EnzoMeal as a fish meal replacer: effects on growth, digestive and oxidative status of yellow perch (*Perca flavescens*)

†Corresponding author: *Vikas Kumar (vikas.kumar@kysu.edu), Division of Aquaculture, College of Agriculture, Food Science and Sustainable Systems, Kentucky State University, Frankfort, KY, 40601, US.* Office: +1-502-597-8130, Fax: +1-502-597-8118. Hanping Wang (wang.900@osu.edu)

Abstract

This experiment was conducted to develop a tandem chemical and enzymatic treatment of soybean meal (SBM) to reduce the antinutritional factors (ANFs), and to produce EnzoMeal (EZM) for fish feed. Using yellow perch (*Perca flavescens*) fingerlings, a 10-week experiment was conducted to evaluate the nutritional quality of the EZM to compare with that of fish meal (FM) and SBM. Fingerlings (297; 11.01 ± 0.19 g) were randomly distributed in nine treatments with three replicates and fed iso-nitrogenous diets (crude protein 41%): control (FM based protein); S_{50} and S_{100} (50% and 100% of FM protein replaced by SBM), and EZM_{50} and EZM_{100} (50% and 100% of FM protein replaced by EZM). These diets were also supplemented with an exogenous enzyme cocktail to reduce ANFs and named as $S_{50+E,}$ $S_{100+E,}$ $EZM_{50+E,}$ and $EZM_{100+E,}$ respectively. All diets were processed in a Brabender single screw extruder, using a 1:1 compression ratio, with processing temperatures of approximately 40^{o}C. The highest ($P < 0.05$) growth performance and nutrient utilization parameters (protein efficiency ratio and protein productive value, PPV) were observed for the EZM_{50+E} group, which were not statistically different to that for control and EZM_{50} groups, and significantly ($P < 0.05$) higher than all other groups. The least ($P < 0.05$) growth performance was observed for the $S_{100,}$ S_{100+E} and EZM_{100} groups. On the other hand, the opposite trend was observed for the feed conversion ratio. Digestive enzymes (amylase, lipase and protease) activity was higher ($P < 0.05$) in the intestine than pyloric caeca of fish in all groups. The highest protease activity (in intestine and pyloric caeca) was observed for the control group, which is significantly similar to EZM_{50}, $EZM_{50+E,}$ and S_{50+E} groups, and the lowest value was observed for 100% replacement of FM protein by SBM and EZM fed groups. However, inclusion of exogenous enzymes in feed showed positive effects in EZM_{50+E} compared to EZM_{50} for PPV and lipid productive value and amylase activity in the intestine. Activity of protein metabolism enzymes i.e., alanine transaminase and aspartate aminotransferase in the

liver were the highest in the control, which was similar ($P < 0.05$) to the EZM_{50+E} and EZM_{50} groups, whereas other groups exhibited lower activity. Antioxidant enzymes (catalase, superoxide dismutase, and glutathione peroxidase) in the liver were the lowest in the control group, which was significantly similar to the group fed the feed with 50% replacement of FM protein, whereas complete (100%) replacement of FM protein exhibited the highest activity. Conclusively, performance of the EZM_{50} and EZM_{50+E} groups were similar to the FM fed group, and better than SBM fed groups, and EnzoMeal has considerable potential as alternatives to fishmeal in aquafeed.

Keywords: EnzoMeal, antinutritional factors, exogenous enzymes cocktail, growth, protein metabolism, antioxidant enzymes, yellow perch.

Introduction

Aquaculture is the fastest-growing sector of food production. Traditionally, fish meal (FM) has been the main source of dietary protein for farmed fish. However, the increasing demand in contrast to limited supply for this finite protein source has caused a significant increase in FM prices in the last decade (Kader *et al*. 2010; Kumar *et al*. 2010a; Kumar *et al*. 2010b; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a). Therefore, it is a matter of urgency that alternative protein sources for fish diets be found to support global aquaculture development (Kader *et al*. 2010; Kumar *et al*. 2011a; Kumar *et al*. 2011b). Several alternative proteins have been tested for fish feed, among them soybean appeared to be the best FM replacer for fish diet. It has been reported that 30–50% FM could be replaced by soybean meal (SBM). Soybean meals have a high content of available protein with a well-balanced amino acid profile, a constant composition, a reasonable price, and there is a steady supply available; however, their methionine level is low, and they also contain approximately 30% of indigestible carbohydrates including non-starch polysaccharides (NSP), and several compounds or anti-nutritional factors (ANFs; protease inhibitors, lectin, and phytate) that may disturb the digestive process (Hernandez *et al*. 2007; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a; Olli *et al*. 1994; Storebakken *et al*. 2000), as many of them have been reported to hinder digestion and absorption of nutrients and restrict their potential as replacements of FM in fish diets (Bureau *et al*. 1998; Hernandez *et al*. 2007; Klein *et al*. 1998; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a; McGoogan & Gatlin 1997; Olli & Krogdahl 1994; Rumsey *et al*. 1994).

Digestion and absorption of nutrients depends on the activity of the digestive enzymes, in particular those located in the intestine, which are responsible for the final stages of breaking down and assimilation of the food (Klein *et al*. 1998; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a; Silva *et al*. 2010). In salmonids, higher inclusion levels of

solvent-extracted SBM led to a marked reduction in the activities of such enzymes in enterocytes of the distal intestine (Bakke-McKellep *et al*. 2000; Krogdahl *et al*. 1995; Krogdahl *et al*. 2003; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a; Silva *et al*. 2010). This suggests that measuring the activities of intestinal enzymes may represent a sensitive tool to study the effects of differently-processed SBMs and cocktail exogenous enzymes on nutrient bioavailability and to ascertain tolerability to certain soy-ANFs in various fish species.

In addition, nutrition plays an important role in animal welfare by maintaining this fragile oxidative balance, either by supplying nutrients that enhance the antioxidant system or avoiding those that would induce an increase of reactive oxygen species (ROS) production by different physiological pathways. The oxidative stress in aquatic organisms is more profound during nutritional deficiency, elevated temperature, hypoxia and exposure to xenobiotics (Avanzo *et al*. 2002; Hwang & Lin 2002; Kolkovski *et al*. 2000; Radhakrishnan *et al*. 2014; Romeo *et al*. 2000). However, either an increase in ROS production above the level that can be removed by antioxidant defenses, or a decrease in the capacity of the antioxidant defenses, could result in oxidative damage to key molecules, including DNA, protein, and lipids (lipid peroxidation) (Halliwell & Gutteridge 1999).

While the predominant concern about the effects of various alternative plant proteins is on fish growth and feed efficiency, relatively few studies have monitored the dietary influence on the biochemical index of fish, such as changes in protein metabolism enzyme activities (Krogdahl *et al*. 2003; Kumar *et al*. 2010b; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a), hepatic metabolism (Vilhelmsson *et al*. 2004) and oxidative status, which have also been used to provide an indication of disturbance by specific ingredient to the metabolic function and nutrient utilization by fish (Kumar *et al*. 2011a; Kumar *et al*. 2011b; Kumar *et al*. 2012a; Lin & Luo 2011).

Yellow perch (*Perca flavescens*) is a major species of the Great Lakes region, particularly in the north central region of the USA, and is a consumer favorite due to low fat content and good taste (Fallahi *et al*. 2012; González *et al*. 2006). However, commercial production of yellow perch has not rapidly developed yet, partially due to limited information on nutritional requirements. A commercially available specialized diet has not been developed for yellow perch. Therefore, it has made it essential to develop suitable complete and supplemental diets for use in yellow perch. It may well be that fish with more omnivorous feeding habits, like yellow perch, could make more efficient use of high levels of dietary SBM.

To our knowledge, no studies have been reported on the use of enzyme-treated SBMs as a FM substitute in yellow perch diets. The aims of this study were to 1) test a modified soybean meal diet (EnzoMeal) against a regular soybean meal diet and a standard fish meal diet using yellow perch, and determine the benefits and deficiencies of the EnzoMeal as a fish food ingredient; and 2) evaluate the possibility of replacing different levels of FM protein by SBM and EZM in the practical diet of yellow perch.

Materials and methods

This study and all experimental procedures involving animals were performed according to the protocol approved by the Ohio State University Institutional Animal Care and Use Committee.

Preparation of EnzoMeal and diet formulations

The EZM was obtained by heating SBM (NUTRASOY® 7B, obtained from Archer Daniels Midland, Decatur, IL, USA) in 3% hydrogen peroxide solution using a proprietary catalyst. The product was isolated and further treated with an enzyme cocktail.

Prior to feed formulation, the proximate composition of SBM, whole wheat meal, EZM, soy protein isolate, and FM were determined. Nine isonitrogenous and isolipidic diets were formulated. Experimental diets containing crude protein 41%, crude lipid 11%, vitamin premix 2%, mineral premix 2%, and Titanium oxide (TiO2) 0.2% were prepared. TiO2 was added for digestibility measurement. Methionine was supplemented at the rate of 0.3% in 100% FM protein replacement diets. Experimental diets are as follows: control (FM based protein); S_{50} and S_{100} (50% and 100% of FM protein replaced by SBM), and EZM_{50} and EZM_{100} (50% and 100% of FM protein replaced by EZM), and SBM and EZM based diets were also supplemented with an exogenous enzyme cocktail (phytase, NSPase, and carbohydrase) and named as S_{50+E}, S_{100+E}, EZM_{50+E} and EZM_{100+E} respectively (Table 1).

Enzyme cocktail supplementation

Enzymes (cocktail) and buffer were mixed only with SBM and EZM and kept at room temperature for three hours to hydrolyze the carbohydrates and phytate. Thereafter, these soybean products (hydrolyzed SBM and EZM) were mixed along with other fish feed ingredients for fish diet preparation.

2.1.2. Feed extrusion: All ingredients were ground with a Wiley mill (Model 4, Thomas Scientific, Swedesboro, NJ, USA) to an average particle size of approximately 500 μm. All components were then combined and mixed for 15 min (Kushlan Products, Inc., Goldendale, WA). After all ingredients were thoroughly homogenized, each blend was adjusted to a desired pre-extrusion moisture content of ~40% by adding adequate amounts of water, then mixed again for 15 min. The extrusion processing of each blend was performed using a single screw extruder (Brabender Plasti-Corder, Model PL 2000, South Hackensack, NJ, USA), which had a compression ratio of 3:1, a screw length-to-diameter ratio of 20:1, and a barrel length of 317.5 mm. The die assembly was conical, and tapered from an initial diameter of

6.0 mm to a diameter of 3.0 mm at the discharge opening. The length of the die was 27.0 mm, which resulted in a die length-to-diameter ratio of 9.0. A 7.5 HP (5.5 kW) motor powered the extruder. The screw speed was set to 100 rpm during extrusion, and the temperature of all extrusion zones (feed, transition, and die) were set to 40°C for the duration of processing. After extrusion, the pelletized feed blends were dried in a laboratory oven (Thelco Precision, Jovan, Winchester, VA) at 50°C for 24 h. After drying, the diets were broken by hand, sieved into proper pellet size, and were stored at −15°C. Enzymes may have been active during the feed drying process, thus hydrolyzing substrates in the feed ingredients.

Experimental system and animals

A mixed strain of yellow perch fingerlings (about 10-12 g) was produced in the breeding program at the Ohio Center for Aquaculture Research and Development at The Ohio State University South Centers, Piketon, OH, USA. These fingerlings (N = 297) were randomly distributed in nine groups with three replicates, each having 11 fish (11.01 ± 0.19 g) in a round tank (55 L capacity). At the start of the experiment, six fish were preserved at −20°C for analysis of the initial body composition. After fish were acclimatized for two weeks in the experimental tanks, fish were trained for different experimental diets for 1 week. The all tanks were supplied with water from a flow-through system. The system was subjected to a photoperiod of 12 h light: 12 h darkness. Water quality was monitored throughout the experiment. All the water parameters were in the optimum range (temperature 17.4 – 20.1°C, pH 7.0 – 7.5, and dissolved oxygen 6.7 – 8.4 mg l^{-1}). Water flow was adjusted to the same level for all tanks. During the experimental period, a ration totaling 3% body weight daily was hand-fed to the fish in three equal proportions at 9:00, 13:00, and 17:00 h. Fish were weighed individually at the beginning of the experiment and at every other week intervals during the experimental period to adjust the feeding level for the subsequent week. On the

designated weigh days, the fish were weighed in the morning, just prior to the 9:00 h feeding, having not had feed for 16 hours prior.

The experiment lasted for 10 weeks. At the end of the experiment, three fish per group were anaesthetized with tricaine methanesulfonate (MS222; 250 mg/L). Anaesthetized fish were carefully dissected to isolate the intestine, pyloric caeca, and liver. These samples were stored at -80°C for determination of activities of digestive, protein metabolism and antioxidant enzymes. Four additional fish per group were killed by a blow to the head with a metal rod and then stored at −20°C for chemical composition analysis. Prior to determination of the proximate composition, the fish were autoclaved at 121°C for 20 min, thoroughly homogenized using an Ultra-Turrax T25, frozen overnight and freeze-dried.

Proximate analysis of feed ingredients, experimental diets, and whole body of fish

All samples were ground to a fine powder by using a grinder prior to analyses. The proximate composition of the feed ingredients, experimental feeds, and of the fish carcasses was determined according to the official methods (AOAC, 1990), i.e., for moisture (oven-drying at 105°C overnight), crude protein by CN analyser (N X 6.25), fat [by extraction according to the method described by Smedes (1999) as modified by Schlechtriem *et al*. (2003) and ash (oven incineration at 480°C overnight).

Growth and nutrient utilization parameters

Growth performance and diet nutrient utilization were assessed in terms of:

— Body mass gain percentage (BMG, %) = [(final body mass - initial body mass) / initial body mass] X 100

— Specific growth rate (SGR, g/day) = [(ln final body mass in g) – (ln initial body mass in g) / number of trial days] X 100

- Metabolic growth rate (MGR, g $kg^{0.8}$ day^{-1}) = (Body mass gain in g) / [{(initial body mass in g / 1000)^0.8 + (final body mass in g / 1000)^0.8}/2] / number of trial days
- Feed conversion ratio (FCR) = dry feed fed (g)/body mass gain (g)
- Protein efficiency ratio (PER) = body mass gain (g)/crude protein fed (g)
- Protein productive value (PPV, %) = [(final fish body protein in g - initial fish body protein in g) / total protein consumed in g] X 100
- Lipid productive value (LPV, %) = [(final fish body lipid in g - initial fish body lipid in g) / total crude lipid consumed in g] X 100

Organ indices

Relative intestinal length (RIL), hepatosomatic index (HSI) and intestinal somatic indexes (ISI) are calculated as indicated below:

RIL = Intestine length (mm)/body mass (g)

HSI = Liver mass (g) X 100 / body mass (g) and

ISI = Intestinal mass (g) X 100 / body mass (g).

Enzyme assays

Digestive enzyme assay

Amylase activity was measured in the pyloric caeca and intestine by using and amylase assay kit (Kit no. # MAK009 SIGMA, Aldrich, USA). Amylase activity was determined using a coupled enzymatic assay, which resulted in a colorimetric product, proportional to the amount of substrate, ethylidene-pNP-G7, cleaved by the amylase. One unit is the amount of amylase that cleaves ethylidene-pNP-G7 to generate 1.0 μmole of p-nitrophenol per minute at 25°C. RayBiotech's Protease Activity Assay Kit (CODE: 68AT-Protease-S100, Ray Bio, USA) was used to determine the protease activity in pyloric caeca and intestine of fish. One

unit is defined as the amount of protease that cleaves the substrate, to yield an amount of fluorescence equivalent to 1.0 nmol of unquenched fluorescein isothiocyanate labeled casein per minute at 25°C. Lipase activity was determined using a coupled enzyme reaction, which resulted in a colorimetric product proportional to the enzymatic activity present in pyloric caeca and intestine of fish. Lipase Activity was measured using an assay Kit (MAK046 Sigma Aldrich, USA). One unit of lipase is the amount of enzyme that will generate 1.0 µmole of glycerol from triglycerides per minute at 37°C.

Protein metabolism enzyme

BioVision's Aspartate aminotransferase (AST) assay Kit (Catalog# K753-100, Bio Vision, USA) was used to determine the AST activity in liver of fish. The principle is amino group is transferred from aspartate to α-ketoglutarate. The products of this reversible transamination reaction are oxaloacetate and glutamate. The glutamate is detected in a reaction that concomitantly converts a nearly colorless probe to color. One unit of AST is the amount of enzyme that will generate 1.0 mmole of glutamate per minute at pH 8.0 at 37°C. Alanine transaminase (ALT) activity was quantified by using an ALT activity Assay Kit (Cayman Chemical Item Number 700260, USA). Measurement of the ALT activity was carried out by monitoring the rate of NADH oxidation in a coupled reaction system employing lactate dehydrogenase (LDH). The oxidation of NADH to NAD^+ was accompanied by a decrease in absorbance. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 µmol of NADH to NAD^+ per minute at 37°C.

Antioxidant enzymes

A superoxide dismutase (SOD, inhibition rate %) assay kit (Catalog # *19160 SIGMA*, Sigma Aldrich, USA) was used to assess the SOD enzyme activity in the liver of fish. SOD was

assayed by utilizing Dojindo's highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl) - 3-(4-nitrophenyl)-5-(2,4-disulfophenyl) - 2H-tetrazolium, monosodium salt) that produces a water-soluble formazan dye upon reduction with a superoxide anion. *Catalase* enzyme activity was determined with a catalase assay kit (Cayman Chemical Item Number 707002, USA). The determination is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured spectrophotometrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which, upon oxidation, changes from colorless to a purple color. One unit is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C. A glutathione peroxidase (GPx) colorimetric assay kit (Catalog # K762-100, Bio Vision, USA) was used to determine the GPx activity in the liver. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 μmol of NADPH to $NADP^+$ under the assay kit condition per minute at 25°C.

Statistical analysis

All data were subjected to a one-way analysis of variance ANOVA and the significance of the differences between means was tested using Tukey's HSD (Honestly Significant Difference) test ($P < 0.05$). The software used was SAS, Version 9.3. Values are expressed as means ± standard deviation.

Results

Proximate composition of feed ingredient and experimental diets

Total crude protein content in the EZM was 58%, and SBM contains 47.5% crude protein (Table 2), which revealed that crude protein amount increased almost 22%, whereas ANFs

especially oligosaccharides, NSPs, and phytate content decreased (>50%) in EZM compared to SBM (data not shown). Heat labile antinutrients such as lectin and trypsin inhibitor were absent in EZM because we have used the roasted SBM.

The composition of experimental diets is shown in Table 1. Proximate composition of feed ingredients and experimental diets are shown in Table 2. Experimental diets contained about 41% crude protein and 15% crude lipid and were isonitrogenous and isolipidic.

Chemical composition of whole body of fish and organ indices

The effects of different inclusion levels of EZM and SBM with and without supplementations of exogenous cocktail enzymes on the body composition of yellow perch are presented in Table 3. The highest ($P < 0.05$) inclusion of EZM and SBM (100% FM protein replacement groups) regardless the inclusion of exogenous enzymes, exhibited higher ($P < 0.05$) moisture content than that of the control and other groups; whereas crude protein and lipid content exhibited an opposite trend. The highest ($P < 0.05$) moisture content was observed in the S_{100} group, which was significantly similar ($P > 0.05$) to the 100% replacement groups (S_{100+E}, EZM_{100} and EZM_{100+E}), whereas the lowest value was found in the control group. Whole body protein content was the highest ($P < 0.05$) in the EZM_{50+E} group, which is statistically similar to $EZM_{50,}$ S_{50+E}, and control groups; whereas the lowest value was observed for 100% replacement groups ($S_{100,}$ $EZM_{100,}$ and S_{100+E}). The highest ($P < 0.05$) crude lipid content was observed in $EZM_{50,}$ which was similar to S_{50} and the control groups, and these values were statistically higher than other groups. Dietary soybean protein and exogenous enzyme supplementation did not significantly ($P > 0.05$) affect the ash content in the whole body of the fish.

Hepato somatic index, GSI, CI, and RIL of yellow perch fed different experimental diets are shown in Table 3. The hepatosomatic indexes were higher in EZM fed groups

(except EZM_{100}+E) than regular SBM fed groups. The relative intestinal length values of plant-fed groups were significantly ($P < 0.05$) higher than the control group. EZM and SBM did not influence ($P > 0.05$) the GSI of fish among the groups.

Fish behavior, feed intake, growth and nutrient utilization

Based on the visual observation during the feeding period, palatability or acceptability of feed was good and the behaviour of fish was normal, except in the 100% FM replacement groups, wherein these groups exhibited lower palatability than other groups.

The effects of the two processed soybean meals (EZM and SBM) with and without the exogenous enzyme cocktail for growth performance, feed intake and nutrient utilization are summarized in Tables 3 and 4, and Figures 1A and 2. The highest ($P < 0.05$) growth performance (FBM, weight gain, SGR, and MGR) were observed for the EZM_{50+E} group, which were not statistically different to that for the control and EZM_{50} groups, and significantly ($P < 0.05$) higher than all other groups. The least ($P < 0.05$) growth performance was observed for the $S_{100,}$ $S_{100+E,}$ and EZM_{100} groups. On the other hand, the opposite trend was observed for the FCR. Feed intake was positively correlated to the growth performance (tables 3 and 4). The highest ($P < 0.05$) PER, PPV, and LPV values were observed for the S_{50+E} group which is statistically similar to the control and EZM_{50} groups. The lowest values for these parameters were observed in the 100% FM replacement groups. Overall, EZM fed groups exhibited higher ($P < 0.05$) growth performance and feed utilization than SBM fed groups; whereas dietary supplementation of exogenous enzymes both in EZM or SBM did not significantly affect the growth performance and feed utilization of yellow perch except PPV and LPV in the EZM_{50+E} group.

Enzymes

Digestive enzyme activities in pyloric caeca and the intestine of yellow perch are presented in Figure 3A, 3B and 3C. Digestive enzymes (amylase, lipase, and protease) activity was higher ($P < 0.05$) in the intestine than in pyloric caeca of fish in all groups. Amylase activity in pyloric caeaca and intestine was the highest ($P < 0.05$) in EZM_{50+E} whereas other groups exhibited lower ($P < 0.05$) value. Inclusion of exogenous enzymes in feed showed positive effects ($P < 0.05$) in EZM_{50+E} compared to EZM_{50} for the amylase activity in the intestine. The highest protease activity (in intestine and pyloric) was observed for the control group, which is significantly similar to the EZM_{50}, $EZM_{50+E,}$ and S_{50+E} groups, and the lowest ($P < 0.05$) value was observed for 100% replacement of FM protein by SBM and EZM fed groups. Lipase activity in the intestine was the highest ($P < 0.05$) in $EZM_{50+E,}$ which was significantly similar ($P > 0.05$) to the control, $EZM_{50,}$ and 50% replacement groups (FM replaced by SBM), whereas 100% FM replacement groups exhibited lower activities of lipase in the intestine. Amylase activity in the pyloric caeca did not differ significantly ($P > 0.05$) among the groups.

Dietary inclusion of EZM and SBM significantly ($P < 0.05$) changes the activities of protein metabolism enzymes (ALT and AST) in liver of yellow perch (Figure 4A). Activities of protein metabolism enzymes i.e., ALT and AST in liver were the highest in control, which was similar ($P < 0.05$) to the EZM_{50+E} and EZM_{50} groups whereas other groups exhibited lower activity. Activities of protein metabolism enzymes were positive correlated with growth performance and feed utilization. Alanine transaminase and AST activities in liver exhibited positive response (not significantly) with dietary inclusion of the exogenous enzyme cocktail in EZM and SBM based diets.

Antioxidant enzyme (catalase, SOD, and GPx) activities in liver were significantly altered by different experimental diets (Figures 1B and 4B). Antioxidant enzymes such as catalase, SOD, and GPx in the liver were the lowest ($P < 0.05$) in the control group, which was significantly similar to the 50% replacement of FM protein fed groups, whereas groups

having complete (100%) replacement of FM protein exhibited the highest activity. Dietary supplementation of exogenous enzyme cocktail in SBM and EZM based diets did not influence ($P > 0.05$) the antioxidant enzyme activity in fish.

Discussion

The use of plant protein products in aquaculture diets is generally limited by their low levels of digestible protein and/or energy (Kumar et al., 2011a; Kumar et al., 2011b). The seed meals of soybeans, lupins, pea, and Jatropha represent some of those plant products that contain a considerable amount of proteins, and efforts have been made to further enhance their protein levels by processing technologies (Glencross *et al*. 2005; Kumar *et al*. 2012a; Makkar & Becker 2009; Overland *et al*. 2009). In the present study we have developed a method to produce soybean meal containing a high amount of protein (58%) with no ANFs (oligosaccharides, NSP, and phytate), and tested efficacy in replacing fish meal in yellow perch. To our knowledge, no studies have been reported on the use of enzyme-treated SBM as a FM substitute in yellow perch diets.

Feed intake, growth and nutrient utilization

The results of the present study clearly show that the nutritive value of complete diets for yellow perch may vary when differently processed soybean meals (roasted SBM and EZM along with or without the inclusion of exogenous enzymes) are used to replace varying amounts of FM protein. In this experiment relative to control (fish meal based diet), palatability was not adversely affected by type of SBM (regular SBM or EZM) inclusion in the diet; however, the level of SBM inclusion adversely affected the feed intake. In general, feed intake has an inverse relationship to higher FM replacement levels with plant proteins in

omnivorous and carnivorous fish (Kader *et al*. 2011; Kader *et al*. 2012; Uyan *et al*. 2006). In the present experiment, feed intake was lower in the 100% FM replacement groups compared to the 50% replacement groups. However, there were no significant effects of supplementation of exogenous enzymes in either EZM or SBM based diets on feed intake and growth performance. In this study, there was a decreasing trend in feed intake with increasing dietary SBM and EZM level, similar to the findings of some previous studies (Espe *et al*. 2006; Kaushik *et al*. 2004). Kaushik *et al*. (2004) used a regression model, while Espe *et al*. (2006) used a total replacement of FM, adding different ingredients to increase voluntary feed intake. The former reported that feeding diets containing only 0.5% FM to European seabass did not affect feed intake and reduce performance on high inclusion of plant ingredients, while in the latter study only one of the groups reduced feed intake, but all reduced growth as compared to the FM control. These results indicate that the effects on the growth response might directly be attributed to the partly even significantly lower feed intake of fish fed the plant protein diets (Espe *et al*. 2006). Hence, in our study, reduced feed intake (consequently a lower intake of essential nutrients and digestible energy) could be the main reason for reduced growth performance of yellow perch as the dietary SBM and EZM level increased.

Reduced nutrient/energy bioavailability and growth in response to complete feeds containing high levels of conventionally-processed SBM and EZM, relative to FM-based diets, has been observed in almost all carnivorous and omnivorous fish species, including yellow perch investigated to date, and various causes and mechanisms have been claimed as possible reasons. Of these, oligosaccharides and NSPs in soy preparations have been suggested to lead to reduced bioavailability of all nutrients and energy through mechanisms involving a binding action with bile salts and/or by an obstructing action on digestive enzymes coupled with changes in digesta viscosity and transit rate (Francis *et al*. 2001; Tibaldi *et al*. 2006). This is supported by findings that soy-protein derivatives obtained

through processes, which ensure partial (50%) removal of non-starch carbohydrate fractions, like EZM or the enzyme-treated SBMs here tested, resulted in higher nutritive value relative to regular SBM when used at 50 and 100% replacement levels (Bureau *et al*. 1998; Hernandez *et al*. 2007; Kumar *et al*. 2010a; Kumar *et al*. 2010b; Kumar *et al*. 2011a; Kumar *et al*. 2011b; McGoogan & Gatlin 1997; Olli & Krogdahl 1994; Rumsey *et al*. 1994). Deficiencies in minerals or essential amino acids may also have impacted the ability of perch to utilize soy products at levels greater than 500 g/kg (Brown *et al*. 1997; Kasper *et al*. 2007; Ketola 1975). In the present study, the use of the EZM with zero oligosaccharides to substitute up to 50% FM protein in the diet resulted in nutrient utilization (FCR, PER, PPV and LPV) values and growth performance, which did not differ from those measured in fish fed the control diet (Refstie *et al*. 1999). Our results are in concurrence with many studies (Denstadli *et al*. 2007; Glencross *et al*. 2005; Kaushik *et al*. 2004; Mambrini *et al*. 1999; Overland *et al*. 2009; Vielma *et al*. 2000), which have shown that soy, lupin, and pea protein isolates and soy protein concentrate can replace 50-75% of FM protein in rainbow trout and Atlantic salmon diets without impairing the growth performance and nutrient utilization. Médale *et al*. (1998) observed that total replacement of FM by soy protein concentrate led to a significant decrease in feed intake and resulted in poor growth partially due to methionine deficiency in the soy protein concentrate based diet.

Soybean meal fed groups exhibited lower growth performance compared to EZM fed groups. Hence, there seems to be some evidence that a higher intake of soy non-starch carbohydrates lowers the nutritive value of yellow perch diets, but the extent to which this depressive effect is attributable to soy oligosaccharides or NSPs could not be established in this experiment.

The weight gain data indicate that SBM/EZM inclusion had no positive impact on production when combined with supplemental exogenous cocktail enzymes (NSPase, phytase

and carbohydrase) but there is increasing trend (not significantly) towards growth and nutrient utilization of feed. Our results concur with other reports suggesting no adverse effects when these enzymes are added to feeds (Allan *et al*. 1998; Kocher *et al*. 2003; Stone *et al*. 2003). However, research with other species has shown a positive impact when a supplemental exogenous cocktail enzyme has been included in aquafeeds wherein a multienzyme (amylase, protease, β- gluconase, β-glucosidase, cellulose and combination of these enzymes) in feed significantly improved weight gain, FCR, and other nutritional parameters (Baas & Thacker 1996; Castañón *et al*. 1997; Cowieson *et al*. 2006; Denstadli *et al*. 2011). The mechanism by which exogenous multienzymes enhance nutrient digestion and utilization in plant proteins have not been identified, but disruption of cell wall integrity and the breakdown of the highly viscous NSP and carbohydrate were thought to be the major factors involved (Sinha *et al*. 2011). The supplemental exogenous enzymes had been shown to decrease digesta viscosity, feed and protein utilization, and improve digestibilities of amino acids, protein, and lipid in fish (Denstadli *et al*. 2011).

Biochemical composition

Efficient protein synthesis requires sufficient availability of all essential amino acids (Dabrowski & Guderly 2002; Kumar *et al*. 2011a; Kumar *et al*. 2011b). Unbalanced amino acid concentrations in a diet resulted in increased protein degradation (Langar *et al*. 1993; von der Decken & Lied 1993), and thereby increased protein turnover (Martin *et al*. 2003). The plant protein (SBM) based diets lower nitrogen retention in salmon and trout because these diets have less digestible energy and an amino acid profile that is suboptimal for muscle growth (Cheng *et al*. 2003; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Pack *et al*. 1995; Refstie *et al*. 2000). A similar pattern was found in the present study. Crude protein content of whole yellow perch was lower in groups fed soy-based protein feeds with 100% FM replacement

than in the control and in the groups fed 50% FM protein, regardless the inclusion of exogenous enzymes. However, interestingly, crude protein content in the whole body was higher in the partially (50% replacement) soy based protein fed groups than in the 100% replacement groups. Similarly, Barrows *et al*. (2008) and Cheng *et al*. (2003) also found that the body protein content increased significantly when SBM replaced FM in trout diet. This indicates that the combination of 50% FM and 50% soy based protein diets contain optimum digestible energy and balanced amino acid profile optimal for yellow perch muscle growth.

In the present study, exogenous enzyme supplementation did not enhance nutrient utilization and nutrient deposition in yellow perch. Our results are in contrast with other studies, which showed that inclusion of exogenous enzymes in soy based diets increased protein synthesis in fish muscle (Ai *et al*. 2007; Danicke *et al*. 2003).

In this study, moisture content exhibited an inverse relationship with lipid content. A similar trend has been reported in a study by Hasan *et al*. (1997), wherein FM protein was replaced by plant protein such as mustard, sesame, linseed, copra, and groundnut oil cakes. Our study also showed that complete (100%) replacement of FM protein by soy protein exhibited lower lipid content in fish than the control group. This suggests that fish were utilizing the dietary lipid for energy purposes and the contribution/utilization of energy from the nitrogen-free extract was minimal. In addition, the change in the efficiency of lipid digestion and absorption from the diet may have contributed to the reduction in whole body total fat level. Partial (50%) replacement of FM protein by soy protein showed higher lipid content in fish than that of 100% FM replacement groups. Similarly, Hasan *et al*. (1997) and Mazurkiewicz (2009) observed that partial FM protein replaced by plant protein in fish diet exhibited higher lipid deposition. There is evidence that partial replacement of FM by plant protein sources such as corn gluten meal and soy protein concentrates increases hepatic lipogenic enzyme activities in seabass that leads to higher whole body lipid amounts (Dias

1999; Kaushik *et al*. 2004). Another possible reason for higher lipid retention could be a higher supply of some of the dispensable amino acids such as glutamic acid in excess by the plant protein fed diets (Barrows *et al*. 2008; Kumar *et al*. 2011a; Kumar *et al*. 2011b). These authors also indicated the involvement of possible metabolic or endocrine mechanisms in eliciting such differences in whole body lipid deposition (Kumar *et al*. 2011a; Kumar *et al*. 2011b). In the present study, a higher value of HSI in the partial FM protein replacement groups suggests higher lipid deposition in liver. Hepatosomatic index values of above 1, as observed here, are common in yellow perch.

Digestive enzyme activities and relative intestinal length (RIL)

Digestion and absorption of nutrients depends on the activity of the digestive enzymes, in particular those located in the pyloric caeca and intestine, which are responsible for the breaking down and assimilation of the feed (Klein *et al*. 1998). The study of digestive enzymes in fish shows a wide range of potential benefits, suggesting that determining of the properties, function and optimal conditions for nutrients (carbohydrate, protein and lipid) hydrolysis of digestive enzymes would provide a more accurate measurement for carbohydrate, protein and lipid digestibility (Klein *et al*. 1998; Silva *et al*. 2010). Therefore, digestive enzyme activities are considered as predictors of potential feed utilization and growth differences in fish (Lin & Luo 2011).

The digestive enzymatic activity measured in the pyloric caeca was significantly lower than that in the intestinal digestive tract of yellow perch (Figure 2) because high enzymatic activity in the intestine plays an important role in absorption of the digested nutrients. Bowyer *et al*. (2013) observed the similar results that the lower digestive enzyme activity in the pyloric caeca than the distal intestine, it appears as a symptom of reduced functionality of the distal intestine and, thus, reduced capacity for reabsorption of digestive

secretions in salmon fed SBM based diets (Bowyer *et al*. 2013; Krogdahl *et al*. 2003). However, our results are in contrast with other studies (Harpaz & Uni 1999; Harpaz *et al*. 2005a; Harpaz *et al*. 2005b; Krogdahl *et al*. 1999), wherein they have observed more of the high enzymatic activity in the pyloric caeca than in the intestine.

In the present study, higher (>50% FM protein replacement) inclusion levels of soy protein (SBM and EZM) led to a marked reduction in the activities of digestive enzymes (amylase, protease, and lipase; except lipase activity in pyloric caeca); and these enzymatic activities were found to decrease with lowering SGR and increasing FCR, which show that digestive processes were affected in both the intestine and pyloric caeca with increasing dietary soy protein (SBM and EZM) levels. Current results suggest that enzyme capacity of fish could be improved using nutrients (low dietary inclusion levels of soy protein, 50% FM protein replacement) that stimulate enzyme secretion. Similar results were observed in Atlantic cod and Atlantic salmon (Bakke-McKellep *et al*. 2000; Bowyer *et al*. 2013; Bureau *et al*. 1998; Hidalgo *et al*. 1999; Krogdahl *et al*. 1995; Krogdahl *et al*. 2003; Lemieux *et al*. 1999). However, high percentages of soy protein (100% replacement of FM by SBM and EZM) led to decreased digestive enzyme activity, and major reasons could be the presence of heat stable antinutrients (phytate, oligosaccharides, and NSPs) in soy based diets. Phytate is known to inhibit activities of digestive enzymes such as pepsin, trypsin, and alpha-amylase (Alarcon *et al*. 1999; Kumar *et al*. 2010a; Kumar *et al*. 2011a; Kumar *et al*. 2011b; Robaina *et al*. 1995), or to form complexes with minerals (Sugiura *et al*. 1999; Teskeredzic *et al*. 1995) and proteins (Moyano *et al*. 1999), thereby modifying digestion processes and impairing intestinal absorption. High content of oligosaccharides and NSP in SBM (15% NSP) and EZM (7% NSP) leads to a significant reduction in the activities of digestive enzymes in the intestine and pyloric caeca (Baeverfjord & Krogdahl, 1996; Bakke-McKellep *et al*. 2000; Bureau *et al*. 1998; Krogdahl *et al*. 1995; Krogdahl *et al*. 2003; Tibaldi *et al*.

2006) and even to diminishing of the carrier-mediated nutrient transport/absorption ability (Nordrum *et al*. 2000; Tibaldi *et al*. 2006). The activity of the intestinal enzymes could provide further insight on possible effects of differently modified SBMs on nutrient bioavailability and on the sensitivity of yellow perch intestinal mucosa functions to certain soy protein ANFs.

There was no significant effect of soy protein (SBM and EZM) levels on amylase activity in pyloric caeca of yellow perch. Similar observation was reported by various researchers (Lopez-Lopez *et al*. 2005; Lin & Luo *et al*. 2011) in other fish species. It is suggested that animals have the capacity to adapt their digestive physiology in response to changes in their nutrient requirements or dietary profile to some extent.

The exogenous enzymes (mixture of NSPase, phytase, and carbohydrase) had been shown to decrease digesta viscosity and improve digestibilities of amino acids, protein, lipid, and starch in domestic animals and fish (Ai *et al*. 2007; Bedford 1995; Classen 1996; Cowieson *et al*. 2006; Sinha *et al*. 2011; Kumar *et al*. 2012b). Also, addition of those enzymes will enhance the utilization of phytate, NSP, and oligosaccharides, which result in higher utilization of protein. However, endogenous digestive enzymes, important for digestion of nutrients, showed contradictory tendency; for example, secretion of pancreatic juice and endogenous digestive enzyme activities increased with increasing viscosity due to viscous NSP (Danicke *et al*. 2003; Ikegami *et al*. 1990). Supplementation of exogenous enzymes significantly decreased the activities of protease, chymotrypsin, amylase, and lipase in fish (Danicke *et al*. 2003; Li *et al*. 2004). However, in our study, supplementation of exogenous enzymes (mixture of NSPase, phytase, and carbohydrase) did not change digestive enzyme activity (except EZM_{50+E} group) in pyloric caeca and intestine. The exact mechanism is not clearly understood in fish. More studies should be conducted to examine the

mechanism by which feed utilization could be improved with exogenous enzyme supplementation.

Overall, our study suggests that measuring the activities of digestive enzymes may represent a sensitive tool to study the effects of differently-processed SBMs on nutrient bioavailability and to ascertain tolerability to soy-oligosaccharides in yellow perch. The lower activity of digestive enzymes in soy protein (SBM and EZM) fed groups were correlated with lower nutrient utilization parameters (PER, PPV, and LPV).

It is well known that carnivorous and omnivorous fish require longer time to digest plant protein based diets compared to animal protein based diets (Buddington *et al*. 1997; Kumar *et al*. 2011a; Kumar *et al*. 2011b). A direct relationship between the amount of dietary plant protein and RIL has been reported earlier in fish (Kramer & Bryant 1995; Kumar *et al*. 2011a; Kumar *et al*. 2011b). In yellow perch, soy protein (SBM and EZM) based diets exhibited higher RIL than the control group. RIL value increases as the soy protein (SBM and EZM) inclusion increases in the fish diets (Overland *et al*. 2009). From a physiological view point, a longer RIL would facilitate an increase in digestibility and retention time by enhancing contact time of the digestive enzymes and the feed components, resulting in an increase in their digestion and absorption (Kumar *et al*. 2011a; Kumar *et al*. 2011b). Omnivorous fish such as yellow perch species showed compensation mechanisms, such as an increase in RIL, and as a result increase in digestive activity, to achieve a digestive balance and growth rates similar to those observed for the FM fed group.

Protein metabolism enzymes

Alanine aminotransferase and AST are two enzymes for protein metabolism, mostly active in the liver, which are quantitatively important in transamination of amino acids. Alanine aminotransferase and AST activities in liver were significantly different among the groups

but supplementation of exogenous enzymes in soy based diets did not change the aminotransferase (ALT and AST) activity in fish. In the present study these enzyme activities were positively correlated with growth performance and feed utilization. In the present study, the activity of protein metabolism enzymes ALT and AST in liver was reduced with increasing dietary SBM and EZM levels, which indicated the utilization of dietary protein decreased and the liver was damaged to a certain extent (Lin *et al*. 2007; Luo 2011). Aminotransferases, such as ALT and AST, catabolize amino acids and transfer amino groups to alpha-keto acids (reversible catalysis) but when the available amino acids are deficient, the keto acids may be reduced, thereby reducing the activity of ALT and AST (Lin and Luo, 2011). Decreasing liver AST and ALT activity and a tendency to lower liver ALT and AST activity in fish given diets containing high level of soy protein compared to the low level of soy protein based diets, indicate reduction in substrates available for transamination as a consequence of reduced dietary levels of these amino acids (Lin & Luo 2011).

Antioxidant enzymes

Reactive oxygen species (ROS), which include hydroxyl radical, superoxide anion, hydrogen peroxide, and singlet oxygen, are physiologically generated in a series of biochemical reactions within cellular compartments and increase in physiological conditions that result in oxidative stress (Dirks *et al*. 1982; Radhakrishnan *et al*. 2014). The increased levels of ROS may lead to irreversible cell damage and eventually to cell death. Antioxidant enzymes play a crucial role in the defense against oxidative cell damage, through catalyzing the breakdown of superoxide anion to oxygen and hydrogen peroxide (McCord & Fridovich, 1988; Radhakrishnan *et al*. 2014). In normal cells, there exists a delicate balance between the prooxidant forces and antioxidant defenses known as redox balance. Previously some

researchers have reported that the overwhelming of antioxidant defenses of cells by prooxidants leads to oxidative stress (Radhakrishnan *et al*. 2014).

The nutrient metabolism alterations induced by NSPs (from SBM and EZM) in fish nutrition, like those observed in Nile tilapia, *Oreochromis niloticus,* and African catfish, in which intestinal volatile fatty acid (VFA) production was increased by NSPs (Amirkolaie *et al*. 2006; Enes *et al*. 2012; Leenhouwers *et al*. 2007; Sinha *et al*. 2011), which may also induce a modification in normal cellular oxidative balance. To minimize ROS related cell damage, fish and other aerobic organisms possess antioxidant defense enzymatic mechanisms, including the key enzymes with antioxidant activity (SOD, CAT and GPx) (Enes *et al*. 2012; Halliwell & Gutteridge, 2007; Martinez-Alvarez *et al*. 2005).

In the present study, the activities of antioxidant enzymes (SOD, CAT and GPx) in partial (50% FM replaced by soy protein) replacement groups did not show any significant increase when compared with the control group; whereas 100% replacement groups exhibited higher antioxidant enzyme activity than other groups. Antioxidant enzyme activities in the liver increased as dietary soy protein increased, which suggests a rise in O_2^- generation related to increased levels of NSP and oligosaccharides in soy based diets. It is known that NSP (from SBM and EZM) tends to increase digesta viscosity and thus delay gastric emptying rate in fish (Amirkolaie *et al*. 2005; Enes *et al*. 2012; Leenhouwers *et al*. 2006; Sinha *et al*. 2011; Storebakken 1985). This delay of digesta passage through the gut may stimulate NSPs fermentation by intestine microbiota, thus leading to an increase of VFA production. These VFA might have been mobilized for energy production, leading to a rise of O_2^- levels and thus an increase of antioxidant enzyme activity (Choct & Kocher 2000; Enes *et al*. 2012). Another possibility for the suggested rise of O_2^- levels would be an increase in intestinal mucosal cell turnover and consequently, the need of energy supply for the process in fish fed increasing levels of SBM (NSP) (Enes *et al*. 2012; Jin *et al*. 1999; Sinha *et al*.

2011). Similar results were observed in other studies wherein plant-containing diets increased the hepatic glutathione redox status as well as antioxidant enzyme activities in fish (Enes et al., 2012). This result indicated that high inclusion of soy protein (SBM and EZM) may exert oxidative stress on yellow perch. In order to eliminate excess free radicals, the fish increased antioxidant enzyme activities to protect the body under normal function (Enes *et al*. 2012; Valko *et al*. 2007). In liver, this was evident by the increase of the GPx activity in a dose-dependent manner with plant protein increment. This higher antioxidant activity could be due to some of the plant constituents such as flavonoids present in soy based diets. Little information about such mechanisms in fish was found in the literature, therefore further research is warranted.

In the present study, dietary supplementation of exogenous enzymes did not significantly change the activities of antioxidant enzymes (SOD, CAT, and GPx) in fish, whereas, Zhu *et al*. (2014) found that supplementation of exogenous enzymes significantly reduced SOD and CAT activities in fish. This suggests that exogenous enzymes could protect the fish body from cell oxidative damage when fed mainly with plant source-protein. Another reason is that the enhancement of aerobic metabolism due to dietary phytase supplementation improves the gastrointestinal digestive enzymes (Nwanna, 2007; Zhu *et al*. 2014), and could lead to the increased risk of oxidative stress (Martinez-Alvarez *et al*. 2005; Zhu *et al*. 2014). However, the real mechanisms underlying the dietary exogenous enzymes actions remain largely speculative and further research is required.

Conclusions

EnzoMeal (EZM) containing 580 g kg^{-1} crude protein and no anti-nutritional factors, has been evaluated as an alternative for fishmeal in the diet of yellow perch with significant success. Higher growth performance and feed utilization was observed for 50% replacement of FM by

EZM fed groups compared to 100% replacement of FM by SBM and EZM fed groups. The activity of protein metabolism enzymes AST and ALT was positively correlated with growth performance and nutrient utilization parameters, whereas antioxidant enzymes (SOD, CAT, and GPx) exhibited an opposite trend. The supplementation of exogenous enzymes (mixture of NSPase, phytase, and carbohydrase) did not exhibit any positive effects on growth performance, nutrient utilization, digestive physiology, protein metabolism, and oxidative status in yellow perch. EnzoMeal with high protein and no ANFs has considerable potential as alternatives to fishmeal in aquafeed. It can be used as one of the promising fishmeal replacers in high-protein fish feeds and substitute 50% - 100% of FM protein without sacrificing fish yield.

Acknowledgments

The research was supported by the Ohio Soybean Council and U.S. Department of Agriculture. Salaries and research support were provided by state and federal funds appropriated to The Ohio State University, Ohio Agricultural Research and Development Center. We thank Paul O'Bryant, Dean Rapp and Hong Yao for their help in the experiment. The authors confirm that there is no conflict of interest.

References

Ai Q., Mai K., Zhang, W., Xu, W., Tan, B., Zhang, C. & Li, H. (2007) Effects of exogenous enzymes (phytase, non-starch polysaccharide enzyme) in diets on growth, feed utilisation, nitrogen and phosphorus excretion of Japanese seabass, *Lateolabrax japonicus*. *Comp. Biochem. Physiol*., **147A**, 502–508.

Alarcon, F.J., Moyano, F.J. & Diaz, M., (1999) Effect of inhibitors present in protein sources on digestive proteases of juvenile sea bream (*Sparus aurata*). *Aquatic Living Resource*, **12**, 233-238.

Allan, G.L., Gleeson, V.P., Evans, A.J. & Stone, D.A.J., (1998) Replacement of fishmeal in diets of silver perch: II. Digestibility of Australian lupins. In: Allan, G.L., Rowland, S.J. (Eds.), Fishmeal Replacement in Aquaculture Diets for Silver Perch Final Report of Project 93/120-01 to the Fisheries Research and Development, Canberra, Australia, pp. 53–71.

Amirkolaie, A.K., Leenhouwers, J.I., Verreth, J.A.J. & Schrama, J.W., (2005) Type of dietary fibre (soluble versus insoluble) influences digestion, faeces characteristics and faecal waste production in Nile tilapia (*Oreochromis niloticus L.). Aquac. Res.*, **36**, 1157–1166.

Amirkolaie, A.K., Verreth, J.A.J. & Schrama, J.W. (2006) Effect of gelatinization degree and inclusion level of dietary starch on the characteristics of digesta and faeces in Nile tilapia (*Oreochromis niloticus (L.)). Aquaculture*, **260**, 194–205.

AOAC (1990) Official Methods of Analysis, 15th edn. Association of Official Analytical Chemists, Arlington, VA.

Avanzo, J.L., de Mendonca Junior, C.X., & Cesar, M.D. (2002) Role of antioxidant system in induced nutritional pancreatic atrophy in chicken. *Comp. Biochem. Physiol.*, Part B **131**, 815–823.

Baas, T.C. & Thacker, P.A. (1996) Impact of gastric pH on dietary enzyme activity and survivability in swine fed β-glucanase supplemented diets. *Canadian J. Animal Science*, **76**, 245-252.

Baeverfjord, G. & Krogdahl, A. (1996) Development and regression of soybean meal induced enteritis in Atlantic salmon, *Salmo salar L.*, distal intestine: a comparison with the intestines of fasted fish. *J. Fish Diseases*, **19**, 375–387.

Bakke-McKellep, A.M., Press, C.M., Baeverfjord, G., Krogdahl, A. & Landsverk, T. (2000) Changes in immune and enzyme histochemical phenotypes of cells in the intestinal mucosa of Atlantic salmon, *Salmo salar L.*, with soybean meal-induced enteritis. *J. Fish Disease,* **23**, 115–127.

Barrows, F.T., Gaylord, T.G., Sealey, W.M., Haas, M.J. & Stroup, R.L. (2008) Processing soybean meal for biodiesel production; effect of a new processing method on growth performance of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture,* **283**, 141–147.

Bedford, M.R., (1995) Mechanism of action and potential environmental benefits from the use of feed enzymes. *Animal Feed Science Technol.,* **53**, 145–155.

Bowyer, J.N., Qin, J.G., Smullen, R.P., Adams, L.R., Thomson, M.J.S. & Stone, D.A.J., (2013) The use of a soy product in juvenile yellowtail kingfish (*Seriola lalandi*) feeds at different water temperatures: 1. Solvent extracted soybean meal. *Aquaculture,* **384–387**, 35–45.

Brown, P.B., Twibell, R.T., Jonker, Y. & Wilson, K.A., (1997) Evaluation of three soybean products in diets fed to juvenile hybrid striped bass, *Morone saxatilis* x *M. chrysops. J. World Aquac. Soc*, **28**, 215–233.

Buddington, R.K., Krogdahl, A. & Bakke-McKellep, A.M., (1997) The intestines of carnivorous fish: structure and functions and the relations with diet. *Acta Physiologica Scandinavica*, **161**, 67–80.

Bureau, D.P., Harris, A.M. & Cho, C.Y. (1998) The effects of purified alcohol extracts from soy products on feed intake and growth of chinook salmon (*Oncorhynchus tshawytscha*) and rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **161**, 27–43.

Castañón, J.I.R., Flores, M.P. & Pettersson, D., (1997) Mode of degradation of non-starch polysaccharides by feed enzyme preparations. *Animal Feed Science Technol.*, **68**, 361–365.

Cheng, Z.J., Hardy, R.W. & Usry, J.L. (2003) Effects of lysine supplementation in plant protein-based diets on the performance of rainbow trout (*Oncorhynchus mykiss*) and apparent digestibility coefficients of nutrients. *Aquaculture*, **215**, 255–265.

Choct, M. & Kocher, A. (2000) Non-starch carbohydrates: digestion and its secondary effects in monogastrics. *Proceedings of the Nutrition Society*, **24**, 31–38.

Classen, H.L. (1996) Cereal grain starch and exogenous enzymes in poultry diets. *Animal Feed Science Technology*, **62**, 21–27.

Cowieson, A.J., Singh, D.H. & Adeola, O. (2006) Prediction of ingredient quality and the effect of a combination of xylanase, amylase, protease and phytase in the diets of broiler chicks. 1. Growth performance and digestible nutrient intake. *British Poultry Science*, **47**, 477–489.

Dabrowski, K. & Guderly, H., (2002) Intermediary metabolism. In: Halver, J.E., Hardy, R.W. (Eds.), Fish Nutrition. Academic Press, London, pp. 310–367.

Danicke, S., Simon, O. & Jeroch, H. (2003) Effects of dietary fat type and nonstarch–polysaccharide-hydrolysing enzyme addition to rye-based diets on muscle protein turnover in broilers. *British Poultry Scieence*, **44**, 245–255.

Denstadli, V., Hillestad, M., Verlhac, V., Klausen, M. & Overland, M. (2011) Enzyme pretreatment of fibrous ingredients for carnivorous fish: Effects on nutrient

utilisation and technical feed quality in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture,* **319**, 391–397.

Denstadli, V., Storebakken, T., Svihus, B. & Skrede, A., (2007) A comparison of online phytase pre-treatment of vegetable feed ingredients and phytase coating in diets for Atlantic salmon *Salmo salar L.* reared in cold water. *Aquaculture,* **269**, 414–426.

Dias, J. (1999) Lipid deposition in rainbow trout (*Oncorhynchus mykiss*) and European seabass (*Dicentrarchus labrax* L.): nutritional regulation of hepatic lipogenesis. Dr thesis, Univ. Porto (Portugal) and Univ. Bordeaux I (France). 190 pp.

Dirks, R.C., Faiman, M.D. & Huyser, E.S. (1982) The roles of lipid free radical initiator; and oxygen on the kinetics of lipid peroxidation. *Toxicology and Applied Pharmacology*, **63**, 21–28.

Enes, P., Pérez-Jiménez, A., Peres, H., Couto, A., Pousão-Ferreira, P. & Oliva-Teles, A. (2012) Oxidative status and gut morphology of white sea bream, *Diplodus sargus* fed soluble non-starch polysaccharide supplemented diets. *Aquaculture*, **358–359**, 79–84.

Espe, M., Lemme, A., Petri, A. & El-Mowafi, A. (2006) Can Atlantic salmon (*Salmo salar*) grow on diets devoid of fish meal? *Aquaculture,* **255**, 255–262.

Fallahi, P., Muthukumarappan, K., Rosentrater, K.A. & Brown, M.L. (2012) Twin-screw extrusion processing of vegetable-based protein feeds for yellow perch (*Perca flavescens*) containing distillers dried grains, soy protein concentrate, and fermented high protein soybean meal. *Journal of food research,* **1(3)**, 230-246.

Francis, G., Makkar, H.P.S. & Becker, K. (2001) Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture,* **199**, 197–227.

Glencross, B., Evansa, D., Dods, K., McCafferty, P., Hawkins, W., Maas, R. & Sipsas, S. (2005) Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss*, using either stripping or settlement faecal collection methods. *Aquaculture*, **245**, 211–220.

González, S, Flick, G.J., O'Keefe, S.F., Duncan, S.E., McLean, E. & Craig, S.R. (2006) Composition of farmed and wild yellow perch (*Perca flavescens*). *Journal of Food Composition and Analysis,* **19**, 720-726.

Halliwell, B. & Gutteridge, J.M.C. (1999) Free Radicals in Biology and Medicine. Oxford Univ. Press, Oxford.

Halliwell, B. & Gutteridge, J.M.C. (2007) Free Radicals in Biology and Medicine, Fourth ed. Oxford University Press, Oxford.

Harpaz, S., Hakim, Y., Barki, A., Karplus, I, Slosman ,T. & Eroldogan, O.T. (2005b) Effects of different feeding levels during day and/or night on growth and brush-border enzyme activity in juvenile *Lates calcarifer* reared in freshwater re-circulating tanks. *Aquaculture,* **248**, 325–335.

Harpaz, .S, Hakim, Y., Slosman, T. & Eroldogan, O.T. (2005a) Effects of adding salt to the diet of Asian sea bass *Lates calcarifer* reared in fresh or salt water recirculating tanks, on growth and brush border enzyme activity. *Aquaculture,* **248**, 315–324.

Harpaz, S., Uni, Z. (1999) Activity of intestinal mucosal brush border membrane enzymes in relation to the feeding habits of three aquaculture fish species. *Comp. Biochem. Physiol.*, A **124**, 155–160.

Hasan, M.R., Macintosh, D.J. & Jauncey, K. (1997) Evaluation of some plant ingredients as dietary protein sources for common carp (*Cyprinus carpio* L.) fry. *Aquaculture*, **151**, 55-70.

Hernandez, M.D., Martinez, F.J., Jover, M., & Garcia, B. (2007) Effects of partial replacement of fish meal by soybean meal in sharpsnout seabream (*Diplodus puntazzo*) diet. *Aquaculture*, **263**, 159-167.

Hew, L.I., Ravindran, V., Mollah, Y. & Bryden, W.L. (1998) Influence of exogenous xylanase supplementation on apparent metabolisable energy and amino acid digestibility in wheat for broiler chickens. *Animal Feed Science Technology*, **75**, 83–92.

Hidalgo, M.C., Urea, E., Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*, **170**, 267–283.

Hwang, D.F. & Lin, T.S. (2002) Effects of temperature on dietary vitamin C requirement and lipid in common carp. *Biochem. Physiol.*, Part B **131**, 1–7.

Ikegami, S., Tsuchihashi, F., Harada, H., Tsuchihashi, N., Nishide, E. & Innami, S. (1990) Effect of viscous indigestible polysaccharides on pancreatic–biliary secretion and digestive organs in rats. *J. Nutri.*, **120**, 353–360.

Jin, L., Reynolds, L.P., Redmer, D.A., Caton, J. & Screnshaw, J.D. (1994) Effects of dietary fibre on intestinal growth, cell proliferation, morphology in growing pigs. *J. Animal Sci*., **72**, 2270–2278.

Kader, M.A., Koshio, S., Ishikawa, M., Yokoyama, S. & Bulbul, M. (2010) Supplemental effects of some crude ingredients in improving nutritive values of low fishmeal diets for red sea bream, *Pagrus major*. *Aquaculture*, **308**, 136–144.

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

Kader Md.A. & Koshio, S. (2012) Effect of composite mixture of seafood by-products and soybean proteins in replacement of fishmeal on the performance of red sea bream, *Pagrus major*. *Aquaculture*, **368–369**, 95–102.

Kasper, C.S., Watkins, B.A. & Brown, P.B. (2007) Evaluation of two soybean meals fed to yellow perch (*Perca flavescens*). *Aquac. Nutri.***, 13**, 431–438.

Kaushik, S.J., Coves, D., Dutto, G. & Blanc, D. (2004) Almost total replacement of fish meal by plant protein sources in the diet of a marine teleost, the European seabass, *Dicentrarchus labrax*. *Aquaculture*, **230**, 391–404.

Ketola, H.G. (1975) Mineral supplementation of diets containing soybean as a source of protein for rainbow trout. *Prog. Fish Cult.*, **37**, 73–75.

Klein, S., Cohn, S.M. & Alpers, D.H. (1998) The alimentary tract in nutrition. In: Shils, M.E., Olson, A.J., Shike, M., Ross, A.C. (Eds.), Modern Nutrition in Health and Disease, pp. 605-630.

Kocher, A., Choct, M., Ross, G., Broz, J. & Chung, T.K. (2003) Effects of enzyme combinations on apparent metabolizable energy of corn–soybean meal-based diets in broilers. *J. Appl. Poultry Research*, **12**, 275–283.

Kolkovski, S., Czesny, S. & Yackey, C. (2000) The effect of vitamin-C and E in (n-3) highly unsaturated fatty acids – enriched *Artemia nauplii* on growth, survival and stress resistance of freshwater walleye, *Stizostedion vitreum* larvae. *Aquac. Nutri.***, 6**, 199–206.

Kramer, D.L., Bryant, M.J. (1995) Intestine length in the fishes of a tropical stream. Relationships to diet—the long and short of a convoluted issue. *Environmental Biology* of *Fishes*, **42**, 129–141.

Krogdahl, A., Bakke-McKellep, A.M. & Baeverfjord, G. (2003) Effects of graded levels of standard soybean meal on intestinal structure, mucosal enzyme activities, and pancreatic response in Atlantic salmon (*Salmo salar* L.). *Aquac. Nutri.*, **9**, 361–371.

Krogdahl, A., Nordrum, S., Sorensen, M., Brudeseth, L. & Rosjo, C. (1999) Effects of diet composition on apparent nutrient absorption along the intestinal tract and of subsequent fasting on mucosal disaccharidase activities and plasma nutrient concentration in Atlantic salmon *Salmo salar* L. *Aquac. Nutri*., **5**, 121–133.

Krogdahl, A., Roem, A. & Baeverfjord, G. (1995) Effects of soybean saponin, raffinose and soybean alcohol extract on nutrient digestibilities, on growth and intestinal morphology in Atlantic salmon. Proc. Intl. Conf. Aquaculture'95 and Satellite Meeting Nutrition and Feeding of Cold Water Species. Eur. Aquacult. Soc. Spec., Trondheim, Norway, pp. 118–119.

Kumar, V., Makkar, H.P.S., Amselgruber, W. & Becker, K. (2010b) Physiological, haematological and histopathological responses in common carp (*Cyprinus carpio* L) fingerlings fed with differently detoxified *Jatropha curcas* kernel meal. *Food and Chemical Toxicology*, **48**, 2063-2072.

Kumar, V., Makkar, H.P.S. & Becker, K. (2010a) Dietary inclusion of detoxified *Jatropha curcas* kernel meal: Effects on growth performance and metabolic efficiency in common carp, *Cyprinus carpio* L. *Fish Physiol. and Biochem*., **36(4)**, 1159-1170.

Kumar, V., Makkar, H.P.S. & Becker, K. (2011a) Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquac. Nutri*., **17**, 313-326.

Kumar, V., Makkar, H.P.S. & Becker, K. (2011b) Nutritional, Physiological and haematological responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas* kernel meal. *Aquac. Nutri*., **17**, 451-467.

Kumar, V., Makkar, H.P.S. & Becker, K. (2012a) Evaluations of the nutritional value of *Jatropha curcas* protein isolate in common carp (*Cyprinus carpio* L.). *J. Animal Physiol. and Animal Nutri*., **96(6)**, 1030-1043.

Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G. & Becker, K. (2012b) Phytate and Phytase in Fish Nutrition: A Review. *Journal of Animal physiology and animal nutrition*, **96**, 335-364.

Langar, H., Guillaume, J., Metailler, R. & Fauconneau, B. (1993) Augmentation of protein synthesis and degradation by poor dietary amino acid balance in European sea bass (*Dicentrarchus labrax*). *J. Nutri*., **123**, 1754–1761.

Leenhouwers, J.I., Adjei-Boateng, D., Verreth, J.A.J. & Schrama, J.W. (2006) Digesta viscosity, nutrient digestibility and organ weights in African catfish (*Clarias gariepinus*) fed diets supplemented with different levels of a soluble non-starch polysaccharide. *Aquac. Nutri*., **12**, 111–116.

Leenhouwers, J.I., ter Veld, M., Verreth, J.A.J. & Schrama, J.W. (2007) Digesta characteristiscs and performance of African catfish (*Clarias gariepinus*) fed cereal grains that differ in viscosity. *Aquaculture*, **264**, 330–341.

Lemieux, H., Blier, P. & Dutil, J.D. (1999) Do digestive enzymes set a physiological limit on growth rate and food conversion efficiency in the Atlantic cod (*Gadus morhua*)? *Fish Physiol. Biochem.*, **20**, 293–303.

Li, W.F., Feng, J., Xu, Z.R. & Yang, C.M. (2004) Effects of non-starch polysaccharides enzymes on pancreatic and small intestinal digestive enzyme activities in piglet fed diets containing high amounts of barley. The *World* Journal of *Gastroenterology,* **10**, 856–859.

Lin, S. & Luo, L. (2011) Effects of different levels of soybean meal inclusion in replacement for fish meal on growth, digestive enzymes and transaminase activities in practical diets for juvenile tilapia, *Oreochromis niloticus* × *O. aureus*. *Animal Feed Science and Technology*, **168**, 80– 87.

Lin, S.M., Mai, K.S. & Tan, B.P. (2007) Effect of soybean meal replacement by rapeseed-cottonseed compound on growth, body composition and immunity of tilapia *Oreochromis niloticus*×*O. aureus*. *Oceanol Limnol Sin*, **38 (2)**, 168–173.

Lopez-Lopez, S., Nolasco, H., Villarreal-Colmenares, H. & Civera-Cerecedo, R. (2005) Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cherax quadricarinatus*. *Aquac. Nutri.*, **11**, 79–85.

Makkar, H.P.S. & Becker, K. (2009) *Jatropha curcas*, a promising crop for the generation of biodiesel and value-added coproducts. *European Journal of Lipid Science and Technology*, **111**, 773–787.

Mambrini, M., Roem, A.J., Crave'di, J.P., Lalle's, J.P. & Kaushik, S.J. (1999) Effects of replacing fish meal with soy protein concentrate and of DL-methionine supplementation in high-energy, extruded diets on the growth and nutrient

utilization of rainbow trout, *Oncorhynchus mykiss*. *Journal of Animal Science*, **77**, 2990–2999.

Martin, S.A.M., Vilhelmsson, O., Mèdale, F., Watt, P., Kaushik, S. & Houlihan, D.F. (2003) Proteomic sensitivity to dietary manipulations in rainbow trout. *Biochimica et Biophysica* Acta, **1651**, 17–29.

Martinez-Alvarez, R.M., Morales, A.E. & Sanz, A. (2005) Antioxidant defenses in fish: biotic and abiotic factors. Reviews in Fish Biology and Fisheries **15**, 75–88.

Mazurkiewicz, J. (2009) Utilization of domestic plant components in diets for common carp *Cyprinus carpio* L. *Archives of Polish Fisheries*, **17**, 5-39.

McCord, J.M. & Fridovich, I. (1998) Superoxide dismutase: the first twenty years (1968–1988). *Free* Radical *Biology* and *Medicine*, **5**, 363–369.

McGoogan, B.B. & Gatlin, D.M. (1997) Effects of replacing fish meal with soybean meal in diets for red drum *Sciaenops ocellatus* and potential for palatability enhancement. *J. World Aquac. Soc*., **28**, 374–385.

Me´dale, F., Boujard, T., Valle´ e, F., Blanc, D., Mambrini, M., Roem, A. & Kaushik, S.J. (1998) Voluntary feed intake, nitrogen and phosphorus losses in rainbow trout (*Oncorhynchus mykiss*) fed increasing dietary levels of soy protein concentrate. *Aquatic Living Resource*, **11**, 239–246.

Moyano, F.J., Martinez, I., Diaz, M., & Alarcon, F.J. (1999) Inhibition of digestive proteases by vegetable meals in three fish species; seabream (*Sparus aurata*), tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*). *Comp. Biochem. Physiol*., **122**, 327–332.

Nordrum, S., Bakke-McKellep, A.M., Krogdahl, A. & Buddington, R.K. (2000) Effects of soybean meal and salinity on intestinal transport of nutrients in Atlantic salmon

(*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol.*, B **125**, 317–335.

Nwanna, L.C. (2007) Effect of dietary phytase on growth, enzyme activities and phosphorus load of Nile tilapia (*Oreochromis niloticus*). *Journal of Engineering and Applied Sciences*, **2**, 972–976.

Olli, J. & Krogdahl, A. (1994) Nutritive value of four soybean products as protein sources in diets for rainbow trout (*Oncorhynchus mykiss*, Walbaum) reared in fresh water. *Acta Agriculturae Scandinavica*, Section A **44**, 185–192.

Olli, J.J., Hjelmeland, K. & Krogdahl, A. (1994) Soybean trypsin inhibitors in diets for Atlantic salmon *Salmo salar*, L: effects on nutrient digestibilities and trypsin in pyloric caeca homogenate and intestinal content. *Comp. Biochem. Physiol.*, **109A**, 923–928.

Overland, M., Sorensen, M., Storebakken, T., Penn, M., Krogdahl, Å. & Skrede, A. (2009) Pea protein concentrates substituting fish meal or soybean meal in diets for Atlantic salmon (*Salmo salar*)—Effect on growth performance, nutrient digestibility, carcass composition, gut health, and physical feed quality. *Aquaculture*, **288**, 305–311.

Pack, M., Rodehutscord, M., Jacobs, S. & Pfeffer, E. (1995) Amino acid requirements of rainbow trout (*Oncorhynchus mykiss*): II. Protein deposition as function of dietary methionine, threonine and arginine. *Journal of Applied Ichthyology*, **11**, 390–394.

Radhakrishnan, S., Bhavan, P., Seenivasan, C., Shanthi, R. & Muralisankar, T. (2014) Replacement of fishmeal with *Spirulina platensis*, *Chlorella vulgaris* and *Azolla pinnata* on non-enzymatic and enzymatic antioxidant activities of *Macrobrachium*

rosenbergii. The Journal of Basic & Applied Zoology (In press). http://dx.doi.org/10.1016/j.jobaz.2013.12.003

Refstie, S., Korsoen, O.J., Storebakken, T., Baeverfjord, G., Lein, I. & Roem, A.J. (2000) Differing nutritional responses to dietary soybean meal in rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*). *Aquaculture*, **190**, 49–63.

Refstie, S., Svihus, B., Shearer, K.D. & Storebakken, T. (1999) Nutrient digestibility in Atlantic salmon and broiler chickens related to viscosity and non-starch polysaccharide content in different soybean products. *Animal Feed Science Technology*, **79**, 331-345.

Robaina, L., Izquierdo, M.S., Moyano, F.J., Socorro, J., Vergara, J.M., Montero, D. & Fernandez-Palacios, H. (1995) Soybean and lupin seed meals as protein sources in diets for gilthead seabream (*Sparus aurata*): nutritional and histological implications. *Aquaculture*, **130**, 219–233.

Romeo, M., Bennani, N., Barelli-Gnassia, M., Lafaurie, M. & Girard, J. (2000) Cadmium and copper display different responses towards oxidative stress in the kidney of the sea bass, *Dicentrarchus labrax*. *Aquatic Toxicology*, **48**, 185–194.

Rumsey, G.L., Siwicki, A.K., Anderson, D.P. & Bowser, P.R. (1994) Effect of soybean protein on serological response, nonspecific defense mechanisms, growth, and protein utilization in rainbow trout. Veterinary *Immunology and Immunopathology*, **41**, 323–339.

Schlechtriem, C., Focken, U. & Becker, K. (2003) Effect of different lipid extraction methods on d13C of lipid and lipid-free fractions of fish and different fish feeds. *Isotopes in Environmental and Health Studies***, 39**, 135–140.

Silva, F.C.P., Nicoli, J.R., Zambonino-Infante, J.L., Le Gall, M.M., Kaushik, S. & Gatesoupe, F.J. (2010) Influence of partial substitution of dietary fish meal on the activity of digestive enzymes in the intestinal brush border membrane of gilthead sea bream, *Sparus aurata* and gold fish, *Carassius auratus*. *Aquaculture*, **306**, 233–237.

Sinha, A.K., Kumar, V., Makkar, H.P.S., De Boeck, G. & Becker, K. (2011) Non-starch polysaccharides and their role in fish Nutrition - A Review. Food Chemistry **127**, 1409-1426.

Smedes, F. (1999) Determination of total lipid using non-chlorinated solvents. Analysts **124**, 1711–1718.

Stone, D.A.J., Allan, G.L. & Anderson, A.J. (2003) Carbohydrate utilization by juvenile silver perch, *Bidyanus didyanus* (Mitchell). IV. Can dietary enzymes increase digestible energy from wheat starch, wheat and dehulled lupin? *Aquac. Res.*, **34**, 135.

Storebakken, T. (1985) Binders in fish feeds. I. Effect of alginate and guar gum on growth, digestibility, feed intake and passage through the gastrointestinal tract of rainbow trout. *Aquaculture*, **47**, 11–26.

Storebakken, T., Refstie, S., Ruyter, B. (2000) Soy products as fat and protein sources in fish feeds for intensive aquaculture. In: Drackley, J.K. (Ed.), Soy in Animal Nutrition. Fed. Anim. Sci. Soc., Savoy, IL, USA, pp. 127–170.

Sugiura, S.H., Raboy, V., Young, K.A., Dong, F.M. & Hardy, R.W. (1999) Availability of phosphorus and trace elements in low-phytate varieties of barley and corn for rainbow trout (*Oncorhynchus mykiss*). *Aquaculture*, **170**, 285–296.

Teskeredzic, Z., Higgs, D.A., Dosanjh, B.S., McBride, J.R., Hardy, R.W, Beames, R.M., Jones, J.D., Simell, M., Vaara, T. & Bridges, R.B. (1995) Assessment of

undephytinized and dephytinized rapeseed protein-concentrate as sources of dietary-protein for juvenile rainbow-trout (*Oncorhynchus mykiss*). *Aquaculture,* **131**, 261–277.

Tibaldi, E., Hakim, T., Uni, Z., Tulli, F., De Francesco, M., Luzzana, U. & Harpaz, S. (2006) Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). *Aquaculture,* **261**, 182-193.

Tibaldi, E., Hakim, T., Uni, Z., Tulli, F., De Francesco, M., Luzzana, U. & Harpaz, S. (2006) Effects of the partial substitution of dietary fish meal by differently processed soybean meals on growth performance, nutrient digestibility and activity of intestinal brush border enzymes in the European sea bass (*Dicentrarchus labrax*). *Aquaculture,* **261**, 182-193.

Uyan, O., Koshio, S., Teshima, S., Ishikawa, M., Thu, M., Alam, M.S. & Michael, F.R. (2006) Growth and phosphorus loading by partially replacing fishmeal with tuna muscle by-product powder in the diet of juvenile Japanese flounder, *Paralichthys olivaceus*. *Aquaculture,* **257**, 437–445.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M.T., Mazur, M. & Telser, J. (2007) Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. *Cell Biology*, **39**, 44–84.

Vielma, J., Ma¨kinen, T., Ekholm, P. & Koskela, J. (2000) Influence of dietary soy and phytase levels on performance and body composition of large rainbow trout (*Oncorhynchus mykiss*) and algal availability of phosphorus load. *Aquaculture,* **183**, 349–362.

Vilhelmsson, O.T., Martin, S.A.M., Médale, F. & Kaushik, S.J. (2004) Dietary plant-protein substitution affects hepatic metabolism in rainbow trout (*Oncorhynchus mykiss*). *British Journal of Nutrition*, **92**, 71–80.

Von der Decken, A. & Lied, E. (1993) Metabolic effects on growth and muscle of soy-bean protein feeding in cod (*Gadus morhua*). British Journal of Nutrition **69**, 689–697.

Zhu, Y., Qiu, X., Ding, Q., Duan, M. & Wang C. (2014) Combined effects of dietary phytase and organic acid on growth and phosphorus utilization of juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture*, **430**, 1–8.

Table 1 Composition of the experimental diets (g kg^{-1} dry matter basis of ingredients) for yellow perch (*Perca flavescens*) fingerlings.

Experimental diets*	**Control**	**S_{50}**	**S_{50+E}**	**S_{100}**	**S_{100+E}**	EZM_{50}	EZM_{50+E}	**EZM_{100}**	**EZM_{100+E}**
Menhaden fish meal[a]	480.0	240.0	240.0	-	-	240.0	240.0	-	-
Wheat meal[b]	340.8	219.0	219.0	90.0	90.0	284.0	284.0	218.0	218.0
Soybean meal (SBM)	-	325.0	325.0	650.0	650.0	-	-	-	-
EnzoMeal (EZM)	-	-	-	-	-	269.0	269.0	538.0	538.0
Soy protein isolate[c]	-	16.0	16.0	28.0	28.0	7.0	7.0	12.0	12.0
Menhaden oil[d]	50.0	78.0	78.0	107.0	107.0	78.0	78.0	107.0	107.0
Soybean oil[e]	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Vitamin premix[f]	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Mineral premix[g]	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0	40.0
TiO_2[h]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Methionine[i]	-	-	-	3.0	3.0	-	-	3.0	3.0
Enzyme cocktail[j]	-	-	+	-	+	-	+	-	+
Total	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**

Treatments*

Control - Fish meal (major source of protein)

S_{50} - 50% fish meal protein replaced by SBM

S_{50} +E - 50% fish meal protein replaced by SBM + enzymes (cocktail)

S_{100} - 100% fish meal protein replaced by SBM

S_{100} +E - 100% fish meal protein replaced by SBM + enzymes (cocktail)

EZM_{50} - 50% fish meal protein replaced by EZM

EZM_{50} +E - 50% fish meal protein replaced by EZM + enzymes (cocktail)

EZM_{100} - 100% fish meal protein replaced by EZM

EZM_{100}+E - 100% fish meal protein replaced by EZM + enzymes (cocktail)

[a]IPC 740; International Proteins Corp., Minneapolis, Minnesota, USA.

[b]Whole wheat meal; Bob's Red Mill Natural Foods, Inc. Milwaukie, OR, USA.

[c]Solae, LLC, St. Louis, USA.

[d]Virginia Prime; Omega Protein, Inc., Reedville, Virginia, USA.

[e]Product number OF1870E; Consumers Supply Distributing, Sioux City, Iowa, USA.

[f]Test Diet; Land O'Lakes Purina Feed, Richmond, Indiana. Pantothenic acid, 4,601 mg/kg; pyridoxine, 823 mg/kg; riboflavin, 3,000 mg/kg; niacin, 5,000 mg/kg; folic acid, 1,800 mg/kg; thiamin hydrochloride, 4,503 mg/kg; biotin, 500 mg/kg; vitamin B12, 200 µg/kg; choline, 50,001 mg/kg, menadione, 1,040 mg/kg; vitamin A, 96.1 international units (IU)/g; vitamin D3, 120 IU/g; vitamin E, 3,000 IU/kg; and ascorbic acid (L-ascorbyl-2-polyphosphate), 17,500 mg/kg.

[g]Test Diet; Land O'Lakes Purina Feed. Calcium, 8.00%; phosphorus, 8.00%; potassium, 5.00%; magnesium, 1.33%; sodium, 3.48%; chloride, 2.80%; fluorine, 144 mg/kg; iron, 1,600 mg/kg; zinc, 1,091 mg/kg; manganese, 276 mg/kg; copper, 126.2 mg/kg; cobalt, 248.1 mg/kg; iodine, 114.68 mg/kg; chromium, 8.0 mg/kg; molybdenum, 5.61 mg/kg; and selenium, 5.02 mg/kg.

[h and i]Sigma Aldrich, Saint Louis, MO, USA.

1 **Table 2** Proximate compositions of feed ingredients and experimental diets (% dry matter

2 basis).

Feed ingredients	**Dry matter**	**Crude protein**	**Crude lipid**	**Nitrogen free extract**	**Ash**
Fish meal	92.45	67.05	10.77	4.45	10.18
Soybean meal	91.24	47.45	2.45	28.58	12.76
EnzoMeal	91.05	58	1.87	24.90	6.28
Soy protein isolate	94.05	91.03	0.74	0.77	4.05
Whole wheat meal	90.19	11.85	2.15	74.53	1.76
Experimental diets*					
Control	95.09	40.78	14.48	27.41	12.42
S_{50}	95.89	41.94	14.56	30.49	8.90
S_{50}+E	94.84	40.48	15.29	30.47	8.60
S_{100}	94.41	41.01	14.34	33.23	5.83
S_{100}+E	95.91	42.16	14.52	33.52	5.71
EZM_{50}	94.6	42.30	14.9	29.23	8.17
EZM_{50}+E	95.27	41.52	15.35	29.71	8.19
EZM_{100}	94.5	41.91	15.09	31.94	4.56
EZM_{100} +E	94.73	42.03	15.27	33.23	4.21

3 * See footnotes to Table 1.

Table 3

Chemical composition of whole body (at the start and at the end of the experiment, g kg $^{-1}$ wet basis ± SD), relative intestinal length (RIL, mm g^{-1}), hepato somatic index (HSI) and feed intake of yellow perch (*Perca flavescens*) fingerlings of different experimental groups.

	Moisture	**Crude protein**	**Crude lipid**	**Ash**	**Calcium**	**Phosphorus**	**Magnesium**	**Potassium**
Initial fish	73.79 ± 0.55	14.55 ± 0.58	3.60 ± 0.34	4.69 ± 0.88	1.69 ± 0.01	2.16 ± 0.01	0.02 ± 0.00	0.21 ± 0.00
Treatment*	**Moisture**	**Crude protein**	**Crude lipid**	**Ash**	**RIL**	**HSI**	**GSI**	**Feed intake (g)**
Control	67.96 ± 1.19^{b}	16.32 ± 0.71^{ab}	9.02 ± 0.35^{ab}	3.86 ± 1.52	0.49 ± 0.03^{c}	1.79 ± 0.37^{ab}	2.49 ± 0.05	36.96 ± 0.86^{a}
S_{50}	68.03 ± 1.38^{b}	16.95 ± 0.86^{ab}	7.27 ± 3.16^{ab}	4.79 ± 0.57	0.64 ± 0.02^{a}	1.32 ± 0.41^{c}	2.07 ± 0.22	36.45 ± 0.77^{a}
S_{50}+E	70.86 ± 2.80^{ab}	17.77 ± 1.05^{ab}	6.20 ± 1.26^{b}	5.09 ± 1.68	0.66 ± 0.01^{a}	1.61± 0.41^{b}	2.43 ± 0.64	35.62 ± 2.93^{a}
S_{100}	73.63 ± 1.19^{a}	14.67 ± 0.89^{b}	5.15 ± 0.91c	3.67 ± 0.71	0.61 ± 0.05^{ab}	1.41 ± 0.21^{b}	2.75 ± 0.28	18.32 ± 0.85^{b}
S_{100}+E	73.37 ± 2.76^{ab}	15.16 ±2.24^{b}	7.64 ± 1.34^{b}	4.92 ± 0.41	0.64 ± 0.04^{a}	1.54 ± 0.05^{b}	2.84 ± 0.29	18.98 ± 0.78^{b}
EZM_{50}	69.15 ± 1.29^{ab}	17.97 ± 0.53^{a}	9.65 ± 0.26^{a}	4.28 ± 1.02	0.55 ±0.05^{b}	1.91 ± 0.06^{a}	2.20 ± 0.24	37.34 ± 1.06^{a}
EZM_{50}+E	69.56 ± 0.84^{ab}	18.04 ± 0.84^{a}	5.87 ± 0.25^{b}	4.41 ± 0.47	0.59 ± 0.04^{b}	2.13 ± 0.06^{a}	2.25 ± 0.32	39.09 ± 0.77^{a}
EZM_{100}	72.86 ± 1.97^{ab}	14.59 ± 1.52^{b}	4.30 ± 0.19^{c}	5.62 ± 0.91	0.61 ± 0.02^{ab}	2.05 ± 0.63^{a}	2.64 ± 0.77	19.46 ± 0.62^{b}
EZM_{100} +E	71.21 ± 2.73^{ab}	14.42 ± 1.16^{b}	4.96 ± 0.52^{c}	3.96 ± 0.79	0.60 ± 0.06^{ab}	1.29 ± 0.04^{c}	2.40 ± 0.24	21.67 ± 2.13^{b}

* See footnotes to Table 1

Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly (P < 0.05).

Table 4

Growth performance of Yellow perch (*Perca flavescens*) fingerlings fed with the experimental diets for ten weeks.

Treatment*	Initial weight (g)	Final weight (g)	Weight gain (g)	Body mass gain (%)	Metabolic growth rate ($gkg^{0.8}$ day^{-1})
Control	10.96 ± 0.06	39.96 ± 2.42^{ab}	29.00 ± 2.41^{ab}	264.65 ± 21.88^{ab}	8.03 ± 0.38^{ab}
S_{50}	11.04 ± 0.07	33.13 ± 2.30^{cd}	22.09 ± 2.25^{cd}	199.98 ± 19.43^{cd}	6.80 ± 0.42^{c}
S_{50}+E	10.91 ± 0.05	36.90 ± 4.88^{bc}	25.99 ± 4.65^{bc}	237.11 ± 74.87^{bc}	7.42 ± 1.49^{bc}
S_{100}	10.98 ± 0.07	22.04 ± 1.65^{e}	11.06 ±1.59^{e}	100.71 ± 13.95^{e}	4.24 ± 0.45^{e}
S_{100}+E	10.99 ± 0.11	22.51 ± 2.54^{e}	11.52 ± 2.38^{e}	104.85 ± 23.82^{e}	4.35 ± 0.76^{e}
EZM_{50}	11.16 ± 0.14	40.02 ± 0.63^{ab}	28.85 ± 0.66^{ab}	258.50 ± 7.28^{ab}	7.96 ± 0.12^{ab}
EZM_{50}+E	11.06 ± 0.13	45.38 ± 1.70^{a}	34.32 ± 1.78^{a}	310.28 ± 18.54^{a}	8.79 ± 0.27^{a}
EZM_{100}	10.71 ± 0.14	24.91 ± 0.85^{e}	14.20 ± 1.18^{e}	133.02 ± 15.78^{de}	5.16 ± 0.38^{de}
EZM_{100} +E	11.18 ± 0.12	27.89 ± 1.71^{de}	16.72 ± 1.61^{de}	149.54 ± 13.48^{d}	5.64 ± 0.35^{e}

* See footnotes to Table 1.

Values are mean (n = 3) ± standard deviation. Mean values in the same column with different superscript differ significantly ($P < 0.05$).

Figure legends

Fig. 1. (A) Specific growth rate (%/day) and (B) antioxidant enzyme (Glutathione peroxidase) activity in liver of yellow perch (*Perca flavescens*).

Values are mean (n = 3) ± standard deviation. Mean values with different superscript differ significantly ($P < 0.05$).

Fig. 2. Nutrient utilization parameters (A) feed conversion ratio and protein efficiency ratio, (B) protein productive value and lipid productive value of yellow perch (*Perca flavescens*).

Values are mean (n = 3) ± standard deviation. Mean values with different superscript differ significantly ($P < 0.05$).

Fig. 3. Digestive enzymes (A) amylase (B) protease and (C) lipase activity in pyloric caeca and intestine of yellow perch (*Perca flavescens*).

Values are mean (n = 3) ± standard deviation. Mean values with different superscript differ significantly ($P < 0.05$).

Fig. 4. (A) Protein metabolism enzymes (alanine amino transferase, ALT and aspartate transferase, AST) activity in liver, (B) antioxidant enzymes (superoxide dismutase and catalase) activity in liver of yellow perch (*Perca flavescens*).

Values are mean (n = 3) ± standard deviation. Mean values with different superscript differ significantly ($P < 0.05$).