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

Gossypol (C_{20}H_{26}O_5) is a toxic polyphenolic pigment present in the cotton plant (Gossypium 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 (Suhuyupfeed 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)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>CS0 †</th>
<th>CS30 ‡</th>
<th>FCS30P ‡</th>
<th>FCS40P ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>White fish meal</td>
<td>54.0</td>
<td>37.8</td>
<td>37.8</td>
<td>32.4</td>
</tr>
<tr>
<td>Soybean meal</td>
<td>0.0</td>
<td>11.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Cottonseed meal ‡</td>
<td>0.0</td>
<td>12.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Fermented soybean meal</td>
<td>0.0</td>
<td>0.0</td>
<td>11.8</td>
<td>15.7</td>
</tr>
<tr>
<td>Fermented cottonseed meal</td>
<td>0.0</td>
<td>0.0</td>
<td>12.7</td>
<td>16.9</td>
</tr>
<tr>
<td>Corn gluten meal</td>
<td>6.6</td>
<td>7.2</td>
<td>7.2</td>
<td>7.4</td>
</tr>
<tr>
<td>Wheat flour</td>
<td>24.0</td>
<td>13.3</td>
<td>13.3</td>
<td>9.72</td>
</tr>
<tr>
<td>Mineral mix ‡</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Vitamin mix ‡</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Squid liver oil</td>
<td>12.0</td>
<td>13.0</td>
<td>13.0</td>
<td>13.3</td>
</tr>
<tr>
<td>CMC</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.0</td>
<td>0.6</td>
<td>0.6</td>
<td>0.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Ferrous Sulfate-7H2O</td>
<td>0.0</td>
<td>0.3</td>
<td>0.3</td>
<td>0.3</td>
</tr>
<tr>
<td>Phytase ‡</td>
<td>0.0</td>
<td>0.0</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>Cellulose</td>
<td>0.9</td>
<td>0.5</td>
<td>0.49</td>
<td>0.49</td>
</tr>
<tr>
<td>Chromic oxide</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Proximate composition

- **Dry matter, %**
  - CS0 †: 88.5
  - CS30 ‡: 88.0
  - FCS30P ‡: 88.8
  - FCS40P ‡: 88.9

- **Protein, % DM**
  - CS0 †: 48.1
  - CS30 ‡: 48.3
  - FCS30P ‡: 51.0
  - FCS40P ‡: 51.2

- **Lipid, % DM**
  - CS0 †: 17.1
  - CS30 ‡: 17.0
  - FCS30P ‡: 17.1
  - FCS40P ‡: 17.5

- **Ash, % DM**
  - CS0 †: 8.8
  - CS30 ‡: 8.2
  - FCS30P ‡: 9.0
  - FCS40P ‡: 8.9

- **Gross energy, MJ/kg DM**
  - CS0 †: 17.9
  - CS30 ‡: 17.9
  - FCS30P ‡: 17.9
  - FCS40P ‡: 17.9

Phytase activity (FTU/ kg DM) ‡

- CS0 †: 0.0
- FCS30P ‡: 0.0
- FCS40P ‡: 1215

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† Cottonseed meal was purchased from Southern Cotton Oil Co., Memphis, Tennessee 38108, USA.
‡ Mineral premix (g/kg of mineral premix): MgSO4·7H2O, 80.0; NaH2PO4·2H2O, 370.0; KCl, 130.0; Ferric citrate, 40.0; ZnSO4·7H2O, 20.0; Ca-lactate, 356.5; CuCl2, 0.2; AlCl3, 6H2O, 0.15; Na2Se2O3, 0.01; MnSO4·H2O, 2.0; CoCl2·6H2O, 1.0.
§ 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-aminobenzoic acid, 18.2; menadione, 1.8; retinyl acetate, 0.73; cholecalciferol, 0.003; cyanocobalamin, 0.003.
¶ Phytase (10,000 FTU/g) was purchased from Easy Bio System, Seoul, Korea.
|| Phytase activity was analyzed according to the method described by Han et al. (1999).
*CS0 = Fish-meal-based diet (control group).
†CS30 = 30% fish meal protein was replaced by unfermented cottonseed meal.
‡FCS30P = 30% fish meal protein was replaced by FCS with phytase supplementation.
§FCS40P = 40% fish meal protein was replaced by FCS with phytase supplementation.
Activity of the extract against the DPPH radicals was calculated as percent inhibition:

\[
\text{Percent inhibition} = 100 \times \left[ \frac{A_0 - A_{10}}{A_0} \right], \quad \text{where } A_0 \text{ and } A_{10} \text{ are the absorbance of the sample at 0 and 10 min, respectively.}
\]

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 x g for 5 min. After centrifugation, an aliquot of the supernatant was diluted with mobile phase [800 mL acetonitrile with 10 mM KH₂PO₄ dissolved in 200 mL water (HPLC grade); adjusted to pH 3.0 with H₃PO₄] to obtain a desirable concentration, centrifuged again at 1,500 x 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.
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

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

<table>
<thead>
<tr>
<th>Diets</th>
<th>CS0</th>
<th>CS30</th>
<th>FCS30P</th>
<th>FCS40P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight gain (WG)</td>
<td>537 ± 19.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>505 ± 48.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>349 ± 23.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>306 ± 31.7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Specific growth rate (SGR)&lt;sup&gt;3&lt;/sup&gt;</td>
<td>1.09 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.06 ± 0.05&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.88 ± 0.03&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.82 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Feed intake (g/g BW)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.94 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.36 ± 0.13&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.28 ± 0.02&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.59 ± 0.43&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Feed conversion ratio (FCR)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.24 ± 0.01</td>
<td>1.30 ± 0.07</td>
<td>1.38 ± 0.04</td>
<td>1.49 ± 0.10</td>
</tr>
<tr>
<td>Protein efficiency ratio (PER)&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1.67 ± 0.01&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.56 ± 0.04&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.13 ± 0.09&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.95 ± 0.07&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Survival (%)</td>
<td>100 ± 0.0</td>
<td>91.7 ± 7.8</td>
<td>98.3 ± 1.3</td>
<td>93.3 ± 8.9</td>
</tr>
</tbody>
</table>

<sup>2</sup>Values are presented as mean ± SD (n=3). Values in the same row having different superscripts are significantly different (*P*<0.05).

<sup>3</sup>WG = 100 × [FBW (g) – IBW (g)] / IBW.

<sup>4</sup>SGR = 100 × [(ln FBW (g) – ln IBW (g)) / days].

<sup>5</sup>FCR = dry feed consumed (g)/ body weight (g).

<sup>6</sup>PER = wet weight gain (g) / total protein given (g).

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

<table>
<thead>
<tr>
<th>Diets</th>
<th>CS0</th>
<th>CS30</th>
<th>FCS30P</th>
<th>FCS40P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Moisture content, %</td>
<td>74.6 ± 0.5</td>
<td>75.1 ± 0.9</td>
<td>75.1 ± 0.4</td>
<td>75.0 ± 0.3</td>
</tr>
<tr>
<td>Protein, % DM</td>
<td>16.6 ± 1.0</td>
<td>16.3 ± 0.8</td>
<td>16.4 ± 0.5</td>
<td>16.5 ± 0.2</td>
</tr>
<tr>
<td>Lipid, % DM</td>
<td>6.0 ± 0.6</td>
<td>5.3 ± 0.4</td>
<td>4.9 ± 0.5</td>
<td>5.1 ± 0.3</td>
</tr>
<tr>
<td>Ash, % DM</td>
<td>2.5 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.4 ± 0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.3 ± 0.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>2</sup>Values are presented as mean ± SD (n=3). Values in the same row having different superscripts are significantly different (*P*<0.05).
Effect of Dietary Supplementation of Fermented Cottonseed and Soybean Meal

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).

Fig. 3. Apparent digestibility coefficient of phosphorus in juvenile olive flounder fed the experimental diets 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.

In our previous study, we found that the fermentation process decreased levels of some essential amino 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 phenomenon 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-scaevenging 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-
Optimization 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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