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Research Article

# Growth Performance and Digestibility Using Proteases and Carbohydrases in Diets for Nile Tilapia *Oreochromis Niloticus*

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## Abstract

This study was conducted to evaluate the production performance and feed digestibility of Nile tilapia, *Oreochromis niloticus* when supplemented with commercial proteases and carbohydrases. Ten practical tilapia diets were formulated, 32% protein and 6% lipids. Six diets were formulated to contain Low-Fiber (LF) and four were High-Fiber (HF) diets. The enzymes supplemented included Free Protease (FP), Protected Protease (PP), Free Carbohydrase (FC), Protected Carbohydrase (PC), and a Mix of Free Protease and Carbohydrases (MFPFC). The level of FP and PP was 175 g/mt, and the level of FC, PC, and MFPFC was 125 g/mt. The diets were offered to sex-reversed juvenile tilapia (mean initial weight 9.29 ± 0.11 g) over a 70-day growth trial. Four replicate groups of 20 fish per 75-L aquaria were offered diets at near satiation. After the growth trial, survival was near 100% and weight gain was around 1000%. In general, fish maintained on the HF diets performed slightly poorer than those on the LF diets. Concerning enzyme supplements, apparent net energy retention was significantly different ($p=0.0001$) in LF diets when FP and PP were added. However, for LF and HF diets there were no significant ($p > 0.05$) differences in final mean weight, percent weight gain, thermal-unit growth coefficients, survival, feed conversion ratio, or apparent net protein retention. Overall, there were no clear advantages detected to the protected enzymes. Dry matter and energy digestibility were significantly improved by the addition of FC and MFPFC when supplemented with LF and HF diets. Therefore, the use of these enzymes in diet formulations for Nile tilapia is an opportunity to increase digestibility while decreasing cost formulation without affecting performance.

**Keywords:** Carbohydrase; Digestibility; Protease; Tilapia

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## Declaration

A previous version of this manuscript has been published as a chapter within the first author's dissertation [1].

## Introduction

The development of commercial feeds for aquaculture has been traditionally based on fishmeal as the main protein source because of its high protein content and essential amino acid profile [2-4]. However, the global fishmeal price has increased more than twofold in recent years [5] due to a shortage in the supply. The use of alternative feed ingredients, including plant sources [6-12], animal sources [13-16], algae [17-19], and restaurant food waste [20] are viable options for decreasing fish meal use and decreasing formulation cost [21-23]. However, the presence of anti-nutritional factors and low digestibility of diets, when some alternative feed ingredients are included, can impair their use as fish meal replacements in aquaculture feeds.

Supplementation of diets with exogenous enzymes is considered effective in eliminating the anti-nutritional factors and improve utilization of dietary energy and amino acids, resulting in improved fish performance [24] and gut health [25]. Dietary proteases and carbohydrases are used in aquatic animals to improve the digestibility of diets when plant-based ingredients are included in the formulation.

Exogenous protease can compensate for the deficiency of endogenous enzymes, especially for young animals, and assist in the breakdown of macromolecular proteins, improving their digestibility [26]. Carbohydrases are used to assist in the breakdown of hemicellulose which are part of the cell wall. As described by Ebringerová [27], among the hemicelluloses are the xyloglycans (xylans) and mannoglucans (mannans). Xylans are the most abundant hemicellulose type in the plant kingdom and mannans are part of the Non-Starch Polysaccharide (NSP) fraction in plant-based feed ingredients. The enzymes required to digest NSP, such as beta-xylans or beta mannans, are very scarce or even absent among fish species [28] and the ability to use NSP by the fish depends on the nature of the microbial population residing in the gut [29]. The NSP fraction influences digesta viscosity, gut morphology, physiology, and mucus layer, affecting the endogenous secretion of water, proteins, electrolytes, and lipids. These changes can lead to reduced nutrient digestibility [29,30].

The addition of exogenous proteases and carbohydrases has been studied in fish. A recent review of the inclusion of protease in aquaculture diets outlined multiple benefits to various fish species [31]. In rainbow trout, *Oncorhynchus mykiss*, the addition of protease to canola, pea-based diets resulted in significant improvements in apparent digestibility for crude protein, energy, lipid, and dry matter [32]. Dalsgaard, et al. [33] supplemented protease to soybean

meal-containing diets for rainbow trout and reported a significant increase in the apparent digestibility of protein, lipid, phosphorus, and dry matter. Farhangi and Carter [34] fed juvenile rainbow trout, diets supplemented with protease and carbohydrase alone or in combination with de-hulled lupin-based feeds. No effects on performance were observed, but the mixed enzyme significantly improved the protein efficiency ratio and the apparent digestibility of dry matter, protein, and gross energy. In contrast, Yigit, et al. [35] reported that rainbow trout supplemented with a mix of beta mannanase and alpha-galactosidase at two levels (1 g/kg and 2 g/kg) to a control diet including soybean meal did not affect growth parameters, feed efficiency, and digestibility. Also, rainbow trout fry diets containing canola and supplemented with cellulase, phytase, pectinase, or an enzyme mix showed no differences in growth parameters, feed conversion ratio, dry matter, protein, or lipid digestibility with enzyme supplementation [36].

Digestibility of crude protein and crude lipid were significantly improved in juvenile Gibel carp, *Carassius auratus gibelio* [37] fed incremental increases in dietary protease up to 300 mg/kg. Carter, et al. [38] reported a positive effect on performance and feed efficiency in Atlantic salmon smolt, Salmo salar L when supplementing a combination of proteolytic enzymes and carbohydrases to a diet containing 340 g/kg soybean meal, no increase was observed in the apparent digestibility of nitrogen and carbon.

In hybrid tilapia, *Oreochromis niloticus* × *Oreochromis aureus*, the digestibility of dry matter and crude protein increased by the supplementation of protease [39]. Lin, Mai, and Tan [24] reported that the addition of a commercial enzyme complex of neutral protease, beta glucanase, and xylanase improved growth performance, but no effect was detected in the apparent digestibility of protein, lipid, and gross energy in hybrid tilapia. Adeoye, et al. [40] fed Nile tilapia probiotics, a mix of enzymes (containing phytase, protease, and xylanase), and the combination of enzymes and probiotics. Tilapia fed diets supplemented with enzymes plus probiotics performed better than tilapia fed only the probiotic supplemented diets in terms of final body weight, feed conversion ratio, and protein efficiency ratio. Hlophe-Ginindza, et al. [41] reported that Mozambique tilapia, *Oreochromis mossambicus* fed a kikuyu-based diet supplemented with a multi enzyme complex composed of cellulase, xylanase, and phytase had improved growth, lower feed conversion rate values, increased protein efficiency, higher protein digestibility and increased activity of fish enzymes up to 0.5 g/kg addition to the diet. The inclusion of beta mannanase improved growth, feed efficiency and feed conversion ratio and increased the intestinal enzyme activity in Nile tilapia [42]. Red hybrid tilapia, *Oreochromis sp.* fed diets containing 40% palm kernel meal did not improve growth and feed utilization when a combination of protease, cellulase, glucanase, pectinase and pure mannanase were added to the diets [43]. Caspian salmon, *Salmo trutta caspius* fed two multienzyme complexes which consisted of a combination of protease, lipase, phytase, alpha amylase, cellulase, amyloglucosidase, beta glucanase, pentosonase, hemicellulase, xylanase, pectinase, acid phosphatase, acid phytase and endo-beta mannanase, amylase, xylanase, cellulose and alpha galactosidase improved growth and feed utilization when enzymes were included in the diet in a multi enzyme complex at levels of 0.5 g/kg and 2.5 g/kg [44]. Nile tilapia can tolerate higher dietary fiber and carbohydrate concentrations than most other cultured fish [45] and has the ability to feed on a wide range of foods. Hence the purpose of this study was to evaluate the efficacy of using commercial protease and carbohydrase enzymes on growth performance, nutrient retention, and nutrient digestibility in practical diets for juvenile Nile tilapia.

## Materials and Methods

### *Experimental diets*

Ten practical tilapia diets were formulated to contain 32% protein and 6% lipids (Table 1). The test diets were formulated to meet the nutritional requirements of the Nile tilapia [46]. Six diets were formulated to contain low levels of fiber, which included a Low-Fiber basal diet (LF) and LF supplemented with free protease (LF-FP), protected protease (LF-PP), free carbohydrase (LF-FC), protected carbohydrase (LF-PC), or a mix of free protease and carbohydrase (LF-MFPFC). Additionally, to evaluate the effects of higher fiber diets, a High-Fiber basal diet (HF) was formulated using 30% distillers dried grains with solubles as a replacement for soybean meal. The HF basal diet was then supplemented with free protease (HF-FP), free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC). The level in the diet of free protease (FP) and protected (PP) was 175 g/mt, the level of free carbohydrase (FC), and protected carbohydrase (PC) and the mix of free protease and carbohydrase (MFPFC) was 125 g/mt. The test diets were prepared at the Aquatic Animal Nutrition Laboratory at the School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University (Auburn, AL, USA). Pre-ground dry ingredients and oil were weighed and then mixed using a food mixer (Hobart Corporation, Troy, OH, USA) for 15 minutes. Boiling water was then blended into the mixture at ~ 30% in order to attain an appropriate consistency for pelleting. Diets were then extruded through a 3-mm diameter die in a meat grinder, air dried at < 50°C to a moisture content of 8-10%, and stored at room temperature. A sample of 150 g of each feed was collected and analyzed for proximate composition (AOAC 930.15, AOAC 990.03, AOAC 2003.05, Ankom Tech, AOAC 942.05 were used for moisture, protein, fat, fiber, and ash analysis respectively) by the Experiment Station Chemical Laboratories, University of Missouri, (Columbia, MO, USA) (Table 2).

The FP complex used in this study is an alkaline serine protease complex produced from bacterial fermentation. The PP is a microencapsulated protease complex composed of vegetable fat and bacterial fermentation extract. The enzymatic activity of both products was 18,000 unit/g. One unit of protease is equivalent to the amount of enzyme that releases 1 nmol of 4-nitroaniline per minute from Succ-AAPF-pNA at pH 9.0 and 40°C. The FC and PC complexes are a combination of xylanase and beta-mannanase. The activity of xylanase in the products was 270 unit/g defined as the quantity of enzyme that releases one micromole of xylose per minute at pH 4.5 and 30°C. The activity of beta-mannanase in the products was 2,790 unit/g defined as the quantity that liberates one micromole of reducing sugar (mannose equivalents) in one minute from a mannan-containing substrate (locust bean gum) at pH 6.0 and 50°C. In the MFP-FC complex, the activity of both carbohydrases was similar to those mentioned above but the protease activity was >5000 unit/g. All the enzymes were supplied by JEFO Nutrition Inc. (Saint-Hyacinthe, Quebec, Canada).

### *Culture methods*

Juvenile sex-reversed Nile tilapia (mean initial weight 9.29 ± 0.11 g) were randomly stocked into 75-L aquaria which are a component of a 2,500-L indoor recirculation system at 20 fish per aquarium at the E.W. Shell Fisheries Center (Auburn, AL, USA). Each diet was randomly assigned to the tanks and offered to fish in four replicate aquaria for the duration of a 70-day growth trial. Samples of fish from the initial stocking were retained for later whole-body analysis.

| | LF | LF-FP | LF-PP | LF-FC | LF-PC | LF-MFPFC | HF | HF-FP | HF-FC | HF-MFPFC |
|---|---|---|---|---|---|---|---|---|---|---|
| Ingredient | Low-Fiber | | | | | | High-Fiber | | | |
| MFM 1 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 | 2.00 |
| MBM 2 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 | 6.00 |
| SBM 3 | 48.00 | 48.00 | 48.00 | 48.00 | 48.00 | 48.00 | 36.80 | 36.80 | 36.80 | 36.80 |
| DDGS 4 | | | | | | | 30.00 | 30.00 | 30.00 | 30.00 |
| Fish oil 5 | 3.30 | 3.30 | 3.30 | 3.30 | 3.30 | 3.30 | 1.55 | 1.55 | 1.55 | 1.55 |
| Lecithin 6 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Corn Starch 7 | 5.60 | 5.582 | 5.582 | 5.587 | 5.587 | 5.587 | 2.850 | 2.832 | 2.837 | 2.837 |
| Corn8 | 30.50 | 30.50 | 30.50 | 30.50 | 30.50 | 30.50 | 15.70 | 15.70 | 15.70 | 15.70 |
| Mineral premix9 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 | 0.50 |
| Vitamin premix10 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 | 0.80 |
| Choline chloride7 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 | 0.20 |
| Stay C 35% active11 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 | 0.10 |
| CaP-dibasic7 | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 | 2.50 | 2.80 | 2.80 | 2.80 | 2.80 |
| Lysine HCl12 | | | | | | | 0.20 | 0.20 | 0.20 | 0.20 |
| Enzyme FP13 | | 0.018 | | | | | | 0.018 | | |
| Enzyme PP13 | | | 0.018 | | | | | | | |
| Enzyme FC13 | | | | 0.013 | | | | | 0.013 | |
| Enzyme PC13 | | | | | 0.013 | | | | | |
| Enzyme MFPFC13 | | | | | | 0.013 | | | | 0.013 |

**Table 1:** Ingredient composition (g/100g as-is) of test diets formulated to contain 32% protein and 6% lipid where six diets included a low-fiber basal diet (LF) and LF supplemented with free protease (LF-FP), protected protease (LF-PP), free carbohydrase (LF-FC), protected carbohydrase (LF-PC), or a mix of free protease and carbohydrase (LF-MFPFC), and four additional diets included a high-fiber basal diet (HF) and HF supplemented with free protease (HF-FP), free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC).

[1] Menhaden Fishmeal, Omega Protein Inc., Houston, TX, USA.

[2] Meat & Bone Meal, Midsouth Milling Co., Memphis TN, USA.

[3] De-hulled solvent extract soybean meal, Bunge Limited, Decatur, AL, USA.

[4] Distillers dried grains with solubles (DDGS) Flint Hills Resources, LLC, Pelham, GA, USA.

[5] Menhaden Fish Oil, Omega Protein Inc., Reedville Houston, VA, USA.

[6] Enhanced D-97, The Solae Company, St. Louis, MO, USA.

[7] MP Biomedicals Inc., Solon, OH, USA.

[8] Faithway Feed Co., LLC., Guntersville, AL, USA.

[9] Trace mineral (g/100g Premix): Cobalt chloride, 0.004; Cupric sulfate pentahydrate, 0.25; Ferrous sulfate, 4.0; Magnesium sulfate anhydrous, 13.86; Manganous sulfate monohydrate, 0.65; Potassium iodide, 0.067; Sodium selenite, 0.010; Zinc sulfate hepahydrate, 13.19; cellulose, 67.96.

[10] Vitamin (g/kg Premix): Thiamin HCl, 0.44; Riboflavin, 0.63; Pyridoxine HCl, 0.91; D-pantothenic acid, 1.72; Nicotinic acid, 4.58; Biotin, 0.21; Folic acid, 0.55; Inositol, 21.05; Menadione sodium bisulfite, 0.89; Vitamin A acetate (500,000 IU g-1), 0.68; Vitamin D3 (400,000 IU g-1), 0.12; DL-alpha-tocoperol acetate (250 IU g-1), 12.63; cellulose 955.59.

[11] Stay C®, (L-ascorbyl-2-polyphosphate 25% Active C), DSM Nutritional Products., Parsippany, NJ, USA.

[12] Ajinomoto Heartland Inc., Chicago, IL, USA.

[13] Jefo Nutrition Inc, Saint-Hyacinthe, QC, Canada.

| | LF | LF-FP | LF-PP | LF-FC | LF-PC | LF-MFPFC | HF | HF-FP | HF-FC | HF-MFPFC |
|---|---|---|---|---|---|---|---|---|---|---|
| | Low fiber | | | | | | High fiber | | | |
| Crude protein* | 32.00 | 29.14 | 27.09 | 30.30 | 30.13 | 29.78 | 31.41 | 31.23 | 32.30 | 31.87 |
| Moisture | 9.17 | 13.28 | 15.15 | 5.91 | 6.70 | 8.31 | 6.95 | 6.23 | 5.88 | 8.10 |
| Crude Fat | 7.35 | 5.33 | 4.70 | 5.19 | 4.54 | 4.82 | 7.07 | 6.50 | 6.77 | 6.45 |
| Crude Fiber | 4.25 | 3.59 | 3.48 | 7.00 | 4.50 | 3.60 | 7.45 | 6.46 | 5.11 | 6.14 |
| Ash | 8.03 | 7.40 | 7.21 | 7.70 | 7.65 | 7.60 | 8.47 | 8.46 | 8.57 | 8.43 |
| Alanine | 1.54 | 1.41 | 1.38 | 1.52 | 1.48 | 1.49 | 1.68 | 1.66 | 1.75 | 1.70 |
| Arginine | 2.14 | 1.96 | 1.86 | 2.08 | 2.01 | 2.04 | 1.89 | 1.91 | 2.00 | 1.96 |
| Aspartic Acid | 3.20 | 2.92 | 2.77 | 3.08 | 3.01 | 3.01 | 2.76 | 2.79 | 2.86 | 2.86 |
| Cysteine | 0.40 | 0.37 | 0.37 | 0.40 | 0.39 | 0.39 | 0.45 | 0.44 | 0.44 | 0.45 |
| Glutamic Acid | 5.27 | 4.85 | 4.67 | 5.14 | 5.02 | 5.01 | 4.79 | 4.80 | 4.90 | 4.84 |
| Glycine | 1.63 | 1.48 | 1.46 | 1.65 | 1.55 | 1.62 | 1.60 | 1.56 | 1.64 | 1.59 |
| Histidine | 0.81 | 0.74 | 0.69 | 0.78 | 0.77 | 0.76 | 0.80 | 0.80 | 0.83 | 0.81 |
| Isoleucine | 1.36 | 1.25 | 1.17 | 1.33 | 1.30 | 1.29 | 1.30 | 1.31 | 1.36 | 1.33 |
| Leucine | 2.45 | 2.28 | 2.16 | 2.40 | 2.35 | 2.32 | 2.65 | 2.62 | 2.75 | 2.69 |
| Lysine | 2.03 | 1.82 | 1.71 | 1.91 | 1.88 | 1.88 | 1.88 | 1.89 | 2.01 | 1.96 |
| Methionine | 0.52 | 0.46 | 0.42 | 0.47 | 0.47 | 0.47 | 0.49 | 0.49 | 0.52 | 0.52 |
| Ornithine | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.02 | 0.03 | 0.03 | 0.03 | 0.03 |
| Phenylalanine | 1.50 | 1.42 | 1.30 | 1.45 | 1.42 | 1.41 | 1.49 | 1.48 | 1.55 | 1.53 |
| Proline | 1.67 | 1.56 | 1.43 | 1.64 | 1.59 | 1.60 | 1.79 | 1.78 | 1.83 | 1.81 |
| Serine | 1.27 | 1.17 | 1.19 | 1.18 | 1.16 | 1.17 | 1.23 | 1.23 | 1.28 | 1.29 |
| Taurine | 0.15 | 0.15 | 0.14 | 0.15 | 0.16 | 0.15 | 0.12 | 0.12 | 0.13 | 0.11 |
| Threonine | 1.18 | 1.07 | 1.04 | 1.12 | 1.09 | 1.10 | 1.13 | 1.14 | 1.18 | 1.18 |
| Tryptophan | 0.44 | 0.42 | 0.39 | 0.41 | 0.42 | 0.42 | 0.42 | 0.43 | 0.43 | 0.40 |
| Tyrosine | 0.98 | 0.93 | 0.86 | 0.94 | 0.93 | 0.93 | 0.97 | 0.99 | 1.04 | 1.01 |
| Valine | 1.56 | 1.42 | 1.35 | 1.53 | 1.50 | 1.49 | 1.56 | 1.55 | 1.62 | 1.57 |

**Table 2:** Proximate composition and amino acid profile of test diets, a low-fiber basal diet (LF) and LF supplemented with free protease (LF-FP), protected protease (LF-PP), free carbohydrase (LF-FC), protected carbohydrase (LF-PC), or a mix of free protease and carbohydrase (LF-MFPFC), and a high-fiber basal diet (HF) and HF supplemented with free protease (HF-FP), free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC) analyzed by Experiment Station Chemical Laboratories, University of Missouri, (Columbia, MO, USA) (% as is basis).

*Crude Protein = %N x 6.25

Water temperature was maintained at around 28˚C using a submerged 3,600-W heater (Aquatic Eco-Systems Inc., Apopka, FL, USA). Dissolved oxygen was maintained near saturation using air stones in each aquarium and the sump tank using a common airline connected to a regenerative blower. Dissolved oxygen and water temperature were measured twice a day using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA) while pH, TAN, and Nitrite-N were measured once per week. The photoperiod was set at 14 h light and 10 h dark.

Diets were offered to fish at 3.0-6.0% BW daily, according to fish size, and divided into two equal feedings each day. Test diets were applied two times per day (0800 and 1600 h) for a 70-day experimental period. Fish were weighed every week for the first two weeks and every other week thereafter. Daily feed rations were calculated based on % body weight. The ration was adjusted each week based on growth and observation of the feeding response. At the end of the growth trial, fish were counted, and group weighed to determine weight gain, survival, and feed conversion ratio. At the conclusion of the trial, four fish were randomly collected from every aquarium and frozen at 20 °C for later biochemical analysis. These whole-body fish samples were homogenized in a food processor and sent to Midwest Laboratories (Omaha, NE, USA) for proximate and mineral analyses as per AOAC procedures (AOAC 930.15, AOAC 990.03, AOAC 954.02, AOAC 942.05 were used for moisture, protein, fat, fiber, and ash analysis respectively; AOAC 985.01 was used for mineral analysis).

Growth performance indexes including weight gain, feed conversion ratio (FCR), survival, apparent net protein retention (ANPR), apparent net energy retention (ANER), Thermal-unit Growth Coefficient (TGC), hepatosomatic index (HI) and Intraperitoneal Fat Index (IFI) were computed using the following calculations:

- Weight gain (g) = Average final weight (g) – Average initial weight (g)
- FCR = dry feed intake/ wet weight gain.
- Survival (%) = (Initial fish number – Final fish number)/Initial fish number × 100
- ANPR (%) = (final weight × final protein content) - (initial weight × initial protein content) × 100 / protein intake

- ANER (%) = (final weight × final energy content) - (initial weight × initial energy content) × 100 / energy intake
- TGC = (final weight^1/3 - initial weight^1/3) / (temperature × day) × 100
- HI = liver weight/fish weight × 100
- IFI= intraperitoneal fat weight/fish weight × 100
- IFI= intraperitoneal fat weight/fish weight × 100

## Digestibility

In order to assess the digestibility of the diets, 1% Chromic Oxide was added to a sub-sample of the LF diets (Table 1, LF, LF-MFPFC, LF-FC, HF, HF-MFPFC, HF-FC). Digestibility coefficients of test diets were determined using groups of 8 fish (~40 g weight). Fish were allowed to acclimate to the various test diets for four days before starting the collection of feces. Before each feeding, the tanks and Fecal Settling Chambers (FSC) were cleaned. Fish were offered two feedings, and all feces were collected using FSC. The feces were stored in sealed plastic containers and stored in a freezer until processed. Samples were collected for four days until a suitable quantity was obtained for analyses (~1 g dry weight). Daily samples were pooled by tank and three replicate aquaria (n=3) were utilized for each treatment. Dry matter, crude protein, and total energy were determined for the fecal and diet samples according to established procedures. Crude protein content was analyzed using the micro-Kjeldahl method [47]. Total energy content using a micro-calorimetric adiabatic bomb calorimeter using benzoic acid as standard (Model 1425, Parr Instrument Co. Moline, IL, USA). Chromic oxide content testing followed the McGinnis and Kasting [48] procedures. Apparent digestibility coefficients of the dry matter (ADDM), protein (ADCP), and energy (ADE) for each diet were calculated according to Cho, et al. [49] using the following formulas:

- ADDM (%)= 100 - [100 x (%Cr2O3 in feed/(%Cr2O3) in feces)]
- ADCP or ADE (%)= 100 - [100 x (%Cr2O3 in feed/(%Cr2O3) in feces x %nutrient feces/(%nutrient) feed)]

### *Statistical analyses*

Statistical analyses were conducted using SAS system for Windows, (V9.4. SAS Institute, Cary, NC, USA). Initial weight, final mean weight, TGC, percent weight gain, FCR, ANPR, ANER, ADDM, ADCP, and ADE were analyzed using a one-way ANOVA to determine significant ($p < 0.05$) differences among the treatment means followed by Student-Newman-Keuls multiple range test to distinguish significant differences between treatment means. Using the paired subset of diets, two-way ANOVA was used to determine interactions between fiber level and enzyme supplementation and protected and free interactions. Survival was analyzed by logistic (binary) regression.

## Results

### *Water quality*

During the experimental period dissolved oxygen, temperature, salinity, pH, total ammonia nitrogen, and nitrite were maintained within acceptable ranges for Nile tilapia at 6.0 ± 0.89 mg/L, 27.70 ± 0.71°C, 1.06 ± 0.64 ppt, 7.0 ± 0.79, 0.06 ± 0.03 mg/L, 0.04 ± 0.02 mg/L over the 70-d trial period.

### *Growth performance*

Parameters of the growth performance of fish offered the LF diets are summarized in (Table 3a). No significant ($p > 0.05$) differences were detected for initial mean weight, final mean weight, percent weight gain, TGC, survival, FCR as FP, PP, FC, PC, and MFPFC were added to the diets. Initial mean weight was not significantly different (p=0.160) for the dietary treatments (9.30 ± 0.10g). The final mean weight was unchanged (p=0.317) with the addition of different enzymes (98.31-104.43 g). Percent weight gain was unchanged (p=0.499) by dietary treatments (954.8-1032.9%). TGC was not significantly different (p=0.398) among treatments (0.129-0.135). Percent survival was unchanged (p=0.701) by dietary treatments (98.75-100.00%). FCR was not affected (p=0.064) by the addition of enzymes (1.15-1.26).

Parameters of growth performance for fish that were fed HF diets are summarized in (Table 3b). Initial mean weight, final mean weight, percent weight gain, TGC, survival, FCR of the fish fed the various diets were not significantly ($p>0.05$) influenced by the addition of FP, FC, or the MFPFC. The initial mean weight (9.26 ± 0.12 g) was not significantly different (p= 0.840) among fish assigned to the various dietary treatments. The final mean weight (95.42-98.83g) was unchanged (p=0.799) by the addition of different enzymes. Percent weight gain was unchanged (p=0.734) by dietary treatments (927.8-966.8%). TGC was not significantly different (p=0.762) among treatments (0.109-0.113). Survival was 100% across all dietary treatments. FCR was not affected (p = 0.498) by the addition of enzymes (1.13-1.18).

### Nutrient retention and body composition

In LF diets, ANER was significantly different (p=0.0001) as FP and PP were added to the diets (32.37, 38.71, 37.79, 33.79, 31.39, 31.07% for LF, LF-FP, LF-PP, LF-FC, LF-PC, LF-MFPFC; respectively). ANPR, (37.58-44.60%) was unchanged (p=0.399) by the inclusion of proteases and carbohydrases (Table 3a). With regards to HF diets, ANPR, (40.12-42.24%, p=0.803) and ANER (29.24-32.567%, p=0.429) were unchanged by the inclusion of protease and carbohydrases (Table 3b). The HI and IFI were unchanged by the addition of enzymes for LF and HF diets (Tables 3a & 3b).

Whole-body fish composition is summarized in (Tables 4a&4b). No differences were observed in crude protein (p=0.786; 14.30-15.43%), dry matter (p=0.547; 24.78-26.98%), fat (p=0.627; 6.38-7.36%) and ash (p=0.323; 3.02-4.98%) in whole-body fish samples among fish fed free, protected or a mix of protease and carbohydrase in LF diets (Table 4a). In diets containing HF, no differences were observed in crude protein (p=0.876; 14.68-15.23), dry matter (p=0.368; 24.85-27.3%), fat (p=0.059; 5.90-7.61%) and ash (p=0.165; 3.50-5.08%) content of whole-body fish samples among fish fed free or a mix of protease and carbohydrase (Table 4b).

## Digestibility

Digestibility values for LF diets are summarized in (Table 5a). The ADDM was significantly different (p=0.0001) in diets containing free carbohydrase and a mix of free protease and free carbohydrase (52.39, 53.34, 59.38% for LF, LF-FC, LF-MFPFC, respectively). ADE was significantly improved (p=0.0003) by the addition of enzymes to the diet (57.17, 63.14, 65.49%, for LF, LF-FC, LF-MFPFC, respectively). ADCP was significantly different (p=0.0002; 77.11, 83.74, 82.30 for LF, LF-FC, LF-MFPFC, respectively).

| Diet | Final mean Weight (g) | Weight Gain (%) | TGC | Survival (%) | FCR | ANPR (%) | ANER (%) | HI | IFI |
|---|---|---|---|---|---|---|---|---|---|
| LF | 104.4 | 1014.9 | 0.134 | 100 | 1.08 | 37.58 | 32.37[b] | 1.73 | 2.03 |
| LF-FP | 105.6 | 1032.9 | 0.135 | 100 | 1.06 | 41.64 | 38.71[a] | 1.79 | 2.06 |
| LF-PP | 102.2 | 999.3 | 0.133 | 98.75 | 1.07 | 43.65 | 37.79[a] | 1.38 | 1.85 |
| LF-FC | 98.3 | 970.9 | 0.129 | 98.75 | 1.08 | 43.7 | 33.79[b] | 1.62 | 1.67 |
| LF-PC | 98.7 | 954.9 | 0.13 | 100 | 1.09 | 44.6 | 31.39[b] | 1.68 | 1.75 |
| LF-MFPFC | 102.2 | 997.4 | 0.132 | 98.75 | 1.07 | 42.58 | 31.07[b] | 1.81 | 1.6 |
| PSE | 2.6 | 29.9 | 0.002 | 0.88 | 0.03 | 2.41 | 0.87 | 0.23 | 0.41 |
| *p*-value | 0.317 | 0.499 | 0.398 | 0.701* | 0.064 | 0.399 | 0.001 | 0.276 | 0.736 |

**Table 3a :** Growth response of juvenile tilapia (9.30 ± 0.10 g) fed for 70 d on six low-fiber diets, low-fiber basal diet (LF) and LF supplemented with free protease (LF-FP), protected protease (LF-PP), free carbohydrase (LF-FC), protected carbohydrase (LF-PC), or a mix of free protease and carbohydrase (LF-MFPFC)+

PSE=Pooled Standard Error, n=4. *Analyzed by binary regression. TGC=thermal-unit growth coefficient, FCR=feed conversion ANPR= Apparent net protein retention, ANER=Apparent net energy retention, HI=Hepatosomatic index, IFI=Intraperitoneal fat index. Significance ($p<0.05$) based on ANOVA followed by Student-Newman-Keuls grouping. Superscripts represent significant differences. +Jefo Nutrition Inc, Saint-Hyacinthe, QC, Canada.

| Diet | Final mean Weight (g) | Weight Gain (%) | TGC | Survival (%) | FCR | ANPR (%) | ANER (%) | HI | IFI |
|---|---|---|---|---|---|---|---|---|---|
| HF | 95.42 | 927.8 | 0.113 | 100 | 1.06 | 41.32 | 30.71 | 1.7 | 1.43 |
| HF-FP | 96.31 | 935.6 | 0.113 | 100 | 1.07 | 42.25 | 29.24 | 1.63 | 1.03 |
| HF-FC | 95.46 | 934.6 | 0.109 | 100 | 1.12 | 40.12 | 30.94 | 1.31 | 1.22 |
| HF-MFPFC | 98.84 | 966.8 | 0.111 | 100 | 1.07 | 40.95 | 32.56 | 1.76 | 1.33 |
| PSE | 2.82 | 26.4 | 0.002 | | 0.03 | 1.53 | 1.36 | 0.25 | 0.41 |
| p-value | 0.799 | 0.734 | 0.762 | | 0.498 | 0.803 | 0.429 | 0.0785 | 0.552 |

**Table 3b:** Growth responses of juvenile tilapia (9.26 ± 0.12 g) fed for 70 d on four high-fiber diets, a high-fiber basal diet (HF) and HF supplemented with free protease (HF-FP), free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC)+.

PSE= Pooled Standard Error, n=4. *Analyzed by binary regression. TGC=thermal-unit growth coefficient, FCR=feed conversion ANPR= Apparent net protein retention, ANER= Apparent net energy retention, HI=Hepatosomatic index, IFI= Intraperitoneal fat index. Significance ($p<0.05$) based on analysis of variance followed by Student-Newman-Keuls grouping. +Jefo Nutrition Inc, Saint-Hyacinthe, QC, Canada.

| | LF | LF-FP | LF-PP | LF-FC | LF-PC | LF-FPFC | PSE | p-value |
|---|---|---|---|---|---|---|---|---|
| Dry matter | 25.23 | 26.4 | 24.93 | 26.98 | 25.53 | 24.78 | 0.87 | 0.547 |
| Protein | 14.3 | 14.8 | 14.88 | 14.98 | 15.43 | 14.6 | 0.54 | 0.786 |
| Fat | 6.52 | 7.36 | 6.88 | 6.77 | 7.05 | 6.38 | 0.42 | 0.627 |
| Energy | 1333 | 1331 | 1357 | 1371 | 1358 | 1330 | 44.94 | 0.977 |
| Ash | 3.61 | 3.77 | 3.02 | 4.98 | 3.25 | 3.18 | 0.63 | 0.323 |
| Calcium % | 1.02 | 1.08 | 1.05 | 1.24 | 1.34 | 1.17 | 0.21 | 0.88 |
| Copper ppm | 1.63 | 1.68 | 1.65 | 1.48 | 1.45 | 1.68 | 0.1 | 0.829 |
| Iron ppm | 15.95 | 14.83 | 22.15 | 13.85 | 15.58 | 15.78 | 2.32 | 0.209 |
| Magnesium % | 0.033 | 0.033 | 0.035 | 0.038 | 0.035 | 0.035 | 0.003 | 0.783 |
| Phosphorus % | 0.608 | 0.653 | 0.643 | 0.72 | 0.77 | 0.688 | 0.1 | 0.881 |
| Potassium % | 0.258 | 0.253 | 0.258 | 0.253 | 0.253 | 0.258 | 0.007 | 0.972 |
| Sodium % | 0.113 | 0.103 | 0.108 | 0.11 | 0.113 | 0.113 | 0.004 | 0.539 |
| Sulfur % | 0.158 | 0.16 | 0.163 | 0.155 | 0.158 | 0.158 | 0.004 | 0.786 |
| Zinc ppm | 19.95 | 19.25 | 20.83 | 19.5 | 20.3 | 20.18 | 1.4 | 0.972 |

**Table 4a:** Proximate composition (%, as is) of whole-body tilapia fed, low-fiber basal diet (LF) and LF supplemented with free protease (LF-FP), protected protease (LF-PP), free carbohydrase (LF-FC), protected carbohydrase (LF-PC), or a mix of free protease and carbohydrase (LF-MFPFC) analyzed by Mid-west Laboratories (Omaha, NE, USA).

| | HF | HF-FP | HF-FC | HF-FPFC | PSE | *p*-value |
|---|---|---|---|---|---|---|
| Dry matter | 24.85 | 26.75 | 27.30 | 26.35 | 0.98 | 0.368 |
| Protein | 14.68 | 14.90 | 15.23 | 15.00 | 0.48 | 0.876 |
| Fat | 6.15 | 5.90 | 7.61 | 7.51 | 0.24 | 0.059 |
| Energy | 1303 | 1177 | 1266 | 1317 | 44.62 | 0.171 |
| Ash | 3.50 | 5.08 | 4.09 | 3.87 | 0.47 | 0.165 |
| Calcium % | 1.00 | 0.85 | 1.11 | 0.97 | 0.14 | 0.611 |
| Copper ppm | 1.23 | 1.37 | 1.70 | 1.43 | 0.12 | 0.161 |
| Iron ppm | 13.65 | 18.35 | 18.80 | 16.05 | 2.34 | 0.416 |
| Magnesium % | 0.030 | 0.030 | 0.035 | 0.033 | 0.003 | 0.552 |
| Phosphorus % | 0.605 | 0.520 | 0.668 | 0.570 | 0.068 | 0.498 |
| Potassium % | 0.243 | 0.253 | 0.248 | 0.253 | 0.011 | 0.894 |
| Sodium % | 0.103 | 0.103 | 0.103 | 0.105 | 0.006 | 0.985 |
| Sulfur % | 0.153 | 0.158 | 0.165 | 0.158 | 0.003 | 0.116 |
| Zinc ppm | 19.53 | 18.43 | 20.70 | 18.25 | 1.15 | 0.434 |

**Table 4b:** Proximate composition (%, as is) of whole-body tilapia fed, high-fiber basal diet (HF) and HF supplemented with free protease (HF-FP), free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC) analyzed by Midwest Laboratories (Omaha, NE, USA).

| Diet | ADDM | ADE | ADCP |
|---|---|---|---|
| LF | 52.39±0.54$^{c}$ | 57.17±3.71$^{b}$ | 77.11±6.07$^{b}$ |
| LF-FC | 53.34±0.32$^{b}$ | 63.14±1.46$^{a}$ | 83.74±1.00$^{a}$ |
| LF-MFPFC | 59.38±0.19$^{a}$ | 65.49±0.58$^{a}$ | 82.30±2.91$^{a}$ |
| PSE | 0.22 | 1.34 | 0.84 |
| P-value | 0.0001 | 0.012 | 0.0002 |

**Table 5a:** Digestibility values of dry matter (ADMM), energy (ADE), and protein (ADCP) in a low-fiber basal diet (LF) and LF supplemented free carbohydrase (LF-FC), or a mix of free protease and carbohydrase (LF-MFPFC)+.

| | ADDM | ADE | ADCP |
|---|---|---|---|
| HF | 52.22 ± 0.91$^{c}$ | 58.92 ± 1.63$^{b}$ | 85.45 ± 0.20$^{b}$ |
| HF-FC | 56.81 ± 0.91$^{a}$ | 62.56 ± 0.77$^{a}$ | 87.02 ± 0.92$^{a}$ |
| HF-MFPFC | 54.47 ± 0.38$^{b}$ | 60.84 ± 1.43$^{ab}$ | 89.07 ± 4.14$^{a}$ |
| PSE | 0.45 | 0.77 | 0.84 |
| P-value | 0.001 | 0.042 | 0.0003 |

**Table 5b:** Digestibility values of dry matter (ADMM), energy (ADE), and protein (ADCP) in a high-fiber basal diet (HF) and HF supplemented free carbohydrase (HF-FC), or a mix of free protease and carbohydrase (HF-MFPFC)+.

Significance ($p<0.05$) determined by one way ANOVA followed by Student-Newman-Keuls grouping. PSE= Pooled Standard Error. Superscripts represent significant differences.

+Jefo Nutrition Inc., Saint-Hyacinthe, QC, Canada.

Digestibility values for HF diets are summarized in (Table 5b). The ADDM was significantly different (p=0.0011) in diets containing free carbohydrase and a mix of free protease and free carbohydrase (52.22, 56.81, 54.47 8% for HF, HF-FC, HF-MFPFC, respectively). ADE was significantly different (p=0.042) by the addition of enzymes to the diet (58.92, 62.56, 60.84%, for HF, HF-FC, HF-MFPFC, respectively). ADCP was significantly different (p=0.004; 85.45, 87.02, 89.07 for HF, HF-FC, HF-MFPFC, respectively).

In LF diets, higher digestibility values were observed when the MFPFC was added to the diets. In contrast, HF diets resulted in higher digestibility values when FC was added to the diet.

## Discussion

The use of exogenous enzymes can be a tool to incorporate different feed ingredients without affecting fish performance by increasing the quality of aquaculture diets [50]. In the present study, diets without enzyme addition presented lower dry matter, energy, and protein digestibility coefficients compared to diets containing carbohydrases and the mix of protease and carbohydrases, indicating that enzymes can improve digestibility in tilapia diets, thus increasing the protein and carbohydrate uptake by the fish. These results agree with the findings reported by Li, Chai, Liu, Chowdhury, and Leng [39] where increased digestibility of dry matter and crude protein was observed by the supplementation of protease in diets for hybrid tilapia. These improvements in digestibility can be due to the increase of free amino acids in the diets by the enzyme becoming active with the help of moisture and temperature during processing [39]. In rainbow trout, Farhangi and Carter [34] indicated that apparent digestibility coefficients of dry matter, crude protein, and energy were improved by a multi enzyme protease and carbohydrase due to increases in nutrient digestibility by the stimulation of the release of bile acids, improving emulsification of non-starch polysaccharides [51]. Dalsgaard, Verlhac, Hjermitslev, Ekmann, Fischer, Klausen and Pedersen [33] reported that supplementing protease to soy-containing diets for rainbow trout significantly increased the apparent digestibility of protein, lipid, phosphorus, and dry matter, the authors explained an improved nutrient uptake in fish fed soybean meal containing diets by targeting proteinaceous anti-nutrients or hydrolyzing antigenic proteins. Carter, Houlihan, Buchanan, and Mitchell [38] reported no effects of dietary supplementation with combinations of enzymes on the apparent digestibility of nitrogen in Atlantic salmon, however, the specific growth rate and feed efficiency were significantly improved. In contrast, Lin, Mai, and Tan [24] reported that the addition of a commercial enzyme complex of neutral protease, beta-glucanase, and xylanase had no detectable effect on the apparent digestibility of

protein, lipid, and gross energy in hybrid tilapia. The variability of these results may be due to the differences in the enzymes and diet formulations used in these studies. Ogunkoya, et al. [52] found no effect on growth and feed efficiency with the addition of graded levels of enzyme cocktail to rainbow trout offered a soybean meal-based diet. The effect of the enzyme inclusion is not always predictable due to the non-specific action of the enzymes on the target substrate [34].

Fish final mean weight, FCR, percent weight gain, TGC, and percent survival were not affected by the inclusion of protease and carbohydrase in the diets. This is most likely the result of high nutrient levels which satisfied the nutrition requirement of the tilapia. Hence, supplementation with enzymes did not show positive effects on fish growth. Similar results were observed in rainbow trout fed lupin plus enzyme supplements [34]. Ng and Chong [43] reported that growth performance was not affected in red hybrid tilapia by diets containing 10 to 40% palm kernel meal and beta mannanase at inclusions of 0.01%, 0.05%, or 0.1%. In contrast, hybrid tilapia supplemented with proteases in low fish meal diets showed improvements in gain and decreased FCR with enzyme addition to the diet [39]. Also, Li, et al. [53] reported improved weight gain and digestibility for hybrid tilapia when diets were supplemented with protease or phytase, individually or in combination. Similarly, growth performance was improved when protease was supplemented and allowed for the reduction of fish meal in diets for Nile tilapia [54]. Also, similar results are reported by Zamini, Kanani, Azam Esmaeili, Ramezani, and Zoriezahra [44], the addition of 0.5 g/kg and 2.5 g/kg of two multienzyme complexes and the combination containing protease, lipase, phytase, alpha amylase, cellulase, amiloglucosidase, beta glucanase, pentosonase, hemicellulase, xylanase, pectinase, acid phosphatase, acid phytase and endo- beta mannanase, amylase, xylanase, cellulose, and alpha galactosidase improved growth and feed utilization in Caspian salmon.

In our study, net energy retention was significantly improved in low fiber diets with the addition of proteases. Likewise, increased energy retention in Gibel carp fed protease supplemented diets was observed by Shi, et al. [55], they also reported improved growth, digestibility, and protein retention. However, Huan, et al. [56] did not see any significant difference in whole body composition or lipid retention in hybrid tilapia fed protease supplemented diets. Dalsgaard, Verlhac, Hjermitslev, Ekmann, Fischer, Klausen and Pedersen [33] observed no improvement in net energy retention when proteases were added to soybean meal containing diets for rainbow trout. Research on fish suggests that the type of enzymes and their concentration (relative to body weight) affects fish response to enzyme supplementation [24].

## Conclusion

Results demonstrate that the inclusion of protease and carbohydrase improved digestibility in LF and HF tilapia diets. The enzyme supplementation did not alter production performance, body tissue composition, or nutrient retentions, except for ANER which was significantly improved in LF with FP and PP. The use of these enzymes in diet formulations for Nile tilapia is an opportunity to increase digestibility while decreasing cost formulation without affecting performance.

## Data Availability

The data for this research trial is available from the corresponding author upon request.

## Ethical Approval

The animal studies were reviewed and approved by Auburn University IACUC protocol #2015-2664.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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