Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, haematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, *Oreochromis niloticus*

by Hassaan, M.S., El-Sayed, A.I.M., Soltan, M.A., Iraqi, M.M., Goda, A.M., Davies, S.J., El-Haroun, E.R. and Ramadan, H.A.

DOI: https://doi.org/10.1016/j.aquaculture.2019.01.009

Hassaan, M.S., El-Sayed, A.I.M., Soltan, M.A., Iraqi, M.M., Goda, A.M., Davies, S.J., El-Haroun, E.R. and Ramadan, H.A. 2019. Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, haematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, *Oreochromis niloticus*. *Aquaculture*, 503, pp.282 – 292.

Partial dietary fish meal replacement with cotton seed meal and supplementation with exogenous protease alters growth, feed performance, haematological indices and associated gene expression markers (GH, IGF-I) for Nile tilapia, *Oreochromis niloticus*

M.S. Hassaan*[1], A.M.I. El-Sayed[2], M.A. Soltan[2], M.M. Iraqi[2], A.M. Goda[1], E.R. El-Haroun[4], H.A. Ramadan[2], S.J. Davies[3],

[1]Aquaculture Division, Fish Nutrition Research Laboratory, National Institute of Oceanography and Fisheries (NIOF), Cairo, Egypt

[2]Animal Production Department, Faculty of Agriculture, Benha University, Benha, Egypt

[3]Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, England, United Kingdom

[4]Animal Production Department, Faculty of Agriculture, Cairo University, Cairo, Egypt

*Corresponding author. Tel.: +20 1229490090; fax: +20 227943226.

E-mail address: ms.hassaan@niof.sci.eg (M.S. Hassaan).

Abstract

A 12-week feeding trial was conducted to evaluate the effect of different ratios of fish meal (FM): cotton seed meal (CSM) without or with inclusion of exogenous protease in diets on growth performance, hematology, digestibility and selected gene expression markers (GH and I (IGF-I) of juvenile Nile tilapia. The experimental diets were categorized into three groups; the first group CSM_1 which contained fish meal protein: cotton seed meal protein (FM: CSM = 2:1), the second group CSM_2 which contained FM: CSM = 1:1 and the third one CSM_3 contained FM: CSM = 1:2 on protein content based. All groups were supplemented with exogenous protease at 0 and 2500 U kg^{-1} diet, respectively. All diets were fed to fish (initial body weight 11.62 ± 0.03 g $fish^{-1}$) in triplicate aquaria twice daily. The higher weight gain (WG), protein efficiency ratio (PER) and best feed conversion ratio (FCR) were recorded by fish fed CSM_1 and CSM_2 and supplemented with 2500 U protease/ kg diet. The highest apparent digestibility coefficient of crude protein, crude lipid and digestible energy, and apparent availability coefficient of essential amino acids were obtained by fish receiving CSM_1 and CSM_2 supplemented with protease (2500 U protease kg^{-1} diet). The highest mean values of Hb, Htc and RBCs were recorded in fish fed CSM_1 and CSM_2 supplemented with protease enzyme (2500 U protease kg^{-1} diet). Serum of alanine and aspartate aminotransferase activities were improved due to dietary protease (2500 U protease kg^{-1} diet) supplementation, also, fish received the diets supplemented with protease 2500 U kg^{-1} diet generally had higher total protein, albumin, calcium and phosphorus than those fed diets without supplement. The highest growth hormone (GH) gene expression in brain and liver of tilapia were obtained in the group fed CSM_3 and un-supplemented with protease enzyme followed by CSM_2 (un-supplemented). On the other hand, tilapia fed CSM_1 and CSM_2 supplemented with protease enzyme showed the highest values of gene expression of insulin like growth factor I (IGF-I) in

brain and liver of tilapia compared to other groups. Results above showed that supplementation of protease can improve growth, nutrient assimilation, and hematology and alter gene expression of GH and IGF-I of Nile tilapia.

Keywords: Nile tilapia (*Oreochromis niloticus*), Cotton seed meal, Growth, hematology, Digestibility, Gene expression, GH, insulin like growth factor I (IGF-I)

1. Introduction

In view of the rapid rise in aquatic animal production (fish and shrimp) there is a global search for cheaper and nutritionally balanced ingredients for the manufacture of commercial diets to meet this growing demand for the aquaculture industry (FAO, 2016). Traditionally fish meal has been included in feeds for many species but the quest for sustainable nutrient dense ingredients is high on the agenda, avoiding the ecological limits of forage fish destined for fed aquaculture species (Froehlich, 2018). Plant proteins might thus be considered as the most viable alternative in this respect for economic fish production in most of the developing countries (Kumar et al., 2011a; Kader et al., 2012; Hassaan et al., 2018). In this manner, it has become an inevitable trend of replacing fish meal with less expensive and locally available plant protein sources (Hassaan et al., 2017; Hassaan et al., 2018). Cotton seed meal (CSM) has been investigated as a potential alternative ingredient to both fish meal and soybean meal due to its cheaper cost, being readily available in some countries, particularly in the USA, China, India and Egypt, although the protein content can be variable (23–53%) depending on how this product is processed (Mbahinzireki et al., 2001; Yue and Zhou 2008). Also, CSM inclusion has been studied in numerous fish species, *Sarotherodon mossambicus* (Jackson et al*.,* 1982), *Oreochromis niloticus* (Yue and Zhou 2008), *Ictalurus punctatus* (Robinson and Tiersch 1995), and *Oncorhynchus mykiss* (Lee et al., 2006). These studies showed positive results at low inclusion levels, but more

usually growth reduction at high inclusion levels. Among the factors which limit incorporation of CSM into aquafeeds are amino-acid imbalance, digestibility and presence of anti-nutritional factors (ANFs) such as gossypol which impair utilization of nutrients resulting in reduced growth, nutrient utilization and feed efficiency **(**Francis et al., 2001; Li and Robinson 2006**)**. To expand the use of plant-based protein for fish, it is essential to develop adequate processing technologies for plant feed ingredients in order to sufficiently remove or degrade these ANFs. There are a variety of techniques available to exclude ANFs from plant feedstuffs including soaking, dehulling, solid state fermentation and germination (Elmaki et al., 1999; Alonso et al., 2000; Idris et al., 2006; Hassan et al., 2018;). However, the use of natural bioactive agents and exogenous enzymes is gaining much attention as reported by Hlophe-Ginindza et al. 2016. The addition of such exogenous enzymes in fish diets containing high inclusion of plant protein can specifically degrade certain ANF's thus greatly enhancing the nutritional value of plant-based protein ingredients in practice (Dalsgaard et al., 2012). Furthermore, exogenous enzymes can allow flexibility in formulated feed through incorporation of lower quality and less expensive plant ingredients (Adeoye et al., 2016 a, Adeoye et al., 2016 b). In addition, exogenous enzymes may alter substrate availability for specific populations of gut microbes, which enhances digestion of nutrients and synthesis of nutrient substances that the fish need for gut integrity and growth (Jiang et al., 2014). Except for phytase, there are a few studies on the use of exogenous enzymes in fish feeds. Lin et al. (2007) reported that supplementation with a commercial enzyme complex (neutral protease, β-glucanase and xylanase) significantly improved the growth performance and feed utilization of juvenile hybrid tilapia *Oreochromis niloticus* × *O. aureus*. Drew et al. (2005) observed an increase in the apparent nutrient digestibility and an improvement in the feed efficiency when supplementing a commercial protease to a rainbow trout (*Oncorhynchus mykiss*) diet containing a mixture of rapeseed

and pea meals. The use of a multi-enzyme complex such as Natuzyme50® may be beneficial in improving the digestibility of Kikuyu leaf meal -based diets (Hlophe and Moyo 2014). More recently, a specific enzyme, exogenous protease, was suggested to be added to the feed to raise efficiency, aimed to improve the dietary protein utilization of Gibel carp (Liu et al., 2018). Consequently, there is a need for further studies to establish the benefits of dietary enzyme supplementation for *in vivo* processing of plant ingredients such as CSM into value added products for fish. Such nutritional investigations can be aided by a better understanding of the underlying physiological and metabolic responses of fish to dietary modulation using more advanced techniques such as nutri-genomics.

Recently, new progresses in nutrition study have allowed for the integration of nutrition and genomics analysis through the nutrigenomics approach, which has added to the understanding of the impact of component of diet on gene expression (qRT-PCR) (Mutch et al., 2005). Further advances have been made with respect to the proteome and metabolomic profile in fish to substantiate the effects of nutrition on protein biosynthesis and metabolic changes. Furthermore, their main mode of action is to stimulate growth, and, though IGFs share this ability with other growth factors such as epidermal growth factor, platelet-derived growth factor, and nerve growth factor IGFs differ from these substances in that they are quite unique in exhibiting endocrine actions in higher vertebrates including the teleost. For example, nutrigenomics studies in cultured fish have addressed the partial replacement of fish meal with plant protein in the diet. These studies have concluded that the growth rates of fish are mediated by the growth hormone (GH)/insulin-like growth factor (IGF) axis (Company et al., 2001; Pérez-Sánchez et al., 2002) as well as the dietary protein sources may be affected the expression of GH-and IGF-1- encoding genes (Kumar et al., 2011b). It has been suggested that both energy and protein as well as amino

119 acid availability are required for maintenance of IGF-I. Serum IGF-I may also serve as a marker
120 for evaluation of nutritional status in humans as shown by several animal models (Ketelslegers et
121 al., 1994). However, changes in the expression of growth-related genes due to replacement of
122 fish meal with cotton seed meal with exogenous protease in tilapia have not been studied before.

123 Sustainable and balanced dietary formulations are essential and dependence on optimizing the
124 use of raw materials such as plant ingredients is critical to successful future production.
125 Therefore, the aim of the present study was to investigate the effects of a protease exogenous
126 enzyme supplement on the response of *O. niloticus* fed CSM as a partial protein concentrate
127 substitute for fish meal in a series of experimental diets under controlled laboratory conditions.
128 The main objectives were to record growth and feed utilization efficiency including digestibility,
129 and specific hematological parameters. Gene expression for growth hormone and insulin like
130 growth factor I (IGF-I) in liver and brain of tilapia was targeted to confirm any longer terms
131 metabolic responses to dietary influences on growth and development.

132 **2. Materials and methods**

133 *2.1. Diets and experimental design*

134 Six isonitrogenous (29.50 % crude protein) and isocaloric (18.76 MJ kg^{-1} gross energy)
135 experimental diets were formulated and the proximate chemical composition of the experimental
136 diets is presented in Table (1). The first group CSM_1 which contained fish meal protein: cotton seed
137 meal protein (FM: CSM = 2:1), the second group CSM_2 which contained FM: CSM = 1:1 and the
138 third one CSM_3 contained FM: CSM = 1:2 on protein content based. All groups were supplemented
139 with exogenous enzyme (protease) at 0 and 0.5 g kg^{-1} diet. The protease (5000 Ug^{-1} product, supplied
140 by Huvepharma, Antwerp, Belgium) was added to the basal diet to provide two concentrations of 0
141 (0.00 g kg^{1}) and 2500 (50 mg kg^{-1}) U protease kg^{-1} diet. Activity of protease was assayed according

142 to the method from the Committee on Food Chemicals Codex (1996). One protease unit was the
143 amount of enzyme that releases 1.0 μ g of phenolic compound, expressed as tyrosine equivalents,
144 from a casein substrate per minute at pH 7.5 and 40 °C. The analyzed activity of protease was
145 4395 U g^{-1}. All dry ingredients i.e. fishmeal, cotton seed meal, soybean meal, yellow corn and wheat
146 bran were blended for 5 mins and thoroughly mixed with soybean oil. Also, each of the diets
147 contained 5 g kg^{-1} chromic oxide (Cr_2O_3) as a marker for nutrient digestibility measurements. The
148 ingredients were mixed well and made into dry pellets using a laboratory pellet mill (California Pellet
149 Mill, San Francisco, CA, USA) and air dried at 37 °C overnight. The pellets (2-mm die) were
150 subsequently stored at -20 °C until subsequent use.

151 *2.2. Determination of gossypol in cotton seed meal*

152 Free gossypol concentration in the experimental diets was determined by high-performance liquid
153 chromatography (HPLC). Extraction of free gossypol by acetone was performed after hydrolysis
154 with hydrochloric acid, followed by separation of the pure compound, as assayed by HPLC
155 according to the method of Luo et al. (2006).

156 2.3. Exogenous protease activity in the experimental diets

157 The activity of exogenous enzymes was estimated according to the method described Shi et
158 al. (2016). In brief, 2 g of diets of CSM_1, CSM_2 and CSM_3 were mixed with 0.5 g of fish meal,
159 respectively. Each group after mixed was incubated with buffer solution ($Na_2B_4O_7 \cdot (H_2O)_{10}$-
160 H_2BO_3, pH 8.5) containing penicillin and streptomycin (200 U ml^{-1}) for 2 h at a temperature of
161 35 °C. Total free amino acid was analyzed comparing with the ammonium sulphate (the standard
162 solution) standard curve using a spectrophotometer at OD 570 nm. The amount of free amino
163 acid hydrolyzed by the exogenous protease in the diets of CSM_1, CSM_2 and CSM_3 (with or

without protease supplementation) and occurred naturally in fish meal were compared. The difference of free amino acid content between diet (with or without protease supplementation) was shown. The exogenous protease activity in original products was 87.9% activity.

2.4. Fish and experimental conditions

Nile tilapia, *O. niloticus* fingerlings (approximately 11-11.5 g) from a private farm (Kafer El-sheekh Governorate, Egypt), were transferred to the Fish Nutrition Laboratory, Faculty of Agriculture, Benha University**,** and kept in two 450 L- capacity tanks for prior acclimatization. Fish were fed daily on the basal diet (30 % crude protein and 18.90 MJ kg^{-1} gross energy). After an acclimatization period of 15 days, 216 fish were randomly distributed into six groups with three replicates, each replicate contained 12 fish (avg. wt. 11.60 ± 0.72 g) in an aquarium (100 L capacity). Fresh water was supplied to each aquarium housed within an artificially illuminated room with a photoperiod of 12 h light: 12 h dark regime. All aquaria were supplied with compressed air for oxygen requirements throughout the experimental period. Six groups of experimental fish were fed close to apparent satiation twice per day at 09:00 and 14:00 h. Total fish weight in each aquarium estimated every 2 weeks to check their growth. About one-third of water volume in each aquarium was replaced daily by fresh water after removing the accumulated feces by siphoning. Water quality was measured throughout the experiment for all essential parameters. During the 84 days of feeding trial, the water-quality parameters averaged as follows: water temperature ranged from 27.85 to 29.33°C: dissolved oxy-gen, ranged between 5.56 and 6.65 mg L^{-1}: water total ammonia ranged from 0.16 to 0.2 mg L^{-1}: and pH, ranged between 8.04 and 8.30. It noticed that, the reported water quality parameters in this study were within the normal ranges for fish growth (Boyd, 1990).

2.5. Growth performance and feed utilization indices

During the feeding period, the fish per aquarium were counted, weighed and measured for body weight individually every two weeks. The following measurements and equations were applied to fish to indicate the growth performance and feed utilization criteria.

Weight gain (WG) = Final body weight (FBW g) - Initial body weight (IBW g); Specific growth rate (SGR) = (ln FBW - ln IBW)/t ×100, Where: ln is natural logarithmic of FBW and IBW; t = time in days; Feed conversion ratio (FCR) = Feed intake (g)/weight gain (g); Protein efficiency ratio (PER) = weight gain (g)/protein intake (g).

2.6. Digestibility measurements

The apparent digestibility coefficients (ADCs) and amino acids apparent availability of different experimental diets were determined using chromic oxide (Cr_2O_3) as an external marker at a level of 0.5% within the diet. After a two-month feeding period for the experimental diets, feces were collected from each aquarium once daily prior to feeding for a one-month period. The collection was done manually by siphoning the faecal matter and straining through a fine-meshed net (Baruah et al., 2007). Faecal matter collected was pooled in each aquarium and subsequently dried in a hot air oven at 60 °C. Dried feces were digested in a mixture of perchloric acid and nitric acid mixture (2:1) at 250 °C, according to the method described by Zhou et al. (2004). After appropriate dilution chromic oxide was determined according to the procedure described by Furukawa and Tsukahara (1966). The following equation determined the ADCs and amino acids apparent availability of the experimental diets: ADCs = [100- (Cr_2O_3 % $_{\text{in diet}}$ / Cr_2O_3 % $_{\text{in faces}}$) × (nutrient % $_{\text{in faces}}$ / nutrient % $_{\text{in diet}}$)] × 100.

208 *2.7. Chemical composition and amino acid*

209 Proximate chemical analyses were made for the experimental diets and samples of fish (five
210 fish in each replicate) at end of the experiment according to standard methods AOAC, (1990) for
211 dry matter, crude protein, ether extract, crude fiber and ash. Dry matter was determined by oven
212 drying at 105 °C until a constant weight was achieved. Crude protein (N × 6.25) was determined
213 using the Kjeldahl method after acid digestion using an Auto Kjeldahl System (UDK 126 D,
214 Italy). Crude lipid was determined by the ether-extraction method using a Soxtec System HT
215 (Soxtec System HT6, Tecator) with diethyl ether (40–60 °C). The ash content was estimated
216 after incineration the samples in a muffle furnace at 550 °C for 24 h. Fiber content of the
217 experimental diets was determined using the method described by Van Soest et al. (1991).
218 Nitrogen-free extract (NFE) was computed by taking the sum of values for crude protein, crude
219 lipid, crude fiber and ash and by subtracting this sum from 100. The samples of diets and fecal
220 for amino acid analysis were ground following by digestion using 10 mL 6N HCl solution at 110
221 °C for 24 h. Amino acids were separated using high performance liquid chromatography (HPLC;
222 Shimadzu Corp., Tokyo, Japan) following the method showed by Kader et al. (2010). The
223 hydrolyzed amino acids composition of the experimental diets was showed in Table 2.

224 *2.8. Hematological and blood chemistry parameters*

225 At the end of the experiment, blood samples (five fish in each replicate) were collected from the
226 caudal vein of all treatments from anaesthetized fish with overdose of tricaine methanesulfonate
227 (MS-222; 1 g L^{-1}). Blood samples were divided into two portions. The first portion was collected
228 with anticoagulant 10% ethylenediaminetetraacetate (EDTA) to determine the hematocrit (Htc),
229 hemoglobin (Hb), erythrocyte counts (RBCs) and total count of white blood cells (WBCs)
230 according to standard methods as described elsewhere by Rawling et al. (2009). The second

portion of the blood sample was allowed to clot overnight at 4°C and then was centrifuged at 3000 rpm for 10 min. The non-hemolysed serum was collected and stored at -20°C until use. Levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) were determined according to the method described by Reitman and Frankel (1957) and serum creatinine was measured by the calorimetric method and enzymatic determination methods as described by Henry et al. (1974). Total serum protein and albumin were determined according to Henry (1964) and Wotton and Freeman (1974), respectively. However, the total serum globulin was calculated by subtracting the total serum albumin from the total serum protein according to Coles (1974). Serum phosphorus and calcium were measured spectrophotometrically using commercial kits produced by Pasteur labs (Egyptian American Co. for Laboratory Services, Egypt).

2.9. Gene expression analysis

2.9.1. RNA extraction

Total RNA was isolated from liver and pituitary samples (three fish in each replicate) using a Promega RNA Isolation Kit (Cat No. Z3100, USA) according to the manufacturer's instructions. The quantity of the RNA was assessed using a Nano-Drop spectrophotometer (NANODROP 1000, Thermo Scientific, USA). The integrity (quality) was checked by denaturing gel electrophoresis (1% agarose gel) and the purity by measuring the OD260/OD280 absorption ratio (>1.95).

2.9.2. First strand cDNA synthesis

cDNA was generated from 1 µg of total RNA using High Capacity cDNA (Thermo Fisher Scientific, Cat. No .436, 8814) reverse transcriptase kit for reverse transcriptase polymerase

chain reaction (RT-PCR) following the manufacturer's protocol. The product of the first strand cDNA synthesis was stored at −80°C until the quantitative RT-PCR (qRT-PCR) runs.

2.9.3. Real-time quantitative RT-PCR

The primers employed for the quantification of the desired genes were purchased from Invitrogen, Germany. The primer sequences and calculated efficiency are enlisted in Table 3. Triplicate qPCR reactions were performed on an AriaMx Real-Time PCR System (Agilent technologies). Reactions containing 5 µl of 5 × diluted cDNA, 10 pmol each of forward and reverse primers, 0.4 µl ROX dye solution (1:500 dilution) and 10 µl SYBR Green PCR MasterMix (Maxima SYBR Green qPCR, Thermo Fisher Scientific, Cat. No # k0251) were performed in a four-step experimental run protocol: a denaturation program (10 min at 95 °C); an amplification and quantification program repeated 40 times (30 s at 95 °C, 50 s at 55°C and 40 s at 72°C); a melting curve program (55–95°C with a heating rate of 0.10°C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, "no-template" controls (i.e. with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation. Moreover, 18S rRNA gene was used as an internal standard. The relative mRNA expression levels were calculated by a standard curve method. The expression levels of genes were normalized to the levels of 18S rRNA gene in the same sample. Standard curves were generated by serial dilution of a random mixture of control samples.

2.10. Statistical analysis

All data were analyzed by using the software SAS, version 6.03 (Statistical Analysis System 1996). One-way analysis of variance (One-way ANOVA) was used to determine whether significant variation existed between the treatments. When overall differences were found, differences between means were tested by Tukey's HSD test. Two-way ANOVA was used for analyzing the individual effects of FM: CSM ratios and protease level and the interaction between them. All differences were considered significant at $P<0.05$ and the results are presented as means with pooled standard error of the mean (Pooled S.E.M).

3. Results

3.1. Relative rate of exogenous protease activity

Relative activity of exogenous protease of CSM1, CSM2 and CSM3 diets were 70.45%, 68.03% and 67.99%, respectively, when compared with the activity of protease in original product (87.9%) (Table 4).

3.2. Growth performance

Body weight gains (g) of tilapia are shown in Figure 1 as affected by different ratios of FM: CSM; 2:1, 2:2 and 1:2 and exogenous protease levels 0 or 2500 U kg^{-1} and their interaction. Mean bi-weekly body mass gain revealed that fourth week onwards; there was differential growth among the treatments, and the lower body mass gain was observed in fish fed CSM_3 without protease. The effects of FM: CSM ratios, protease and their interaction on the growth performance and feed utilization for treated groups are presented in Table 5. All indices of growth and feed utilization were significantly affected by FM: CSM ratios, protease and their interaction, except FI ($P = 0.288$, $P = 0.097$ and $P = 0.790$, respectively). Although, there was a significant interaction between FM:CSM ratio and protease, fish fed the diets supplemented with protease 2500 U kg^{-1} diet generally had greater

295 WG, FCR and PER than those fed the basal diets; growth performance and feed utilization
296 generally decreased with decreasing FM:CSM ratios. The highest WG, FCR and PER were
297 recorded by fish fed CSM_1 and CSM_2 and supplemented with 2500 U protease kg^{-1} diet.

298 *3.3. Apparent digestibility coefficient*

299 Results of the apparent digestibility coefficient (ADCs) of dry matter, protein lipid and digestible
300 energy, are shown in Table 6. The ADCs of dry matter, crude protein, crude lipid and digestible
301 energy (DE) were significantly affected by FM: CSM ratios, protease and their interaction.
302 Generally, ADC of dry matter ($P = 0.001$), crude protein ($P = 0.017$), crude lipid ($P = 0.021$) and
303 digestible energy ($P = 0.013$) were improved in fish fed diet supplemented with 2500 U protease
304 kg^{-1} diet compared with un-supplemented diet. The highest ADC of dry matter crude protein,
305 crude lipid and digestible energy was obtained by fish fed CSM_1 and CSM_2 supplemented with
306 protease (2500 U protease kg^{-1} diet).

307 3.4. *Amino acids apparent* availability

308 The effects of protease enzyme, different ratios of FM: CSM and their interaction on the
309 essential amino acid apparent availability of Nile tilapia are shown in Table 7. Apparent
310 availability of essential amino acids was significantly ($P < 0.05$) affected by dietary different
311 ratio of FM:CSM, supplementation with exogenous protease and their interaction. Although
312 there was a significant interaction between FM:CSM ratio and protease, fish fed the diets
313 supplemented with protease 2500 U kg^{-1} diet generally had greater of apparent availability of
314 essential amino acids than those fed the basal diets. The highest apparent availability of essential
315 amino acids was noted in fish fed CSM_1 and CSM_2 supplemented with protease 2500 U protease
316 kg^{-1} diet.

3.5. *Chemical composition*

Data of the proximate chemical composition of whole fish are presented in Table 8. No significant differences due to FM: CSM ratios, protease and their interaction were observed for all indices of chemical composition, except crude protein (P = 0.035). The lowest content of crude protein was recorded by fish fed CSM_2 without supplemented exogenous protease. Both CSM_1 groups without and with supplemental protease had significantly higher crude protein than other groups.

3.6. *Hematology indices*

The effects of protease enzyme, different ratios of FM: CSM and their interaction on the hematology parameters of Nile tilapia are shown in Table 9. With exception of WBC (P = 0.781; P = 0.051; P = 0.072), FM:CSM ratios, exogenous protease and their interaction had significant effect on Hb, Htc and RBCs of Nile tilapia. Hb (P = 0.026), Htc (P = 0.041) and RBCs (P =0.049) values were significantly higher in fish fed diet supplemented with exogenous protease 2500 U kg^{-1} diet in comparison with the other diet without supplemented.

3.7. *Blood biochemistry*

The effects of FM: CSM ratios, exogenous protease and their interaction on serum of ALT, AST activities, total protein, albumin, globulin, calcium and phosphorus for Nile tilapia are presented in Table 10. With exception, globulin (P = 0.121; P = 0.321; P = 0.221), FM: CSM ratios, exogenous protease and their interaction had significant effect on serum of ALT, AST, total protein, albumin, calcium and phosphorus of Nile tilapia. Although there was a significant interaction between FM:CSM ratio and protease, fish received diets supplemented with protease 2500 U kg^{-1} diet generally had lower ALT, AST and higher total protein, albumin, calcium and

339 phosphorus than those fed the basal diets (without supplemented). The best ALT, AST, total
340 protein, albumin, calcium and phosphorus were recorded by fish fed CSM_1 and CSM_2
341 supplemented with 2500 U protease kg^{-1} diet.

342 *3.8. Gene expression*

343 Table 11, Figure 2 and 3 illustrated gene expression of growth hormone (GH) in brain and
344 liver of tilapia as influenced by different ratios of FM: CSM and exogenous protease levels and
345 their interaction. Gene expression of growth hormone (GH) in brain and liver were significantly
346 affected by different ratios of FM: CSM and exogenous protease levels and their interaction
347 (Table 10). Relative growth hormone (GH) gene expression was significantly down-regulated in
348 pituitary ($P = 0.012$) and liver ($P = 0.021$) of fish fed different ratios of FM: CSM supplemental
349 with exogenous protease after 84 days (Figure 2). Furthermore, the highest GH expression in
350 brain and liver of tilapia were observed in fish fed CSM_3 without supplemental exogenous
351 protease. Gene expression of insulin like growth factor I (IGF-I) in brain and liver of tilapia are
352 shown in Figure 3 which was affected by different ratios of FM: CSM and exogenous protease
353 enzyme level and their interaction. Fish fed different ratios of FM: CSM supplemented with
354 protease enzyme showed the highest expression of IGF-I gene as compared to other treatments.

355 **4. Discussion**

356 In the present study, Nile tilapia fed different ratios of FM:CSM; CSM_1, CSM_2 and CSM_3 and
357 supplemented with 2500 U exogenous protease kg^{-1} diet yielded the highest growth performance
358 and feed utilization compared to fish fed the similar diets with no added exogenous protease.
359 Inclusion levels of dietary cotton seed meal (CSM) that can be used as a plant protein source for
360 tilapia diets depend mainly on the level of free gossypol and available lysine content (El-Saidy

and Gaber, 2004). The reduction of growth performance in this study for Nile tilapia fed varying inclusion ratio of CSM, i.e. CSM_1, CSM_2 and CSM_3 without supplemented with protease levels (Table 1) were consistent with Nile tilapia fed dietary levels of CSM at 240 g kg^{-1} diet (Robinson et al. 1984) and rainbow trout fed up 200 g kg $diet^{-1}$ with CSM (Cheng and Hardy 2002) and these results may be attributed to the presence of gossypol and low biological availability of lysine (Francis et al., 2001; Ofojekwu and Ejike (1984). On the contrary, there were no significant effects found in hybrid tilapia and rainbow trout with dietary levels of CSM (337.6 and 588 g kg^{-1} diet, respectively (Yue and Zhou, 2008; Lee et al., 2006). In the present study, supplementation of protease enzyme (2500 U protease kg^{-1} diet) mitigated some of these negative effects. Many studies have reported that exogenous enzyme supplementation can eliminate the effect of ANFs (Hlophe-Ginindza et al., 2016; Adeoye et al., 2016) and enhance the utilization of protein and amino acids, resulting in improved growth performance of fish (Farhangi and Carter 2007; Lin et al. 2007; Baruah et al. 2007; Soltan 2009; Hussain et al. 2015). In addition, exogenous proteases may increase endogenous peptidase production, raise protease activity and subsequently improve the digestibility of dietary protein leading to fast assimilation and increased growth as well as being capable of increasing accessibility of nutrients by breaking down and disrupting layers of complex proteins in plant cell walls (Caine et al., 1998). In contrast, Dalsgaard et al. (2012) found no significant differences in growth performance of rainbow trout fed three different plant-based feedstuffs (soybean, rapeseed, sunflower) a supplemented with mixture of exogenous enzymes (β-glucanase, xylanase and protease).

In this study, the highest apparent digestibility of crude protein, crude lipid and gross energy in diets were attributed to the protease supplementation with the enzyme assisting in minimizing the action of Anti-Nutritional Factors such as gossypol and releasing more protein for

384 assimilation. Likewise, Liu et al. (2018) showed that supplementing 400 mg kg^{-1} protease, to a
385 low protein diet, could save 20 g kg^{-1} dietary protein, improve Apparent Digestibility
386 Coefficients (ADC) of crude protein and crude lipid and having no harmful effects on juvenile
387 Gibel carp (*Carassius auratus gibelio*) health. Similarly, Drew et al. (2005) showed that
388 supplementation with 250 mg/kg protease to a diet containing coextruded canola and pea meal
389 (1:1) improved ADC of protein, lipid, energy and dry matter of rainbow trout with similar
390 supplementation enzyme levels used in the present study with tilapia. In contrast, enzyme
391 supplementation to feed had no noticeable impact on aquatic animal production as viewed by
392 Divakaran and Velasco (1999) and Miller et al. (2008). This may be due to exogenous enzymes
393 being thermally degraded during feed processing such as with extrusion causing deactivation of
394 their activities. These differences in results might be explained by diet composition, including
395 the nutrition level and plant ingredient inclusion level and conditions of storage.

396 The present data clarified that, no significant ($P > 0.05$) effect were detected among different
397 fish groups for whole body composition, except protein content FM:CSM, exogenous protease
398 supplementation and their interaction. This finding agrees with the study of Lin et al. (2007) who
399 revealed that tilapia fed exogenous commercial enzyme complex (neutral protease, b-glucanase
400 and xylanase) have displayed no significant differences in whole body moisture, protein, lipid
401 and ash.

402 Hematological parameters are useful for monitoring fish general health and physiological
403 responses to stress, reducing Htc and HB of fish in one of the most common indicators of
404 harmful effect of free gossypol (Mbahinzireki et al., 2001; Garcia-Abiado et al., 2004). In the
405 current study, Hb, Htc, RBCs and WBCs were higher in tilapia fed different ratios of CSM_1 and
406 CSM_2 and supplemented with protease enzyme (2500 U protease kg^{-1} diet) than counterpart diets

not supplemented with exogenous protease, which indicated that exogenous protease could inhibit the deleterious effect of free gossypol in diets (Table 6). To the best of our knowledge, there are no studies describing the effects of dietary protease supplementation on hematological parameters of fish when fed a diet containing gossypol. Although, Goda et al. (2012) found that red blood cell count, hematocrit and hemoglobin were significantly ($P < 0.05$) elevated in all treatments fed supplemented diets with mixtures of exogenous digestive enzymes (pepsin, papain and α-amylase). On the other hand, supplementation with a mixed enzyme cocktail had no effects on hematological parameters of Nile tilapia as reported recently by Adeoye et al. (2016a).

Moreover, the measurement of AST and ALT is indicative of general systemic nutritional status as well as the integrity of the vascular system and liver function (Kumar et al., 2011a). Increased activities of serum AST and ALT in fish may reveal possible leakage of enzymes across damaged plasma membranes and/or increased synthesis of enzymes by the liver (Yang and Chen, 2003). In a study with Gilthead sea bream (*Sparus aurata*) Gómez-Requeni et al. (2004) reported that dietary treatment did not alter the hepatic activity of amino acid catabolizing enzymes AST, ALT, glutamate dehydrogenase when fish meal was replaced up to 100% with a mixture of plant protein concentrates. Direct hepatic measurements of these enzymes were not performed in this study with tilapia, although plasma activities were undertaken to indirectly assess liver status.

The present study with tilapia showed that the plasma activity of AST and ALT in fish fed high inclusion level of CSM without protease supplementation was higher than those fed high inclusion level of CSM and supplemented with exogenous protease. These results indicated that dietary protease could improve the metabolic processes of liver and kidney of fish when challenged with elevated plant ingredients in the diet. This is in contrast to the findings of Cai et

al. (2011) observed that dietary inclusion of CSM up to a concentration of 400 g kg^{-1} diet did not alter plasma levels of ALT and AST of crucian carp (Carassius auratus gibelio ♀ × Cyprinus carpio ♂). However, Liu et al. (2018) found that diets for rainbow trout containing CSM supplemented with 600 mg kg^{-1} protease achieved the minimum serum level of ALT and AST activities. These serum biochemical indices are usually employed to assess the nutritional and health status of fish (Hassaan et al., 2017). The increase rate of anabolic processes in fish may be due to increases in serum protein level to meet increased metabolic demands in fast growing fish, and the cyclic nature of the total serum protein is an indicator of the changes taking place in the serum globulin fraction (Helmy et al., 1974). Increases in proteinogram levels are thought to be associated with a stronger innate response in fish (Jha et al., 2007). Globulin level is very often used as an indicator of immune responses and a source of antibody production (Blazer and Wolke, 1984). In the present study, protease supplementation appeared to increase the levels of total protein, globulin and albumin in the serum of Nile tilapia fed diets with high inclusion level of CSM and supplemented with exogenous protease (Table 7).

Growth hormone (GH) initiates many of its growth-promoting actions by binding to GH receptors (GHRs) and stimulating the synthesis and secretion of insulin-like growth factor-I (IGF-I) from the liver (Reindl *et al.,* 2011). Cao et al. (2009) reported that IGF-1 is an important hormone involved in the growth and development of carp. In the present study, relative growth hormone (GH) gene expression was significantly ($P < 0.05$) down-regulated in pituitary and liver of fish fed different ratios of FM: CSM and supplemented with exogenous protease. Furthermore, there was a negative correlation between GH gene and growth performance.

The highest growth performance value was recorded by tilapia fed CSM_1 and CSM_2 and supplemented with 2500 U protease/ kg diet, but the expression of GH gene exhibited the

453 opposite trend. This finding was also confirmed by Pierce et al. (2005) who reported that
454 transcription of the GH gene was significantly ($P < 0.05$) higher during extended periods of fasting
455 or feed restrictions for Chinook salmon (*Oncorhynchus tshawytscha*). In our study, tilapia received
456 their nutritional requirements according to apparent satiation, but fish fed CSM_2 and CSM_3 and un-
457 supplemented with protease showed lowered feed utilization than other diets which were
458 supplemented with exogenous protease. These diets, in turn led to elevated expression of GH in
459 both brain and liver of fish possibly indicating a lower plane of nutrition and growth rate.

460 In this context, GH has important functions during inferior nutritional conditions and may serve
461 to spare protein use for energy and preferentially mobilize energy from stored lipid (Björnsson et
462 al., 2002). Protein malnutrition not only decreases IGF-I production rate, but also enhances its
463 serum clearance and degradation. Our results with tilapia are consistent the findings of Gómez-
464 Requeni et al. 2004 with Gilthead sea bream indicating that the activity of the GH-liver axis was
465 affected by dietary treatment. In comparison to fish fed a 100% FM diet, these latter investigators
466 reported increased circulating GH levels paralleled the decrease in circulating IGF-I levels.
467 However, a limitation in our study with tilapia was the lack of information regarding the plasma
468 level of these hormones for direct comparison.

469 In the present study, tilapia fed different FM: CSM ratios (2:1 and 1:1) and supplemented
470 with a protease enzyme showed the highest expression of IGF-I gene as compared to the other
471 treatment groups. From this data, there appeared a negative correlation between GH gene
472 expression and that of the IGF-I gene. Our results are therefore similar to those obtained by Duan,
473 (1998) who also reported there are negative correlations between IGF-I and GH in fish and the
474 high secretion level of GH was detected in fish during starvation to promote lipolysis. Our results
475 are also consistent with those acquired by Gómez-Requeni et al. (2004), Dyer et al. (2004).

476 Gómez-Requeni et al. (2004) and with Aksnes et al. (2006) who also concluded that, rainbow
477 trout and gilthead seabream fed diets containing 75% of plant protein mixtures replacing fish
478 meal protein gave the highest GH gene expression in liver probably caused by lower growth
479 rates on such diets due mainly to essential amino acid imbalance, and reduced availability of
480 protein for effective biosynthesis and anabolic pathways. There is evidence for selective organ
481 resistance to the growth-promoting effects of IGF-I in protein-restricted rats (Thissen et al.,
482 1994). All this revealed a state of GH-liver desensitization, a characteristic feature of catabolic
483 states. In the fasting rat model, liver growth hormone (GH) binding is decreased, providing one
484 explanation for decreased IGF-I (Ketelslegers et al., 1995). This may be more acute in
485 carnivorous fish compared to species like tilapia with a better ability to assimilate such low
486 protein diets. Gabillard et al. (2002) reported that although temperature seems to promote growth
487 through IGF-I secretion by the liver following GH stimulation, an impairment of nutritional
488 status would prevent the IGF-I stimulation and this may validate our findings with tilapia albeit
489 reared at a constant 28 °C. Gómez-Requeni et al. (2004) were the first to describe simultaneous
490 and nutritionally regulated changes in mRNA transcripts of GHR and IGF-I in fish species in
491 their experiments with seabream; although in our study we did not attempt to explore the GH
492 Receptor transcript for tilapia under the experimental conditions. It should be cautioned however,
493 that gene expression data does not always imply a functionality response in terms of the generation
494 of 'active' proteins synthesized during the post-translation of mRNA at the ribosomal level and
495 where proteins are modified such as in glycosylation, phosphorylation, and methylation by
496 enzymatic processes within the cell. It will be important to measure actual circulating hormonal
497 and associated metabolites directly in fish for further clarification as stated previously.

However, to the authors' knowledge, this is the first-time gene expression was measured in tilapia fed diets with different ratios of FM:CSM supplemented with exogenous protease. This requires further studies to establish the effect of exogenous digestive enzymes on tilapia gene expression that relate to growth and feed utilization as well as many other metabolic factors and to provide practical as well as scientific value to design more efficient feed formulations for tilapia and other fish species.

In conclusion, this investigation has provided good evidence for the benefits associated with the addition of an exogenous enzyme (protease) in association with high plant ingredient inclusion, namely CSM as one example. This is becoming an important technology to realizing the promise of greatly enhancing the nutritional quality and value of plant by-products in practical fish diets. Indeed, exogenous enzymes are being used successfully as functional feed additives and supplements to enhance digestion and growth in fish in the commercial sector. These will need to take into account processing techniques such as extrusion technologies where the higher temperatures encountered can modify and reduce enzyme activities and effects on any beneficial bioactive components that may be thermo-labile. It is evident that further research and development is needed in these areas to fully appraise these products as viable dietary supplements for fish and especially for tilapia.

References

Adeoye, A.A., Jaramillo-Torres, A., Fox, S.W., Merrifield, D.L. Davies, S.J., 2016a. Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: Overall performance and effects on intestinal histology and microbiota. Animal Feed Science and Technology 215, 133-143.

520 Adeoye, A.A., Yomla, R., Jaramillo-Torres, A., Rodiles, A., Merrifield, D. L., Davies, S.J.,
521 2016b. Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis*
522 *niloticus*) growth, intestinal morphology and microbiome. Aquaculture 463, 61-70.
523 Aksnes, A., Hope, B., Jönsson, E., Björnsson, B. T., Albrektsen, S., 2006. Size-fractionated fish
524 hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus mykiss*) fed high plant protein
525 diets. I: Growth, growth regulation and feed utilization. Aquaculture 261(1), 305-317.
526 Alonso, R., Aguirre, A., Marzo, F., 2000. Effects of extrusion and traditional processing methods
527 on anti-nutrients and in vitro digestibility of protein and starch in faba and kidney beans. Food
528 Chemistry 68, 159-165.
529 AOAC. (1990). Official methods of analysis. Virginia: Arlington. 1117p.
530 Baruah, K., Sahu, N.P., Pal, A.K., Jain, K.K., Debnath, D., Mukherjee, S.C., 2007. Dietary
531 microbial phytase and citric acid synergistically enhances nutrient digestibility and growth
532 performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. Aquaculture
533 Research 38(2), 109-120.
534 Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T.,
535 Jönsson, E., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and
536 mode of action. Fish Physiology and Biochemistry 27(3-4), 227-242.
537 Blazer, V.S., Wolke, R.E. 1984. The effects of α-tocopherol on the immune response and non-
538 specific resistance factors of rainbow trout (*Salmo gairdneri* Richardson). Aquaculture 37(1),
539 1-9.
540 Boyd, C.E., 1990. Water quality in ponds for aquaculture. Alabama Agricultural Experiment
541 Station, Auburn University. Alabama P462.

542 Björnsson, B.T., Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T.,
543 Jönsson, E., 2002. Growth hormone endocrinology of salmonids: regulatory mechanisms and
544 mode of action. Fish Physiology and Biochemistry 27(3-4), 227-242.
545 Cai, C., Li, E., Ye, Y., Krogdahl, A., Jiang, G., Wang, Y., Chen, L., 2011. Effect of dietary
546 graded levels of cotton seed meal and gossypol on growth performance, body composition
547 and health aspects of allogynogenetic silver crucian carp, *Carassius auratus gibelio* ♀ ×
548 *Cyprinus carpio* ♂. Aquaculture nutrition 17(4), 353-360.
549 Caine, W., Verstegen, M., Sauer, W., Tamminga, S., Schulze, H., 1998. Effect of protease
550 treatment of soybean meal on content of total soluble matter and crude protein and level of
551 soybean trypsin inhibitors. Animal Feed Science and Technology 71, 177-183.
552 Cao, Y., Chen, X., Wang, S., Chen, X. C., Wang, Y.X., Chang, J.P., Du, J.Z., 2009. Growth
553 hormone and insulin-like growth factor of naked carp (*Gymnocypris przewalskii*) in Lake
554 Qinghai: expression in different water environments. General and Comparative
555 Endocrinology 161, 400–406.
556 Cheng, Z. J., Hardy, R.W., 2002. Apparent digestibility coefficients and nutritional value of
557 cotton seed meal for rainbow trout (*Oncorhynchus mykiss*). Aquaculture 212(1), 361-372.
558 Coles, M. G., 1974. Physiological activity and detection: The effects of attentional requirements and
559 the prediction of performance. Biological Psychology 2(2), 113-125.
560 Committee on Food Chemicals Codex, 1996. Food Chemicals Codex, Fourth ed. National Academy
561 Press, Washington, DC.
562 Company, R., Astola, A., Pendón, C., Valdivia, M. M., Pérez-Sánchez, J., 2001. Somatotropic
563 regulation of fish growth and adiposity: Growth hormone (GH) and somatolactin (SL)
564 relationship. Comparative Biochemistry and Physiology 130C, 435–445.

565 Dalsgaard, J., Verlhac, V., Hjermitslev, N., Ekmann, K. S., Fischer, M., Klausen, M., Pedersen,
566 P. B., 2012. Effects of exogenous enzymes on apparent nutrient digestibility in rainbow trout
567 (*Oncorhynchus mykiss*) fed diets with high inclusion of plant-based protein. Animal Feed
568 Science and Technology 171(2-4), 181-191.
569 Divakaran, S., Velasco, M., 1999. Effect of proteolytic enzyme addition to a practical feed on growth of
570 the Pacific white shrimp, *Litopenaeus vannamei* (Boone). Aquaculture Research 30, 335-339.
571 Drew, M. D., Racz, V. J., Gauthier, R., Thiessen, D. L., 2005. Effect of adding protease to
572 coextruded flax: Pea or canola: Pea products on nutrient digestibility and growth performance
573 of rainbow trout, *Oncorhynchus mykiss*. Animal Feed Science and Technology 119, 117-128.
574 Duan, C., 1998. Nutritional and developmental regulation of insulin-like growth factors in fish.
575 Journal of Nutrition 128, 306S–314S.
576 Elmaki, H.B., Babiker, E.E., El-Tinay, A.H., 1999. Changes in chemical composition, grain
577 malting, starch and tannin contents and protein digestibility during germination of sorghum
578 cultivars. Food Chemistry 64, 331-336.
579 El-Saidy, D.M.S.D., Gaber, M.M.A., 2004. Use of cotton seed meal supplemented with iron for
580 detoxification of gossypol as a replacement of fish meal in Nile tilapia, *Oreochromis niloticus*
581 (L.) diets. Aquaculture Research 35, 859–869.
582 FAO, I., 2016. WFP (2015), The State of Food Insecurity in the World 2015. Meeting the 2015
583 international hunger targets: taking stock of uneven progress. Food and Agriculture
584 Organization Publications, Rome.
585 Farhangi M., Carter, C.G., 2007. Effect of enzyme supplementation to dehulled lupin-based diets
586 on growth, feed efficiency, nutrient digestibility and carcass composition of rainbow trout,
587 Oncorhynchus mykiss (Walbaum). Aquaculture Research 38, 1274-1282.

588 Francis, G., Maakar, H.P.S., Becker, K., 2001. Anti-nutritional factors present in plant-derived
589 alternate fish feed ingredients and their effects in fish. Aquaculture 199, 197-227.
590 Froehlich, H. E., Jacobsen, N. S., Essington, T. E., Clavelle, T., Halpern, B. S., 2018. Avoiding
591 the ecological limits of forage fish for fed aquaculture. Nature Sustainability 1(6), 298.
592 Furukawa, A., Tsukahara, H., 1966. On the acid digestion method for the determination of
593 chromic oxide as an index substance in the study of digestibility of fish feed. Bulletin of the
594 Japanese Society of Scientific Fisheries 32, 502-504.
595 Gabillard J.C., Weil, C, Rescan, P.Y., Navarro, I., Gutiérrez, J., Le Bail P. Y., 2002. Effects of
596 environmental temperature on IGF1, IGF2, and IGF type I receptor expression in rainbow
597 trout (*Oncorhynchus mykiss*). General Comparative Endocrinology 133 (2), 233-42.
598 Gómez-Requeni, P., Mingarro, M., Calduch-Giner, J. A., Médale, F., Martin, S. A. M., Houlihan,
599 D. F., Pérez-Sánchez, J., 2004. Protein growth performance, amino acid utilization and
600 somatotropic axis responsiveness to fish meal replacement by plant protein sources in gilthead
601 sea bream (*Sparus aurata*). Aquaculture 232(1-4), 493-510.
602 Goda, A.M.A., Mabrouk, H.A.-H.H., Wafa, M.A.E.-H., El-Afifi, T.M., 2012. Effect of using
603 baker's yeast and exogenous digestive enzymes as growth promoters on growth, feed
604 utilization and hematological indices of Nile tilapia, *Oreochromis niloticus* fingerlings.
605 Journal of Agricultural Science and Technology B 2, 15-28.
606 Hassaan, M. S., Goda, M. A-S., Kumar, V., 2017. Evaluation of nutritive value of fermented de-
607 oiled Physic nut, Jatropha curcas seed meal for Nile tilapia, *Oreochromis niloticus* fingerlings.
608 Aquaculture nutrition 23(3), 571-584.
609 Hassaan, M. S., Soltan, M. A., Mohammady, E. Y., Elashry, A. M., El-Haroun, E. R., Davies, S.
610 J., 2018. Growth and physiological responses of Nile tilapia, *Oreochromis niloticus* fed

611 dietary fermented sunflower meal inoculated with *Saccharomyces cerevisiae* and *Bacillus*
612 *subtilis*. Aquaculture 495, 592-601.
613 Helmy, A.M., Badawi, H. K., El-Bishry, A., 1974. Seasonal variations in the protein composition
614 of blood serum of Anguilla vulgaris and *Mugill cephalus*. Egyptian Journal of Aquatic
615 Research 4, 369-375.
616 Henry, R. J., 1964. *Clinical Chemistry: Principles and Technics.* [With a Bibliography]. Hoeber
617 Medical Division, Harper & Row.
618 Henry, R. J., Cannon, D. C., Winkleman, J. W., 1974. Automated determination of serum and
619 urine creatinine. Clinical chemistry, principles and techniques. New York, NY: Harper and
620 Row.
621 Hlophe S.N., Moyo, N.A.G., 2014. Evaluation of kikuyu grass and moringa leaves as protein
622 sources in *Oreochromis mossambicus* diet. African Journal of Aquatic Sciences 39, 305–312.
623 Hlophe-Ginindza, S. N., Moyo, N. A., Ngambi, J. W., Ncube, I., 2016. The effect of exogenous
624 enzyme supplementation on growth performance and digestive enzyme activities in
625 *Oreochromis mossambicus* fed kikuyu-based diets. Aquaculture Research 47(12), 3777-3787
626 Hussain, S. M., Afzal, M., Javid, A., Hussain, A. I., Ali, Q., Mustafa, I., Chatha, S. A. S., Shah, S.Z.
627 H., Hussain, M. Ullah, M.I., 2015. Efficacy of phytase supplementation on growth performance
628 and mineral digestibility of *Labeo rohita* fingerlings fed on cotton seed meal based diet. Pakistan
629 Journal of Zoology 47(3), 699- 709.
630 Idris, W.H., Abdel Rahaman, S.M., ElMaki, H.B., Babiker, E.E., El Tinay, A.H., 2006. Effect of
631 malt pretreatment on phytate and tannin level of two sorghum (*Sorghum bicolor*) cultivars.
632 International Journal of Food Science and Technology 41, 1229-1233.

633 Jackson, A.J., Capper, B.S., Matty, A.J., 1982. Evaluation of some plant proteins in complete
634 diets for the tilapia *Sarotherodon mossambicus*. Aquaculture 27, 97-109.
635 Jha, A. K., Pal, A., Sahu, N., Kumar, S., Mukherjee, S. 2007. Haemato-immunological responses
636 to dietary yeast RNA, ω-3 fatty acid and β-carotene in *Catla catla* juveniles. Fish and
637 Shellfish Immunology 23, 917–927.
638 Jiang, T.T., Feng, L., Liu, Y., Jiang, W.D., Jiang, J., Li, S.H., Zhou, X.Q., 2014. Effects of
639 exogenous xylanase supplementation in plant protein-enriched diets on growth performance,
640 intestinal enzyme activities and microflora of juvenile Jian carp (*Cyprinus carpio* var. Jian).
641 Aquaculture Nutrition 20(6), 632-645.
642 Kader, A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., Nguyen, B.T., Gao, J., Laining,
643 A., 2012. Can fermented soybean meal and squid by-product blend be used as fishmeal
644 replacements for Japanese flounder (*Paralichthys olivaceus*) Aquaculture Research 43, 1427–
645 1438.
646 Kader, M. A., Koshio, S., Ishikawa, M., Yokoyama, S., Bulbul, M., (2010). Supplemental
647 effects of some crude ingredients in improving nutritive values of low fishmeal diets for
648 red sea bream, Pagrus major. *Aquaculture, 308*(3-4), 136-144.
649 Ketelslegers, J.M, Maiter, D., Maes, M., Underwood, L.E., Thissen J. P., 1995. Nutritional
650 regulation of insulin-like growth factor-I. Metabolism 44, 50-7.
651 Kumar, V., Makkar, H., Becker, K., 2011a. Nutritional, physiological and haematological
652 responses in rainbow trout (*Oncorhynchus mykiss*) juveniles fed detoxified *Jatropha curcas*
653 kernel meal. Aquaculture Nutrition 17, 451–467.
654 Kumar, V., Khalil, W. K. B., Weiler, U., Becker, K., 2011b. Influences of incorporating
655 detoxified *Jatropha curcas* kernel meal in common carp (*Cyprinus carpio* L.) diet on the

656 expression of growth hormone-and insulin-like growth factor-1-encoding genes. Journal of
657 Animal Physiology and Animal Nutrition 97, 97−108.

658 Lee, K. J., Rinchard, J., Dabrowski, K., Babiak, I., Ottobre, J.S., Christensen, J. E., 2006. Long-
659 term effects of dietary cotton seed meal on growth and reproductive performance of rainbow
660 trout: three-year study. Animal Feed Science and Technology 126, 93-106.

661 Li, M. H. Robinson, E. H. 2006. Use of cotton seed meal in diets of aquatic animals: a review.
662 North American Journal of Aquaculture 68, 14-22.

663 Li, M. H., Peterson, B.C., Janes, C.L., Robinson, E.H., 2006. Comparison of diets containing
664 various fish meal levels on growth performance, body composition, and insulin-like growth
665 factor-I of juvenile channel catfish *Ictalurus punctatus* of different strains. Aquaculture 253,
666 628–635.

667 Lin S., Mai K., Tan B., 2007. Effects of exogenous enzyme supplementation in diets on growth
668 and feed utilization in tilapia, *Oreochromis niloticus* × *Oreochromis aureus*. Aquaculture
669 Research 38, 1645-1653.

670 Liu, W., Wu, J., Li, Z., Duan, Z., Wen, H., 2018. Effects of dietary coated protease on growth
671 performance, feed utilization, nutrient apparent digestibility, intestinal and hepatopancreas
672 structure in juvenile Gibel carp (*Carassius auratus* gibelio). Aquaculture Nutrition 24(1), 47-
673 55

674 Luo L., Xue, M., Wu X., Cai X., Cao, H., Liang, Y., 2006. Partial or total replacement of fish
675 meal by solvent-extracted cotton seed meal in diets for juvenile rainbow trout (*Oncorhynchus*
676 *mykiss*). Aquaculture Nutrition 12, 418-424.

677 Mbahinzireki, G. B., Dabrowski, K., Lee, K. J., El-Saidy, D., Wisner, E. R., 2001. Growth, feed
678 utilization and body composition of tilapia (*Oreochromis sp*.) fed cotton seed meal-based
679 diets in a recirculating system. Aquaculture Nutrition 7(3), 189-200.
680 Miller, D.R., Granzin, B.C., Elliott, R., Norton, B.W., 2008. Effects of an exogenous enzyme,
681 Roxazyme G2 Liquid, on milk production in pasture fed dairy cows. Animal Feed Science
682 and Technology 145, 194-208.
683 Mutch, D. M., Wahli, W., Williamson, G. 2005. Nutrigenomics and nutrigenetics: The emerging
684 faces of nutrition. The FASEB Journal 19, 1602–1616.
685 Anh, N.T.L., Kunhareang, S., Duangjinda, M., 2015. Association of chicken growth hormones
686 and insulin-like growth factor gene polymorphisms with growth performance and carcass
687 traits in Thai broilers. Asian-Australasian journal of animal sciences 28(12), 1686.
688 Ofojekwu, P.C., Ejike, C., 1984. Growth response and feed utilization in the tropical cichlid
689 *Oreochromis niloticus* (Linn.) fed on cotton seed-based artificial diets. Aquaculture, 42(1),
690 27-36.
691 Pérez-Sánchez, J., Calduch-Giner, J. A., Mingarro, M., de Celis, S. V. R., Gómez-Requeni, P.,
692 Saera-Vila, A., Valdivia, M. M., 2002. Overview of fish growth hormone family. New
693 insights in genomic organization and heterogeneity of growth hormone receptors. Fish
694 Physiology and Biochemistry 27(3-4), 243-258.
695 Pierce, A.L., Shimizu, M., Beckman, B.R., Baker, D.M., Dickhoff, W.W., 2005. Time course of
696 the GH/IGF-I axis response to fasting and increased ration in Chinook salmon (*Oncorhynchus*
697 *tshawytscha*). General and Comparative Endocrinology 140, 192–202.

698 Rairakhwada, D., Pal, A. K., Bhathena, Z. P., Sahu, N. P., Jha, A., Mukherjee, S. C., 2007.
699 Dietary microbial levan enhances cellular non-specific immunity and survival of common
700 carp (*Cyprinus carpio*) juveniles. Fish & shellfish Immunology 22(5), 477-486.
701 Rawling, M.D., Merrifield, D.L., Davies, S.J., 2009. Preliminary assessment of dietary
702 supplementation of Sangrovit on red tilapia (*Oreochromis niloticus*) growth performance and
703 health. Aquaculture 294, 118-122.
704 Reitman, S. Frankel, S., 1957. A colorimetric method for the determination of serum glutamic
705 oxaloacteic and glutamic pyruvic transaminases. American. Journal of Clinical. Pathology 28,
706 56.
707 Robinson, E.H., Rawles, S.D., Oldenburg, P.W., Stickney, R.R., 1984. Effects of feeding
708 glandless or glanded cotton seed products and gossypol to *Tilapia aurea*. Aquaculture 38(2),
709 145-154.
710 Robinson, E.H., Tiersch, T.R., 1995. Effects of long-term feeding of cotton seed meal on growth,
711 testis development, and sperm motility of male channel catfish *Ictalurus punctatus* brood fish.
712 Journal of the World Aquaculture Society 26, 426-431
713 Santiago, C. B., Lovell, R.T., 1988. Amino acid requirements for growth of Nile tilapia. The
714 journal of nutrition 118 (12), 1540-1546.
715 Shi, Z., Li, X.Q., Chowdhury, M.K., Chen, J.N., Leng, X.J., 2016. Effects of protease
716 supplementation in low fish meal pelleted and extruded diets on growth, nutrient retention and
717 digestibility of gibel carp, *Carassius auratus gibelio*. Aquaculture 460, 37-44.
718 Soltan M.A., 2009. Effect of dietary fish meal replacement by poultry by- product meal with
719 different grain source and enzyme supplementation on performance, faeces recovery, body
720 composition and nutrient balance of Nile tilapia. Pakistan Journal of Nutrition 8, 395–407.

721 Thissen J.P., Ketelslegers, J.M., Underwood, L.E., 1994. Nutritional regulation of the insulin-
722 like growth factors. Endocrine Reviews 15 (1), 80-101.
723 Thissen, J.P., Underwood, L., Ketelslegers, J.M., 1998. Nutritional Regulation of Hepatic
724 Production of IGF-1. In Nutritional Aspects of Osteoporosis (pp. 119-130). Springer, New
725 York, NY.
726 Van Soest, P. V., Robertson, J., Lewis, B., 1991. Methods for dietary fiber, neutral detergent
727 fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science
728 74 (10), 3583-3597.
729 Wotton, I. D., and Freeman, H., 1974. Microanalysis in medicinal biochemical. Churchill
730 Livingstone Press, Edinburgh, London.
731 Yang, J. L., Chen, H. C., 2003. Serum metabolic enzyme activities and hepatocyte ultrastructure
732 of common carp after gallium exposure. Zoological Studies 42, 455-461.
733 Yue, Y. R., Zhou, Q.C., 2008. Effect of replacing soybean meal with cotton seed meal on
734 growth, feed utilization, and hematological indexes for juvenile hybrid tilapia, *Oreochromis*
735 *niloticus* × *O. aureus*. Aquaculture 284(1-4), 185-189.
736

Table 1 Formulation and proximate composition of the experimental diets (g kg^{-1} dry matter)

	Experimental diets					
	¶CSM_1		†CSM_2		‡CSM_3	
	FM:CSM;2:1	FM:CSM;2:1+protease	FM:CSM; 1:1	FM:CSM; 1:1+protease	FM:CSM;1:2	FM:CSM;1:2+protease
Fish meal	150	150	110	110	80	80
Soybean meal	300	300	300	300	300	300
Cotton seed meal†	120	120	160	160	230	230
Yellow corn	220	220	220	220	220	220
Wheat bran	150	150	150	150	110	110
soybean oil	40	40	40	40	40	40
Vitamin &Minerals[1]	14.50	14.00	14.50	14	14.50	14
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5
Chromic oxide	5	5	5	5	5	5
Protease	-	0.5	-	0.5	-	0.5
Proximate analysis						
Dry matter	900.10	898.10	897.00	892.90	898.90	895.60
Crude protein	298.50	297.00	296.00	295.00	296.20	295.10
Ether extract	75.00	72.40	70.00	67.90	72.40	73.50
Ash	60.20	59.10	58.60	58.20	59.10	59.60
Fiber content	51.00	49.00	50.00	51.00	52.90	52.80
NFE[2]	515.30	522.50	525.40	527.90	519.40	519.00
Gross energy ($MJkg^{-1}$)[3]	18.85	18.84	18.77	18.71	18.76	18.78
Free gossypol (mg kg^{-1})	230.16	230.19	300.88	300.85	430.03	430.04

[1]Vitamin and mineral mixture kg^{-1} of mixture contains: 4800 I.U. Vit A, 2400 IU cholecalciferol (vit. D), 40 g Vit E, 8 g Vit K, 4.0 g Vit B_{12}, 4.0 g Vit B2, 6 g Vit B6, 4.0 g, Pantothenic acid, 8.0 g Nicotinic acid, 400 mg Folic acid, 20 mg Biotin, 200 gm Choline, 4 g Copper, 0.4 g Iodine, 12 g Iron, 22 g Manganese, 22 g Zinc, 0.04 g Selenium. folic acid, 1.2 mg; niacin, 12 mg; d-calcium pantothenate, 26 mg; pyridoxine. HCl, 6 mg; riboflavin, 7.2 mg; thiamin. HCl, 1.2 mg; sodium chloride (NaCl, 39% Na, 61% Cl), 3077 mg; ferrous sulfate ($FeSO_4.7H_2O$, 20% Fe), 65mg; manganese sulfate ($MnSO_4$, 36% Mn), 89 mg; zinc sulfate ($ZnSO_4.7H_2O$, 40% Zn), 150 mg; copper sulfate ($CuSO_4.5H_2O$, 25% Cu), 28 mg; potassium iodide (KI, 24% K, 76% I), [2]NFE (Nitrogen free extract) =100-(crude protein + lipid + ash +fiber content). [3]Gross energy calculated using gross calorific values of 23.63, 39.52 and 17.15 kJ g^{-1} for protein, fat and carbohydrate, respectively according to Brett (1973).

¶CSM_1 = (FM:CSM, 2:1) ; †CSM_2 = (FM:CSM, 1:1) ; ‡CSM_3 = (FM:CSM, 1:2)

Table 2 Hydrolyzed amino acids composition of experimental diets (%)

Essential amino acid	Experimental diets			Requirements of tilapia[#]
	CSM_1	CSM_2	CSM_3	
Arginine	2.12	2.02	1.96	1.18
Histidine	0.87	0.88	0.84	0.48
Lysine	2.11	1.95	1.78	1.43
Methionine	1.24	1.23	1.19	0.75
Leucine	2.42	2.44	2.35	0.87
Isoleucine	1.12	1.02	0.96	0.87
Threonine	1.59	1.54	1.52	1.05
Phenylalanine	1.49	1.53	1.51	1.05
Valine	1.53	1.51	1.45	0.78

[#]Requirements as percentage of dry diet for tilapia (Santiago and Lovell 1988)

Table 3 List of real time qPCR assays used in this experiment

Gene	Primers	Amplicon (bp)	GenBank no.
18s rRNA	F: GGTTGCAAAGCTGAAACTTAAAGG R: TTCCCGTGTTGAGTCAAATTAAGC	85	AF497908.1
IGF-I	F: GTTTGTCTGTGGAGAGCGAGG R: GAAGCAGCACTCGTCCACG	97	Y10830.1
GH	F: TCGACAAACACGAGACGCA R: CCCAGGACTCAACCAGTCCA	75	M2916

F: Forward primer

R: Reverse primer

Table 4 ~~mino acid hydrolyzed by the exogenous protease addition and the r~~Relative protease activity in the experimental diets CSM_1, CSM_2 and CSM_3 (Means ± SD; n = 4)

	Experimental diets		
	CSM_1 (FM: CSM; 2:1)	CSM_2 (FM: CSM; 1:1)	CSM_3 (FM: CSM; 1:2)
Without protease (mg mL^{-1})	15.92±0.51	14.86±0.56	13.78±0.32
With protease (mg mL^{-1})	21.13±0.38	18.89±0.68	17.46±0.49
Difference (mg mL^{-1})	5.21±0.35	4.12±0.18b	3.86±0.24
Relative activity of protease† %	70.45±3.19	68.03±2.54	67.99±3.69

Table 5 Growth response and feed utilization of Nile tilapia fed experimental diets for 84 days

FM:CSM ratios	Protease U kg^{-1}	Growth performance		Feed utilization		
		IBW[1] (g $fish^{-1}$)	WG[2] (g $fish^{-1}$)	FI [3] (g $fish^{-1}$)	FCR[4]	PER[5]
Individual treatment means[†]						
CSM_1 (2:1)	0	11.54	29.92[c]	40.92	1.37[a]	2.43[b]
CSM_1 (2:1)	2500	11.62	34.56[a]	41.92	1.21[b]	2.75[a]
CSM_2 (1:1)	0	11.63	27.58[d]	38.72	1.41[a]	2.38[c]
CSM_2 (1:1)	2500	11.67	33.23[a]	41.12	1.25[b]	2.69[a]
CSM_3 (1:2)	0	11.96	26.96[d]	39.05	1.45[a]	2.30[c]
CSM_3 (1:2)	2500	11.50	31.45[b]	40.61	1.29[b]	2.58[a]
Pooled S.E.M[¶]		0.02	1.18	0.99	0.063	0.123
Two-way ANOVA (p-value)						
FM: CSM		0.968	0.045	0.288	0.045	0.042
Protease		0.874	0.004	0.097	0.011	0.025
FM: CSM × Protease		0.961	0.041	0.790	0.032	0.021

[†]Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M[¶] = pooled standard error of the mean. Means followed by the same letter are not significantly different.

IBW[1] = initial body weightWG[2] = weight gain; FI[3] = feed intake g^{-1}fish; FCR[4] = feed conversion ratio; PER[5] = protein efficiency ratio.

Table 6 Apparent digestibility coefficient (%) of Nile tilapia fed experimental diets for 84 days

FM:CSM ratios	Protease U kg^{-1}	Apparent digestibility coefficient (%)			
		Dry matter	Crude protein	Crude lipid	Digestible energy
Individual treatment means†					
CSM_1 (2:1)	0	92.65^c	88.40^b	90.31^b	84.62^b
CSM_1 (2:1)	2500	94.25^a	90.46^a	91.75^a	87.00^a
CSM_2 (1:1)	0	93.50^b	86.66^c	90.17^b	83.20^b
CSM_2 (1:1)	2500	94.20^a	89.55^a	92.05^a	86.50^a
CSM_3 (1:2)	0	93.45^b	85.35^c	89.57^c	83.65^b
CSM_3 (1:2)	2500	93.22^b	88.40^b	90.16^b	86.18^a
Pooled S.E.M¶		0.113	0.493	0.556	0.749
Two-way ANOVA (p-value)					
FM: CSM		0.022	0.006	0.412	0.425
Protease		0.069	0.002	0.012	0.021
FM: CSM × Protease		0.001	0.017	0.021	0.013

†Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M¶ = pooled standard error of the mean. Means followed by the same letter are not significantly different.

Table 7 Apparent availability coefficients (%) of essential amino acids in experimental diets for Nile tilapia

Variables	Protease U kg^{-1}	Apparent availability coefficient (%)								
		Arginine	Histidine	Lysine	Methionine	Leucine	Isoleucine	Threonine	Phenylalanine	Valin
Individual treatment means[†]										
CSM_1 (2:1)	0	96.65[b]	86.23[b]	87.52[b]	86.23[b]	94.15[b]	93.63[b]	86.17[b]	96.65[b]	86.22[b]
CSM_1 (2:1)	2500	98.05[a]	88.15[a]	90.16[a]	93.14[a]	97.09[a]	97.60[a]	89.35[a]	98.05[a]	88.55[a]
CSM_2 (1:1)	0	95.53[c]	84.13[c]	81.25[c]	84.29[c]	93.53[c]	92.82[c]	84.43[c]	95.53[c]	85.43[c]
CSM_2 (1:1)	2500	97.15[a]	88.65[a]	89.97[a]	88.50[a]	96.21[a]	96.87[a]	87.51[a]	97.15[a]	87.11[a]
CSM_3 (1:2)	0	94.15[c]	83.17[c]	79.57[c]	83.01[c]	92.35[c]	89.47[c]	84.25[c]	94.15[c]	83.15[c]
CSM_3 (1:2)	2500	96.32[b]	86.40[b]	86.32[b]	85.18[b]	95.82[b]	93.33[b]	84.99[c]	96.32[b]	85.33[c]
Pooled S.E.M[¶]		0.683	0.963	0.96	0.921	0.988	0.963	0.978	0.890	0.992
Two-way ANOVA (p-value)										
FM: CSM		0.001	0.006	0.011	0.011	0.006	0.001	0.035	0.001	0.001
Protease		0.012	0.002	0.018	0.032	0.032	0.001	0.001	0.014	0.011
FM: CSM × Protease		0.032	0.017	0.031	0.014	0.002	0.013	0.021	0.001	0.002

[†]Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M[¶] = pooled standard error of the mean. Means followed by the same letter are not significantly different

Table 8 Proximate composition (g kg^{-1} dry matter) of Nile tilapia fed diet fed experimental diets for 84 days

FM:CSM ratios	Protease U kg^{-1}	Dry matter	Crude protein	Total lipid	Ash
Individual treatment means†					
CSM_1 (2:1)	0	272.60	153.10[a]	51.60	38.50
CSM_1 (2:1)	2500	261.30	146.70[a]	49.40	39.10
CSM_2 (1:1)	0	251.20	138.90[c]	47.20	35.20
CSM_2 (1:1)	2500	256.70	141.30[b]	51.10	35.70
CSM_3 (1:2)	0	255.00	142.30[b]	52.80	33.50
CSM_3 (1:2)	2500	259.10	142.50[b]	49.55	35.40
Pooled S.E.M¶		0.960	0.250	0.570	0.460
Two-way ANOVA (p-value)					
FM: CSM		0.563	0.011	0.231	0.113
Protease		0.121	0.035	0.657	0.322
FM: CSM × Protease		0.425	0.035	0.092	0.123

†Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M¶ = pooled standard error of the mean. Means followed by the same letter are not significantly different.

Table 9 Hematological parameters, differential red blood and white blood cells of Nile tilapia fed the experimental diets for 84 days.

FM:CSM ratios	Protease U kg^{-1}	Hemoglobin (g l^{-1})	Hematocrit (%)	RBCs ($\times 10^6$ mm^{-3})	WBCs ($\times 10^5$ mm^{-3})
Individual treatment means†					
CSM_1 (2:1)	0	16.25[a]	24.50[b]	1.75[c]	36.80
CSM_1 (2:1)	2500	16.80[a]	25.95[a]	2.15[a]	37.60
CSM_2 (1:1)	0	15.75[b]	21.95[c]	1.80[b]	36.25
CSM_2 (1:1)	2500	16.70[a]	27.05[a]	2.04[a]	37.00
CSM_3 (1:2)	0	13.50[c]	23.35[c]	1.84[b]	36.95
CSM_3 (1:2)	2500	15.95[b]	24.80[b]	2.03[a]	35.75
Pooled S.E.M¶		0.534	0.515	0.051	0.920
Two-way ANOVA (p-value)					
FM: CSM		0.041	0.425	0.042	0.781
Protease		0.021	0.025	0.032	0.051
FM: CSM × Protease		0.026	0.041	0.049	0.072

†Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M¶ = pooled standard error of the mean. Means followed by the same letter are not significantly different.

Table 10 Blood chemistry parameters of Nile tilapia fed the experimental diets for 84 days.

FM:CSM ratios	Protease U kg^{-1}	ALT (u l^{-1})[#]	AST (u l^{-1})[†]	Total protein (g dl^{-1})	Albumin (g dl^{-1})	Globulin (g dl^{-1})	Calcium (mg dl^{-1})	Phosphorus (mg dl^{-1})
Individual treatment means[†]								
CSM_1 (2:1)	0	25.05[a]	14.80[a]	4.74[b]	1.91[b]	2.83	8.21[b]	4.2[a]
CSM_1 (2:1)	2500	21.80[b]	12.70[b]	5.03[a]	2.20[a]	2.83	9.50[a]	4.7[a]
CSM_2 (1:1)	0	25.30[a]	15.80[a]	4.69[b]	1.83[c]	2.86	7.20[c]	3.15b
CSM_2 (1:1)	2500	22.20[b]	12.75[b]	5.35[a]	2.94[a]	2.86	9.18[a]	4.25a
CSM_3 (1:2)	0	25.75[a]	17.80[a]	4.86b	1.90[b]	2.95	8.15[b]	2.85[c]
CSM_3 (1:2)	2500	21.85[b]	13.80[b]	4.73b	1.95[b]	2.78	9.01[a]	3.51b
Pooled S.E.M[¶]		0.604	0.491	0.062	0.048	0.421	0.121	0.031
Two-way ANOVA (p-value)								
FM: CSM		0.0421	0.045	0.001	0.004	0.121	0.031	0.001
Protease		0.001	0.001	0.003	0.001	0.321	0.022	0.012
FM: CSM × Protease		0.021	0.031	0.042	0.002	0.221	0.001	0.001

[†]Treatments means represent the average values of three aquaria per treatment. Duncan multiple range test was conducted for individual means only if there was a significant interaction (ANOVA: $P < 0.05$). Pooled S.E.M[¶] = pooled standard error of the mean Means followed by the same letter are not significantly different

[#]ALT = Alanine aminotransferase; [†]AST = aspartate aminotransferase

Table 11 Two-way ANOVA (P-values) results of experimental diets on growth hormone (GH) and insulin like growth factor I (IGF-I) gene expression of Nile tilapia

Mohamed delete this table :**Commented [E1]**

Parameters	Probability (**P–value**)		
	FM: CSM	Protease	FM: CSM× Protease
GH in brain	0.001	0.001	0.012
GH in liver	0.012	0.011	0.021
IGF-I in brain	0.001	0.001	0.001
IGF-I in liver	0.001	0.014	0.011