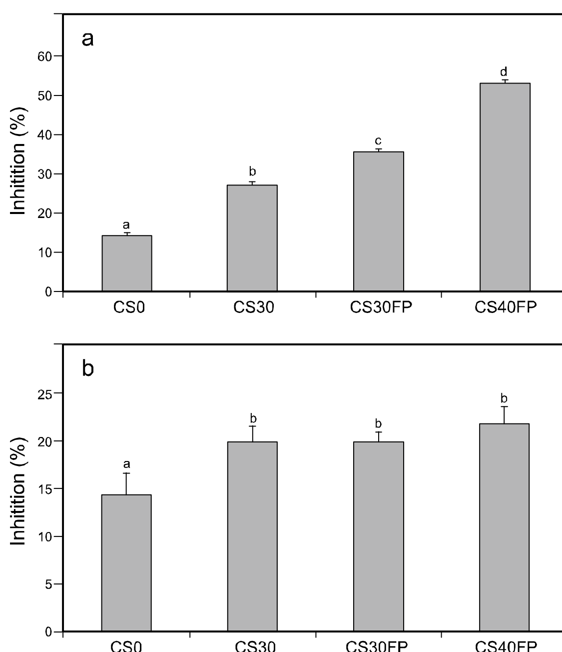

Fig. 2. DPPH radical-scavenging activity in diets (a) and liver (b) of juvenile olive flounder fed experimental diets for 10 weeks. Absorbance was measured at 517 nm. Bars represent mean±standard errors of three fish from each of triplicate groups. The bars with different letters are significantly different (P< 0.05).

physical and chemical properties of FCS in the diets. In our previous study, we found that the fermentation process decreased levels of some essential amino

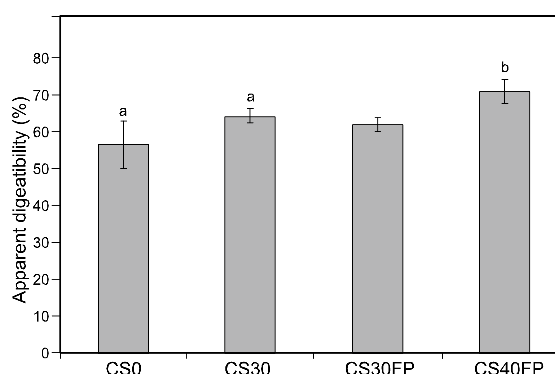

Fig. 3. Apparent digestibility coefficient of phosphorus in juvenile olive flounder fed the experimental diets fed for 10 weeks. Bars represent mean± standard errors of three fish from each of triplicate groups. The bars with different letters are significantly different (P<0.05).

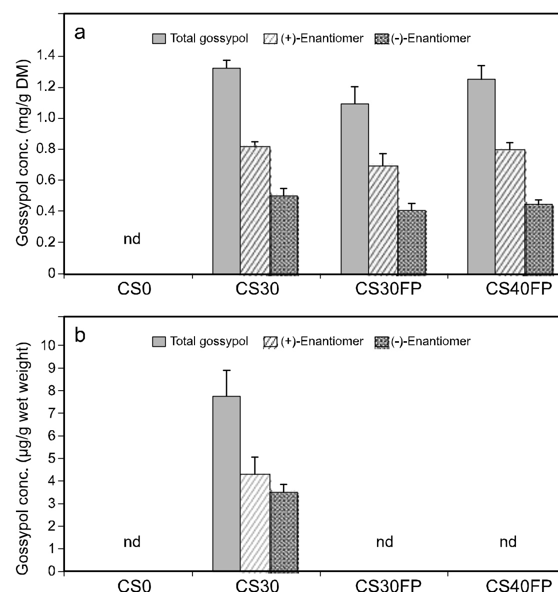

Fig. 4. Total and (+) and (-) enantiomers of gossypol in diets (a) and liver (b) of juvenile olive flounder fed experimental diets for 10 weeks. Bars represent mean±standard errors of three fish from each of triplicate groups. Treatments with different letters are significantly different (P<0.05). nd: not detected.

acids in CS and resulted in higher daily feed intake in parrot fish (data not published). The finding in the present study is partly in agreement with the results reported by Dabrowski et al. (2007) and suggests that the CS fermentation process can change amino acid profiles and reduce the concentration of specific amino acids, thereby increasing feed intake of fish to compensate for the deficiency. The fish, however, did

not catch up with the growth of fish fed either fishmeal-based or regular CS-containing diets. Reasons for this should be examined in future studies.

In the current study, whole-body composition was not affected by the fermentation process or phytase supplementation, except for whole-body ash content. The increase in whole-body ash content may be related to an excess of inorganic phosphorus in FCS30P and FCS40P diets by the supplementation of phytase. Sajjadi and Carter (2004) reported an interaction between phytase and inorganic phosphorus in bone ash, bone phosphorus, and whole-body ash contents. In that study, Atlantic salmon fed diets containing phytase had higher ash content than did fish fed diets containing phytic acid. The fermentation process may be another reason for the increased ash content in FCS30P and FCS40P diets. Filamentous fungi, including *A. oryzae,* are potent tools in solid-state fermentation and have been commonly used for the production of phytase (Ramachandran et al., 2005). Therefore, dietary supplemental phytase and the CS fermentation are considered to be the main reasons for the increased whole-body ash content in the current study.

The current study clearly demonstrated a beneficial effect of fermentation of CS on polyphenolic compound concentrations. The increase in dietary total polyphenols after incorporation of the fermented material is in agreement with the findings of other studies (Lin et al., 2006; Randhir et al., 2004; Vattem and Shetty, 2002). Jayabalan et al. (2008) also reported through an *in vitro* test that polyphenolic compounds from green tea, black tea, and tea waste material were increased by fermentation. This phenolmenon could reflect that β-glucosidase produced from microorganisms catalyzes the release of aglycones from substrates during fermentation and increases their polyphenol concentration.

Most antioxidants in feed are polyphenolic compounds, acting as reducing agents and metal chelators (Mathew and Abraham, 2006). Skerget et al. (2005) reported that DPPH radical-scavenging activities were linearly correlated with the concentration of polyphenolic compounds. Thus, in the current study, the higher antioxidant activities in CS or FCS diets and in the livers of fish fed the diets is likely related to their high total polyphenol concentration. Flavones and isoflavones in plant protein sources are known to have strong antioxidant capacities against free radicals (Birt et al., 2001). Birt et al. (2001) reported that the bioavailability of flavones or isoflavones is influenced by their chemical form in the products (mostly glycoside conjugates) and by their hydrophobicity and susceptibility to degradation by microbial flora. However, fish cannot utilize the isoflavones in glycoside conjugates, which can only be hydrolyzed by sulfatase and glucuronic acid (Piskula and Terao, 1998). It is believed that the microorganism *A. oryzae* in the fermented products hydrolyzed the isoflavone glycosides into isoflavone aglycones, which can be utilized by fish. Therefore, this is considered to be the cause of the increased dietary polyphenols and improved antioxidant capacities in diets and fish tissue in this study.

In the current study, phytase was supplemented in the experimental diets, as suggested by Yoo et al. (2005). The higher ADC of phosphorus might be attributed to either supplementation of microbial phytase or the fermentation process. Increased ADC of phosphorus in diets containing plant protein sources with supplementation of exogenous phytase has been reported in many fish species including rainbow trout (Sugigura et al., 2001; Cheng and Hardy, 2003; Vielma et al., 2004), Atlantic salmon (Sajjadi and Carter, 2004), Korean rockfish (Yoo et al., 2005), striped bass (Paratryphon and Soares, 2001), African catfish (Van et al., 1999), channel catfish (Li et al., 2004), olive flounder (Masumoto et al., 2001), and Nile tilapia (Portz and Liebert, 2004). Additionally, the fermentation process has been reported to increase phosphorus availability in plant protein sources due to production of relatively large amounts of extracellular phytase during microbial fermentation (Chelius and Wodzinski, 1994). Liang et al. (2008) reported that fermentation was the most effective treatment in decreasing phytic acid (56-96% removal) in brown rice. Matsui et al. (1996) found that fermentation with *Aspergillus usami* improved phosphorus availability of soybean meal for chicks and proposed that dietary supplementation of inorganic phosphorus is not necessary for chicks fed fermented soybean meal. Therefore, it can be concluded that a fermentation process and phytase supplementation (1,000 FTU/kg) can reduce phytic acid, liberate phosphorus from phytate, and consequently improve its availability in fish fed CS-containing diets.

The absence of detectable gossypol in the livers of fish fed FCS-containing diets clearly demonstrates that the microbial fermentation of cottonseed meal can reduce the toxicity of dietary gossypol to fish (Fig. 4b). Gossypol degradation may be caused by either dietary supplemental iron as ferrous sulfate or the fermentation processing of the cottonseed meal. Ferrous sulphate has been used to counteract the toxic effect of free gossypol in fish (Sealey et al., 1997; Barros et al., 2002). Gossypol detoxification by microbial fermen-

tation was previously reported, and an *in vitro* test indicated that microbial fermentation could greatly decrease free gossypol concentrations in cottonseed meal, although the effectiveness depends on the species of microorganism used (Zhang et al., 2006; Zhang et al., 2007). They chose six fungi strains, including *A. oryzae,* for microbial fermentation of cottonseed meal and concluded that *Candida tropicalis* was the most effective among the six strains in detoxifying free gossypol. The decrease in gossypol concentrations found both in diets and in fish livers in the present study seemed to be due to the binding of free gossypol to amino acids secreted by microorganisms or by the microorganism itself, which contains several exoenzymes that degrade gossypol molecules (Brock et al., 1994). In the current study, ferrous sulfate was equally supplemented in diets containing both regular and fermented CS (CS30, FCS30P, and FCS40P). Therefore, it is clear that the degradation of dietary gossypol and the failure to detect it in fish tissue were due to the effect of the fermentation process only.

In conclusion, fermentation of plant protein sources and phytase supplementation could improve phosphorus availability of CS-containing diets for juvenile olive flounder. The fermentation process of CS by *A. oryzae* could increase antioxidant activities in feed and in the fish fed the diets, effectively reducing toxic gossypol in cottonseed meal for fish. These preliminary results on microbial fermentation of cottonseed meal provide insights to improve the quality of fish feeds, which usually contain high levels of oxidation-susceptible polyunsaturated fatty acids and to utilize cottonseed meal at relatively high levels for dietary inclusion. However, the slow growth rate of fish fed FCS needs to be studied further.

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References

Arndt RE, Hardy RW, Sugiura SH and Dong FM. 1999. Effects of heat treatment and substitution level on palatability and nutritional value of soy defatted flour in feeds for Coho Salmon, *Oncorhynchus*. Aquaculture 180, 129-145.

Barraza ML, Coppock CE, Brooks KN, Wilks DL, Saunders RG and Latimer GW. 1991. Iron sulfate and feed pelleting to detoxify FG in cottonseed diets for dairy cattle. J Dairy Sci 74, 3457-3467.

Barros MM, Lim C and Klesius PH. 2002. Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response and resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. Aquaculture 207, 263-279.

Birt DF, Hendrich S and Wang WQ. 2001. Dietary agents in cancer prevention: flavonoids and isoflavonoids. Pharmacol Therapeut 90, 157-177.

Biswas AK, Kaku H, Ji SC, Seoka M and Takii K. 2007. Use of soybean meal and phytase for partial replacement of fish meal in the diet of red sea bream, *Pagrus major*. Aquaculture 267, 284-291.

Brock TD, Madigan MT, Martinko JM and Parker J. 1994. Biology of Microorganisms, seventh ed. Prentice-Hall, Englewood Cliffs, NJ.

Canella M and Sodini G. 1977. Extraction of gossypol and oligosaccharides from oil seed meals. Food Sci 42, 1218-1219.

Chelius MK and Wodzinski RJ. 1994. Strain improvement of *Aspergillus niger* for phytase production. Appl Microbiol Biot 41, 9-83.

Cheng ZJ and Hardy RW. 2003. Effects of extrusion and expelling processing, and microbial phytase supplementation on apparent digestibility coefficients of nutri-tents in full fat soybeans for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 218, 501-514.

Cherry JP and Gray S. 1981. Methylene chloride extraction of gossypol from cottonseed products. Food Sci 46, 1726-1733.

Dabrowski K, Arslan M, Terjesen BF and Zhang YF. 2007. The effect of dietary indispensable amino acid imbalances on feed intake: Is there a sensing of deficiency and neural signaling present in fish? Aquaculture 268, 136-142.

Dalsgaard J, Ekmann KS, Pedersen PB and Verlha V. 2009. Effect of supplemented fungal phytase on performance and phosphorus availability by phosphorus-depleted juvenile rainbow trout (*Oncorhynchus mykiss*), and on the magnitude and composition of phosphorus waste output. Aquaculture 286, 105-112.

Fujita J, Yamane YI, Fukuda H, Kizaki Y, Wakabayashi S, Shigeta S, Suzuki O and Ono K. 2003. Production and properties of phytase and acid phosphatase from a sake koji mold, *Aspergillus oryzae*. J Biosci Bioeng 95, 348-353.

Furukawa H and Tsukahara H. 1966. On the acid digestion method for the determination if chromic oxide as an index substance in the study of digestibility of fish fed. Bull Jpn Soc Sci Fish 32, 502-508.

Gatlin III DM, Barrows FT and Brown P. 2007. Expanding the utilization of sustainable plant products in aquafeeds: a review. Aquac Res 38, 551–579.

Han Y, Wilson DB, Lei XG (1999) Expression of an *Aspergillus niger* phytase gene (phyA) in *Saccharomyces cerevisiae*. Appl Environ Microb 65: 1915-1918.

Hong KJ, Lee CH and Kim SW. 2004. *Aspergillus oryzae* GB-107 fermentation improves nutritional quality of food soybean and feed soybean meals. J Med Food 7, 430-436.

Jayabalan R, Subathradevi P, Marimuthu S, Sathishkumar M and Swaminathan K. 2008. Chang in free-radical scavenging ability of kombucha tea during fermentation. Food Chem 109, 227-234.

Jung KO, Park SY and Park KY. 2006. Longer aging time increases the anticancer and antimetastatic properties of *Deonjang*. Nutrition 22, 539-545.

Kaushik SJ. 1990. Use of alternative protein sources for the intensive rearing of carnivorous fishes. In: Flos, R., Tort, L., Torres, P. (ed) Mediterranea Aquaculture. InEllis Horwood, UK, pp 125-138.

Kim SS, Galaz GB, Pham MA, Jang JW, Oh DH, Yeo IK and Lee KJ. 2009. Effects of dietary supplementation of a Meju, fermented soybean meal, and *Aspergillus oryzae* for juvenile parrot fish (*Oplegnathus fasciatus*). Asian-Aust J Anim Sci 22, 849-856.

Lee KJ and Dabrowski K. 2002. Tissue gossypol and gossypolone isomers in rainbow trout fed low and high levels of dietary cottonseed meal. J Agri Food Chem 50, 3056-3061.

Leske KL and Coon CC. 1999. A bioassay to determine the effect of phytase on phytate phosphorus hydrolysis and total phosphorus retention of feed ingredients as determined with broilers and laying hens. Poultry Sci 78, 1151-1157.

Li MH, Manning BB and Robinson EH. 2004. Summary of phytase studies for channel catfish. Res Report 23, 1-5.

Liang JL, Han BZ, Nout MJR and Hamer RJ. 2008. Effects of soaking, germination and fermentation on phytic acid, total and in vitro soluble zinc in brown rice. Food Chem 110, 821-828.

Lim SJ and Lee KJ. 2008. Supplementation iron and phosphorus increase dietary inclusion of cottonseed and soybean meal in olive flounder (*Paralichthys olivaceus*). Aquacult Nutr 14, 423-430.

Lim SJ and Lee KJ. 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. Aquaculture 290, 283-289.

Lin CH, Wei YT and Chou CC. 2006. Enhanced anti-oxidative activity of soybean koji prepared with various filamentous fungi. Food Microbiol 23, 628-633.

Masumoto T, Tamura B and Shimeno S. 2001. Effects of phytase on bioavailability of phosphorus in soybean meal-based diets for Japanese flounder, *Paralichthys olivaceus*. Fisheries Sci 67, 1075-1080.

Mathew S and Abraham TE. 2006. In vitro antioxidant activity and scavenging effects of *Cinnamomum verum* leaf extracts assayed by different methodologies. Food Chem Toxicol 44, 198-206.

Matsui T, Hirabayashi M, Iwama Y, Nakajima T, Yano F and Yano H. 1996. Fermentation of soya-bean meal with *Aspergillus usami* improves phosphorus availability in chicks. Anim Feed Sci Tech 60, 131-136.

Nagalakshmi D, Sastry VRB and Agrawal DK. 2002. Detoxification of undecorticated cottonseed meal by various physical and chemical methods. Anim Nutr Feed Tech 2, 117-126.

Nagalakshmi D, Sastry VRB and Pawde A. 2003. Rumen fermentation patterns and nutrient digestion in lambs fed cottonseed meal supplemental diets. Anim Feed Sci Tech 103, 1-14.

Paratryphon E and Soares JH. 2001. The effect of phytase on apparent digestibility of four practical plant feed stuffs fed to striped bass, *Morone saxatilis*. Aquacult Nutr 7, 161-167.

Peres H, Lim C and Klesius PH. 2003. Nutritional value of heat-treated soybean meal for channel catfish (*Ictalurus punctatus*). Aquaculture 225, 67-82.

Pham MA, Lee KJ, Dang TM, Lim SJ, Lee BJ, Ko GY, Eo JN and Oh DH. 2008. Improved apparent digestibility coefficient of protein and phosphorus by supplementation of microbial phytase in diets containing cottonseed and soybean meal for juvenile olive flounder (*Paralichthys oliveceus*). Asian-Aust J Anim Sci 21, 1367-1375.

Pham MA, Lee KJ, Lim SJ and Park KH. 2007. Evaluation of cottonseed and soybean meal as partial replacement for fishmeal in diets for juvenile Japanese olive flounder *Paralichthys olivaceus*. Fisheries Sci 7, 760-769.

Piskula M and Terao J. 1998. Quercetin`s solubility affects its accumulation in rat plasma after oral administration. J Agr Food Chem 50, 3056-3061.

Portz L and Liebert F. 2004. Growth, nutrient utilization and parameters of mineral metabolism in Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758) fed plant-based diets with graded levels of microbial phytase. J Anim Physiol An N 88, 311-320.

Rahardjo YSP, Weber FJ, Haemers S, Tramper J and Rinzema A. 2005. Aerial mycelia of *Aspergillus oryzae* accelerate alpha-amylase production in a model solid state fermentation system. Enzyme Microb Tech 36, 900-902.

Ramachandran S, Roopesh K, Nampoothiri KM, Szakacs G and Pandey A. 2005. Mixed substrate fermentation for the production of phytase by *Rhizopus* spp. using oilcakes as substrates. Process Biochem 40, 1749-1754.

Randhir R, Vatterm D and Shetty K. 2004. Solid-state bioconversion of fava bean by *Rhizopus oligosorus* for

enrichment of phenolic antioxidants and L-DOPA. Innovative Food Sci Emerg 5, 235-244.

Sajjadi M and Carter CG. 2004. Effect of phytic acid and phytase on feed intake, growth, digestibility and trypsin activity in Atlantic salmon (*Salmo salar,* L). Aquacult Nutr 10, 135-142.

Sandoval M, Okuhama NN, Angeles FM, Melchor VV, Condezo LA, Lao J and Miller MJS. 2002. Antioxidant activity of the cruciferous vegetable Maca (*Lepidium meyenii*). Food Chem 79, 207-213.

Sealey WM, Lim C and Klesius PH. 1997. Influence of the dietary level of iron from iron methionine and iron sulfate on immune response and resistance of channel catfish to *Edwardsiella ictaluri.* J World Aquacult Soc 28, 142-149.

Skerget M, Kotnik P, Hadolin M, Hras AR, Simonic M and Knez Z. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and the antioxidant activities. Food Chem 89, 191-198.

Storebakken T, Shearer KD and Roem AJ. 1998. Availability of protein, phosphorus and other elements in fish meal, soy-protein concentrate and phytase-treated soy-protein-concentrate-based diets to Atlantic salmon, *Salmo salar.* Aquaculture 161, 365-379.

Sugigura SH, Gabaudan J, Dong FM and Hardy RW. 2001. Dietary microbial phyatse supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout (*Oncorhynchus mykiss*) fed soybean meal-based diets. Aquac Res 32, 583-592.

Van WJH, Khalaf KHA, Aartsen FJ and Tijssen PAT. 1999. Balance trials with catfish *Clarias gariepinus* fed phytase treated soybean meal-based diets. Aquacult Nutr 5, 135-142.

Vattem D and Shetty K. 2002. Solid-state production of phenolic antioxidants from cranberry pomace by *Rhizopus oligosporus*. Food Biotechnol 16, 189-210.

Vielma J, Ruohonen K, Gabaudan J and Vogel K. 2004. Top-spraying soybean meal based-diets with phytase improve protein and mineral digestibilities but not lysine utilization in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Aquac Res 35, 955-964.

Willard ST, Neuendorff DA, Lewis AW and Randel RD. 1995. Effect of FG in the diet of pregnant and postpartum Brahman cows on calf development and cow performance. Anim Sci 73, 496-507.

Yamamoto T, Akimoto A, Kishi S, Unuma T and Akiyama T. 1998. Apparent and true availabilities of amino acids from several protein sources for fingerlings rainbow trout, common carp, and red sea bream. Fisheries Sci 64, 448-458.

Yoo GY, Wang X, Choi S, Han K, Kang JC and Bai SC. 2005. Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish *Sebastes schelegeli* fed diets containing soybean meal. Aquaculture 243, 315-322.

Zhang WJ, Xu ZR, Zhao SH, Jiang, JF, Wang YB and Yan XH. 2006. Optimization of process parameters for reduction of gossypol levels in cottonseed meal by *Candida tropicalis* ZD-3 during solid substrate fermentation. Toxicon 48, 221-226.

Zhang WJ, Xu ZR, Zhao SH, Sun JY and Yang X. 2007. Development of a microbial fermentation process for detoxification of gossypol in cottonseed meal. Anim Feed Sci Tech 135, 176-186.

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