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Commercial enzyme complex (Allzyme®) improves feed digestibility for pre-growout Nile tilapia

Complexo enzimático comercial (Allzyme®) melhora a digestibilidade da ração para tilápias do Nilo em crescimento

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ABSTRACT

Recent studies have evidenced exogenous enzymes as nutritional tool to elaborate low-polluting aquafeeds. This study aimed to evaluate effects of enzyme complex (EC) on apparent digestibility coefficients (ADC) of energy and nutrients, including amino acids, as well nitrogen (N) and phosphorus (P) loss in pre-growout Nile tilapia (*Oreochromis niloticus*). Diets without EC (Control) or with EC supplemented at 225 (EC225) or 450 mg/kg diet (EC450) and fed to Nile tilapia (*n* = 135, 150 ± 20 g) distributed in an entirely randomized design of three treatments and three replicates of 15 fish each. Fish fed diet EC450 showed higher digestibility of energy, protein, amino acids and mineral, and increased digestible energy (DE; +221.25 kcal/kg diet), digestible protein (DP; +10.54 g/kg diet) contents of diets, whereas reduced N (−23.82%) and P (−18.46%) loss relative to fish fed diet control. This study evidenced that EC at 450 mg/kg diet optimizes the ADC of multiple nutrients, including amino acids, and identified its potential to enhance the nutritive value of feeds and elaborate sustainable feeds for Nile tilapia.

Index terms: Feed additive; exogenous enzyme; nitrogen and phosphorus balance; nutritive value; *Oreochromis niloticus*.

RESUMO

Estudos recentes têm evidenciado enzimas exógenas como ferramenta nutricional para elaboração de dietas aquáticas pouco poluentes. Este estudo teve como objetivo avaliar os efeitos de um complexo enzimático (CE) nos coeficientes de digestibilidade aparente (CDA) de energia e nutrientes, incluindo aminoácidos, bem como perda de nitrogênio (N) e fósforo (P) em tilápia-do-nilo, *Oreochromis niloticus* em crescimento. Dietas sem CE (Controle) ou com EC suplementado em 225 (CE225) ou 450 mg/kg dieta (CE450) foram fornecidas para tilápias do Nilo (n = 135, 150 ± 20 g) distribuídas em um desenho inteiramente aleatório de três tratamentos e três repetições de 15 peixes cada. Peixes alimentados com a dieta EC450 apresentaram maior digestibilidade da energia, proteína, aminoácidos e minerais, e aumento nos conteúdos de energia digestível (ED; +221,25 kcal/kg dieta) e proteína digestível (PD; +10,54 g/kg dieta) das dietas, enquanto houve redução na perda de N (−23,82%) e P (−18,46%) em relação aos peixes alimentados com a dieta de controle. Este estudo evidenciou que o CE a 450 mg/kg de dieta otimiza o CDA de múltiplos nutrientes, incluindo aminoácidos e identificou seu potencial para melhorar o valor nutritivo dos alimentos, assim como elaborar dietas sustentáveis para tilápias do Nilo.

Termos para indexação: Aditivo alimentar; enzima exógena; balanço de nitrogênio e fósforo; valor nutritivo; *Oreochromis niloticus*.

INTRODUCTION

Cereal and legume-based feedstuffs, including their co-products, are cost-effective and sustainable non-competitive feed alternatives to traditional feed sources for tilapia feed (Tacon; Metian; Mcnevin, 2022). However, a significant challenge in utilizing vegetable ingredients is the presence of various anti-nutritional factors, which can negatively impact nutrient utilization (Francis; Makkar; Becker, 2001). Plant-derived feedstuffs contain phytate and non-starch polysaccharides as primary antinutrients that impair nutrient utilization in Nile tilapia, *Oreochromis niloticus* (Jiang et al., 2022; Liu; Li; Wu, 2022; Tachibana et al., 2010). These studies demonstrate that exogenous enzymes may increase nutrient utilization by alleviating the adverse effects of several anti-nutritional factors in plant feedstuffs.

Vegetable ingredients are a practical approach in the aquafeeds' fishmeal replacement inevitability (Jannathulla et al., 2019). Nevertheless, lower-cost vegetable ingredients contain several anti-nutritional factors impairing nutrient utilization (Montoya-Camacho et al., 2019). A practical approach to the problem involves supplementing exogenous enzyme complex (EC) can alleviate the adverse effects of anti-nutritional factors in Nile tilapia fed plant-rich diets.

Previous studies have shown the potential benefits of EC on nutrient digestibility and growth performance in Nile tilapia (Martins et al., 2018; Moura et al., 2012). The synergistic effects of multiple enzymes on the apparent digestibility coefficients (ADC) of energy and nutrients in this fish species have been well-documented (Novelli et al., 2017; Nakamura et al., 2022; Adeoye et al., 2016; Maas et al., 2018). Consistently, multienzyme supplementation may optimize enzyme effects by targeting multiple substrates (Castillo; Gatlin, 2015). However, the impact of EC on the ADC of amino acids, nitrogen (N), and P loss in fish is not well understood in Nile tilapia.

Tilapia aquaculture plays a vital role in social and economic development worldwide, as previously described by the Food and Agriculture Organization (Food and Agriculture Organization - FAO, 2021). However, there is growing concern among public opinion and regulatory authorities for more sustainable aquaculture practices (Risius; Hamm; Janssen, 2019). To address this, safe, natural, and eco-friendly feed additives, such as exogenous enzymes, are being considered as a potential alternative to reduce the environmental impact of fish culture (Gule; Geremew, 2022). Thus, exogenous enzymes may be and nutritional tool to increase the sustainability of aquaculture by decreasing N and P loads in the water body. Reducing N and P loads may also assist tilapia farming certification and accreditation schemes. However, whether the EC regulates N and P balance are largely unknown in Nile tilapia. Therefore, this study aimed to evaluate the effects of Allzyme® on the ADC of energy and nutrients, N and P outputs in pre-growout Nile tilapia.

MATERIAL AND METHODS

The feeding trial was approved by the Animal Care and Use Committee of Universidade Estadual de Ponta Grossa (Protocol n° 5577/2018).

Experimental diets

Tables 1 and 2 present the formulation and analyzed composition of the experimental diets, respectively. A basal diet (Control) was formulated based on common vegetable feed ingredients currently used in tilapia feeds (Maas et al., 2020) to meet the dietary requirements of Nile tilapia previously established by the National Research Council (National Research Council - NRC 2011).

Two other diets were formulated from the control diet by supplementing exogenous EC at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450), added at the expense of corn. The select dose of the enzyme additive was established based on a previous published value in Nile tilapia to maximize the body weight gain of fish (Moura et al., 2012). A commercially available thermo-resistant EC (Allzyme® SSF®, Alltech Inc., Nicholasville, Kentucky, USA) containing pectinase 4.000.000 IU/kg, protease 700.000 IU/kg, phytase 300.000 IU/kg, β-glucanase 200.000 IU/kg, xylanase 100.000 IU/kg, cellulase 40.000 IU/kg and amylase 30.000 IU/kg of product, as declared by the manufacturer, was used. The EC was derived from a select strain of non-GMO *Aspergillus niger*, developed through solid-state fermentation.

The ingredients were ground to a particle size of 800-µm using a mesh before mixing in an automatic mixer (MA200; Marconi, Piracicaba, SP, Brazil). All diets were extruded in a single-screen experimental feed mill (Exteec, Ribeirão Preto, SP, Brazil). The die temperature was maintained at 86 ± 2 °C, and the extrudates were oven-dried at 55 °C for 24 h. After drying, the extrudates, with a moisture content of approximately 7%, were stored in polyethylene bags until laboratory analysis.

Digestibility assay

A total of 135 Nile tilapia (initial body weight of 150 ± 20 g; mean ± SD) were randomly distributed into 9-150 L conical fiberglass aquaria with 15 fish per aquarium. The feces pool from each aquarium was collected for 21 days and treated as a replicate. A constant photoperiod of 12 hours light and 12 hours dark through artificial light was maintained throughout the digestibility assay. Water quality parameters were monitored daily, and the data of mean values ± standard error of the mean (SEM) were as follows: water temperature was 27.3 ± 0.4 °C, total ammonia was 0.11 ± 0.001 mg/L, and pH was 7.13 ± 0.01. During the experimental trial, the dissolved oxygen level of the water was maintained at 6.2 ± 0.2 mg/L using a central blower. A two-week acclimation period was implemented before the digestibility trial (21 days) to ensure the fish were acclimated to the laboratory handling and feeding conditions. The fish were hand-fed a commercial extruded diet containing 320 g/kg of crude protein to apparent satiety six times per day.

Table 1: Formulation of the experimental diets (g/kg, fed-basis).

Ingredient	Diet[a]		
	Control	EC225	EC450
Corn[b]	276.10	275.875	275.650
Wheat bran[b]	124.00	124.00	124.00
Soybean meal[b]	150.00	150.00	150.00
Meat and bone meal[b]	140.00	140.00	140.00
Fish meal[b]	90.00	90.00	90.00
Poultry by-products meal[b]	100.00	100.00	100.00
Feather meal[b]	20.00	20.00	20.00
Low tannin sorghum[b]	40.00	40.00	40.00
Soybean oil[c]	34.30	34.30	34.30
Blood meal[b]	13.90	13.90	13.90
Salt[d]	4.00	4.00	4.00
DL-methionine (99%)[e]	1.80	1.80	1.80
Mineral and vitamin mix[f]	4.00	4.00	4.00
Antioxidant[g]	0.10	0.10	0.10
Antifungal[h]	0.80	0.80	0.80
Chromium oxide (Cr_2O_3)[e]	1.00	1.00	1.00
Multienzime preparation[i]	n.s.[j]	0.225	0.450

[a] Diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450). [b] Alisul Alimentos S.A, Maringá Grossa, PR, Brazil. [c] Cocamar, Maringá, PR, Brazil. [d] Jasmine, Curibita, PR, SP, Brazil. [e] Sygma-Alldrich Brasil Ltda, 99.5%, São Paulo, SP, Brazil. [f] Mineral and vitamin mix supplied per kg of diet: Vitamin A (retinyl acetate), 6,000 IU; vitamin D_3, (cholecalciferol), 1,000 IU; vitamin E (DL-α-tocopheryl acetate), 60 mg; vitamin K_3 (menadione Na-bisulphate), 12 mg; vitamin B_1 (thiamine HCl), 24 mg; vitamin B_2 (riboflavin), 24 mg; vitamin B_6 (pyridoxine HCl), 20 mg; vitamin B_{12} (cyanocobalamin), 0.05 mg; folic acid, 6 mg; D-calcium pantothenate, 60 mg; ascorbic acid (ascorbyl polyphosphate), 240 mg; d-biotin, 0.24 mg; choline chloride, 325 mg; niacin, 120 mg; ferrous sulfate ($FeSO_4.H_2O.7H_2O$), 50 mg; copper sulfate ($CuSO_4.7H_2O$), 3 mg; manganese sulfate ($MnSO_4.H_2O$), 20 mg; zinc sulfate ($ZnSO_4.7H_2O$), 30 mg; potassium iodide (KI), 0.4 mg; cobalt sulfate ($CoSO_4.4H_2O$), 0.25 mg; sodium selenite (Na_2SeO_3), 0.1 mg. [g] Butylated hydroxytoluene (BHT), Sygma-Alldrich Brasil Ltda, 99.5%, São Paulo, SP, Brazil. [h] *Calcium propionate,* Sygma-Alldrich Brasil Ltda, 99.5%, São Paulo, SP, Brazil. [i] Allzyme® SSF®, Alltech, Kentucky, USA. [j] Non-supplemented.

Fecal collection in each digestibility aquarium followed the modified Guelph protocol previously established (Guimarães et al., 2012). The fish were hand-fed until apparent satiety with four daily meals and then transferred and kept overnight in cylindrical-conical feces collection aquaria. The feeding and feces-collecting aquaria were individually supplied with continuous aeration (6.2 ± 0.2 mg/L), and the water temperature was maintained at 27.5 ± 0.5 °C by electrical heaters coupled with a thermostat. At the beginning and end of the experimental trial, fish were bulked-weighed to determine the body weight gain and feed conversion ratio responses.

Chemical analysis

The composition analysis of diets and feces included dry matter, crude protein, crude fiber, crude lipid, and ash content and was performed according to methods described by the Association of Official Analytical Chemists (Association of Official Analytical Chemists - AOAC, 1995). Dry matter was determined by oven-drying at 105 °C until constant weight, and crude protein (N × 6.25) analysis was performed by the Kjeldahl method after acid digestion (Tecnal, MA-036, Piracicaba, SP, Brazil). Crude fiber analysis was determined by the loss on ignition method of dried lipid-free residues following digestion

with 1.25% H_2SO_4 and 1.25% NaOH. Crude lipid was determined by ether-extraction method in Soxhlet extractor System (Tecnal, TE-044, Piracicaba, SP, Brazil).

Table 2: Analyzed composition of the experimental diets (g/kg, dry matter basis).

Item	Diet*		
	Control	EC225	EC450
Dry matter	929.8	928.4	928.2
Gross energy, kcal/kg	19.96	19.98	20.03
Crude protein	333.1	333.1	333.3
Starch	260.9	263.9	267.8
Lipids	81.7	81.5	82.3
Crude fiber	28.5	28.4	28.6
Calcium	25.4	25.1	25.1
Phosphorus	15.1	15.3	15.1
Essential amino acid			
Arginine	21.1	21.5	19.8
Histidine	8.6	8.8	8.4
Isoleucine	12.7	13.1	12.9
Leucine	25.9	26.5	26
Lysine	19.3	19.4	18.7
Methionine	6.0	5.9	6.0
Phenylanalnine	15.7	15.3	15.6
Threonine	12.9	12.9	13.2
Tryptophan	3.9	3.9	4.0
Valine	16.1	16.2	15.4
Non-essential amino acid			
Alanine	18.9	19.5	18.8
Aspartic acid	18.4	18.9	18.3
Cysteine	3.6	3.4	3.2
Glycine	19.6	19.6	18.5
Glutamic acid	52.5	52.3	50.4
Serine	12.3	12.5	12.5
Tyrosine	8.8	8.9	8.8

*Diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450).

Mineral content was obtained by combusting in a muffle furnace at 600 °C for 5 hours (Tecnal, 2000B, Belo Horizonte, MG, Brazil). Gross energy was determined using an adiabatic bomb calorimeter (Parr, Moline, IL, USA). Starch content in diets and feces was determined using hydrolysis (Varns; Sowokinos, 1974) and glucose assay (Miller, 1959). After acid digestion, the dietary calcium content was analyzed using an atomic absorption phase spectrophotometer (Spectra-55, Varian). The vanadium–molybdate method was used to determine P following the standard method of the Association of Official Analytical Chemists (AOAC, 1995).

Chromic oxide concentrations were determined by flame atomic absorption spectrophotometer, following the combustion of the sample in a muffle furnace before and after digestion in nitric acid, as previously established by the Association of Official Analytical Chemists (AOAC, 1995). Amino acids analyses were performed by high-performance liquid chromatography - HPLC (Hitachi, Tokyo, Japan), after perchloric digestion. Tryptophan analysis followed the hydrolysis of the sample with lithium hydroxide. All analyses were performed in duplicate.

Calculations

The ADC were calculated using chromic oxide as an inert marker (Furukawa; Tsukahara, 1966) and were calculated as follows: ADC (%) = 100 − [100 x (M_d/M_f)/(N_f/N_d)], where M_d is % of marker in the diet; M_f, is % of marker in feces; N_f is nutrient (%) or energy (MJ) in feces and N_d is nutrient or energy in the diet (Maynard; Loosli, 1969).

Digestible energy and digestible protein content of diets

The digestible energy (DE) and digestible protein (DP) contents of diets were calculated as the product of the gross energy and crude protein in the diets, respectively, considering their respective ADC, as follows: DE = GE of diet x (ADC_d/100), and DP = CP of diet (kcal/kg diet) x (ADC/100), where DE is digestible energy (kcal/kg diet); GE is gross energy of respective diet (MJ kg^{-1} diet); ADC is apparent digestibility coefficient of respective diet; DP is digestible protein (g/kg diet); CP_d is crude protein of respective diet (g/kg diet); ADC is apparent digestibility coefficient of respective diet.

Nitrogen and phosphorus balance

The N and P balance was expressed as grams of N or P per kilogram of body weight gain (BWG) using a

modified method previously established for Nile tilapia (Saravanan et al., 2012) Gross N or P intake (g/kg of BWG) was calculated by multiplying the feed conversion ratio by the quantity (g/kg) of N or P content in the diet. Digestible N and P intake were determined as the product of gross N intake and the ADC of (%) N or P. Fecal N and P loads (g/kg BWG) were calculated as the difference between N or P gain (g/kg BWG) and digestible N or P intake. Retained N and P were estimated as the difference between the N or P of the final and initial whole-body composition of fish. Branchial and urinary N and P loss (g/kg BWG) were obtained as the difference between digestible N or P intake and N or P retained.

Statistical analysis

Data normality and homogeneity of variance were checked using Shapiro-Wilk's and Levene's tests, respectively. Statistical differences between all treatments were determined using a one-way ANOVA followed by Tukey's post hoc test. Data are presented as the mean ± standard deviation of the mean within each treatment. Percentage data were arcsine transformed before statistical analysis. The P-value considered was 0.05. Data were analyzed using the statistical software Minitab version 19 (Minitab, Inc., State College, PA, USA).

RESULTS AND DISCUSSION

Table 3 shows the effects of EC on ADC of dry matter, gross energy and nutrients. The ADC of dry matter, gross energy, and P were significantly higher in fish fed the EC450 diet than those fed other diets ($P<0.05$), but similar in fish offered diets control and EC225. Compared to the control group, fish fed the EC450 diet revealed higher ADC of crude protein and starch ($P<0.05$), but did not differ in fish fed diets control and EC225. However, dietary treatments did not affect the ADC of lipids ($P=0.084$).

The current study identified EC supplementation at 450 ppm (EC450) as a prominent digestibility enhancer of multiple nutrients. Consequently, fish revealed increased body weight gain and enhanced feed efficiency ratio than those fed other diets. These results align with previous findings in Nile tilapia (Moura et al., 2012) and red sea bream (*Pagrus major*) (Matsukura et al., 2017) fed plant-based diets supplemented with the same EC used in this study. In our study, we aimed to expand upon the previous findings of Moura et al. (2012) regarding the digestibility of sucrose, glucose, and fructose. Additionally, we investigated the effects of EC on the digestibility of energy and nutrients, including amino acids. This study provides new information that can further contribute to our understanding of the effects of EC on nutrient digestibility. However, it is important to note that the composition of the diet, including the types and levels of anti-nutritional factors, may affect the effects of exogenous EC on nutrient utilization in fish. Previous studies have supported this hypothesis, showing that the type and levels of anti-nutritional factors can vary significantly among the plant feedstuffs commonly used in aquafeeds (Tacon; Metian; Mcnevin, 2022; Francis; Makkar; Becker, 2001; Sinha et al., 2011).

Table 3: Apparent digestibility coefficients (%) of dry matter, gross energy, and nutrients of the experimental diets fed to pre-growout Nile tilapia[1].

Parameter	Diet[2]			P-value
	Control	EC225	EC450	
Dry matter	64.60±0.58^{b}	66.10±1.15^{b}	69.73±0.55^{a}	0.004
Gross energy	68.94±0.48^{b}	71.13±1.18^{b}	81.64±1.47^{a}	<0.001
Crude protein	85.83±0.29^{b}	87.01±0.49^{ab}	88.99±1.16^{a}	0.020
Starch	90.76±0.27^{b}	92.07±0.44^{ab}	94.03±0.79^{a}	0.007
Lipids	89.43±0.80	89.65±0.54	91.35±0.73	0.084
Phosphorus	61.84±1.07^{b}	64.95±0.48^{b}	70.10±0.89^{a}	<0.001

[1] Values represent mean ± standard deviation of three replicate aquaria. [2] Diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450). Diets without (Control) or with enzyme complex supplemented at 225 (EC225) and 450 mg/kg diet (EC450).
[a,b,c] Means within the same row with no common superscript differ significantly ($P<0.05$; Tukey's test).

The current study identified the effects of EC inclusion on the ADC of gross energy, protein, and P, thereby improving DE, DP and available P contents of diets. These findings align with previous studies that have demonstrated that exogenous enzymes produced through solid-state fermentation technology can enhance the ADC of energy and nutrients in Nile tilapia feeds (Novelli et al., 2017). The improvement in the ADC of energy and nutrients observed in this study may be due to the mechanism by which multiple exogenous enzymes mitigate the anti-nutritional effects of cereal and legume ingredients in the diet.

The higher ADC of P observed in the current study is primarily attributed to the efficacy of phytase in releasing phytate-bound P and improving its availability, as reported in previous studies (Verlhac-Trichet et al., 2014). Additionally, it has been shown that phytase can improve the digestibility of other nutrients, such as protein (Liebert; Portz, 2007) and amino acids (Pontes et al., 2021; Nakamura et al., 2022), as demonstrated in this work.

The effects of EC on ADC of essential and non-essential amino acids are shown in Table 4. Fish fed the EC450 diet showed significantly higher ADC of histidine, lysine, mean of essential amino acids, alanine, and mean of non-essential amino acids than those fed other diets ($P<0.05$), but there was no significant difference in fish fed diets control and EC225. Fish offered the EC450 diet revealed higher ADC of isoleucine, leucine, phenylalanine, tryptophan, cysteine, and serine than those fed the control diet ($P<0.05$), but similar to fish fed the EC225 diet. However, dietary treatments did not affect the ADC of arginine, methionine, valine, aspartic acid, glutamine acid, glycine, and tyrosine ($P>0.05$).

Table 4: Apparent digestibility coefficients (%) of essential and non-essential amino acids of the experimental diets fed to pre-growout Nile tilapia[1].

Amino acid	Diet[2]			P-value
	Control	EC225	EC450	
Essential amino acid				
Arginine	94.62±0.34	94.54±0.48	96.01±0.58	0.052
Histidine	89.00±0.37^{b}	89.46±0.5^{b}	91.31±0.57^{a}	0.010
Isoleucine	89.57±0.47^{b}	90.24±0.52^{ab}	91.68±0.50^{a}	0.019
Leucine	88.03±0.56^{b}	89.67±0.29^{ab}	90.86±0.66^{a}	0.008
Lysine	91.01±0.09^{b}	91.15±0.58^{b}	92.50±0.26^{a}	0.019
Methionine	91.53±0.88	92.17±0.20	92.95±0.15	0.161
Phenylalanine	90.04±0.45^{b}	90.93±0.33^{ab}	91.74±0.20^{a}	0.014
Threonine	83.96±0.46^{b}	87.04±0.40^{a}	88.29±0.66^{a}	0.001
Tryptophan	88.27±0.25^{b}	89.77±0.38^{a}	90.66±0.41^{a}	0.003
Valine	85.56±0.46	87.53±0.22	87.92±0.83	0.436
MEAA	89.16±0.21^{b}	90.25±0.28^{b}	91.39±0.40^{a}	0.001
Non-essential amino acid				
Alanine	84.98±0.09^{b}	84.82±0.29^{b}	87.71±0.60^{a}	0.001
Aspartic acid	91.36±0.61	91.43±0.54	92.57±0.47	0.149
Cysteine	85.51±1.25^{b}	87.74±1.03^{ab}	89.72±0.50^{a}	0.023
Glutamic acid	94.19±0.21	91.13±4.50	94.99±0.16	0.396
Glycine	87.84±1.12	88.52±0.69	90.09±0.71	0.117
Serine	90.00±0.42^{b}	90.52±0.43^{ab}	91.64±0.36^{a}	0.032
Tyrosine	92.56±0.74	91.95±1.15	94.20±0.42	0.113
MNEAA	89.49±0.45^{b}	89.44±0.84^{b}	91.56±0.33^{a}	0.030

Abbreviations: MEAA, mean of essential amino acids; MNEAA, mean of non-essential amino acids.
[1] Values represent mean ± standard deviation of three replicate aquaria. [2] Diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450).
[a,b] Means within the same row with no common superscript differ significantly ($P<0.05$; Tukey's test).

The detrimental effect of phytic acid on ADC of amino acids is thought to be related to an increase in endogenous amino acid loss in the intestine rather than a direct impact on dietary protein digestibility (Cowieson; Acamovic; Bedford, 2004). It is well established that phytate increases mucin secretion into the gut, increasing endogenous amino acid flux (Selle et al., 2012). Another potential explanation is that the adverse effects of phytate on the ADC of amino acid may also occur through binding to the amino acid, forming binary or ternary protein-phytate complexes at pH levels below or above their isoelectric points (Selle et al., 2012).

In addition to the adverse effects of phytate on amino acid utilization, non-starch polysaccharides increase intestinal viscosity and decrease the accessibility of digestive enzymes to protein (Jiang et al., 2022; Liu; Li; Wu, 2022; Hassaan et al., 2019b). Previous work has reported higher ADC of amino acid utilization in Nile tilapia fed diet with mixture of xylanase and β-glucanase (Brito et al., 2021). Similarly, earlier research has described increased ADC of amino acids in Atlantic salmon (*Salmo salar*) fed EC containing protease, xylanase, and (Jacobsen et al., 2018). However, the effects of exogenous enzymes on amino acid utilization can be influenced by various factors, including the gastrointestinal characteristics of fish, the concentration and sources of enzymes, and the application method (Vielma et al., 2004).

Compared to the control, fish fed the EC450 diet exhibited markedly higher dietary DE ($P<0.001$) and DP ($P=0.002$) contents by 221.25 kcal/kg diet and 10.54 g/kg diet, respectively. However, the DE and DP contents were similar in fish fed diets control and EC225 (Figure 1).

The blend of XB is a suitable NSP-degrading enzyme containing xylanase, β-glucanase, amylase, pectinase, and cellulose, which might improve the ADC of energy. The benefits of xylanase and β-glucanase on increased DE and DP content of diets may be attributed to their direct effect on reducing digesta viscosity, as reported in previous studies in Nile tilapia (Brito et al., 2021; Tachibana et al., 2010). Of note, soluble NSPs have been shown to correlate with high intestinal viscosity and low activity of digestive enzymes in Nile tilapia (Hassaan et al., 2019b). Additionally, previous research has identified that phytate may decrease amylase activity and impair starch digestion (Li; Li; Wu, 2009). As a result, adding phytase to the diet may improve starch digestibility, thus enhancing dietary energy utilization and DE contents of the diet, as previously reported in Nile tilapia (Maas et al., 2018). Previous studies have identified the benefits of protease on protein utilization in Nile tilapia (Hassaan et al., 2019a; Hassaan et al., 2021), which may justify the higher DP content observed in fish fed the EC-added diet in this study. Interestingly, previous research has not identified significant differences in Nile tilapia fed diet supplemented with cellulase (Yigit; Olmez, 2011). Therefore, the benefits observed on DE and DP in this study may primarily be attributed to the effects of other exogenous carbohydrases and phytase contained in the EC used in this work.

The N and P balance of fish fed the experimental diets are shown in Table 5. The gross N and gross P intake were lower, while the fecal N and fecal P were lower in fish offered diet EC450 than those fed other diets ($P<0.05$).

The total N and P loss were markedly lower ($P<0.05$) in fish fed diet EC450 than those fed other diets, but similar in fish fed control and EC225 diets, as shown in Figure 2. However, dietary treatments did not affect digestible N intake, N retention, digestible P intake, and branchial and urinary P loss ($P>0.05$).

These results indicated that EC improved the efficiency of N and P utilization and significantly reduced their outputs, which aligns with previous results using a mixture of phytase and xylanase on N and P loss in Nile tilapia (Maas et al., 2018). In addition, previous study in our laboratory revealed that Nile tilapia fed an enzyme cocktail composed of phytase, xylanase, and β-glucanase reduced N and P excretion by 27% and 58%, respectively, relative to fish fed control diet without exogenous enzymes (Nakamura et al., 2022). These findings indicate that EC may improve the efficiency of dietary N and P utilization, thereby reducing their potential for eutrophication. Furthermore, EC would be useful for tilapia farming certification and accreditation schemes following low N and P loads sustainability indicators.

To summarize, the EC450 diet revealed compelling effects by boosting the apparent ADC of multiple nutrients. New generations of thermostable enzymes that can be thoroughly mixed in the feed before extrusion has been proposed as a practical way for large-scale industrial aquafeed production (Cian et al., 2018). It is worth noting that in the current study, the extrusion process was performed on a small-scale laboratory extruder where the die temperature reached a mean value of 86 °C. Additionally, the drying process was also

performed at relatively low temperature of 55 °C. Thus, further research using large-scale industrial conditions would be needed to analyze the impacts on the residual activity of exogenous enzymes. This study highlighted the potential of exogenous multienzymes to improve the nutritive value of vegetable-based feeds and contribute to developing eco-friendly diets for Nile tilapia. This research contributes to the advancement of aquaculture practices and the sustainable management of Nile tilapia farming

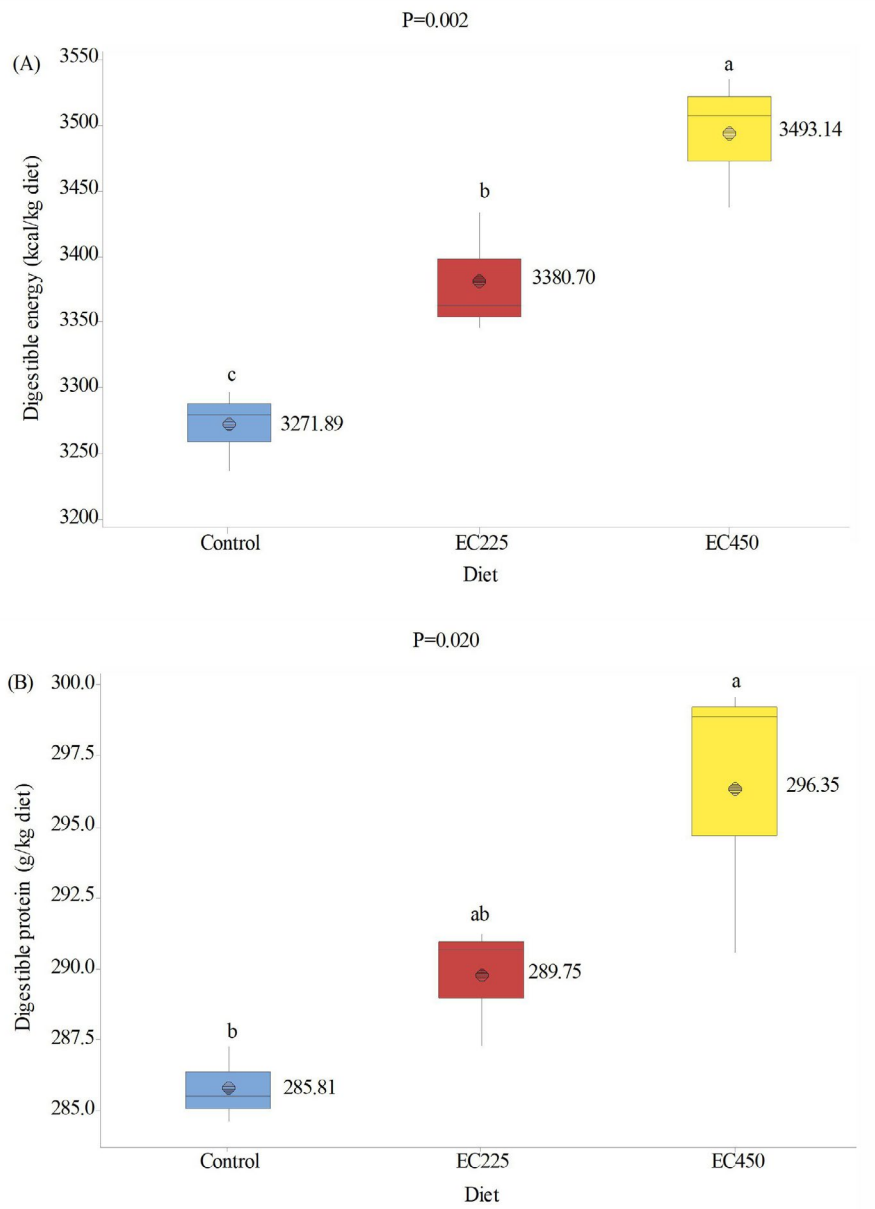

Figure 1: Boxplots of digestible energy (A) and digestible protein (B) contents in extruded diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg kg^{-1} diet (EC225) and 450 mg/kg diet (EC450) fed to pre-grow-out Nile tilapia. Individual data points are presented as box plots, showing mean (dot points), median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers). Different small letters on the bars indicated significant differences. Data were analyzed by one−way ANOVA and Tukey's HSD test ($P<0.05$).

Table 5: Nitrogen and phosphorus balance in pre-growout Nile tilapia fed the experimental diets[1].

Parameter	Diet[2]			P-value
	Control	EC225	EC450	
N-balance				
GNi	74.69±2.63[a]	68.72±2.90[a]	64.08±2.37[b]	0.031
DNi	64.11±2.21	59.81±2.85	57.06±2.83	0.129
BUN_l	42.56±1.27[a]	39.06±2.12[ab]	33.46±1.44[c]	0.009
FN_l	10.58±0.42[a]	8.91±0.18[b]	7.02±0.46[c]	<0.001
TN_l	53.14±1.69[a]	47.97±2.17[a]	40.48±1.24[b]	0.002
N_r	21.55±0.94	20.75±0.73	23.61±1.45	0.122
NR_e	33.59±0.51[b]	34.73±0.79[b]	41.35±0.89[a]	<0.001
P-balance				
GP_i	21.11±0.61[a]	20.81±0.20[a]	19.33±0.27[b]	0.015
DP_i	15.18±0.67	15.60±0.25	15.49±0.36	0.709
BUP_l	7.45±0.54	7.59±0.18	7.07±0.28	0.461
FP_l	5.94±0.06[a]	5.21±0.07[b]	3.84±0.12[c]	<0.001
TP_l	13.38±0.49[a]	12.81±0.16[a]	10.91±0.20[b]	0.001
P_r	7.73±0.13[b]	8.00±0.07[b]	8.42±0.08[a]	0.002
PR_e	51.00±1.38[b]	51.32±0.37[ab]	54.40±0.78[a]	0.035

Abbreviations: N, nitrogen; GN_i, gross N intake [g/kg body weight gain (BWG)]; DNi, digestible N intake (g/kg BWG); BUN_l, branchial and urinary N loss (g/kg BWG); FN_l, fecal N loss (g/kg BWG); FN_l, fecal N loss (g/kg BWG); TN_l, total N loss (branchial, urinary and fecal) (g/kg BWG); N_r, N retention (g/kg BWG); NR_e, N retention efficiency (%); P, phosphorus; GP_i, gross P intake (g/kg BWG); DP_i, digestible P intake (g/kg BWG); BUP_l, branchial and urinary P loss (g/kg BWG); FP_l, fecal P loss (g/kg BWG); TP_l, total P loss (branchial, urinary and fecal) (g/kg BWG); P_r, P retention (g/kg BWG); PR_e, P retention efficiency (%).

[1] Values represent mean ± standard deviation of three replicate aquaria. [3] Diet without enzyme complex (Control), and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450).

[a,b,c] Means within the same row with no common superscript differ significantly (P<0.05; Tukey's test).

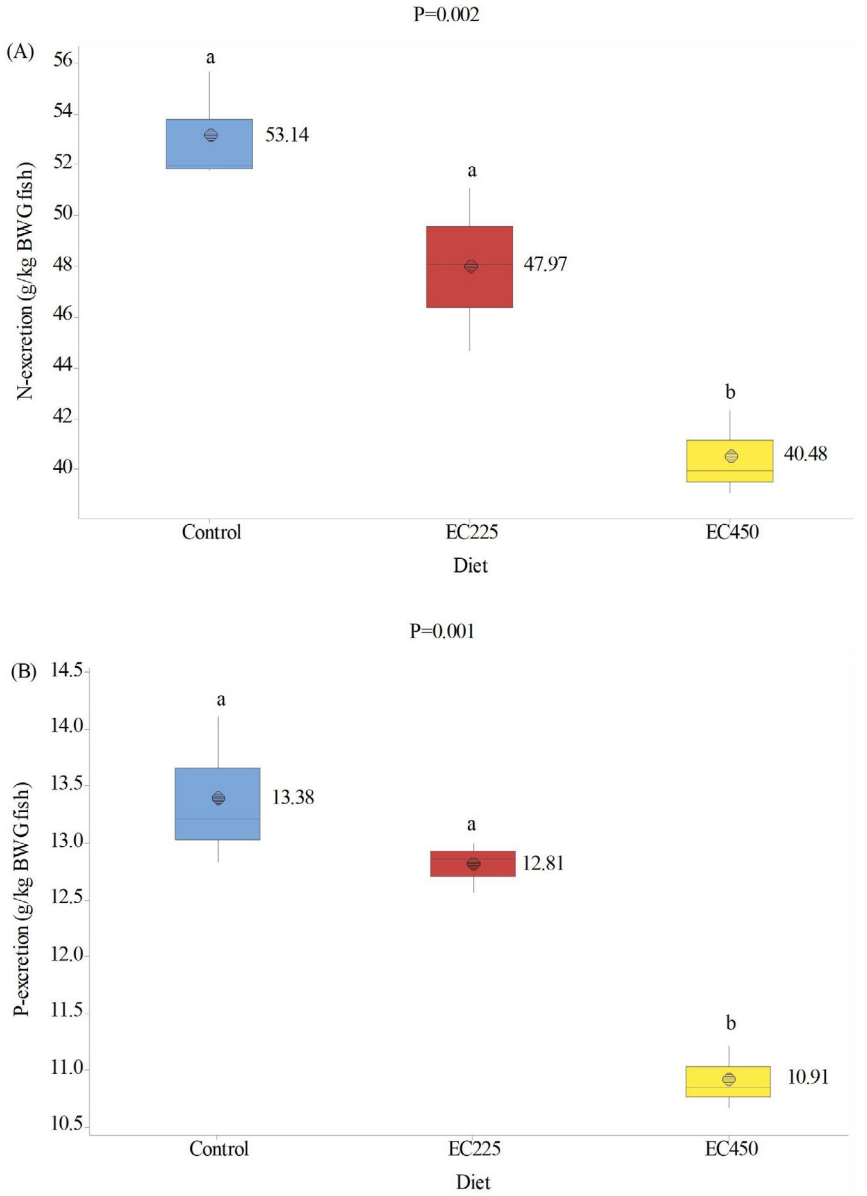

Figure 2: Boxplots of nitrogen (N) loss (A) and phosphorus (P) loss (B) of pre-grow-out Nile tilapia fed diet without enzyme complex (Control) and diets supplemented with enzyme complex at 225 mg/kg diet (EC225) and 450 mg/kg diet (EC450). Individual data points are presented as box plots, showing mean (dot points), median (horizontal lines), the lower and upper quartiles (lower and upper borders of the boxes), and minimum and maximum values (lower and upper whiskers). Different small letters on the bars indicated significant differences. Data were analyzed by one−way ANOVA and Tukey's HSD test (P<0.05).

CONCLUSIONS

The results of this study indicate that EC supplementation at 450 mg/kg diet improves nutrient digestibility, reduces nitrogen and phosphorus output in pre-growout Nile tilapia. These findings provide a comprehensive understanding of the benefits of multiple exogenous enzyme supplementation in improving the nutritive value of feeds and could have significant implications for the formulation of cost-effective and environmentally-sustainable diets in tilapia farming operations.

AUTHOR CONTRIBUTIONS

Conceptual Idea: Furuya, W.M.; Methodology design: Brandes, A.; da Cruz, T.P.; Michelato, M.; Data analysis and interpretation: Furuya, W.M.; Michelato, M.; Data analysis and interpretation: Furuya, W.M.; Michelato, M.; Furuya, V.R.B, and Writing and editing: Furuya, W.M.; Brandes, A.; da Cruz, T.P; Furuya, W.M.; Michelato, M.

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