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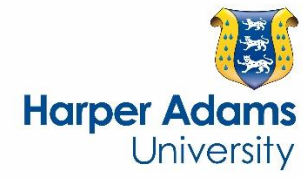

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Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: overall performance and effects on intestinal histology and microbiota

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Abstract

This study was conducted to evaluate the effects of exogenous enzymes on Nile tilapia (*Oreochromis niloticus*) growth and general health status. Tilapia (38.7 g) were fed one of four plant-based diets (408 g kg^{-1}protein, 78 g kg^{-1} lipid); one of which was a control and the remaining three were supplemented with exogenous enzymes (phytase, protease and carbohydrase at 300 mg kg^{-1}, 200 mg kg^{-1}, and 300 mg kg^{-1}, respectively). Tilapia fed the phytase supplemented diet displayed higher final body weight, FBW (94.9 g $fish^{-1}$) and specific growth rate, SGR (2.48 % day^{-1}) compared to tilapia fed the control diet (82.6 g $fish^{-1}$ FBW and 2.11 % day^{-1} SGR) ($P < 0.05$). In terms of feed conversion ratio, FCR and protein efficiency ratio, PER, tilapia fed diet supplemented with phytase (1.36 FCR and 1.08 PER) performed better ($P < 0.05$) than tilapia fed the control diet (1.68 FCR and 0.80 PER). However, the dietary treatments had no significant effect on tilapia somatic indices ($P > 0.05$). The level of circulatory red blood cells was higher ($P < 0.05$) in tilapia fed the carbohydrase supplemented diet (1.98 X 10^6 μL^{-1}) compare to those fed the control diet. Dietary treatments did not affect the mid-intestinal perimeter ratio, goblet cell abundance and intraepithelial leucocytes abundance. However, the microvilli density

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of the mid-intestine was higher ($P < 0.05$) in tilapia fed the phytase (15.6) and carbohydrase (16.0) supplemented diets compared to those fed the control (10.4) and protease (11.5) supplemented diets. The intestinal bacterial community profile of tilapia fed the carbohydrase supplemented diet was significantly altered in contrast to those fed the control diet ($P < 0.05$). The supplementation of diets with phytase has the potential to enhance tilapia growth without detrimental impacts on intestinal health.

Keywords: Phytase, protease, carbohydrase, histology, microbiota, microscopy

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Abbreviations: ANFs, anti-nutritional factors; NSPs, non-starch polysaccharides; IBW, initial body weight (g); FBW, final body weight (g); FI, feed intake (g); T, duration of feeding (days); WG, wet weight gain (g); PI, protein ingested (g); FL, final length (cm); LW, liver weight (g); VW, viscera weight (g); SGR, specific growth rate (% day^{-1}); FCR, feed conversion ratio; PER, protein efficiency ratio; K, condition factor; HSI, hepatosomatic index; VSI, viscero-somatic index; PCV, packed cell volume; RBC, red blood cells; WBC, white blood cells; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin concentration; MGG, May-Grünwald Giemsa; H&E, haematoxylin and eosin; AU, arbitrary units; IELs, intraepithelial leucocytes; M, microvilli foreground; B, microvilli background; MD, microvilli density; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; DGGE, denaturing gradient gel electrophoresis; BLAST, basic local alignment search tool; ANOVA, analysis of variance; OTU, operational taxonomic unit; SEM, pooled standard error of the mean.

Introduction

Aquaculture is recognized as the fastest growing agri-business sector and has thus become an important component of global food supply (FAO, 2014). However the increased production of intensively reared fish species necessitates the supply of high quantity and sustainable feed ingredients in balanced formulated diets for warmwater fish species. Tilapia production volumes rank second only to the carps, contributing significantly to global aquaculture supply (FAO, 2011; Wang and Lu, 2015). To support expanding tilapia production, there is a need for sustainable feed production. Plant by-products are of particular relevance within commercial diets for tilapia; oilseed meals (e.g. soybean, copra, rapeseed, etc.), legumes and pulses (e.g. peas, beans, etc.) as well as lupins and cereal by-products such as corn and gluten are commonly used ingredients. However, there are limitations on the inclusion levels of plant ingredients for most fish species due to the presence of anti-nutritional factors (ANFs) which impair utilisation of nutrients resulting in reduced growth, nutrient utilisation and feed efficiency (Francis et al., 2001; Khattab and Arntfield, 2009).

For example, phytate, protease-inhibitors and non-starch polysaccharides (NSPs) are important anti-nutritional factors often present in plant ingredients. Phytate is an indigestible form of phosphorus that has a low bioavailability for tilapia (NRC, 2011) due to absence of an intestinal phytase. In addition, phytate is capable of binding to positively charged proteins, amino acids and minerals in plants (Suhairin et al., 2010) thus reducing the bioavailability of nutrients. NSPs (typically cellulose, arabinoxylan and mixed-linked β-glucans) are major components of plant cell walls and are indigestible to fish. They may cause increased viscosity in the gut which consequently results in a reduced rate of digestion, nutrient absorption and

reduced feed intake (Zijlstra et al., 2010). Protease-inhibitors, abundant in seed and storage tissues of plants, impairs protein digestibility thereby resulting in adverse physiological effects and reduced growth in fish (Olli et al., 1994).

Supplementing tilapia diets with exogenous enzymes (phytases, NSPases and proteases) offers potential for better utilisation of nutrients from plant ingredients based on findings of enzyme applications in swine and poultry diets (Adeola and Cowieson, 2011). The application of phytase has been successful in breaking down phytate to increase mineral and nutrient digestibility in fish (Cao et al., 2007; Cao et al., 2008; Kiarie et al., 2010; Kiarie et al., 2013). NSP-degrading enzymes (e.g. cellulase, xylanase, etc.) are capable of disrupting plant cell wall integrity thereby reducing molecular size characteristics of NSPs. Consequently, this enhances rapid digestion by reducing viscosity in the gut (Zijlstra et al., 2010; Bedford and Cowieson, 2012). Supplementing diets with proteases has the potential to increase utilisation of crude proteins from plant ingredients by increasing crude protein digestibility. In addition, the application of exogenous enzymes can allow flexibility in diet formulation through incorporation of lower quality and less expensive plant ingredients. Apart from the potential of exogenous enzymes to promote growth and nutrient utilisation (Adeola and Cowieson, 2011), they may alter substrates availability for specific populations of gut microbes, thus, potentially altering bacterial community composition or activities (Bedford and Cowieson, 2012; Kiarie et al., 2013; Zhou et al., 2013; Jiang et al., 2014).

Although exogenous enzymes have been applied to enhance the utilisation of plant nutrients in aquaculture diets, the reported results have been inconsistent as reviewed by Adeola and Cowieson (2011), Kumar et al. (2012) and Castillo and Gatlin (2015). Consequently, there is a need for further investigations to establish the

benefits of dietary enzyme supplementation for fish. To the authors' knowledge, previous studies have not investigated the effects of exogenous enzymes on the intestinal microbiota and general health of tilapia. Given the growing body of literature which demonstrates that feed ingredients can impact fish intestinal health and micro-ecology (Dimitroglou et al., 2011; Zhou et al., 2013; Jiang et al., 2014; Merrifield and Carnevali, 2014), elucidating the effects of dietary enzymes on intestinal status is both timely and novel. Therefore, the objective of the present study was to investigate the effects of selected exogenous enzymes (phytase, protease and carbohydrase) on tilapia growth performance, haematoimmunology and intestinal health.

2. Materials and methods

The experiment was conducted under the UK Home Office project license PPL30/2644 and personal license PIL30/10510. All investigation complied with the UK Animals (Scientific Procedure) Act 1986 and the Plymouth University Animal Welfare & Ethical Review Committee.

2.1 Diet preparation

Four iso-nitrogenous and iso-lipidic diets were formulated (Table 1) according to the known nutritional requirements of tilapia (NRC, 2011). The three exogenous enzymes used for the trial were RONOZYME® Hiphos (phytase), RONOZYME® ProAct (protease), and ROXAZYME® G2 (carbohydrase) from DSM Nutritional Products. Three of the formulated diets were supplemented with the exogenous enzymes (phytase, protease, and carbohydrase at 300 mg kg^{-1}, 200 mg kg^{-1} and 300 mg kg^{-1}, respectively) at the expense of corn starch and the basal diet served as

control diet. The feed ingredients were thoroughly mixed, moistened with warm water (400 mL kg^{-1}) and then cold press extruded to produce 2 mm pellets using a PTM extruder system (model P6, Plymouth, UK). The diets were dried to ca. 5g kg^{-1} moisture in an air convection oven set at 45°C and their proximate composition analysed (Table 1) using AOAC protocols (AOAC, 1995)**.** After drying, the diets were stored in airtight containers prior to use.

2.2 Experimental design

Genetically male tilapia (*Oreochromis niloticus*) were obtained from North Moore Tilapia, Goxhill, UK and stocked in fibreglass tanks (72 L capacity each) for a period of 4 weeks to acclimatize. Thereafter, three hundred and sixty fish were randomly distributed into 12 tanks with three replicate tanks per dietary treatment (30 fish per tank; average weight = 38.7 ± 0.51 g) containing aerated recirculated freshwater. Tilapia were fed the experimental diets at 20 - 50 g feed kg^{-1} biomass per day in equal rations at 09.00, 13.00 and 17.00 hours for six weeks. Daily feed was adjusted on a weekly basis by batch weighing following a 24-h starvation period. Fish were held at 26.3 ± 0.8°C with a 12:12 h light: dark photoperiod. Water quality parameters were monitored daily and maintained at pH 6.2 ± 0.7 (adjusted with sodium bicarbonate as necessary) and dissolved oxygen > 6.0 mg L^{-1}. Ammonium, nitrite and nitrate levels were monitored weekly and water changes (~444.6 L, an equivalence of ~20% system volume) were undertaken weekly to minimise accumulation of these compounds.

2.3 Growth, feed utilisation and somatic indices

Growth performance, feed utilisation and somatic indices were assessed by specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER), condition factor (K), hepatosomatic index (HSI) and viscera-somatic index (VSI). Calculations were carried out using the following formulae:

$$SGR = 100 ((\ln FBW - \ln IBW)/T)$$

Where; FBW = final body weight (g) and IBW = initial body weight (g)

$$FCR = FI/WG$$

Where; FI = feed intake (g) and WG = wet weight gain (g)

$$PER = WG/PI$$

Where = WG = wet weight gain (g) and PI = protein ingested (g),

$$K = (100 \times FW)/ FL^3$$

Where FL = FL = final length (cm)

$$HSI = 100 (LW/ BW)$$

Where; LW = liver weight (g) and BW = body weight (g)

$$VSI = 100 (VW/ BW)$$

Where; VW = visceral weight (g)

All fish were euthanized with an overdose of buffered tricaine methanesulfonate, MS222 (Pharmaq Ltd. Hampshire, UK) at a concentration of 200 mg L^{-1} followed by destruction of the brain prior to sampling. For proximate composition analysis (AOAC, 1995), at the onset of the trial 12 fish were pooled to constitute three samples and at the end of the trial, three fish per tank were sampled.

The fish were also used to record viscera weight and whole body weight in order to calculate the hepatosomatic index (HSI) and visceromatic index (VSI).

2.4 Haemato-immunological parameters

Blood from three fish per tank was taken from the caudal arch using a 25 gauge needle and a 1 mL syringe after fish were anaesthetized with MS222 (Pharmaq Ltd. Hampshire, UK) at 150 mg L^{-1}. Blood smears were prepared for the determination of differential leucocyte counts and additional blood was left to clot for a period of 12 h (at 4°C) to isolate serum. Serum was isolated by centrifugation at 3600 g for 5 min and was stored at −80 °C. Haematocrit (measured and read as % packed cell volume; PCV), haemoglobin, red blood cells (RBC), serum lysozyme activity, white blood cells (WBC) and differential leucocyte proportions were determined according to standard methods as described by Rawling et al. (2009).

2.5 Intestinal histology

At the end of the trial, three fish per tank were sampled for histological appraisal (light and scanning electron microscopy) of the mid-intestine (n = 9). For light microscopy examination, the samples were fixed in 10% formalin, dehydrated in graded ethanol concentrations and embedded in paraffin wax. In each specimen, multiple sets of sections (5mm thick) were stained with May-Grünwald Giemsa (MGG), haematoxylin and eosin (H&E) and Alcian-Blue-PAS (Dimitroglou et al., 2010; Ferguson et al., 2010). The intestinal perimeter ratios (arbitrary units, AU) were assessed after Dimitroglou et al. (2009) and the numbers of intra epithelial leucocytes (IELs) and goblet cells in the epithelium, across a standardized distance of 100 µm (10 folds per specimen), was then calculated by averaging the cell

numbers from all specimens (Ferguson et al., 2010). For scanning electron microscopy examination, the samples were processed and analysed as described by (Merrifield et al., 2009a). The scanning electron microscope images were analysed for microvilli density (MD) of the enterocytes on top of the villi. The ratio between the microvilli covered area (M, foreground) to the gaps between the microvilli (B, background) was calculated (MD = M/B, AU). All images were analysed with ImageJ version 1.47 (National Institute of Health, USA).

2.6 Intestinal microbiota: PCR - DGGE

The intestines from two fish per tank (n = 6) were aseptically excised and the digesta from the posterior section removed. DNA extraction and PCR amplification of V3 region of 16S rRNA gene was undertaken as described by Merrifield et al. (2009b). The PCRs was conducted using the forward primer P3 which include a GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2 (5'-ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). PCR reactions (50 µL) contained 25 µL BioMix™ Red Taq [Bioline, UK], 1 µL of each primer [50 pmol/µL each MWG-Biotech AG, Germany], 1 µL DNA template and 23 µL sterile Milli-Q water). Positive and negative control templates were included in each assay; negative control (sterile, using molecular grade water as template) and positive control (DNA from cultured *Pediococcus acidilactici*). Touchdown thermal cycling was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA), under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for 1 min, 65 °C for 2 min, 72 °C for 3 min (Muyzer et al., 1993). The annealing temperature decreased by 1 °C every second cycle until 55 °C and then remained at

55 °C for the remaining 10 cycles. The PCR products were used to obtain DNA fingerprints of the bacterial communities present in the fish intestines on a 40-60% DGGE using a BioRad DGGE system (DCode™ System, Italy) as described elsewhere (Merrifield et al., 2009b). Selected dominant bands were then excised and DNA was eluted in TE buffer at 4 °C overnight before re-PCR. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen) according to manufacturer's instructions and sequenced by GATC laboratories (GATC-biotech laboratories, Germany). In order to obtain the taxonomic classification from the partial 16S rRNA sequences, a BLAST search in GenBank database (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed. The highest similarities were used to assign the taxonomic description of each sequence.

2.7 Statistical analysis

Data were analysed using one-way analysis of variance (ANOVA). Multiple comparisons were performed using Tukey post-hoc test. Differences were considered significant at a value of $P < 0.05$. The statistical analysis was carried out using SPSS for Windows (SPSS Inc., 22.0, Chicago, IL, USA). DGGE banding patterns were transformed into presence/ absence matrices based on band peak intensities (Quantity One® version 4.6.3, Bio-Rad Laboratories, CA, USA). The band intensities were measured (Quantity One® 1-D Analysis Software, Bio-Rad Laboratories Ltd., Hertfordshire, UK). Group differences for the microbiota were calculated using PERMANOVA of Bray-Curtis distances using PRIMER V6 software (PRIMER-E Ltd., Ivybridge, UK) (Anderson et al., 2008).

3. Results

3.1 Growth, feed utilisation and somatic indices

Growth, feed utilisation and somatic indices are presented in Table 2. Tilapia fed the phytase supplemented diet performed significantly better ($P < 0.05$) than those fed control and protease supplemented diets in terms of FBW. Tilapia fed the phytase supplemented diet also displayed better feed utilisation in terms of FCR and PER when compared to tilapia fed the control and protease supplemented diets. The fish fed phytase and carbohydrase supplemented diets have similar growth performance; there was no significant difference ($P < 0.05$) in their FBW and SGR. Good survival was recorded in all the treatments (i.e. ≥ 90%) but higher in phytase and protease treatments. The dietary treatments did not affect ($P > 0.05$) the fish somatic indices assessed.

3.2 Whole body composition

The whole body composition of tilapia fed the experimental diets is displayed in Table 3. The body moisture content of tilapia fed the protease supplemented diet was higher ($P < 0.05$) than those fed the control diet. However, there was no difference ($P > 0.05$) in the body moisture contents of tilapia fed the enzymes supplemented diets. The dietary treatment did not affect body lipid, protein or ash levels.

3.3 Haemato-immunological parameters

Haematological and immunological parameters are displayed in Table 4. Haematocrit, haemoglobin, leucocyte levels, mean corpuscular haemoglobin, mean corpuscular haemoglobin concentration and serum lysozyme activity were unaffected by the dietary treatments. However, red blood cell levels were higher ($P <$

0.05) in the blood of tilapia fed the carbohydrase diet compare to fish fed the control diet. There were no differences ($P > 0.05$) in the other haemato-immunological parameters measured.

3.4 Intestinal histology

Light and scanning electron microscopy revealed a normal and healthy morphology of the mid-intestines of tilapia fed the experimental diets. The intestines of the fish showed intact epithelial barrier with well organised villi-like mucosal folds, abundant IELs and goblet cells (Figures 1a–1h). The dietary treatments had no significant effects ($P > 0.05$) on the intestinal perimeter ratio, number of goblet cells or IELs (per 100µm) of the fish fed the experimental diets (Table 5). The fish intestines displayed healthy brush border with well organised and tightly packed microvilli revealing no signs of damage (Fig 1i–1l). However, the microvilli of the brush border of tilapia fed control and protease supplemented diets appeared to be less tightly packed (Figures 1i & 1k) compared to those fed phytase and carbohydrase supplemented diets (Figures 1j & 1l). Consequently, the microvilli density of the fish intestines was significantly different among tilapia fed the experimental diets; the microvilli density of tilapia fed the phytase and carbohydrase supplemented diets were significantly higher ($P < 0.05$) than that of tilapia fed the control and protease supplemented diets (Table 5).

3.5 Intestinal microbiota

The bacterial community of tilapia fed the experimental diets were analysed by PCR-DGGE. The DGGE banding patterns of the 16S rRNA V3 region from the fish intestinal digesta is displayed in Figure 2 and the taxonomic affiliation of the

DGGE bands is displayed in Table 6. A total of eleven OTUs (operational taxonomic units) were selected from the DGGE for sequencing. OTU #9 was detected in all tilapia fed the experimental diets and had 100% sequence alignment to *Clostridium ghonii*. OTU #5 was uniquely detected in tilapia fed carbohydrase supplemented diet and was identified as belonging to *Acinetobacter schindleri* (97%). OTU #7 was common in tilapia fed phytase and protease supplemented diets and had 99% sequence alignment to *Arthrobacter russicus.* OTU #8 was common in tilapia fed protease and carbohydrase supplemented diets and had 99% sequence alignment to *Sporosarcina aquimarina.* OTUs #10 and #11 were common in tilapia fed control and phytase supplemented diets and both had 99% sequence alignment to *Austwickia chelonae* and *Intrasporangium calvum,* respectively. OTUs #1 and #3 were present in all tilapia fed the exogenous supplemented diets and had 96% and 83% sequence alignment to *Aquisphaera giovannonii* and uncultured bacterium clone AMD-A65, respectively. OTUs #2, #4 and #6 were common in tilapia fed the control diet and had 93%, 81% and 99% sequence alignment to *Marinobacter hydrocarbonoclasticus, Desulforegula conservatrix, and Arthrobacter russicus,* respectively. Firmicutes was the most frequently identified phylum across all the treatments and high frequency of the OTUs from the *Clostridium* genus was also evident. In terms of the number of OTUs, species richness and diversity, no significant differences were observed among the treatments (Table 7). However, high variability in the bacterial community structure was observed among individuals in the same group in all the treatments; the control group showed the greatest differences (Figure 3). The dietary treatments did not affect ($P > 0.05$) the species diversity of PCR-DGGE fingerprints. However, Permanova analysis revealed a

significant difference in bacterial community composition of fish fed the control and carbohydrase supplemented diets (Table 7).

4. Discussion

The potential of exogenous enzymes to enhance aquaculture production by liberating potentially unavailable plant nutrients within specific plant feed ingredients warrants more study to validate their effectiveness in fish feed. To this end, the present trial was conducted to establish and document the effects of dietary phytase, protease and carbohydrase on Nile tilapia production and health when supplemented to diets containing narrow-leafed lupin and soybean proteins. Diet supplementation with exogenous enzymes, especially phytase and carbohydrase, may neutralise some of the negative effects of anti-nutritional factors, increase nutrient bioavailability and consequently improve diet nutritional quality. In the present study, improved growth (FBW and SGR) of fish fed the phytase supplemented diet infers improved nutrient bioavailability. Confirming this, fish fed the phytase diet displayed significantly better FCR and PER values than the control group. This could be attributed to better utilisation of previously sequestered nutrients released by the effect of phytase on phytate-bound nutrients such as phosphorus. Cao et al. (2008) reported the same effect when Nile tilapia were fed with a phytase supplemented diet; the phytase supplemented diet gave better growth performance, FCR and PER compared with the control group. This is also in agreement with previous findings from Portz and Liebert (2004) and Nwanna (2007) on improved digestibility and growth performance effects of phytase on plant-based diets fed to Nile tilapia. However, there are some reports of non-effects of dietary phytase provision on growth performance and nutrient utilisation in fish (Cao et al., 2007). This could

possibly be due to the fact that removal of phytate could enhance the influence of other anti-nutritional factors and shield amino acids from degradation or reduce leaching of water soluble components (Cao et al., 2007). This could also be attributed to enzyme dosage (activity) and substrates available for enzymatic reaction. Although not undertaken in the present study, a future digestibility trial may validate this possibility for tilapia to provide more detailed information on nutrient and mineral availability.

Tilapia fed the carbohydrase supplemented diet had similar growth performance (FBW & SGR) with tilapia fed the control diet which is in agreement with the findings by (Yigit and Olmez, 2011) who reported no benefits on growth when tilapia were fed a carbohydrase supplemented diet. It was hypothesised by the authors that protease supplementation could degrade complex proteins in the diet into usable amino acids and peptides thereby resulting in improved protein digestibility and growth performance. In the current study however, growth performance and nutrient utilisation of fish fed the protease supplemented diet were not significantly different from the fish fed control diet. Contrary to this, Dias et al. (2014) reported a positive effect of protease on tilapia growth performance fed a lower crude protein diet compared to the higher crude protein diet in the present study. It could be inferred from this report that the protease effect is likely to be more pronounced in a low crude protein and low fishmeal diet. The non-effect of protease in a relatively higher crude protein diet could be as a result of non-beneficial effects of digestible protein when the level exceeds the requirement for fish maintenance and growth.

Haematological parameters are useful for monitoring fish general health and physiological responses to stress. In this study, an elevated red blood cell count was

observed in fish fed the carbohydrase supplemented diet. The increased red blood cells could infer better immune response (Jiang et al., 2007). As there is no clear understanding of established interaction between exogenous enzymes and fish haematological status, further study is required to establish the mode of action between exogenous enzymes and haematological parameters. In terms of gastrointestinal morphology, there was no significant difference in mid-intestine with respect to perimeter ratios, goblet cells levels and IELs levels, but significantly higher microvilli density (a measure of absorptive intestinal surface area) was observed in tilapia fed the phytase and carbohydrase supplemented diets. This is in line with improved growth performance and nutrient utilisation mentioned earlier and may have been a contributory factor to the observed growth parameters.

To the authors' knowledge, this is the first study investigating the effect of exogenous enzymes (phytase, protease and carbohydrase) on the intestinal microbiota of tilapia in a feeding trial. Previous studies have demonstrated that different feed additives such as antibiotics (He et al., 2010), probiotics (He et al., 2013; Standen et al., 2013; Standen et al., 2015) and prebiotics (Qin et al., 2014) can modulate the gut microbiota in tilapia. Zhou et al. (2013) reported significant changes in bacteria species and density of the intestinal microbiota of grass carp (*Ctenopharyngodon idella*) fed cellulase supplemented duckweed-based diets. In the present study, the predominant allochthonous bacterial species in the intestine of tilapia was *Clostridium ghonii,* which was the only phylotype found in all the individual tilapia regardless of the dietary treatment. Other authors have also found members of Family *Clostridiaceae* in tilapia's intestine suggesting that members of this family may be adapted to play an important role in the tilapia gut system (Zhou et al., 2011). There were some phylotypes that were only present in the intestine of

fish fed with certain exogenous enzymes. For instance, OTU #1 which had 96% similarity with *Aquisphaera giovannonii* was present in intestines of tilapia fed phytase, protease and carbohydrase supplemented diets but was not detected in the intestine of tilapia fed control treatment. The occurrence of specific bacterial members of *Proteobacteria* and *Actinobacteria* were selectively associated to particular dietary treatment. The *Proteobacteria* was present in the control and carbohydrase treatments while the *Actinobacteria* was detected in control, phytase and protease treatments. The presence of *Proteobacteria* and *Actinobacteria* in the intestine of tilapia is in agreement with previous research that used molecular techniques to assess gut microbiota in tilapia (Standen et al., 2015). Permanova analysis revealed that inclusion of exogenous carbohydrase in diet of tilapia altered significantly the bacterial community composition in the intestine of fish in contrast to that of tilapia fed the control diet. This finding suggests that this specific enzyme may have a modulating effect on the diet substrate profile thereby promoting or decreasing certain bacterial groups in the intestine.

In conclusion, tilapia fed diet supplemented with phytase exhibited superior growth performance in contrast to fish fed the control diet. This change did not have detrimental impacts on the haematological, intestinal morphological or intestinal microbiological parameters investigated. A significant difference was observed in the intestinal microbiota of tilapia fed the carbohydrase supplemented diet when compared to those fed the control diet. Although the microbiota species diversity parameters were not affected by dietary treatment, Permanova analysis revealed differences in the community profiles. Further quantitative studies are necessary to confirm how exogenous enzymes (especially carbohydrase) modulate intestinal

424 microbiota and if these modulations contribute towards the improved growth
425 performance of the host.
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Table 1 Formulation and composition of the experimental diets

Ingredients (g kg^{-1})	Control	Phytase	Protease	Carbohydrase
Soybean protein[a]	353.0	353.0	353.0	353.0
Narrow-leafed lupin meal[b]	250.0	250.0	250.0	250.0
Corn starch[c]	210.0	209.7	209.8	209.7
Herring meal LT94[d]	100.0	100.0	100.0	100.0
Corn oil	21.7	21.7	21.7	21.7
Fish oil	20.0	20.0	20.0	20.0
Lysamine pea protein[e]	20.0	20.0	20.0	20.0
Vitamin & mineral premix[f]	20.0	20.0	20.0	20.0
CMC-binder[c]	5.00	5.00	5.00	5.00
Phytase[g]	0.00	0.30	0.00	0.00
Protease[h]	0.00	0.00	0.20	0.00
Carbohydrase[i]	0.00	0.00	0.00	0.30
BHT[f] (mg)	75.0	75.0	75.0	75.0
Ethoxyquin[f] (mg)	7.50	7.50	7.50	7.50
Alpha tocopherols[f]	0.20	0.20	0.20	0.20
Total	1000	1000	1000	1000
Composition (g kg^{-1} dry weight basis)				
Moisture	70.4	74.3	64.9	59.8
Crude protein	406.3	408.6	406.5	410.1
Lipid	77.7	74.9	82.4	78.5
Ash	63.5	64.8	65.0	64.6
Energy (MJ kg^{-1})	19.2	19.2	19.1	19.3
NFE[j]	190.3	185.7	190.3	193.6

[a]Hamlet HP100, Hamlet Protein, Denmark
[b]Soya UK
[c]Sigma- Aldrich Ltd., UK
[d]Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK
[e]Roquette Frêres, France
[f] Premier nutrition vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorus 5.2 g kg^{-1}
[g] RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products
[h] RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products
[i] ROXAZYME® G2 (contains 2700U g^{-1} xylanase, 700U g^{-1} β-glucanase and 800U g^{-1} cellulose) from DSM Nutritional Products
[j]Nitrogen - free extracts (NFE) = dry matter – (crude protein + crude lipid + ash).

582 **Table 2** Growth, feed utilisation and somatic indices of fish fed the experimental diets for 6 weeks

	Control	Phytase	Protease	Carbohydrase	Pooled SEM	P-value
IBW (g fish^{-1})	38.6	38.9	38. 6	38.9	0.16	0.88
FBW (g fish^{-1})	82.6^{a}	94.9 ^{b}	85.6 ^{a}	89.4 ^{ab}	1.61	0.001
SGR (% day^{-1})	2.11^{a}	2.48^{b}	2.21^{ab}	2.31^{ab}	0.05	0.02
FCR	1.68^{a}	1.36^{b}	1.55^{a}	1.50^{ab}	0.04	0.00
PER	0.80^{a}	1.08±^{b}	0.88^{a}	0.94^{ab}	0.04	0.01
HSI	1.65	1.50	1.68	1.73	0.05	0.48
K-factor	1.97	1.93	2.02	1.94	0.03	0.82
VSI	11.5	10.1	10.5	10.2	0.25	0.22
Survival (%)	90.0^{a}	100^{b}	100^{b}	97.8^{ab}	1.66	0.08

583 Means in the same row with different superscripts are significantly different ($P < 0.05$). IBW, initial mean body weight; FI, daily feed
584 intake; FBW, final mean body weight; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein
585 efficient ratio; HIS, hepatosomatic index and VSI, viscera-somatic index

586

587 **Table 3** Whole body composition of tilapia fed the experimental diets (g kg^{-1} wet weight)

	Control	Phytase	Protease	Carbohydrase	Pooled SEM	P-value
Moisture	730.4[a]	740.0[ab]	747.0[b]	738.3[ab]	0.22	0.03
Protein	152.8	155.6	145.5	154.0	0.30	0.74
Lipid	87.6	70.5	74.1	81.6	0.27	0.13
Ash	23.2	30.4	25.7	27.3	0.28	0.08

588 Means in the same row with different superscripts are significantly different ($P < 0.05$).

589

590 **Table 4** Haematological and immunological parameters of fish fed the experimental diets after 6 weeks

	Control	Phytase	Protease	Carbohydrase	Pooled SEM	P-value
Haematocrit (%PCV)	40.5	41.1	41.4	40.9	1.81	0.78
Haemoglobin (g dL^{-1})	6.60	6.94	7.80	6.93	0.26	0.47
RBC (10^6 μL^{-1})	1.50^a	1.66^a	1.64^a	1.98^b	0.06	0.01
WBC (10^3 μL^{-1})	24.3	24.1	21.2	28.4	1.68	0.57
MCV (fL)	260.9	250.7	232.5	183.8	12.6	0.12
MCH (pg)	46.3	42.3	48.1	35.5	2.34	0.24
MCHC (g dL^{-1})	16.4	16.9	17.2	16.9	0.50	0.96
Serum lysozyme (U)	73.6	74.7	100.9	80.2	5.92	0.37
Lymphocytes (10^3 μL^{-1})	22.6	21.9	19.5	25.9	1.53	0.59
Monocytes (10^3 μL^{-1})	0.8	1.09	0.7	1.13	0.08	0.11
Granulocytes (10^3 μL^{-1})	0.92	1.09	0.97	1.33	0.08	0.28

591 Figures in each row with similar superscript are not significantly different (*P > 0.05*). RBC, red blood cells; WBC, white blood cells;
592 MCV, mean corpuscular volume (haematocrit (%PCV)*10)/RBC 106 μL^{-1}); MCH, mean corpuscular haemoglobin (haemoglobin (g
593 dL^{-1})*10)/RBC (106 μL^{-1}); MCHC, mean corpuscular haemoglobin concentration (haemoglobin (g dL^{-1})*100)/haematocrit (%PCV); U,
594 lysozyme activity unit
595

Table 5 Intestinal histology of fish fed the experimental diets

	Control	Phytase	Protease	Carbohydrase	Pooled SEM	P-value
Perimeter ratio (AU)	4.86	6.22	5.84	5.84	0.39	0.70
Goblet cells (per 100µm)	7.35	6.87	7.26	7.75	0.17	0.34
IELs (per 100µm)	47.7	49.1	53.5	46.3	2.45	0.81
Microvilli density (AU)	10.4^{a}	15.6^{b}	11.5^{a}	16.0^{b}	0.77	0.00

Values with different superscripts indicate significant differences ($P < 0.05$). AU, arbitrary units and IELs, Intraepithelial leucocytes

599 **Table 6** Taxonomic affiliation of DGGE bands sequenced from intestinal digesta of Nile tilapia fed the experimental diets

Band #	Phylum	Nearest neighbour identified by BLASTn	Similarity (%)	Treatment
1	Planctomycetes	*Aquisphaera giovannonii*	96	Protease (4) Carbohydrase (3)
2	Proteobacteria	*Marinobacter hydrocarbonoclasticus*	93	Control (4)
3	Unidentified bacteria	*Uncultured bacterium clone AMD-A65*	83	Phytase (3) Protease (1) Carbohydrase (3)
4	Proteobacteria	*Desulforegula conservatrix*	81	Control (2)
5	Proteobacteria	*Acinetobacter schindleri*	97	Carbohydrase (4)
6	Actinobacteria	*Arthrobacter russicus*	99	Control (4)
7	Actinobacteria	*Arthrobacter russicus*	99	Phytase (1) Protease (2)
8	Firmicutes	*Sporosarcina aquimarina*	99	Protease (2) Carbohydrase (3)
9	Firmicutes	*Clostridium ghonii*	100	Control (4) Phytase (5) Protease (3) Carbohydrase (6)
10	Actinobacteria	*Austwickia chelonae*	99	Control (5) Phytase (3) Protease (3)
11	Actinobacteria	*Intrasporangium calvum*	99	Phytase (4)

600 Numbers in parenthesis represents number of replicates, out of 6, where the respective OUT (operational taxonomic unit) was

601 detected

602

603 **Table 7** Microbial community analysis from PCR-DGGE fingerprints of the intestinal bacteria of tilapia. n = 6

Treatment	OTUs[1]	Richness[2]	Evenness[3]	Diversity[4]	SIMPER (%)	Permanova P (perm)	Similarity (%)
Control	16.3 ± 2.36	3.3 ± 0.51	0.95 ± 0.00	2.6 ± 0.16	25.7		
Phytase	16.0 ± 2.21	3.2 ± 0.48	0.94 ± 0.01	2.5 ± 0.19	36.3		
Protease	20.2 ± 1.85	4.1 ± 0.40	0.94 ± 0.00	2.8 ± 0.10	39.4		
Carbohydrase	19.2 ± 1.65	4.0 ± 0.36	0.95 ± 0.01	2.8 ± 0.09	43.7		
Control vs phytase						0.260	28.5 ± 17.5
Control vs protease						0.187	27.6 ± 13.0
Control vs carbohydrase						0.029	24.8 ± 12.8
Phytase vs protease						0.464	34.8 ± 16.4
Phytase vs carbohydrase						0.086	34.7 ± 17.6
Protease vs carbohydrase						0.085	36.5 ± 17.1

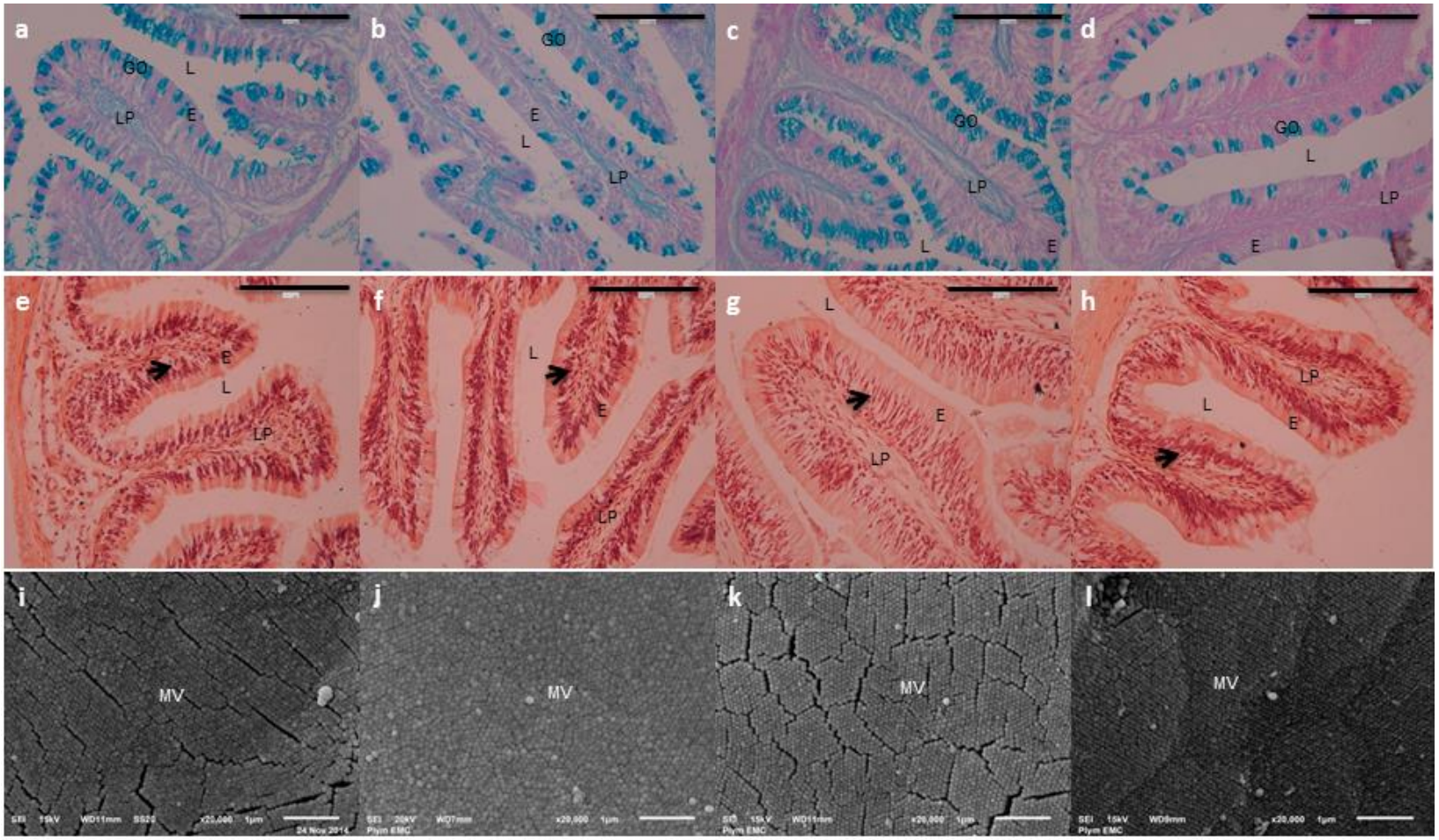
604 Results are presented as mean ±SD in each group (n=6). There were no significant differences between the treatments.

605 [1]Operational taxonomic unit (OTU)

606 [2]Margalef species richness: $d = (S - 1) / \log (N)$

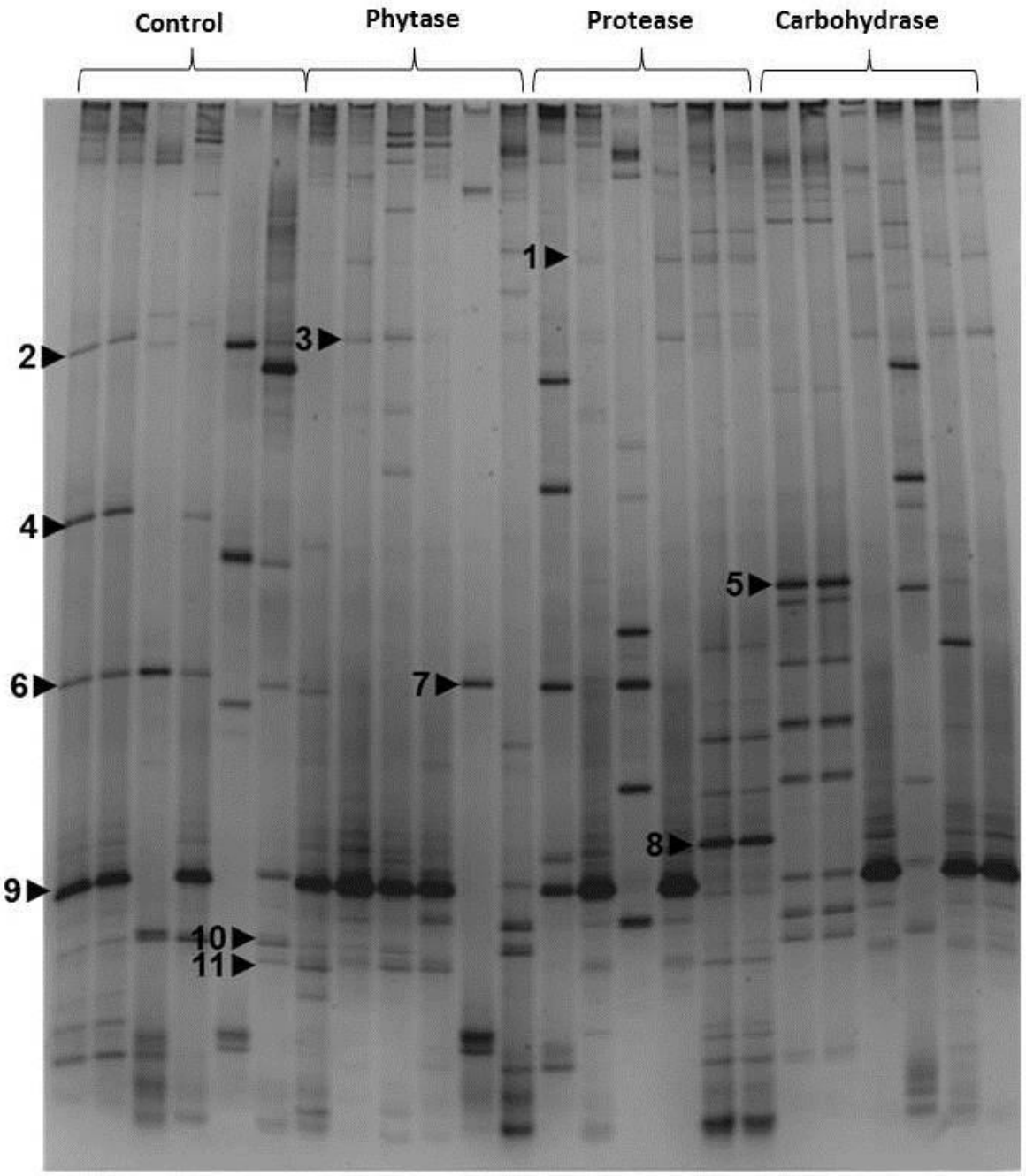
607 [3]Pielou's evenness: $J' = H' / \log(S)$

608 [4]Shannons diversity index: $H' = - \Sigma (pi(\ln pi))$



609

610 **Figure 1** Light (a-h) and scanning electron (i-l) micrographs of the mid-intestine of tilapia fed the control (a, e and i), phytase (b, f
611 and j), protease (c, g and k) and carbohydrase (d, h and l) diets. Goblet cells (GO) are filled with abundant acidic mucins (blue; a-d)
612 in all treatments and abundant IELs (arrows) are present in the epithelia. Abbreviations are E enterocytes, LP lamina propria, L
613 lumen, GO goblet cell and, MV microvilli. Light microscopy staining: [a-d] Alcian Blue-PAS; [e-h] H & E. Scale bars = 100 µm (a-h)
614 or 1 µm (i-l).



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616 **Figure 2** 40 - 60% DGGE banding patterns of V3 region of 16S rRNA fragments

617 from tilapia distal intestinal digesta.

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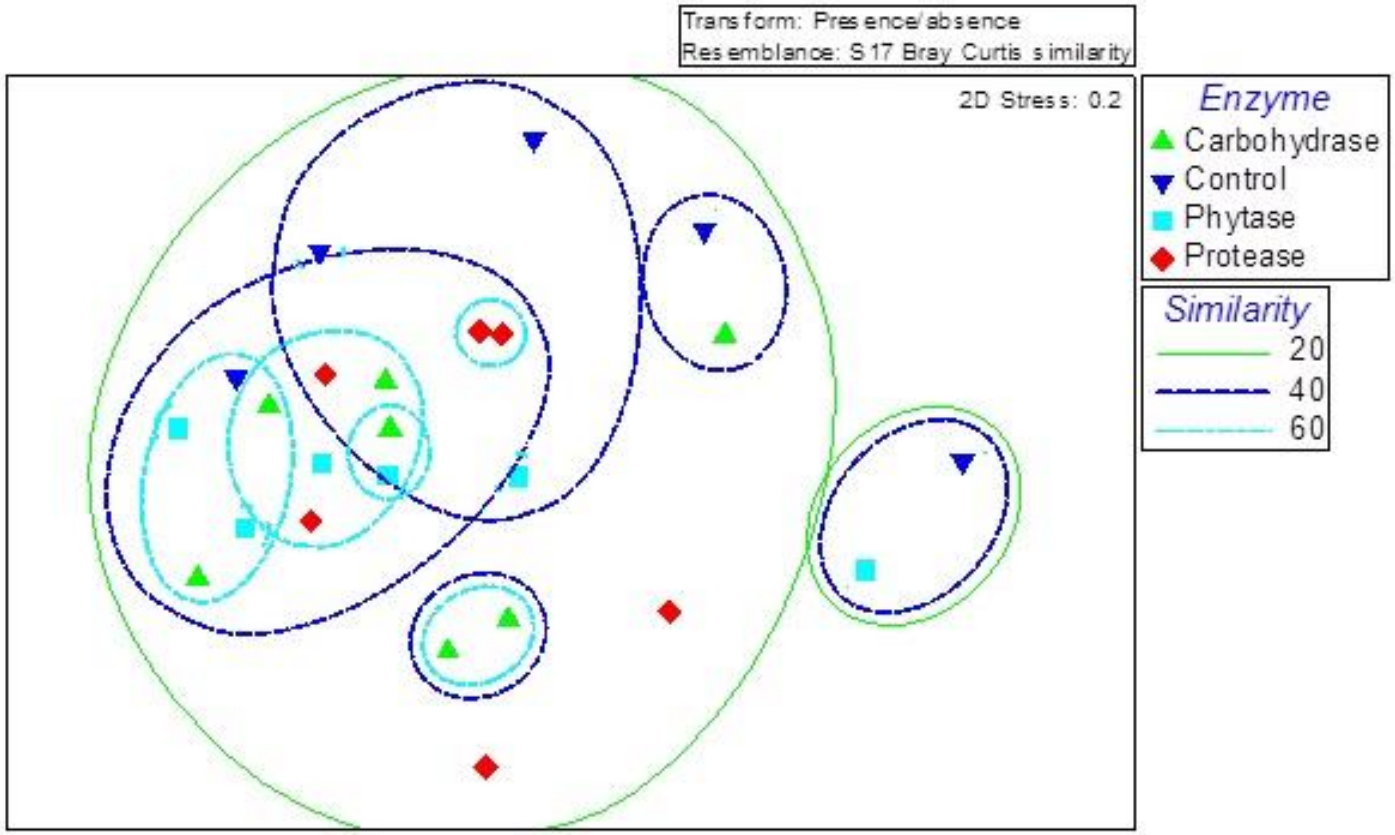

624

625 **Figure 3** Cluster analysis based on the DGGE profiles of V3 region fragments of 16S rRNA from distal intestinal digesta of fish fed
626 the experimental diets after 6 weeks. A. Cluster B. nMDS. Dietary treatments: C = control; PH = phytase; PR = Protease; CA
627 Carbohydrase.