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USE OF EXOGENOUS ENZYMES IN DIETS FOR JUVENILE POMPANO *Trachinotus marginatus*: GROWTH AND LIVER AND INTESTINE MORPHOPHYSIOLOGY

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ABSTRACT

This study investigated the use of exogenous enzymes in diets of juvenile pompano *Trachinotus marginatus*. Six diets (43% protein, 8% lipid), containing 0 (TC), 25 (T25), 50 (T50), 100 (T100), 250 (T250), and 500 (T500) mg kg^{-1} of multiple enzymes Rovabio® MAX AP were produced. The effects on growth performance, body composition, liver glycogen, triglyceride levels, and intestinal and hepatic morphological alterations were assessed. The results showed significant differences ($p < 0.05$) in growth performance among fish fed diets T100, T250, and T500, with high liver triglyceride accumulation and changes in morphology in the fish fed diets T250 and T500. The calcium concentration in the bones showed a significant difference ($P < 0.05$) only in fish fed diets T50, T100, and T250 in relation to control. However, there were no significant differences in body composition, intestinal morphology, or liver glycogen concentration. These results suggest that 100 mg enzyme per kg diet was the optimal level of inclusion for juvenile pompano, favoring improved growth performance and calcium concentration in the bones without significant effects on liver triglyceride levels.

Key words: carbohydrase; growth; phytase; proteinase.

USO DE ENZIMAS EXÓGENAS EM DIETAS PARA JUVENIS DE PAMPO *Trachinotus marginatus*: CRESCIMENTO E MORFOFIOLOGIA DO FÍGADO E INTESTINO

RESUMO

Este estudo investigou o uso de enzimas exógenas em dietas para juvenis de pampo prateado *Trachinotus marginatus*. Foram preparadas seis dietas (43% de proteína, 8% de lipídio), contendo 0 (TC), 25 (T25), 50 (T50), 100 (T100), 250 (T250) e 500 (T500) mg kg^{-1} do complexo enzimático Rovabio® Max AP. Avaliou-se os efeitos sobre o crescimento, composição de carcaça, níveis de glicogênio e triglicerídeo no fígado e possíveis alterações na morfologia do intestino e fígado. Os resultados mostraram diferença significativa ($p < 0,05$) no desempenho de crescimento dos peixes dos tratamentos T100, T250 e T500, com grande concentração de triglicerídeos e alteração na morfologia do fígado nos peixes dos tratamentos T250 e T500. A concentração de cálcio nos ossos também teve um aumento significativo ($p < 0,05$) nos peixes dos tratamentos T50, T100 e T250 em relação ao controle. Entretanto, não houve diferença significativa ($p > 0,05$) na composição de carcaça, morfologia do intestino e concentração de glicogênio no fígado. Esses resultados indicam que o uso de 100 mg kg^{-1} de enzimas na dieta mostrou ser o melhor nível de inclusão para juvenis de pampo prateado, favorecendo o crescimento e a concentração de cálcio nos ossos. Sem efeitos significativos sobre os níveis de triglicerídeos no fígado.

Palavras-chave: carboidrases; crescimento; fitase; protease.

INTRODUCTION

Although the contribution of plant-based ingredients to reducing the use of fishmeal in the diets of aquatic organisms is very promising (SILVA *et al*., 2007; DALSGAARD *et al*., 2012), its use as the main protein source for fish, especially those with a typically carnivorous diet and a low capacity to digest carbohydrates, presents limitations (NRC, 2011; FRACALOSSI and CYRINO, 2013). In addition to physiological factors

of the fish, the low digestibility of carbohydrates is also influenced by the ingredient nutritional factors (KAMALAM *et al*., 2017). The nutritional aspect of vegetables are very important, as these, in addition to contributing valuable nutrients, may also contain antinutritional substances, which may produce negative effects on the digestibility and nutritional balance of foods when present in the diet (DREW *et al*., 2007).

Non-starch polysaccharides (NSP) are important components of plants, having structural roles in the cell wall (CAPRITA *et al*., 2010). When present in fish feed, NSP, depending on the level of inclusion and the physiology of the species, can be considered antinutritional factors because fish do not have endogenous enzymes to break down beta-glycosidic bonds (CHOCT, 2015). As an important consequence, dietary viscosity increases, slowing gastric emptying, altering digestive tract morphology and physiology, and affecting nutrient absorption (SINHA *et al*., 2011).

Another important consideration is the presence of antinutritional factors, especially phytic acid, that can negatively affect nutrient bioavailability, even if the diet is nutritionally adequate (ADEOLA and COWIESON, 2011; CASTILLO and GATLIN III, 2015). Phytic acid has in its structure six molecules of phosphorus, representing a reserve of this element for plants (PAPATRYPHON *et al*., 1999). Moreover, this compound can be chelated to cations of nutritional importance, such as calcium and magnesium, making them unavailable to the organism. To be used efficiently, phytic acid must be cleaved by the enzyme phytase, releasing inositol and phosphorus, in addition to the other cations complexed to it (SINGH and SATYANARAYANA, 2015). Fish do not naturally possess phytase, and therefore, phosphorus is not absorbed but is excreted into the environment.

Studies testing the use of carbohydrases in diets based on vegetable protein have shown that their addition improves digestibility and nutrient utilization (CASTILLO and GATLIN III, 2015). Of the carbohydrases, xylanase (endo-1,4-β-xylanase) and glucanase (endo-1,3(4)-β-glucanase) are the most studied. The use of multiple enzymes (carbohydrases, phytase, and proteases) acting on different substrates aims to maximize the benefits provided by enzymatic supplementation (ADEOLA and COWIESON, 2011). In the diets of fish, the combined use of multiple enzymes has shown improvements, for example, in growth (GHOMI *et al*., 2012; ALI ZAMINI *et al*., 2014) and apparent nutrient digestibility (DALSGAARD *et al*., 2012).

Fish of the genus *Trachinotus* have potential utility in aquaculture, presenting the biological characteristics of tolerance to extreme environmental conditions and fast growth (JORY *et al*., 1985). The pompano (*Trachinotus marginatus*) has good tolerance to a wide range of salinity, varying from 7‰ to 58‰ (SAMPAIO *et al*., 2003). It presents high tolerance to toxic ammonia and nitrite, withstanding concentrations of up to 1.87 mg L^{-1} and 116.68 mg L^{-1}, respectively (COSTA *et al*., 2008), and an isosmotic point of 13‰ (ABOU ANNI *et al*., 2016). It consumes a carnivorous diet, has a protein requirement of 43%, and can tolerate a diet of up to 40% soy protein (SILVA *et al*., 2015).

The present work aimed to evaluate the growth, carcass composition, morphological alterations of the liver and intestine, and biochemical composition of the liver of pompano juveniles fed diets with different levels of the Rovabio ® Max AP enzymatic complex.

METHODS

The experiment was conducted in the Marine Aquaculture Station (EMA) – FURG and was approved by the Ethics Committee on Animal Use – CEUA FURG (Protocol CEUA P044/2016). Six diets were prepared and oven dried with forced air circulation at 50 °C for a period of 6 hours and then stored in a freezer at –20 °C. A total of 270 pompanos juveniles were collected at Cassino beach, Rio Grande-RS, and kept in 200-L tanks for acclimatization and maintenance until the beginning of the experiment (SISBIO Authorization No 52073-1). During this acclimation period (2 weeks), fish were fed exclusively on the control diet (without enzymatic supplementation). Water quality was maintained by means of daily renewals of almost 90% of the volume of the tank. The temperature and salinity were maintained at 25 °C and 30 ppm, respectively. At the beginning of the experiment, the fish were redistributed among the experimental tanks, and feeding with the respective diets commenced.

All diets were prepared from the same basic formulation, containing approximately 43% crude protein and 8% lipid, varying only the amount of enzymes (Table 1). One diet was prepared without the addition of enzymes and called the control diet (TC). The other

Table 1. Formulation and proximal composition of the experimental diets.

Ingredients (%)		
Fishmeal	8.0	
Poultry byproduct meal	15.0	
Soybean meal	40.0	
Wheat bran	10.0	
Gelatin	3.0	
Starch	16.0	
Fish oil	7.0	
Vitamin and Mineral mixture[a]	1.0	
Proximal Composition (%)		
Dry matter	94	
Crude Protein	41	
Ether extract	8	
Ash	8	
NNE	34	
Phosphorus	1,134	
Calcium	0.97	
Enzymatic Activity		
Inclusion levels (mg/kg)	Xylanase (U kg^{-1})	Phytase (U kg^{-1})
0	-	
25	694	416
50	950	540
100	1762	947
250	4991	2203
500	9240	4404

[a]Premix M. Cassab: Vit. A (500000 UI kg^{-1}), Vit. D3 (250000 UI kg^{-1}), Vit. E (5000 mg kg^{-1}), Vit. K3 (500 mg kg^{-1}), Vit. B1 (1000 mg kg^{-1}), Vit. B2 (1000 mg Kg^{-1}), Vit. B6 (1000 mg kg^{-1}) Vit. B12 (2000 mg kg^{-1}), Niacin (2500 mg kg^{-1}), Calcium pantothenate (4000 mg kg^{-1}), Folic acid (500 mg kg^{-1}), Biotin (10 mg kg^{-1}), Vit. C (10000 mg Kg^{-1}). Coline (100000mg kg^{-1}), Inositol (1000 mg Kg^{-1}). Se (30 mg kg^{-1}), Fe (5000 mg kg^{-1}), Cu (5000 mg kg^{-1}), Mn (5000 mg kg^{-1}), Zn (9000 mg kg^{-1}), Co (50 mg kg^{-1}), I (200 mg kg^{-1}).

five diets were supplemented with 25 (T25), 50 (T50), 100 (T100), 250 (T250), and 500 mg kg^{-1} (T500) of the Rovabio® Max AP enzymatic complex (endo-1,4-xylanase, α-arabinofuranosidase, β-xylosidase, feruloyl esterase, endo-1,5-arabinase, endo-1,4-glucanase [cellulase], cellobiohydrolase, β-glucosidase, polygalacturonase, pectinesterase, rhamnogalacturonase, endo-1,4β-mannanase, 6-phytase, aspartate protease, and metalloprotease). Experimental diets were prepared by mixing the ingredients from the lowest to the highest inclusion level, with the addition of oil at the end of the preparation. The enzymes were diluted in distilled water (heated to 40 °C) and added to the feed preparation. After complete homogenization of the blend, it was formed into pellets using a meat grinder. To verify the presence of enzymes in the diets, enzymatic activity analysis was performed for xylanase and phytase at the Center for Analysis, Research and Applied Technology (Adisseo, Commentry, France) (Table 1).

After the acclimation period and to start the experiment, all fish, weighing approximately 1.00 ± 0.15 grams, were anesthetized with 50 ppm benzocaine and distributed among the experimental units (18 tanks). The density used was 15 fish per tank (50 L). Each treatment was replicated three times, and the replicates were randomly distributed among the tanks. The experiment was carried out in a water recirculation system composed of a biological filter, UV filter (18 w Philips), and skimmer, with constant aeration and a photoperiod of 14 hours light/10 hours dark. The feces and the dietary remains were siphoned periodically, always preceding the feeding times, and the water losses were restored whenever necessary. Food was offered four times a day (8:00, 11:00, 14:00, and 17:00 hours) until apparent satiation.

During the experiment, dissolved oxygen and temperature were measured using a multiparameter digital oximeter (YSI 50A, Yellow Springs, OH, USA). A refractometer (Atago®, model 103, Tokyo, Japan) was used for salinity measurements. To verify the pH value, a digital pH meter (± 0.01, YSI®-pH100, Yellow Springs, OH, USA) was used. The alkalinity, ammonia, and nitrite analyses were performed according to the methodology described by UNESCO (1983).

The experimental period was 80 days. At the conclusion of the experiment, all fish to be used in the proximal, biochemical, and histological analyses were euthanized with buffered benzocaine hydrochloride (500 ppm). Three fish livers and intestines were collected per tank for histological analysis and another three livers for biochemistry. Three fish carcasses were collected per tank for determinations of proximal analysis. Liver samples to be used for biochemical determinations were preserved in an ultra-freezer (−80 °C). Liver and intestine samples for histological analysis were preserved in 10% buffered formalin. Carcasses for proximal analysis were preserved in a freezer (–20 °C).

For the determination of carcass and diet composition (n = 3), proximal composition analysis was performed according to the AOAC (1999) method. Dry matter (DM) analysis was carried out in an oven at 102 °C for 5 h; for ash (MM), the samples were precalcined and then heated in a muffle furnace to 600 °C for 5 h. Crude protein (CP) analysis was performed according to the Kjeldahl methodology, with previous digestion of the samples and subsequent distillation and titration of the nitrogen, multiplying the result by 6.25. The ethereal extract (EE) value was obtained using the Soxhlet extractor and petroleum ether as solvent, for 5 h.

At the end of the experiment, all fish were weighed, and the following parameters were analyzed:

1. Feed conversion rate (FCR) = feed consumed/weight gain;
2. Specific growth rate (SGR) = [(ln final weight – ln initial weight)/days of experiment] × 100;
3. Protein efficiency ratio (PER): weight gain (g)/protein intake (g);
4. Survival (%) = (final number fish/initial number of fish) × 100.

For analyses of triglycerides and hepatic glycogen (n = 3 per tank), the samples were homogenized for 40 min in a sonicator with perchloric acid (6%) in a volume of 7.5 times the sample weight (LÁIZ-CARRIÓN *et al*., 2012; modified by ZAMORA-SILLERO *et al*., 2013). After sonication, the homogenates were neutralized with the same volume of potassium bicarbonate (1M), and centrifuged (13,000 x g for 30 min). After centrifugation, the supernatant was used for the analyses. Total triglyceride levels were measured using commercial kits (Liquid Enzymatic Triglycerides, Doles, Goiânia, Brazil).

The liver glycogen content (n = 3 per tank) was measured in duplicate (CARR and NEFF, 1984; modified by NERY and SANTOS, 1993). The glycogen content was obtained through the enzymatic degradation of glucose (amyloglucosidase, Sigma). The resulting product was assayed with a commercial kit (Enzymatic Glucose; Doles, Goiânia, Brazil). All measurements were performed spectrophotometrically at a wavelength of 490 nm in a microplate reader (ELx800; Biotek Instruments Inc., Winooski, VT).

The solution for analysis of Ca and P in the bones (n = 3 per tank) was prepared according to the methodology of Santos (2009) through nitro-perchloric digestion. Calcium was determined using a commercial kit (Calcio Arsenazo, Doles, Goiânia, GO, Brazil) and read in a spectrophotometer (Biospectro, Spectrophotometer SP-22) at a wavelength of 670 nm. Total phosphorus was quantified spectrophotometrically at 420 nm (AOAC, 2010).

Samples of liver and intestine (n = 3 per tank) were collected and fixed in 20% buffered formaldehyde and later processed in an automatic tissue processor (LUPE PT 05). The tissues were embedded in Paraplast. Five-micrometer-thick cuts were performed on a microtome (LUTETEC MRPO3). The slides were stained with Hematoxylin-Eosin and observed with the aid of a Zeiss Primo Star microscope.

For the observation and evaluation of possible morphological alterations in the liver, a scale of 1 to 3 was adopted, as described by MCFADZEN *et al*. (1997). Grade 1 represents a liver in good condition, with sparse granules, small and distinct nuclei, and a hepatocyte cytoplasmic structure varying in texture and containing dispersed granules; grade 2 indicates an intermediate condition in which the nucleoli are broad or amorphous, and the cytoplasm is homogeneous and vacuolated to a very limited degree; grade 3 corresponds to a degraded liver in which many nuclei are pyknotic, small, and black, and the cytoplasm is hyaline with a lack of texture.

In the observation and evaluation of intestinal samples, the criteria suggested by KROGDAHL *et al*. (2003) were applied, which classify possible changes in intestinal morphology as follows: 1) enlargement or shortening of intestinal folds; 2) loss of supranuclear vacuolation in the absorptive cells (enterocytes) in the intestinal epithelium; 3) widening of the central lamina, within the intestinal folds, with an increase in the amount of connective tissue; and 4) presence of a mixed population of leukocytes in the central lamina of the intestinal and submucosal folds.

In this paper all data are presented as mean ± standard deviation. Prior to analysis of variance, the normality of the data was verified with the Shapiro-Wilk test and the homogeneity of the variances, with the Levene test. Once these principles were confirmed, the results were submitted to analysis of variance (ANOVA). When significant differences ($P < 0.05$) were detected between treatments, Tukey's test was applied at the 5% level of significance.

RESULTS

During the experimental period, mean dissolved oxygen concentrations throughout the study were 6.2 ± 0.3 mg L^{-1}, and the temperature was 25.6 °C ± 0.2 °C. The pH value remained constant, averaging 7.97 ± 0.09. The alkalinity presented an average of 136.41 ± 17.79 mg L^{-1} $CaCO_3$. The mean values for total ammonia and nitrite were 0.14 ± 0.14 mg L^{-1} and 0.11 ± 0.11 mg L^{-1}, respectively.

Survival rates were equal to or greater than 90%, with no significant difference between treatments (Table 2). Growth and weight gain were significantly higher ($P < 0.05$) in the T100 and T500 treatments than in the TC. However, there was no significant difference ($P > 0.05$) for the parameters of feed intake, protein efficiency rate, specific growth rate, and apparent feed conversion between treatments.

There was no significant difference ($P > 0.05$) between treatments for dry matter, crude protein, ethereal extract, and ash (Table 3). Mineral concentrations showed a significant difference ($P < 0.05$) only for the total calcium readings, which presented the lowest values for the TC and T25 treatments, while the highest values were presented by the fish in treatment T250. The total phosphorus concentration did not present a significant difference ($P > 0.05$) between treatments (Table 3).

The hepatic glycogen concentration did not show a significant difference between treatments. The liver triglyceride content showed a significant difference ($P < 0.05$) between treatments (Table 4): the TC treatment had the lowest triglyceride value, while the T250 and T500 treatments had the highest values.

Table 2. Effects of enzyme supplementation on the performance parameters of juveniles of *Trachinotus marginatus*, fed experimental diets for 80 days.

Diets	TC	T25	T50	T100	T250	T500	*CV* (%)
Initial weight (g)	1.00 ± 0.17	1.00 ± 0.13	1.00 ± 0.11	0.99 ± 0.14	0.99 ± 0.13	0.99 ± 0.13	13.4
Final weight (g)	7.78 ± 2.08^{b}	8.30 ± 2.43^{ab}	8.73 ± 1.86^{ab}	9.21 ± 2.55^{a}	8.77 ± 2.27^{ab}	9.23 ± 2.14^{a}	26.3
Weight gain (g)	6.78 ± 2.08^{b}	7.30 ± 2.44^{ab}	7.73 ± 1.87^{ab}	8.21 ± 2.55^{a}	7.77 ± 2.27^{ab}	8.23 ± 2.13^{a}	29.5
Feed Consumption (g)	278.87 ± 4.90	267.23 ± 30.17	283.33 ± 13.76	280.83 ± 14.25	281.53 ± 3.52	282.33 ± 4.36	5.1
SGR[1]	2.56 ± 0.14^{a}	2.60 ± 0.26^{a}	2.70 ± 0.12^{a}	2.79 ± 0.07^{a}	2.71 ± 0.11^{a}	2.78 ± 0.01^{a}	6.3
FCR[2]	2.51 ± 0.27	2.69 ± 0.63	2.33 ± 0.16	2.30 ± 0.20	2.35 ± 0.17	2.24 ± 0.05	13.8
PER[3]	0.87 ± 0.10	0.84 ± 0.22	0.95 ± 0.08	0.97 ± 0.08	0.94 ± 0.08	0.99 ± 0.02	12.0
Survival (%)	98.00 ± 0.04	90.00 ± 0.04	98.00 ± 0.04	89.00 ± 0.14	95.00 ± 0.04	95.00± 0.04	7.0

Values are expressed as means ± SD with n = 3. Different letters on the same line indicate significant difference between treatments, significance of $p < 0.05$. [1]Specific Growth Rate; [2]Feed Conversion; [3]Protein Efficiency Rate. TC = Control Diet, T25 = 25 mg/kg, T50 = 50 mg/kg, T100= 100 mg/kg, T250 = 250 mg/kg, T500 = 500 mg/kg of enzymes. CV = Coefficient of Variation.

Table 3. Proximal carcass analysis of *Trachinotus marginatus* (% wet weight) fed diets supplemented with increasing levels of enzymes for 80 days.

Diets	TC	T25	T50	T100	T250	T500	*CV* (%)
Dry matter	37.49 ± 0.01	35.86 ± 0.01	37.47 ± 0.03	38.52 ± 0.01	37.53 ± 0.01	37.59 ± 0.02	4.9
Crude Protein	18.56 ± 0.01	19.17 ± 0.01	18.44 ± 0.01	18.78 ± 0.01	19.11 ± 0.01	19.22 ± 0.01	3.9
Ethereal Extract	14.68 ± 0.02	14.82 ± 0.01	15.54 ± 0.03	16.61 ± 0.01	15.57 ± 0.01	15.80 ± 0.01	13.7
Ashes	4.27 ± 0.003	4.70 ± 0.003	4.44 ± 0.005	4.29 ± 0.004	4.17 ± 0.003	4.45 ± 0.004	9.2
P*	10.70 ± 0.39	10.73 ± 0.28	10.27 ± 0.49	10.00 ± 0.68	10.02 ± 0.53	10.15 ± 1.15	6.7
Ca*	31.21 ± 2.54^{c}	31.52 ± 1.71^{bc}	35.23 ± 1.85^{ab}	35.31 ± 2.11^{ab}	36.77 ± 2.89^{a}	33.07 ± 3.31^{bc}	9.1

Values are expressed as means ± SD with n = 3. Different letters on the same line indicate significant difference between treatments, significance of $p < 0.05$. *Measured in bones. TC = Control Diet, T25 = 25 mg/kg, T50 = 50 mg/kg, T100= 100 mg/kg, T250 = 250 mg/kg, T500 = 500 mg/kg of enzymes. CV = Coefficient of Variation.

Table 4. Effects of enzyme supplementation on the concentration of glycogen and triglycerides in the liver of juveniles of the *Trachinotus marginatus*, fed experimental diets for 80 days.

Diets	TC	T25	T50	T100	T250	T500	*CV* (%)
Glycogen (mmol L^{-1})	333 ± 34	285 ± 85	296 ± 75	332 ± 28	331 ± 36	259 ± 42	19.2
Triglycerides (mg L^{-1})	83 ± 6^{c}	120 ± 26^{bc}	120 ± 23^{bc}	138 ± 21^{ab}	178 ± 13^{a}	184 ± 17^{a}	28.4

Values are expressed as means ± SD of three replicate groups. Different letters on the same line indicate significant difference between treatments, significance of $p < 0.05$. TC = Control Diet, T25 = 25 mg/kg, T50 = 50 mg/kg, T100= 100 mg/kg, T250 = 250 mg/kg, T500 = 500 mg/kg of enzymes. CV = Coefficient of Variation.

The fish livers of the T250 and T500 treatments had a higher number of vacuoles in the hepatocytes, due to high lipid accumulation (Table 5). However, the liver cells of fish in the TC treatment did not present alterations (Figure 1).

There were no differences in intestinal morphology between treatments (Figure 2). All the analyzed fish were in degree I of alteration, presenting slight inflammation of the lamina propria and moderate inflammation of the epithelial layer.

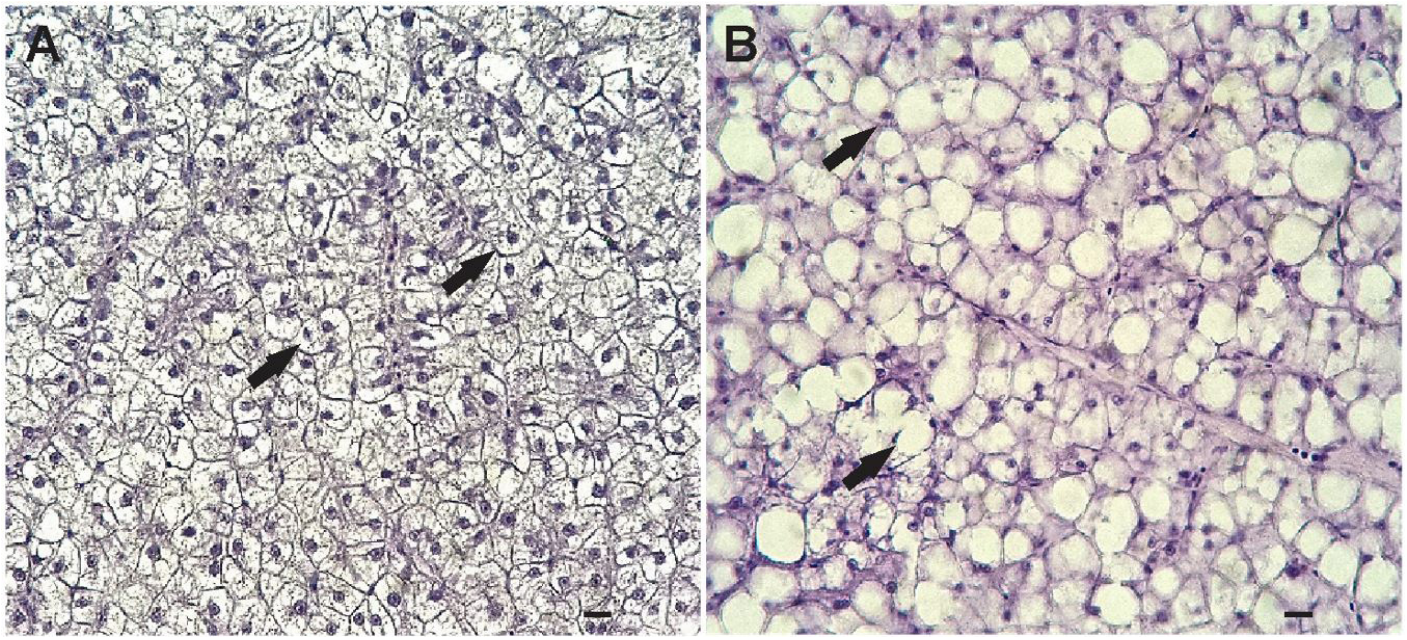

Figure 1. (A) Fish liver fed with control diet, the arrow points to the cells at normal stage, with no apparent changes; (B) Fish liver of the T500 treatment, the arrow points the cells with formation of lipid vacuoles, displacement of the nucleus to the ends of the cell and with greater cytoplasmic volume. H & E bar = 20 µm. T500 = 500 mg/kg of enzymes.

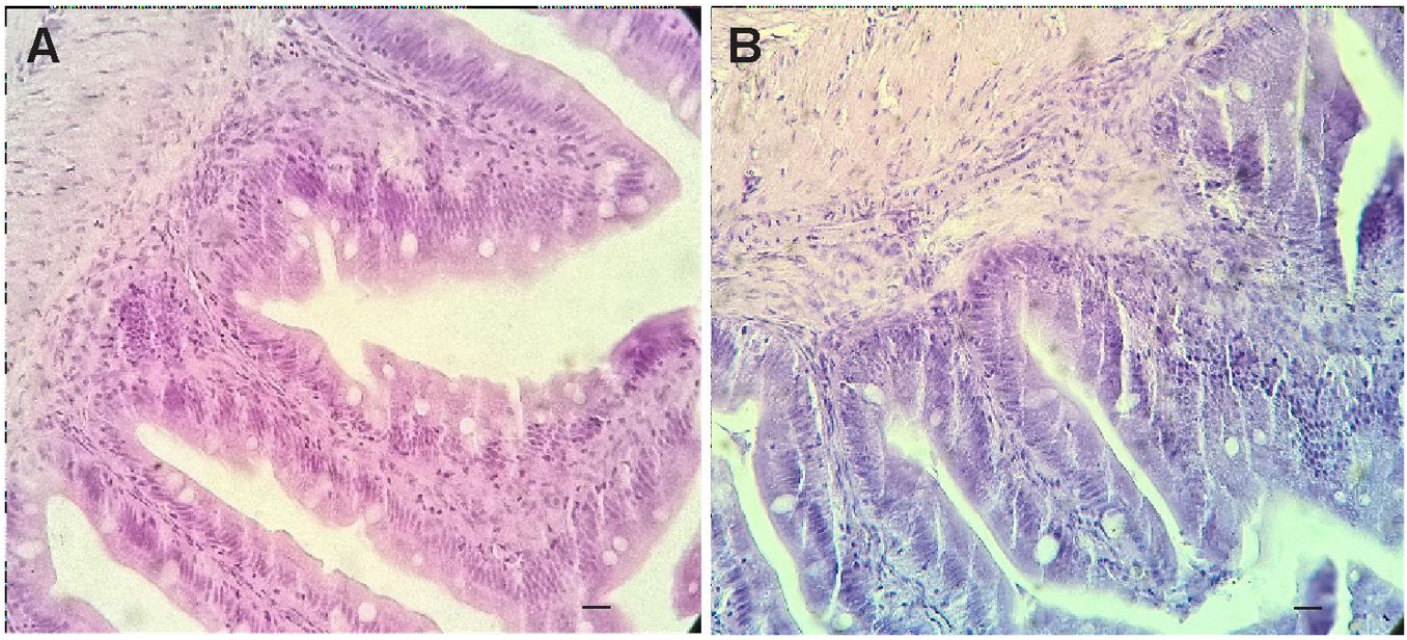

Figure 2. (A) Fish intestine fed with diets without enzymes; (B) Intestine of fish fed with diet containing 500 mg kg-1 of enzymes. There was no significant difference in fish intestinal morphology between treatments. H & E bar = 20 µm.

Table 5. Morphological index of the liver of *Trachinotus marginatus* fed for 80 days with diets supplemented with different enzymatic levels.

Diet	Degree 1	Degree 2	Degree 3
	Normal Structures	Moderate alterations	Severe Alterations
TC	8	1	-
T25	4	5	-
T50	4	5	-
T100	5	4	-
T250	1	4	4
T500	-	5	4

TC = Control Diet, T25 = 25 mg/kg, T50 = 50 mg/kg, T100= 100 mg/kg, T250 = 250 mg/kg, T500 = 500 mg/kg of enzymes.

DISCUSSION

In the present study, the growth of fish in the T100 and T500 treatments was positively influenced by enzymatic supplementation. Inclusion of the enzymatic complex improved the growth performance of *Trachinotus marginatus*. This result corroborates the results of other authors who also used an enzymatic complex and obtained an improvement in growth, such as ALI ZAMINI *et al*. (2014) in *Salmo trutta caspius*, AI *et al*. (2007) in *Lateolabrax japonicus,* and LIN *et al*. (2007) in juveniles of hybrid tilapia *Oreochromis niloticus* x *O. aureus*.

Some authors have explained growth improvement in fish with the use of enzymes as the effect of carbohydrase and protease activity against non-starch polysaccharides. It is known that soluble NSPs, such as hemicellulose and pectins (present at high levels in soybean meal), increase the viscosity of the diet, leading to slower gastric emptying, causing possible alterations in the morphology and physiology of the digestive tract, and consequently, altering nutrient absorption (SINHA *et al*., 2011; RAMOS *et al*., 2017).

Positive results of the use of carbohydrases were observed by JIANG *et al*. (2014), who tested the effects of xylanase supplementation on *Cyprinus carpio* fed diets containing vegetable protein. In addition to improved performance, the authors observed an increase in the activity of intestinal trypsin, chymotrypsin, lipase, and amylase enzymes and an increase in the *Lactobacillus* counts and a reduction in the *Aeromonas* and *Escherichia coli* counts in the intestine. Although neither enzyme activities nor the intestinal microbiota were evaluated in the present study, the use of exogenous enzymes in the diet may have provided an improvement in these aspects, which would result in a greater breakdown of the NSPs, directly favoring fish performance (CASTILLO and GATLIN III, 2015). In fact, the fish of the T100 and T 500 treatments presented higher mean final weight than the fish of the TC control treatment.

In this study it was also observed that supplementing fish diets with inclusion levels of 50, 100, and 250 mg kg^{-1} of the enzymatic complex caused a high concentration of Ca in the bones. This difference may be directly related to the presence of the enzyme phytase in the diet, which favored the absorption of this mineral, as observed by ADEOYE *et al*. (2016). The same result was observed by RAMOS *et al*. (2017), who used the same enzymatic complex in diets for *Mugil liza* juveniles. In both works there was no variation in phosphorus deposition. A possible explanation is the disproportion of the Ca:P ratio present in the diets, since the high level of Ca could have interfered in the absorption of P and the reduction of phytase activity (CAO *et al*., 2007).

Liver histology showed excessive hepatic lipid deposition in the T250 and T500 treatments, which was also evidenced by liver triglyceride analysis. In diets with high energy levels, changes in liver morphology caused by accumulation of lipids are common. High levels of lipids or energy in the diet, which exceed the capacity of liver cells to oxidize fatty acids, leads to a massive increase in the synthesis and deposition of lipids in fish liver (MARTÍNEZ-LLORENS *et al*., 2012). This trend of lipid accumulation in the liver may have been the result of a greater incorporation of energy from the diet, provided by enzyme supplementation, leading to the formation of lipid vacuoles in the hepatocytes. The use of exogenous enzymes improves the digestibility of plant ingredients by promoting the breakdown of NSPs and antinutritional factors such as phytate, allowing nutritional reformulation with a reduction in the energy level of the diet. As the energy levels of the enzyme-supplemented diets in this experiment were not adjusted, such an excess may lead to a scenario of excessive accumulation of lipids in the liver, as observed in this work. In this way, CASTILLO and GATLIN III (2015) suggest that carbohydrases should be included at sub-optimal levels precisely to avoid excessive energy input.

CONCLUSION

The inclusion level of 100 mg kg^{-1} of the enzyme complex was shown to be optimal for use in the diet of juvenile pompano. In addition to its growth-promoting properties, it also favored greater retention of calcium in the bones, without causing excessive accumulation of triglycerides in the liver.

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