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The effect of phytase, xylanase and their combination on growth performance and nutrient utilization in Nile tilapia

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ABSTRACT

Increasing the inclusion rate of plant ingredients will increase the content of non-starch polysaccharides (NSP) and phytate in the fish feed. Both NSP and phytate are undesired in fish feed due to their anti-nutritional properties. The main objective of the present study was to assess the impact of exogenous enzyme supplementation on growth, body composition, digestibility and the energy, nitrogen and phosphorus balances in Nile tilapia. Four experimental diets were tested in a 2 × 2 factorial arrangement of treatments. The first factor was phytase supplementation at a dose of either 0 or 1000 FTU/kg and the second factor was xylanase supplementation at a dose of 0 or 4000 U/kg. This resulted in a control diet (CON-CON) without enzymes, phytase diet (PHY-CON), xylanase diet (CON-XYL) and a diet with both xylanase and phytase (PHY-XYL). In total 24 tanks (6 replicates/treatment) were used with 30 (mean initial body weight 42 g) fish each. Fish were restrictively (80% of expected satiation) fed the experimental diets for 38 days. Growth was significantly affected by the interaction between phytase and xylanase supplementation ($P < 0.05$), showing a synergism between both enzymes. Growth at the CON-CON and CON-XYL diets were similar, whereas fish fed the PHY-CON had an improved growth. The effect of phytase supplementation on growth was further enhanced when xyalanse was supplemented (PHY-XYL diet). Phytase significantly improved the digestibility of dry matter, crude protein, carbohydrates, energy, ash, phosphorus and calcium ($P < 0.001$). Xylanase enhanced the digestibility of dry matter, crude protein, carbohydrates and energy significantly ($P < 0.05$). In contrast to growth, there was no significant synergetic effect of the combination of phytase and xylanase on the digestibility ($P > 0.05$). The significant synergetic effect of the combination of phytase and xylanase on growth was not reflected on the digestibility ($P > 0.05$). The nitrogen balance showed that the synergism on growth was predominantly due the significant synergistic effect of phytase and xylanase on the protein retention ($P = 0.005$). Both xylanase and phytase showed to be an effective tool to improve the nutrient availability and growth in Nile tilapia. Fish fed the diet supplemented with both phytase and xylanase had a significantly higher growth than all other treatments.

1. Introduction

The expected future growth of the aquaculture sector increases the pressure of using more sustainable and novel feed ingredients in aqua feeds (Tacon and Metian 2015). Plant ingredients are nowadays increasingly used to replace fishmeal to reduce cost, to improve the sustainability and to keep up with the demand of high quality protein. This results in a steady decline of dietary fish meal inclusion levels in aquafeeds (Carter and Hauler 2000; Shepherd and Jackson 2013). However, with the inclusion of plant ingredients such as soybean and rapeseed meal, the content of non-starch polysaccharides (NSP) as well as phytate will increase in the fish feed. Both NSP and phytate are undesired in fish diets through their anti-nutritional properties (Choct 1997; Francis et al. 2001; Sinha et al. 2011).

The NSP fraction generally remains undigested, as the enzymes to hydrolyse the glycosidic bonds are scarce or non-existing in the gastrointestinal tract of fish (Kuz'mina 1996; Choct 1997; Stone et al. 2003; Sinha et al. 2011). Besides that, the NSP fraction may influence the gut morphology, physiology and mucus layer, affecting the endogenous secretion of water, proteins, electrolytes and lipids. These changes can lead to a reduced nutrient digestibility (Choct 1997; Sinha et al. 2011). Phosphorus (P) in fishmeal is highly available for growth and

Abbreviations: NSP, non-starch polysaccharides; MEm, energy requirements for maintenance; REp, energy retained as protein; REf, energy retained as fat; PHY, phytase; XYL, xylanase; FCR, Feed conversion ratio

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metabolism compared to P from plants. In plants P is mostly present in the form of phytate, which is poorly available (Francis et al. 2001; Lall and Hardy 2003; Lall and Lewis-McCrea 2007). Increasing levels of dietary phytate reduced the performance of fish (Spinelli et al. 1983; Richardson et al. 1985; Francis et al. 2001). The main effects of phytate are through reduced bio-availability of minerals like Fe, Zn, Mg, Cu, Ca and P and the formation of phytic acid-protein complexes, reducing the solubility of protein (Nolan et al. 1987; Francis et al. 2001) and leading to increased endogenous amino acid losses. Moreover, a low P availability leads to an increasing discharge of P in the water. Enrichment of P in natural waters can contribute to eutrophication, negatively affecting the aquatic ecosystem by stimulating primary production, which can lead to anoxic conditions (Tyrrell 1999; Mainstone and Parr 2002; Peuhkuri 2002).

The use of enzymes like phytase, xylanase or β-glucanase in pigs and poultry is a common way to reduce the negative impact of phytate and NSP. With the breakdown of phytate and NSP through enzyme supplementation, the digestibility of the feed improves (Bedford and Schulze 1998). Recently, the use of exogenous carbohydrases in aqua feeds is getting more attention. Multiple responses have been addressed to exogenous carbohydrases and phytase supplementation like improved feed intake, improved growth rate and nutrient digestibility (Lin et al. 2007; Pimentel-Rodrigues and Oliva-Teles 2007; Goda et al. 2012; Castillo and Gatlin 2015). However, besides the improved nutrient digestibility and growth, enzymes may improve energy efficiency in general (i.e. by lowering energetic cost for digestion or an alteration in body composition). The energy spend on digestion is part of the energy requirements for maintenance (MEm) of an animal. Besides environmental factors, nutritional factors can influence the MEm of fish (Schrama et al. 2012). For example, the dietary electrolyte balance (dEB) of the diet showed to affect the MEm in Nile tilapia, using nutritional identical diets (Saravanan et al. 2012). In pigs and poultry, dietary phytase supplementation has been shown to affect the energy utilization (Kies et al. 2001). However, in fish information is lacking on the impact of enzyme application on the growth composition and energy partitioning and balance, in particular the effect on the MEm (i.e. energetic cost of digestion). In addition, synergy between enzymes have been observed on P and calcium (Ca) retention (Juanpere et al. 2005) and abdominal fat content (Tahir et al. 2005) in broilers. Similarly, synergy between different types of enzymes on fish can be expected.

Considering the above, the main objective of the presented study is to assess the impact of phytase and xylanase supplementation to the diet on the growth, body composition, digestibility and the energy, nitrogen and P balances. This was tested in a two by two factorial arrangement in Nile tilapia, to quantify the individual effects and the combination of the two enzymes.

2. Materials and methods

The experiment was approved by the Ethical Committee judging Animal Experiments of Wageningen University, The Netherlands, and carried out according to the Dutch law on animal experiments.

2.1. Diets

Four experimental diets were formulated in a 2 × 2 factorial arrangement. The first factor was phytase and the second was xylanase. This resulted in a control diet (CON-CON) without enzymes, a phytase diet (PHY-CON, *Buttiauxella* sp. Phytase at 1000 FTU/kg, Danisco Animal Nutrition, Marlborough, UK), a xylanase diet (CON-XYL, Danisco xylanase at 4000 U/kg, Danisco Animal Nutrition, Marlborough, UK) and a diet with both phytase and xylanase (PHY-XYL). The experimental diets were identical in ingredient composition (Table 1). Yttrium oxide (Y_2O_3) was included as an inert marker for digestibility studies. Diets were produced by SPAROS Lda. (Portugal), using extrusion. After extrusion, the respective enzyme in liquid form was coated onto the pellets prior to oil coating under vacuum. The experimental diets were formulated to meet the requirements of the essential nutrients according to the NRC (2011). Table 2 shows the analysed proximate composition and the measured enzyme activity of the experimental diets. The proximate composition was identical for all diets. The enzyme supplementation was successful with the intended recovery. Diets with (PHY-CON and PHY-XYL) and without (CON-CON and CON-XYL) phytase supplementation had an average measured

Table 1
Ingredient composition of the experimental diets.

Ingredient (%)	Diet
Soybean meal	20.0
Wheat DDGS[a]	10.0
Wheat gluten	10.0
Rapeseed meal	10.0
Sunflower meal	10.0
Wheat	7.43
Maize	7.50
Wheat bran	8.50
Full-fat rice bran	8.50
Fish oil	2.00
Soy oil	1.50
Monocalcium phosphate	0.95
Lime ($CaCO_3$)	1.50
Lysine	0.50
Methionine	0.40
L-Threonine	0.20
Premix[b]	1.00
Yttrium oxide	0.02

[a] Dried Distillers Grains with Solubles.
[b] Premix for freshwater fish, PREMIX Lda, Portugal. Vitamins (IU or mg/kg diet): DL-alpha tocopherol acetate, 200 mg; sodium menadione bisulphate, 25 mg; retinyl acetate, 20,000 IU; DL-cholecalciferol, 1500 IU; thiamin, 20 mg; riboflavin, 30 mg; pyridoxine, 15 mg; cyanocobalamin, 0.05 mg; nicotinic acid, 1750 mg; folic acid, 30 mg; ascorbic acid, 200 mg; inositol, 1000 mg; biotin, 2.5 mg; calcium panthotenate, 50 mg; choline chloride, 1000 mg, betaine, 500 mg. Minerals (g or mg/kg diet): cobalt carbonate, 2 mg; copper sulphate, 7.5 mg; ferric sulphate, 50 mg; potassium iodide, 0.3 mg; manganese oxide, 5.6 mg; sodium selenite, 0.1 mg; zinc sulphate, 5 mg; magnesium hydroxide, 10 mg; sodium chloride, 400 mg; potassium chloride, 900 mg; calcium carbonate, 2.15 g; excipient wheat middlings.

Table 2
Analysed chemical composition and enzyme activity of the experimental diets.

	Diet			
	CON		PHY	
	CON	XYL	CON	XYL
Analysed nutrient content (g/kg DM)				
Dry matter (DM, g/kg)	915	902	905	916
Crude protein	370	373	368	371
Crude fat	88	89	90	93
Carbohydrates[a]	460	456	460	455
Energy (kJ/g)	20.4	20.4	20.2	20.4
Ash	82	82	82	82
Phosphorus	9.5	9.5	9.4	9.4
Calcium	14.8	14.7	14.5	14.5
Yttrium oxide	0.186	0.180	0.161	0.159
Enzyme activity				
Phytase (FTU/kg)	130	101	1125	1477
Xylanase (U/kg)	–	4228	< 100	4241

PHY, phytase; XYL, xylanase; CON-CON = control diet; CON-XYL = diet supplemented with xylanase; PHY-CON = diet supplemented with phytase; XYL-PHY = diet supplemented with xylanase and phytase.
[a] Carbohydrates calculated as 1000 - (crude protein + crude fat + ash).

phytase activity of 1301 and 116 FTU/kg. Diets with (CON-XYL and PHY-XYL) and without (CON-CON and PHY-CON) xylanse had an average measured xylanase activity of 4235 and < 100 U/kg (Table 2). The diet formulation aimed to have a high phytate and NSP content and to magnify the response of the applied enzymes. Therefore, the protein in the diets was solely of plant origin. Sunflower meal, rapeseed meal and wheat DDGS (Dried Distillers Grains with Solubles) and a substantial amount of rice bran and wheat bran were included in the diets.

2.2. Fish, rearing and housing facilities

The experiment was performed at the Aquaculture Research Facility (ARF) of the Wageningen University, The Netherlands. Nile Tilapia (*Oreochromis niloticus*) from an all-male GIFT silver strain produced by Til-Aqua International BV (Someren, The Netherlands) were obtained. Fish were stocked in 24 rectangular 120 L tanks. Fish were fed a commercial diet prior to the experiment (Skretting E-1P Stella, containing 47% crude protein and 14% crude fat). The 24 tanks were divided over 3 racks of 8 tanks each. Each tank was randomly stocked with 30 fish. All tanks were connected to the same recirculation system, resulting in a common water supply and ensuring the same water quality for the inflow of each tank. The system consisted of a sump, settling tank and trickling filter with a water refreshment of 300 L/day. Each individual tank was connected to a swirl separator (AquaOptima AS, column height 44 cm; diameter 24.5 cm). The swirl separators, with detachable glass bottles were used to collect faeces and count feed spills for each tank separately. The flow through each tank was set at 7 L/min using a hand held liquid rotameter. All tanks were equipped with a cylinder shaped air stone. The air stone and water flow ensured sufficient dissolved oxygen.

Water quality parameters were checked regularly to ensure that the water quality remained within pre-set ranges. During the experiment, the temperature ranged from 26.8–28.3 °C, pH 6.8–7.9, dissolved oxygen between 4.7 and 7.5 mg/L, total ammonia nitrogen < 0.8 mg/L, nitrite < 0.15 mg/L and nitrate 100–250 mg/L, all within the pre-set ranges. Conductivity was 8000 μS/cm at stocking as a precaution, which was gradually lowered to 3000 μS/cm after week one. The photoperiod was set to 12 h light: 12 h dark (lights on 7:00, lights off 19:00).

2.3. Experimental procedure

The experiment was conducted for 38 days. The four experimental diets were assigned randomly per rack of eight tanks. This resulted in two replicates per rack and six replicates per treatment. From a common batch fish were caught and randomly assigned to one of the 24 tanks. In total 30 fish were stocked per tank with an average initial weight of 42 g. Fish per tank were group weighted while mildly sedated using (0.25 mL/L) 2-phenoxyethanol. At the end of the experiment all fish per tank were batch weighed and counted again while mildly sedated to determine final weight and calculate growth parameters. At the start of the experiment 20 fish from the same batch as the fish stocked were euthanized using an overdose of 2-phenoxyethanol (1.0 mL/L) for initial body composition determination. At the end of the experimental period, 10 fish per tank were randomly selected and euthanized for final body composition. Fish samples were stored at − 20 °C until further analysis.

The aim of the experiment was to test the effect of phytase, xylanase and their combination on growth, body composition, digestibility and the energy, nitrogen and P balances. Therefore, the fish were restrictedly fed to keep the amount of feed on dry matter (DM) basis per tank per day equal. Fish were fed 16 g $kg^{-0.8}$ BW day^{-1}, except the first six days when the daily ration was stepwise increased from 20% to 100% of the intended feed ration. The daily amount of feed was increased throughout the experiment by predicting fish growth and weight, using the average start weight of the fish (all treatments) and an expected feed conversion ratio (FCR) of 1.1. The daily feed ration was divided into two equal portions fed at 8:30 and 15:00 h. Fish were hand fed, feeding was completed within 1 h per tank. Feed spills recovered from the settling units were recorded per tank after each feeding moment. The diets were kept under refrigerated (4 °C) conditions throughout the experiment. Weekly a feed sample of 100 g was taken from each diet. The feed samples were pooled per diet and stored (4 °C) until further analysis.

Faeces were collected for digestibility studies from week 4 onwards, using the swirl separators for 5 days per week (not the weekends). The glass bottles were submerged in ice to prevent bacterial degradation of the faeces. Faeces were collected between the afternoon and morning feeding (16:30 PM–8:00 AM). Faeces were pooled per week and stored in aluminium trays at − 20 °C until further analysis.

3. Analytical procedures and calculations

3.1. Performance

The absolute growth (g) was calculated as the difference between the average individual initial (W_i) and final (W_f) body weight per fish. The specific growth rate (SGR) was calculated as $SGR = (LnW_f - LnW_i \times 100)/t$, where t is the duration of the experiment in days (d). The feed intake was recorded daily by weighing the feed given and refusals. In addition, the feed pellets recovered in the settling unit after feeding were counted. The FCR was calculated using the feed intake and weight gain per tank. The survival of fish per tank was calculated as $(N_f/N_i) \times 100$, where N_f is the final number of fish and N_i the initial number.

3.2. Digestibility

The apparent digestibility coefficient (ADC) of DM, crude protein (CP), crude fat, total carbohydrate, gross energy, ash, P and Ca was calculated, with the use of Yttrium as inert marker. The ADC was calculated according to the following formula (Cheng and Hardy 2002); $ADC\ (\%) = 100 \times [1 - (Y_i \times \text{amount nutrient in faeces})/(Y_f \times \text{amount nutrient in feed})]$, where Y_i is the concentration of Yttrium in the feed and Y_f the concentration of Yttrium in the faeces. The total amount of carbohydrates in feed and faeces was calculated as: DM − (crude protein + crude fat + ash).

3.3. Analyses

Frozen fish samples (− 20 °C) were ground twice using a meat mincer (Gastromaschinen, GmbH model TW-R 70; Feuma) with a 4.5 mm die and homogenised. Samples for the determination of DM and CP were taken from fresh samples, samples for crude fat and energy were freeze dried prior to the analyses. Collected faeces were oven dried at 70 °C. Feed, faeces and fish samples were analysed according to the same methods. DM content was determined by drying samples for at least 4 h at 103 °C until constant weight (ISO 6496, 1983). Ash content by incineration using a muffle furnace for 4 h at 550 °C (ISO 5984, 1978). CP (N × 6.25) was analysed by the Kjeldahl method (ISO 5983, 1979). Crude fat was measured by petroleum-ether extraction (Soxhlet method, ISO 5986). Energy content was measured bomb calorimetric by direct combustion (IKA® werke, C7000; IKA analysentechnik, Weitershem, Germany). Yttrium, P and Ca in feed and faeces were analysed using inductively coupled plasma-mass spectrometry (ICP-OES) according to the standard NEN 15510 (2007).

The phytase and xylanase activity of feed samples were analysed by Danisco Innovation Laboratories (Brabrand, Denmark). Phytase was analysed using the methods described by Yu et al. (2012). One FTU phytase was defined as the amount of enzyme required to release 1 μmol of inorganic P per minute from sodium phytate at pH 5.5 at 37 °C. Xylanase was analysed using the methods described by Romero

et al. (2013). One xylanase unit (U) was defined as the amount of enzyme that releases 0.48 μmol of reducing sugar as xylose from wheat arabinoxylan per minute at pH 4.2 and 50 °C.

3.4. Energy, nitrogen and P balance

The energy, P and nitrogen (N) balance parameters were calculated per tank and expressed on per fish basis as kJ/d, mg/d and mg/d respectively. The parameters were calculated as described by Saravanan et al. (2012). Generally balance parameters are expressed per metabolic body weight, however in the present study, fish were fed equal amount of DM of diets identical in nutrient composition and thus similar amount of energy, P and nitrogen. The final weights of the fish were affected by the enzyme supplementation (see result), using metabolic body weight would already create differences in balance parameters due to the differences in final body weight.

For the N balance, N intake was calculated as the product of feed intake and the dietary N content; digestible N intake as N intake times the digestibility coefficient of N; retained N as the difference between final and initial N body mass; branchial urinary N losses as the digestible N intake minus retained N. The N efficiency was calculated as retained N (RN) divided by digestible N (DN). The P balance was calculated according to the same principle as the N balance. For the energy balance, energy intake was calculated as the product of feed intake and dietary energy content; digestible energy (DE) intake as energy intake times the energy digestibility coefficient; brachial urinary energy (BUE) losses as branchial urinary N losses times the energy content of NH_3-N (24.9 kJ N/g), assuming that all N was excreted as NH_3-N; metabolisable energy (ME) intake as DE minus BUE; (RE) as the difference between final and initial body energy content; and heat production (HE) as ME minus RE. The MEm was calculated from the ME and the energy retained as protein (REp) and fat (REf). The following formula was used to calculate the MEm = ME − ((REp/0.5) + (REf/0.9)). In this calculation an energetic utilization efficiency of ME for protein gain of 50% and an energetic utilization efficiency of ME for fat gain of 90% was assumed (Lupatsch et al. 2003).

3.5. Statistical analysis

Statistical analyses were performed using the Statistical Analysis Systems (SAS) statistical software package version 9.3 (SAS Institute, Cary, NC, USA). All data were analysed for the effect of phytase and xylanase supplementation and their interaction by two-way ANOVA using PROC GLM. When the interaction between the enzymes phytase and xylanase was significant ($P < 0.05$), individual treatment means were compared using Tukey HSD (honest significant difference). All data were expressed as mean per treatment of the six replicates.

4. Results

4.1. Performance

Mean fish performance over the 38-day experimental period is given in Table 3. The average initial weight of the fish was 42 g and statistically identical ($P > 0.1$) among treatments. The survival of the fish during the experiment was high with a mean survival of 98.8%. Conform to the experiment set-up the absolute feed intake was uniform among treatments (1.56 g DM/d). The daily growth (g/d) and the specific growth rate (SGR %/d) were significantly affected by phytase and xylanase supplementation ($P < 0.05$). There was synergism between phytase and xylanase on growth (g/d) and the SGR, indicated by a significant interaction effect ($P < 0.05$). The effect of xylanase on daily growth was small, but in combination with phytase supplementation, xylanase enhanced growth. Combined phytase and xylanase had the highest growth (1.58 g/d). The average FCR for diets without xylanase (CON-CON and PHY-CON) was 1.10 versus 1.06 for the diets with xylanase (CON-XYL and PHY-XYL). For diets without phytase (CON-CON and XYL-CON) the average FCR was 1.14 versus 1.02 for the diets with phytase (PHY-CON and PHY-XYL). The FCR was the lowest for the diet (PHY-XYL) supplemented with both phytase and xylanase (0.99), there was no significant interaction effect between phytase and xylanase on the FCR ($P = 0.138$).

4.2. Digestibility

Apparent digestibility coefficient (ADC) of DM, CP, fat, carbohydrates, minerals and energy was significantly enhanced by the supplementation of phytase (Table 4, $P < 0.001$). The supplementation of xylanase only significantly enhanced the DM and macro nutrient digestibility ($P < 0.05$), with a tendency for significance for ash ($P = 0.064$) and P ($P = 0.053$) digestibility. The PHY supplementation had a large impact on the mineral ADCs, with an increase in ADC of ash, P and Ca by 120, 72 and 275%, respectively. Although phytase and xylanase act directly on phytate and carbohydrates, also the ADC of crude fat and CP increased with the supplementation of these enzymes. Phytase supplementation (average CON-CON and CON-XYL versus average PHY-CON and PHY-XYL) increased the ADC from 90.1 to 91.2% for CP, 92.9 to 93.9% for crude fat and 49.7 to 56.7% for carbohydrates. Xylanase supplementation (CON-CON and PHY-CON versus CON-XYL and PHY-CON) increased the ADC from 90.4 to 91.0% for CP, 93.2 to 93.6% for crude fat and 51.9 to 54.4% for carbohydrates. There was no significant interaction between phytase and xylanase supplementation on the digestibility of nutrients ($P > 0.05$), but a trend towards significance ($P = 0.068$) for interaction on P digestibility was present. Xylanase supplementation alone did not affect P digestibility but in combination with phytase, xylanase further increased P digestibility.

4.3. Body composition

The protein content was unaffected by enzyme supplementation ($P > 0.1$; Table 5). The phytase supplementation lowered the body fat content significantly with 11 g/kg fresh weight ($P < 0.001$). This corresponded with the significant lower DM ($P = 0.017$) and energy ($P < 0.001$) content for the fish fed the phytase diets. The DM, CP and fat content of the body were unaffected by the xylanase supplementation ($P > 0.05$). The phytase supplementation had a large impact on the whole body mineral content of the fish. Both the ash, P and Ca content of the body was significantly higher with phytase ($P < 0.001$). The xylanase supplementation slightly reduced the Ca contend of the fish significantly ($P = 0.046$) and had a tendency for significance ($P = 0.061$) to reduce the P content. There was no significant interaction found on the body composition between phytase and xylanase supplementation ($P > 0.05$). However, a tendency for a significant interaction effect on the Ca content was present ($P = 0.093$).

4.4. Balances

The nitrogen, energy and P balance expressed on fish basis as mg/d are displayed in Table 6. Parallel to the CP digestibility, the digestible N intake was significantly increased both by phytase ($P = 0.019$) and xylanase ($P < 0.001$) supplementation. There was a significant interaction effect ($P = 0.005$) on the N retention between the two enzymes, indicating its synergistic properties. Compared to the CON-CON diet the increase in N retention was 2.3, 7.4 and 17.9% higher for the CON-XYL, PHY-CON and PHY-XYL diets respectively. In particular, the increase of N efficiency resulted in an increase in N retention, besides reduced branchial and urinary N losses. Phytase and xylanase supplementation had a significant synergistic effect ($P = 0.011$) on N efficiency, inducing the synergetic effect between the enzymes on the N retention. The N efficiency was comparable for the CON-CON and CON-XYL diet (42.7 and 42.9%), the PHY-CON diet had a higher efficiency (45.7%),

Table 3
Effect of dietary enzyme supplementation on the performance of Nile tilapia over 38 days.

	Diet							
	CON		PHY		pooled	*P*-values		
	CON	XYL	CON	XYL	SEM	PHY	XYL	PHY * XYL
Survival (%)	98.3	96.7	100	100	–	0.133	0.607	0.607
Initial body weight (g)	41.7	42.3	41.8	41.4	0.300	0.206	0.862	0.105
Final body weight (g)	93.2	94.6	98.1	101.4	0.800	< 0.001	0.009	0.250
Feed intake abs. (g DM/d)	1.55	1.56	1.55	1.56	0.003	–	–	–
Growth (g/d)	1.36[a]	1.38[a]	1.48[b]	1.58[c]	0.018	< 0.001	0.004	0.048
SGR (%/d)	2.12[a]	2.12[a]	2.25[b]	2.36[c]	0.020	< 0.001	0.009	0.012
FCR	1.15	1.13	1.04	0.99	0.014	< 0.001	0.014	0.138

PHY, phytase; XYL, xylanase; CON-CON = control diet; CON-XYL = diet supplemented with xylanase; PHY-CON = diet supplemented with phytase; XYL-PHY = diet supplemented with xylanase and phytase; Feed intake abs., feed intake absolute; SGR, specific growth rate; FCR, feed conversion ratio. Values are means and the pooled standard error of the mean (SEM). Means within the same row not sharing a common letter are significantly different ($P < 0.05$).

Table 4
Effect of dietary enzyme supplementation on the apparent digestibility (ADC) coefficient of Nile tilapia. Values are means and the pooled standard error of the mean (SEM).

	Diet							
	CON		PHY		pooled	*P*-values		
	CON	XYL	CON	XYL	SEM	PHY	XYL	PHY * XYL
ADC (%)								
Dry matter	65.0	67.4	71.2	72.8	0.580	< 0.001	0.003	0.449
Crude protein	89.8	90.4	90.9	91.5	0.147	< 0.001	< 0.001	0.898
Crude fat	92.6	93.1	93.7	94.1	0.153	< 0.001	0.014	0.853
Carbohydrates	47.9	51.4	55.8	57.4	0.902	< 0.001	0.010	0.311
Energy	72.4	74.6	76.0	77.5	0.428	< 0.001	< 0.001	0.427
Ash	18.6	23.8	44.5	48.6	2.276	< 0.001	0.053	0.812
Phosphorus	39.3	39.3	66.1	68.9	0.726	< 0.001	0.064	0.068
Calcium	7.2	18.3	44.5	51.0	6.490	< 0.001	0.189	0.729

PHY, phytase; XYL, xylanase; CON-CON = control diet; CON-XYL = diet supplemented with xylanase; PHY-CON = diet supplemented with phytase; XYL-PHY = diet supplemented with xylanase and phytase.

whereas xylanase and phytase (PHY-CON diet) combined had the best N efficiency (49.1%). This pattern of increasing N efficiency for the PHY-CON and PHY-XYL diet was reflected in significant lower branchial and urinary N losses ($P < 0.05$).

Both DE and ME increased significantly with phytase and xylanase supplementation ($P < 0.001$), without a significant interaction ($P > 0.1$). REp is by definition identical to the N retention multiplied by 23.7 kJ/g. The REf increased significantly with XYL supplementation ($P = 0.011$), whereas phytase supplementation significantly decreased REf ($P = 0.001$). The phytase supplementation resulted in a leaner fish growth, which is reflected in the increase of the REp and REf ratio from 1.05 to 1.36. In contrast, xylanase supplementation increased both the REp and REf, but with minor impact on the protein deposition (Table 5). The total energy retention had a tendency to show a significant interaction effect ($P = 0.074$). Only xylanase supplementation significantly increased ($P = 0.001$) the RE energy. The effect on RE was the strongest for the fish fed the XYL-PHY diet. MEm was also expressed in kJ/kg$^{0.8}$/d since MEm is proportionally related to the metabolic body weight of fish. Phytase had a tendency to significantly affect MEm in kJ/d ($P = 0.065$), with numerically the highest MEm for the PHY-CON diet. Expressed in kJ/kg$^{0.8}$/d there are no significant main effects of phytase and xylanase supplementation, however combined there is an tendency for an significant interaction effect ($P = 0.092$). The CON-CON, CON-XYL and XYL-PHY diets had comparable energy

Table 5
Effect of dietary enzyme supplementation on body composition (on fresh weight basis) of Nile tilapia. Values are means and pooled standard error of the mean (SEM).

	Diet							
	CON		PHY		pooled	*P*-values		
	CON	XYL	CON	XYL	SEM	PHY	XYL	PHY * XYL
Unit in g/kg								
Dry matter	285	287	278	284	1.9	0.017	0.059	0.477
Crude protein	159	159	157	160	1.0	0.123	0.123	0.263
Crude fat	95	97	83	87	2.1	< 0.001	0.213	0.530
Energy (kJ/g)	7.3	7.4	6.7	7.0	0.08	< 0.001	0.039	0.479
Ash	30	30	36	35	0.60	< 0.001	0.134	0.527
Phosphorus	5.0	4.9	6.1	5.9	0.09	< 0.001	0.061	0.366
Calcium	7.7	7.6	10.4	9.6	0.21	< 0.001	0.046	0.093

PHY, phytase; XYL, xylanase; CON-CON = control diet; CON-XYL = diet supplemented with xylanase; PHY-CON = diet supplemented with phytase; XYL-PHY = diet supplemented with xylanase and phytase.

Table 6
Effect of dietary enzyme supplementation on nitrogen, energy and phosphorous balances of Nile tilapia.

	Diet							
	CON		PHY		pooled	*P*-values		
	CON	XYL	CON	XYL	SEM	PHY	XYL	PHY * XYL
Nitrogen balance (mg/d)								
Gross N intake	91.8	92.8	91.1	92.5	0.19	–	–	–
Digestible N intake	82.5	83.9	82.8	84.6	0.21	0.019	< 0.001	0.321
Branchial and urinary N loss	47.3[a]	47.9[a]	45.0[b]	43.0[b]	0.51	< 0.001	0.241	0.023
N retention	35.2[a]	36.0[a]	37.8[b]	41.5[c]	0.46	< 0.001	< 0.001	0.005
N efficiency (RN/DN, %)	42.7[a]	42.9[a]	45.7[b]	49.1[c]	0.57	< 0.001	0.005	0.011
Energy balance (kJ/d)								
Gross E intake	31.7	31.8	31.3	31.8	0.065	–	–	–
Digestible E intake	22.9	23.7	23.8	24.7	0.14	< 0.001	< 0.001	0.697
Branchial and urinary E loss	1.17[a]	1.19[a]	1.12[b]	1.07[b]	0.013	< 0.001	0.241	0.023
Metabolisable E	21.8	22.5	22.7	23.6	0.14	< 0.001	< 0.001	0.538
Heat E	11.7	12.0	13.2	12.8	0.25	< 0.001	0.891	0.200
Retained E	10.1	10.5	9.5	10.8	0.22	0.648	0.001	0.074
Retained E as protein	5.2[a]	5.3[a]	5.6[b]	6.2[c]	0.069	< 0.001	< 0.001	0.005
Retained E as fat	4.8	5.2	3.9	4.6	0.18	0.001	0.011	0.293
E maintenance	5.9	6.1	7.1	6.1	0.30	0.065	0.182	0.076
E maintenance (kJ $kg^{-0.8}$ d^{-1})	54.9	55.6	64.1	54.7	2.87	0.157	0.144	0.092
Phosphorus balance (mg/d)								
Gross P intake	14.7	14.7	14.6	14.6	0.030	–	–	–
Digestible P intake	5.8[a]	5.8[a]	9.6[b]	10.1[c]	0.11	< 0.001	0.040	0.032
Branchial and urinary P loss	0.3	0.5	0.7	1.3	0.29	0.069	0.219	0.437
P retention	5.4	5.3	9.0	8.8	0.25	< 0.001	0.602	0.952
P efficiency (RP/DP, %)	94.0	92.0	93.0	88.0	3.70	0.448	0.282	0.704

PHY, phytase; XYL, xylanase; CON-CON = control diet; CON-XYL = diet supplemented with xylanase; PHY-CON = diet supplemented with phytase; XYL-PHY = diet supplemented with xylanase and phytase; RN, retained nitrogen; DN, digestible nitrogen; RP, retained phosphorus; DP, digestible phosphorus. Values are means and pooled standard error of the mean (SEM). Means within the same row not sharing a common letter are significantly different ($P < 0.05$).

requirements (kJ/$kg^{0.8}$/d) for maintenance, while numerically the energy requirements for maintenance where higher for the PHY-CON diet.

The effect of enzyme supplementation on P digestibility (Table 4) was reflected in the digestible P intake. There was a significant interaction effect ($P = 0.032$) between phytase and xylanase on the digestible P intake with the highest digestible P intake (10.1 mg/fish/d) for the PHY-XYL diet. Xylanase supplementation only increased the digestible P in combination with phytase, indicated by a significant interaction effect (P = 0.032). A significant interaction effect of phytase and xylanase was not present on the P retention ($P = 0.952$), only phytase supplementation significantly increased the P retention (66%; $P < 0.001$). The P efficiency remained unaffected by the enzyme supplementation. The P efficiency was high with an average of 92% among treatments.

5. Discussion

The diets where formulated to meet the available P requirements for growth, estimated to be 4.5 g/kg DM feed by meta-analysis across fish species (Antony Jesu Prabhu et al. 2013). With the measured P levels of the diet (Table 2) and the ADC of P (Table 4), the available P level was calculated as 3.7, 3.7, 6.2 and 6.5 g/kg DM of feed for the CON-CON, CON-XYL, PHY-CON, and XYL-PHY diets, respectively. Therefore, the diets without phytase supplementation (CON-CON and CON-XYL) had available P levels below the requirement of 4.5 g/kg DM. The total amount of P in the diet of 9.5 g/kg DM feed (Table 2) is well above the NRC (2011) of 4.0 g/kg total P for tilapia. Due to the large impact of phytase supplementation on growth (Table 3), P body content (Table 5) and P retention (Table 6), it can be questioned whether the 3.7 g available P/kg DM of feed was sufficient to meet the P requirements. In addition, the whole P content of the fish fed the diets without phytase was approximately 30–38% lower compared to whole body P content of tilapia reported by Schneider et al. (2004), where the available P in the feed was minimal 6 g/kg DM. However, branchial and urinary losses (no distinction made between branchial and urinary losses) occurred, while with insufficient dietary P levels, one would expect waterborne P uptake, resulting in negative values for branchial and urinary losses. Even Schneider et al. (2004) measured small P uptake from the water, while the body P content was higher than in the present study. Moreover, when fish are fed a diet deficient in P, resulting in a decrease in P body content, P stored in bones can be used for ATP synthesis in order to realize growth by remineralisation. This can lead to bone deformities when the bone structure is not able to support the growth (Sugiura and Ferraris 2004; Lall and Lewis-McCrea 2007). The ability to use P stored in the bones (buffering effect) to realize growth can result in a delayed growth response when feeding a P deficient diet. It was seen in rainbow trout that the growth can stay unaffected until 80% of the initial body reserves of P is depleted (Hardy et al. 1993; Antony Jesu Prabhu et al. 2013). Therefore one could suggest that the possible effect of a P deficiency is minimal in the present experiment, as the experiment only lasted for 38 days and the body P content of the control diet was only 21% lower compared to the diets high in available P (phytase supplemented). Based on the observed strong impact of phytase supplementation on performance and increased whole body P content, it can be argued whether the present stated (available) P requirement in literature for Nile tilapia is correct.

Results of the present study showed that growth performance of the fish fed the control diet was relative low when comparing the FCR (1.15) with other studies performed at the research facilities (Wageningen University), using fish of comparable weight and feeding level (Schneider et al. 2004; Amirkolaie et al. 2006; Leenhouwers et al. 2007; Saravanan et al. 2012; Schrama et al. 2012). This higher FCR is most likely related to the high level of NSP and inositol bound phosphate in the diet, as those components are considered poorly digestible for monogastric animals and fish (Choct 1997; Stone 2003; Liebert and Portz 2005; Tahoun and Hammouda 2009; Sinha et al. 2011). The dietary high levels of NSP and phytate were incorporated to potentiate the possible effect and synergy between the enzymes xylanase and phytase.

Phytase supplementation in fish is studied to a larger extend

compared to carbohydrases like xylanase. Studies with phytase show improved P digestibility which is generally reflected in improved growth (Kumar et al. 2012). The effect of phytase supplementation on P digestibility depends on the dose of phytase, development stage of the fish, phytate level and type in the diet, which makes comparisons with other studies difficult (Satoh et al. 2002; Kumar et al. 2012). For Nile tilapia a dose of 750–1000 FTU/kg is recommended (Portz and Liebert 2004; Liebert and Portz 2005; Kumar et al. 2012), concurring with 1000 FTU/kg used in our study, which raised P digestibility by 68% (from 39.3 to 67.5%).

Studies assessing the effect of carbohydrases on growth and nutrient digestibility in fish are minimal compared to pigs and poultry (Castillo and Gatlin 2015). Studies testing the effect of xylanase generally apply the enzyme in an enzyme cocktail/mix. Hereby testing xylanase in combination with other enzymes like β-glucanase, cellulase, pectinase and protease (Stone 2003; Ogunkoya et al. 2006; Lin et al. 2007; Yildirim and Turan 2010; Ghomi et al. 2012). Results of various studies, which assessed the impact of carbohydrase supplementation on fish performance and nutrient digestibility, are not always in line with each other. Most studies found improved nutrient digestibility and performance (Lin et al. 2007; Yildirim and Turan 2010; Ghomi et al. 2012), while other studies found no or minimal effect on performance and nutrient digestibility (Ogunkoya et al. 2006; Yigit and Olmez 2011). The design of the present study allowed us to determine the impact on performance, digestibility, body composition and the balances of energy, nitrogen and P of the individual enzymes xylanase and phytase as well as the combination. Dalsgaard et al. (2012) tested the main effect of xylanase supplementation on rainbow trout (*Oncorhynchus mykiss*), using four diets: control diet (fishmeal), soybean meal, sunflower meal and rapeseed meal. In line with the present study, in Dalsgaard et al. (2012) the xylanase supplementation enhanced lipid and CP digestibility. However, the effect of xylanase was depending on the diet composition (soybean, sunflower or rapeseed based), most likely the presence of xylans. Whereby, the increase in nutrient digestibility by xylanase did not result in improved growth parameters ($P > 0.05$). In Jiang et al. (2014), xylanase, when applied in the right dose resulted in an significant ($P < 0.05$) higher feed intake, accompanied with an increased SGR in juvenile Jian carp (*Cyprinus carpio* var. Jian).

Besides the main effects, both enzymes combined resulted in a synergic effect on growth performance, increasing the growth (g/d) from 1.36 to 1.58 compared to the control diet. The effect of applying xylanase without phytase on growth was minor, although significant ($P = 0.004$), but in combination with phytase supplementation, xylanase enhanced growth. Tahir et al. (2005), showed synergistic effects between cellulase and hemicellulase on abdominal fat and viscosity using broilers, indicated by an interaction effect. Another study using broilers (Juanpere et al. 2005), observed interaction between phytase and glycosidase on P and Ca retention. Hitherto, to our knowledge the synergic effects between enzymes on performance have not been seen in fish. The synergic effect on performance is not explained by the digestibility of the macronutrients, with only a tendency for synergy on the P digestibility ($P = 0.068$). The N balance showed that the synergic effect on growth was predominately due to the significant synergistic effects on N efficiency ($P = 0.011$) and N retention ($P = 0.005$). This was also reflected in the energy balance, showing interaction between phytase and xylanase on the REp (P = 0.005). What caused this increased protein deposition needs further elucidation.

The increase in growth with both phytase and xylanase was related to an improved macro nutrient digestibility and for phytase to an improved Ca and P digestibility. The xylanase supplementation increased the protein, fat and total carbohydrate ADC by 0.6, 0.4, and 2.5%, respectively. Phytase increased supplementation the protein, fat and total carbohydrate ADC by 0.9, 1.0 and 7.0%, respectively. For the carbohydrate digestibility, it can be assumed that the increase in digestibility is due to the increase in NSP digestibility. The remaining carbohydrate components, starch and sugars in extruded diets are almost 100% digested in Nile tilapia (Amirkolaie et al. 2006; Haidar et al. 2016). Xylanase hydrolyses arabinoxylans, which are part of the NSP fraction (Dornez et al. 2009). Phytase liberates phosphate from the inositol in the phytate molecule by hydrolysis (Kumar et al. 2012). Without phosphate the inositol becomes available for digestion, improving the NSP digestibility. However, the level of phytate and thus inositol as part of the NSP fraction in the diet is minimal. The effect of xylanase and phytase supplementation on the fat and protein digestibility can be explained by, i.e. increasing the access to protein and fat for digestive protease and lipase (Adeola and Cowieson 2011). In addition, the possible induced negative effects of NSP on gut morphology, physiology and mucus layer, affecting the endogenous secretion of water, proteins, electrolytes and lipids will be lowered with improved NSP digestibility (Choct 1997; Sinha et al. 2011).

The energy balance showed that the available energy for the fish was used differently for the two studied enzymes (Table 6). Both enzymes increased the DE intake as well as the ME intake. The xylanase supplementation resulted in an increase in REf and REp, whereas phytase supplementation resulted in an alteration in the ratio between REp and REf. Here the REf decreased whereas the REp increased, resulting in leaner fish (Table 5). Since the gain of protein goes together with a higher water gain compared to fat gain (FAO 2001), the impact of phytase supplementation on growth on weight basis was larger compared to the xylanase supplementation. What caused this altered protein to fat deposition ratio in fish fed diets supplemented with phytase requires further research. In fish, it was observed that P deficiency led to an accumulation of body fat (Hardy 2010). If the available P level was deficient for the CON-CON and CON-XYL treatments it might explain the altered protein to fat deposition by fish fed diets supplemented with phytase.

Assessing the effect of enzyme supplementation on the MEm was one of the goals of the presented study. It was suggested that with enzyme supplementation the MEm could be lowered, by lowering the energetic cost for digestion or by an altering the body composition. In this study, the MEm was on average 57.3 ± 3.9 kJ $kg^{-0.8}$ d^{-1}. The MEm found were close to the estimate of 60 kJ $kg^{-0.8}$ d^{-1} by Lupatsch et al. (2010), and slightly lower than the average of 64.5 kJ $kg^{-0.8}$ d^{-1} found by Schrama et al. (2012) for Nile tilapia. The hypothesis of this study was that enzymes could improve the growth through reduced MEm (energetic cost of digestion). However, the enzyme supplementation did not significantly affect MEm ($P < 0.05$; Table 6). Numerically the energy requirements were higher for the PHY-CON diet, contrary to our expectation. The higher requirements for maintenance for the PHY-CON diet could be related to the observed increase in ash and P digestibility, increasing mineral uptake and possible affecting osmoregulation. Likewise, in Saravanan et al. (2013), a high dietary electrolyte balance induced by altering the ratio between Na, K and Cl resulted in significant higher MEm, suggesting that the increased MEm is due to an extra demand for energy to maintain the overall acid-base homeostasis. Although not directly involved in osmoregulation, P influences the functioning of the kidney, an important osmoregulatory organ. However, the XYL-PHY treatment with slightly higher ash and P digestibility did not show increased MEm compared to the other treatments. Information about P absorption in fish is lacking. Further studies are needed to elucidate the effect of P and phytase supplementation on osmoregulation in fish.

In summary, the supplementation of both xylanase and phytase showed to be an effective tool to improve the nutrient availability of the diet, enhancing the growth performance in Nile tilapia. The MEm was not reduced with the use of enzyme supplementation. The combination of xylanase and phytase resulted in positive synergetic effects on growth, explained by the interaction effect on protein retention and efficiency.

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