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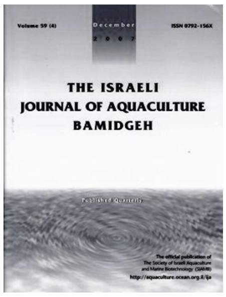

Effects of Total Replacement of Fishmeal with *Spirulina* Powder and Soybean Meal on Juvenile Rainbow Trout (*Oncorhynchus mykiss* Walbaum)

Gerardo Hernández Flores, Luis Héctor Hernández Hernández*, Mario Alfredo Fernández Araiza, Omar Angeles López

Laboratorio de Producción Acuícola, UNAM FES Iztacala, Av. de los Barrios 1, Los Reyes Iztacala, Tlalnepantla, Estado de México, C.P. 54090, México

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Key words: rainbow trout, soybean meal, *Spirulina* powder, growth performance, nitrogen excretion, phosphorus excretion

Abstract

Juvenile rainbow trout (*Oncorhynchus mykiss*) were fed diets in which fishmeal was totally replaced by mixtures of *Spirulina* powder and soybean meal. Three experimental diets were formulated with 75% *Spirulina* and 25% soybean meal, 50% *Spirulina* and 50% soybean meal, and 25% *Spirulina* and 75% soybean meal. A commercial diet and a diet with 100% fishmeal served as controls. Diets were fed to triplicate groups of 10 juveniles with an initial weight of 6.8±0.4 g for 50 days. At the end of the experiment, growth performance, oxygen consumption, nitrogen and phosphorus excretion, protein digestibility, and serum protein content and lysozyme activity were evaluated. Data were analyzed by one-way ANOVA. Growth performance was not significantly affected by the experimental diets, but values tended to be lower as the level of dietary soybean meal increased. Among the experimental diets, the 75% *Spirulina*/25% soybean diet produced the best growth performance. Further, in fish fed this diet, the apparent coefficient of digestibility of protein was higher than 98% (as with all diets), oxygen consumption was lower and lysozyme activity was higher than in fish fed other replacement diets, and dissolved phosphorus and phosphorus in feces were similar or lower than in the controls. The present research shows the possibility of using mixtures of *Spirulina* powder and soybean meal as total substitutes of fishmeal in diets for juvenile rainbow trout.

* Corresponding author. Tel./fax: +52-55-5623-1197, e-mail: luish3@yahoo.com

Introduction

Fishmeal is the main protein source in feeds for aquatic animals of economic importance (Drew et al., 2007; Gatlin et al., 2007). The rapid growth of aquaculture is accompanied by increasing demands for fishmeal, a scarce and expensive ingredient. Feeds have a negative effect on the environment since fishmeal increases the nutrient load in wastewater, particularly of nitrogen and phosphorus (Hardy, 2002). One of the main challenges to the aquaculture industry is to find alternative ingredients that are readily available, cheaper, and more environmentally friendly than fishmeal (Gatlin et al., 2007).

Meals of plant origin are an alternative to fishmeal (Hardy, 2010; González-Felix et al., 2011), but substitution levels range 25-33% (Slawski et al., 2011) in commercial diets (Hardy, 1996). There are several reasons why substitution of fishmeal is limited: plant meals usually contain high levels of fiber, starch, non-soluble carbohydrates, and anti-nutrients that affect digestibility and fish growth (Gatlin et al., 2007; Krogdahl et al., 2010). To alleviate this problem, enzymes are added (Cheng et al., 2004; Wang et al., 2009) or mixtures of different plant meals are used (Gomes et al., 1995).

Spirulina powder and soybean meal have potential as protein sources in fish diets particularly because of their crude protein content and well-balanced amino acids (Hardy, 1996; Nandeesha et al., 1998). Soybean meal can be used to a level of 75% when the enzyme phytase is added to diets for rainbow trout (Cruz et al., 2011). *Spirulina* powder has been included in diets for silver seabream (*Rhabdosargus sarba*; El-Sayed, 1994), tilapia (*Oreochromis mossambicus*; Olvera-Novoa et al. 1998), sturgeon (*Acipenser baeri*; Palmegiano et al. 2005), and common carp (*Cyprinus carpio*; Nandeesha et al., 1998; Ramakrishnan et al., 2011), but total substitution of fishmeal by *Spirulina* is suitable only in carp feeds. The objective of the present research was to evaluate the effects of diets with *Spirulina* powder and soybean meal in total replacement of fishmeal on growth, nitrogen and phosphorus excretion, and lysozyme activity in juvenile rainbow trout.

Materials and Methods

Fish and diets. Rainbow trout (*Oncorhynchus mykiss*) 60 days post-hatching were obtained from a private farm in the State of México and transported to the Laboratorio de Producción Acuícola, Facultad de Estudios Superiores Iztacala, UNAM. Fish were maintained in 500-l tanks and fed a commercial pellet diet for trout (Api-trucha 1, 50% protein, Malta-Cleyton de México) until the beginning of the trial.

Three experimental diets were formulated in which 100% of the fishmeal was substituted with mixtures of *Spirulina* powder (crude protein 55.0±2%; Pronaquim S.A. de C.V., México) and soybean meal (crude protein 50.6±1%; Pronasoya S.A. de C.V., México). The diet formulations were based on species requirements (NRC, 2011), with a minimum of 40% protein (Table 1). Besides the meals, cod liver oil (Drotasa S.A. de C.V., México) and soybean lecithin (Abastecedora de Productos Naturales S.A. de C.V., Mérida, México) were used as lipid sources, and dextrin (Sigma Aldrich Co., St. Louis, MO, USA) was added as a source of carbohydrate. A vitamin-mineral mix (Micro Rovimix for carnivorous fish; DSM Nutritional Products de México S.A. de C.V., Guadalajara, México) and wheat gluten (Sigma Aldrich Co., St. Louis, MO, USA) as a binder were added; α-cellulose (Sigma Aldrich Co., St. Louis, MO, USA) was added to fill the diet to 100%. Test diets contained 0.8 g phytase (Phytase Ronozyme P5000, DSM Nutritional Products de México S.A. de C.V., Guadalajara, México) per kg diet. Diets were prepared according to Cruz et al. (2011) by mixing the powdered ingredients with the oils and distilled water (40%). The wet dough was passed through a meat chopper to produce 5-mm diameter pellets that were dried at 60°C in a constant temperature oven for 60 min and stored at -20°C until use. The experimental diets were compared with a control diet containing 100% fishmeal and a commercial diet (Api-trucha 1, 50% protein, Malta-Cleyton de México).

Feeding trial. Ten juveniles (6.8±0.4 g) were randomly stocked in each of fifteen 100-l plastic tanks (filled with 80 l water) in a recirculation system. Each test diet was fed to triplicate groups at 7% of their body weight daily, divided into two equal feedings at

Table 1. Formulation and proximate composition (means±SE) of diets containing *Spirulina* and soybean meal as replacements of fishmeal for juvenile rainbow trout.

	Diet (%spirulina/%soybean)			
	75/25	*50/50*	*25/75*	*Control*
Ingredient (g/kg diet)				
Fishmeal	0	0	0	600
Soybean meal	150	300	450	0
Spirulina powder	450	300	150	0
α-cellulose	109.2	109.2	109.2	110
Dextrin	100	100	100	100
Cod liver oil	50	50	50	50
Soybean lecithin	50	50	50	50
Gluten	50	50	50	50
Vitamin-mineral mix*	40	40	40	40
Phytase	0.8	0.8	0.8	0
Proximate composition (%)[2]				
Protein	43±2	44±3	42±1	40±2
Lipid	12.5±0.5	11.1±0.4	11.3±0.3	12.7±1
Ash	11±1	11±0.7	12±0.9	12±0.8
Moisture	10±1	10±1.7	11±1	11±0.7

* Vitamin and mineral mix (g/kg): ρ-aminobenzoic acid 1.45; biotin 0.02; myo-inositol 14.5; nicotinic acid 2.9; Ca-pantothenate 1.0; pyridoxine-HCl 0.17; riboflavin 0.73; thiamine-HCl 0.22; menadione 0.17; α-tocopherol 1.45; cyanocobalamine 0.0003; calciferol 0.03; L-ascorbyl-2-phosphate-Mg 0.25; folic acid 0.05; choline chloride 29.65; retinol 0.015; NaCl 1.838; $MgSO_4 \cdot 7H_2O$ 6.85; $NaH_2PO_4 \cdot 2H_2O$ 4.36; KH_2PO_4 11.99; $Ca(H_2PO_4)_2 \cdot 2H_2O$ 6.79; Fe-citrate 1.48; Ca-lactate 16.35; $AlCl_3 \cdot 6H_2O$ 0.009; $ZnSO_4 \cdot 7H_2O$ 0.17; $CuCl_2$ 0.0005; $MnSO_4 \cdot 4H_2O$ 0.04; KI 0.008; $CoCl_2$ 0.05

08:00 and 15:00. The juveniles were weighed every 10 days and the ration size adjusted accordingly. Dead fish were not replaced during the experiment. Thirty minutes after feeding, unconsumed feed was removed and quantified to determine feed intake. Tank bottoms were siphoned to remove fecal matter and determine their phosphorus contents. Water quality was: dissolved oxygen 6.5±1.0 mg/l, pH 7.9±0.5, and temperature 14±1°C. Water flow to each tank was 1.5 l/min and tanks were maintained under a natural photoperiod. The trail was conducted for 50 days from December 2008 to January 2009.

At the end of 50 days, fish were starved for 24 h and weighed to determine growth performance. Five randomly chosen fish were returned to their tanks and fed their respective diets supplemented with 1% chromium oxide for another 10 days to determine the apparent digestibility of the feeds. The remaining fish were used to evaluate oxygen consumption, and nitrogen and phosphorus excretion. Finally, the fish were anesthetized with MS-222 (ethyl 3-aminobenzoate, methanesulfonic acid, Sigma Aldrich Co., St. Louis, MO, USA) at 200 mg/l, four fish were randomly chosen from each tank, and blood samples were collected from the caudal vein. The blood was allowed to clot at 4°C. Three h later, serum was separated by centrifugation at 3000 rpm for 10 min, pooled, and frozen at -20°C until used to measure protein content and lysozyme activity.

Oxygen consumption, nitrogen and phosphorus excretion. Fish were fed their respective diets 24 h before the tests. A closed recirculation system of twenty 1-l flasks connected in a series by plastic tubes was slowly filled with water and one fish was placed in each flask. Initial oxygen, nitrogen, and phosphorus concentrations were measured, then the flasks were hermetically closed. Fish were maintained in that condition for 30 min after which the flasks were opened, dissolved oxygen was again measured, and water samples were taken to determine nitrogen and phosphorus concentrations. Dissolved oxygen was determined using an oxymeter (model 85, YSI Inc., OH, USA) and oxygen consumption was calculated by subtracting the final concentration of oxygen from the initial. Total phosphorus (as PO_4^{-3}) was determined by the molybdovanadate method and nitrogen (as NH_3-N) by the Nessler method (Clescerl et al., 1995). Total phosphorus contents of the feces were determined by the molybdovanadate method with acid persulfate digestion (Clescerl et al., 1995).

Apparent digestibility of protein. Apparent digestibility coefficients (ADC) of protein were determined using chromic oxide (Cr_2O_3 powder; J.T. Baker, Phillipsburg, NJ, USA) as an inert marker at a dietary concentration of 1%. The fish rapidly accepted the diets containing chromic oxide and no conditioning period was necessary. The juveniles were fed 10 days. Feces were collected daily and dried in an oven. The protein content was determined by the AOAC (1990), while the amount of chromic oxide was digested in a mixture of perchloric acid and nitric acid at 250°C. Chromic oxide was quantified by the equation (Y - 0.0032)/0.2089/4, where Y = absorbance at 350 nm (Furukawa and Tsukahara, 1966). ADC (%) was calculated as 100 - 100(%MF/%MH)(%PF/%PH), where MF and MH = marker in feed and feces, respectively, and PF and PH = protein in feed and feces, respectively.

Serum protein content and lysozyme activity. The protein content of the blood serum was determined using a 0.01-g sample and a Micro Lowry, Peterson's Modification Total Protein Kit (Sigma Aldrich Co., St. Louis, MO, USA). Lysozyme activity was determined according to Caruso et al. (2002) by adding 100 µl blood serum to 900 µl aqueous solution of *Micrococcus lysodeikticus* (lyophilized cells, Sigma-Aldrich Chemical, St. Louis, MO, USA). The mix was incubated at 25°C and absorbance was read at 530 nm exactly 0.5 and 4.5 min after adding the sample. One unit of lysozyme activity (U) was defined as the amount of enzyme that caused a decrease in absorbance of 0.001 min/l.

Statistical analysis. Data were tested for normality and homoscedasticity with the Shapiro and Wilk W test and Barlett's test, respectively (Zar, 1999). In case of survival, percentages were arcsine transformed and then tested. Since all data were normal and homoscedastic, they were compared by one-way ANOVA (package Minitab 15, Minitab Inc., State College PA, USA). Significant differences between treatments were evaluated by Tukey multiple comparison test (Zar, 1999). Differences were considered statistically significant at an error of 5% ($p<0.05$).

Results

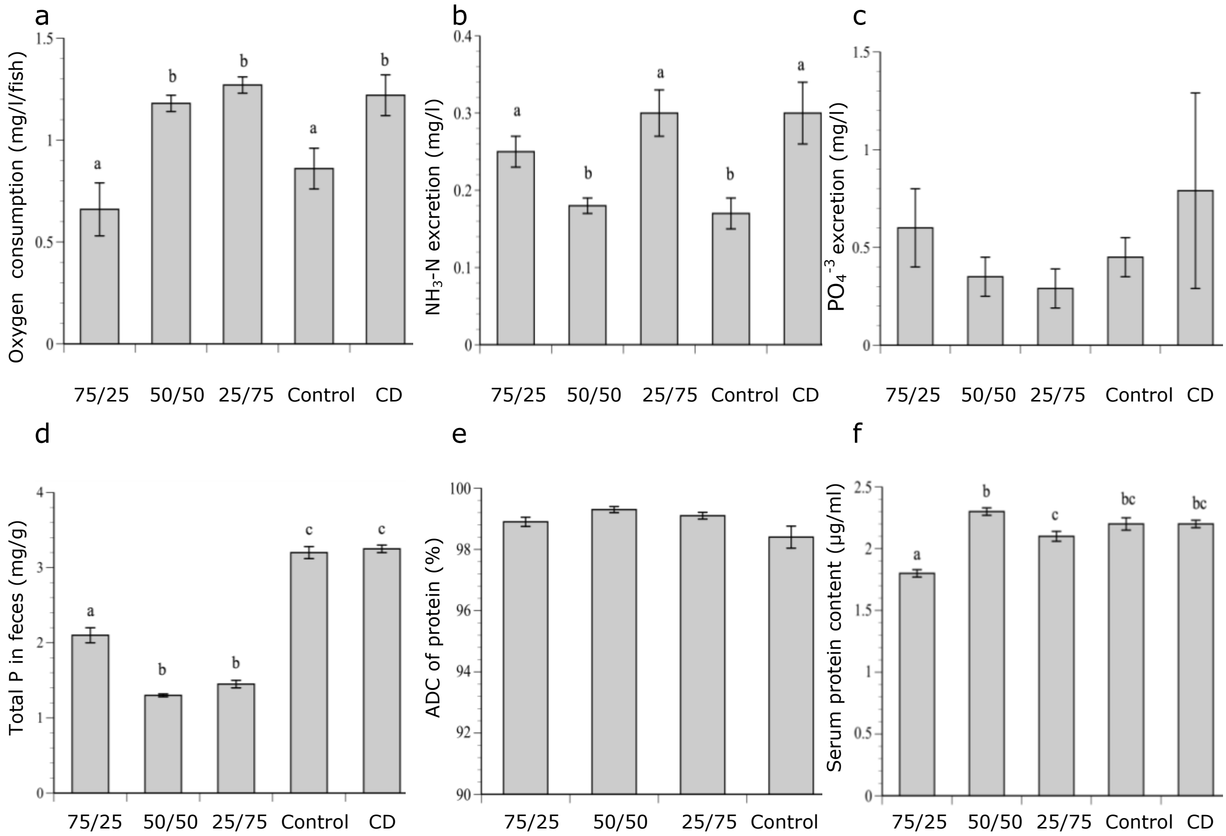
There were no statistical differences between groups in growth performance (Table 2). Fish fed the control diet had the highest growth while, amongst groups fed the replacement diets, fish fed the 75%*Spirulina*/25%soybean diet had the best growth. Survival was higher than 80% in all groups and mortality was unrelated to treatment (some fish jumped out of their tanks and died).

Fish fed the replacement diets showed a tendency for higher oxygen consumption as the percent soybean meal increased (Fig. 1). There was no tendency regarding nitrogen excretion. There were no significant differences in metabolic phosphorus excretion although it tended to drop as the proportion of soybean meal rose. The phosphorus content in the feces was significantly lower in the replacement diets than in the control and commercial diets; especially when soybean represented more 50% or more of the diet. Protein digestibility coefficients were higher than 98% and tended to rise with the percent soybean. Serum protein content was significantly lower in fish fed the 75%*Spirulina*/25%soybean diet while serum lysozyme activity was significantly higher.

Table 2. Weight gain, specific growth rate, feed conversion ratio, protein conversion efficiency, and feed intake (FI) of juvenile rainbow trout fed diets with *Spirulina* powder and soybean meal as replacements of fishmeal (means±SE, n = 3).

	Diet (%Spirulina/%soybean)				
	75/25	*50/50*	*25/75*	*Control*	*Commercial diet*
Wt gain (%)[1]	557±86	470±43	445±6	649±31	525±40
SGR (%/d)[2]	3.7±0.2	3.4±0.1	3.4±0.02	4.0±0.1	3.6±0.1
FCR[3]	0.9±0.1	0.8±0.07	0.8±0.02	0.7±0.03	0.8±0.09
PCE[4]	4.1±0.3	3.3±0.3	3.2±0.03	4.61±0.5	4.0±0.5
FI (g/fish/d)	0.44±0.03	0.40±0.06	0.40±0.01	0.36±0.03	0.37±0.04
Survival	80±6	84±12	97±4	87±4	90±0

[1] Wt gain = 100(final body wt - initial body wt)/initial body wt)
[2] Specific growth rate = 100(ln final body wt - ln initial body wt)/50
[3] Feed conversion ratio = wt gain/total feed intake on dry weight basis
[4] Protein conversion efficiency = wt gain/total protein intake on dry weight basis



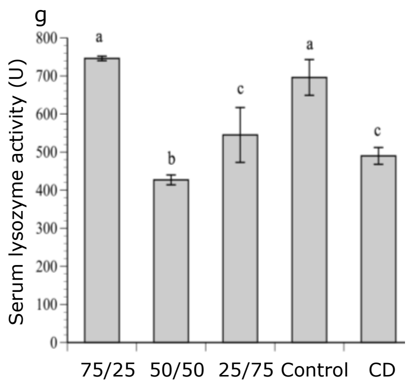


Fig. 1. (a) Oxygen consumption, (b) nitrogen excretion, (c) phosphorus excretion, (d) total phosphorus in feces, (e) apparent digestibility coefficient of protein, (f) serum protein content, and (g) serum lysozyme activity in juvenile rainbow trout fed diets containing *Spirulina* powder and soybean meal as total replacements for fishmeal for 50 days, means±SE, n = 3. 75/25 = 75% *Spirulina* + 25%soybean, 50/50 = 50% *Spirulina* + 50%soybean, 25/75 = 25% *Spirulina* + 75%soybean, CD = commercial diet. Bars with different letters differ significantly ($p<0.05$).

Discussion

Aquaculture faces an increasingly limited supply of fishmeal. Fishmeal can be replaced by grain and oilseed meals (Hardy, 1996, 2010; Gatlin et al., 2007; Kaushik and Seiliez, 2010), as well as microalgae (El-Sayed, 1994). Here, total substitution of fishmeal with *Spirulina* powder and soybean meal in diets for juvenile rainbow trout was tested. Results suggest that 75%*Spirulina*/25%soybean has a positive effect on growth performance.

Growth performance was higher than reported for rainbow trout juveniles fed soybean meal as the sole protein source, with inclusion of phytase (Gomes et al., 1995; Wang et al., 2009; Cruz et al., 2011). However, growth performance dropped as the proportion of soybean meal increased. Even with a high protein content, soybean meal does not meet the requirements of some carnivorous aquatic species for indispensable amino acids such as methionine, lysine, and threonine (Gatlin et al., 2007). But, the incorporation of *Spirulina* powder improved the growth of the juvenile trout, suggesting that *Spirulina* (Olvera-Novoa et al., 1998) includes indispensable amino acids reported for most fish species (NRC, 2011). This agrees with studies of this microalgae as a substitute for fishmeal in diets for silver seabream (El-Sayed, 1994), tilapia (Olvera-Novoa et al., 1998), and sturgeon (Palmegiano et al., 2005).

Our ADC values for diets containing *Spirulina* powder and soybean meal are similar to those of rainbow trout fed a diet containing soybean and phytase (Cheng et al., 2004) and higher than those obtained in juvenile tilapia fed a 100% *Spirulina* diet (Olvera-Novoa et al., 1998). Moreover, fish fed the 75%*Spirulina*/25%soybean diet had a lower oxygen consumption, which is used to estimate the oxidation of nutrients in the diet, especially protein (Bureau et al., 2002). Low values usually indicate that less energy is spent on oxidation, thus protein digestion is more efficient.

Phosphorus tends to be lower as the concentration of soybean meal increases when feeds are supplemented with phytase (Sugiura et al. 2001; Cruz et al., 2011). Likewise in our study, total phosphorus in feces was lower in fish fed the *Spirulina*/soybean diets that contained phytase than in fish fed the control diet. Both excreted phosphorus and phosphorus in feces are major concerns in aquaculture (Bureau and Cho, 1999). In the present study, phosphorus in the feces was significantly lower in the replacement diets than in the control and commercial diets, reducing eventual eutrophication of effluent-receiving water bodies.

Protein is essential for adequate growth and health in fish (Gatlin, 2002). In the present study, except for fish fed the 75%*Spirulina*/25%soybean diet, protein levels in the blood plasma of fish fed the replacement diets was similar to those fed the control or commercial diets and to reports on healthy rainbow trout (Banaee et al., 2011).

Lysozyme is an important molecule of the non-specific immune system and is often used to evaluate the condition of fish (Saurabh and Sahoo, 2008). In the present study, lysozyme levels of fish fed the 50%/50% or commercial diets were lower than previously reported although fish fed the other diets were within the normal range (Verlhac et al., 1996). Soybean products can increase lysozyme in the digestive system of Atlantic salmon (*Salmo salar*; Krogdahl et al., 2000). It seems therefore that lysozyme activity was influenced by the replacement diets. Results do not show a clear tendency.

In conclusion, the present research shows the possibility of using a mixture of 75% *Spirulina* powder and 25% soybean meal to replace 100% fishmeal in diets for juvenile rainbow trout. Growth performance was not affected, the dissolved phosphorus and phosphorus in feces were reduced, and lysozyme activity improved. *Spirulina* powder is expensive, but the results presented here could serve as an incentive to enhance its supply and reduce its price.

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