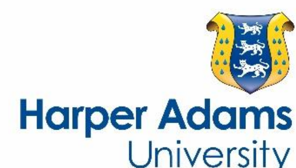
Supplementation of formulated diets for tilapia (*Oreochromis niloticus*) with selected exogenous enzymes: Overall performance and effects on intestinal histology and microbiota

by Adeoye, A.A., Jaramillo-Torres, A., Fox, S.W., Merrifield, D.L. and Davies, S.J.

Copyright, Publisher and Additional Information: This is the author accepted manuscript. The final published version (version of record) is available online via Elsevier
Please refer to any applicable terms of use of the publisher.

DOI: http://dx.doi.org/10.1016/j.anifeedsci.2016.03.002



Adeoye, A.A., Jaramillo-Torres, A., Fox, S.W., Merrifield, D.L. and Davies, S.J. 2016. 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, pp.133-143.

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

A.A. Adeoye[a*], A. Jaramillo-Torres[a], S. W. Fox[b], D. L. Merrifield[a] and S. J. Davies[c]

[a]School of Biological Sciences, Plymouth University, UK
[b]School of Biomedical and Health Sciences, Plymouth University, UK
[c]Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, UK

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 (1.98×10^6 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

[*]Corresponding author. Tel: +44 1752 84684; EM: ayodeji.adeoye@plymouth.ac.uk

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

36

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

38

39 *Corresponding author. Tel: +44 1752 84684; EM: ayodeji.adeoye@plymouth.ac.uk

40

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

55

56 **Introduction**

57 Aquaculture is recognized as the fastest growing agri-business sector and has
58 thus become an important component of global food supply (FAO, 2014). However
59 the increased production of intensively reared fish species necessitates the supply of
60 high quantity and sustainable feed ingredients in balanced formulated diets for
61 warmwater fish species. Tilapia production volumes rank second only to the carps,
62 contributing significantly to global aquaculture supply (FAO, 2011; Wang and Lu,
63 2015). To support expanding tilapia production, there is a need for sustainable feed
64 production. Plant by-products are of particular relevance within commercial diets for
65 tilapia; oilseed meals (e.g. soybean, copra, rapeseed, etc.), legumes and pulses (e.g.
66 peas, beans, etc.) as well as lupins and cereal by-products such as corn and gluten
67 are commonly used ingredients. However, there are limitations on the inclusion
68 levels of plant ingredients for most fish species due to the presence of anti-nutritional
69 factors (ANFs) which impair utilisation of nutrients resulting in reduced growth,
70 nutrient utilisation and feed efficiency (Francis et al., 2001; Khattab and Arntfield,
71 2009).

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

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

84 Supplementing tilapia diets with exogenous enzymes (phytases, NSPases
85 and proteases) offers potential for better utilisation of nutrients from plant ingredients
86 based on findings of enzyme applications in swine and poultry diets (Adeola and
87 Cowieson, 2011). The application of phytase has been successful in breaking down
88 phytate to increase mineral and nutrient digestibility in fish (Cao et al., 2007; Cao et
89 al., 2008; Kiarie et al., 2010; Kiarie et al., 2013). NSP-degrading enzymes (e.g.
90 cellulase, xylanase, etc.) are capable of disrupting plant cell wall integrity thereby
91 reducing molecular size characteristics of NSPs. Consequently, this enhances rapid
92 digestion by reducing viscosity in the gut (Zijlstra et al., 2010; Bedford and Cowieson,
93 2012). Supplementing diets with proteases has the potential to increase utilisation of
94 crude proteins from plant ingredients by increasing crude protein digestibility. In
95 addition, the application of exogenous enzymes can allow flexibility in diet
96 formulation through incorporation of lower quality and less expensive plant
97 ingredients. Apart from the potential of exogenous enzymes to promote growth and
98 nutrient utilisation (Adeola and Cowieson, 2011), they may alter substrates
99 availability for specific populations of gut microbes, thus, potentially altering bacterial
100 community composition or activities (Bedford and Cowieson, 2012; Kiarie et al., 2013;
101 Zhou et al., 2013; Jiang et al., 2014).

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

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

116

117 **2. Materials and methods**

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

122

123 *2.1 Diet preparation*

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

131 control diet. The feed ingredients were thoroughly mixed, moistened with warm water
132 (400 mL kg^{-1}) and then cold press extruded to produce 2 mm pellets using a PTM
133 extruder system (model P6, Plymouth, UK). The diets were dried to ca. 5g kg^{-1}
134 moisture in an air convection oven set at 45°C and their proximate composition
135 analysed (Table 1) using AOAC protocols (AOAC, 1995). After drying, the diets were
136 stored in airtight containers prior to use.
137

138 *2.2 Experimental design*

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

153

154 *2.3 Growth, feed utilisation and somatic indices*

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

159 $SGR = 100 ((\ln FBW - \ln IBW)/T)$

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

161 $FCR = FI/WG$

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

163 $PER = WG/PI$

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

165 $K = (100 \times FW)/ FL^3$

166 Where FL = FL = final length (cm)

167 $HSI = 100 (LW/ BW)$

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

169 $VSI = 100 (VW/ BW)$

170 Where; VW = visceral weight (g)

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

176 The fish were also used to record viscera weight and whole body weight in order to
177 calculate the hepatosomatic index (HSI) and visceromatic index (VSI).
178

179 *2.4 Haemato-immunological parameters*

180 Blood from three fish per tank was taken from the caudal arch using a 25
181 gauge needle and a 1 mL syringe after fish were anaesthetized with MS222
182 (Pharmaq Ltd. Hampshire, UK) at 150 mg L^{-1}. Blood smears were prepared for the
183 determination of differential leucocyte counts and additional blood was left to clot for
184 a period of 12 h (at 4°C) to isolate serum. Serum was isolated by centrifugation at
185 3600 g for 5 min and was stored at −80 °C. Haematocrit (measured and read as %
186 packed cell volume; PCV), haemoglobin, red blood cells (RBC), serum lysozyme
187 activity, white blood cells (WBC) and differential leucocyte proportions were
188 determined according to standard methods as described by Rawling et al. (2009).
189

190 *2.5 Intestinal histology*

191 At the end of the trial, three fish per tank were sampled for histological
192 appraisal (light and scanning electron microscopy) of the mid-intestine (n = 9). For
193 light microscopy examination, the samples were fixed in 10% formalin, dehydrated in
194 graded ethanol concentrations and embedded in paraffin wax. In each specimen,
195 multiple sets of sections (5mm thick) were stained with May-Grünwald Giemsa
196 (MGG), haematoxylin and eosin (H&E) and Alcian-Blue-PAS (Dimitroglou et al.,
197 2010; Ferguson et al., 2010). The intestinal perimeter ratios (arbitrary units, AU)
198 were assessed after Dimitroglou et al. (2009) and the numbers of intra epithelial
199 leucocytes (IELs) and goblet cells in the epithelium, across a standardized distance
200 of 100 µm (10 folds per specimen), was then calculated by averaging the cell

201 numbers from all specimens (Ferguson et al., 2010). For scanning electron
202 microscopy examination, the samples were processed and analysed as described by
203 (Merrifield et al., 2009a). The scanning electron microscope images were analysed
204 for microvilli density (MD) of the enterocytes on top of the villi. The ratio between the
205 microvilli covered area (M, foreground) to the gaps between the microvilli (B,
206 background) was calculated (MD = M/B, AU). All images were analysed with ImageJ
207 version 1.47 (National Institute of Health, USA).
208
209 *2.6 Intestinal microbiota: PCR - DGGE*
210 The intestines from two fish per tank (n = 6) were aseptically excised and the
211 digesta from the posterior section removed. DNA extraction and PCR amplification of
212 V3 region of 16S rRNA gene was undertaken as described by Merrifield et al.
213 (2009b). The PCRs was conducted using the forward primer P3 which include a GC
214 clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG
215 GCC TAC GGG AGG CAG CAG-3') and the reverse primer P2 (5'-
216 ATTACCGCGGCTGCTGG-3') (Muyzer et al., 1993). PCR reactions (50 µL)
217 contained 25 µL BioMix™ Red Taq [Bioline, UK], 1 µL of each primer [50 pmol/µL
218 each MWG-Biotech AG, Germany], 1 µL DNA template and 23 µL sterile Milli-Q
219 water). Positive and negative control templates were included in each assay;
220 negative control (sterile, using molecular grade water as template) and positive
221 control (DNA from cultured *Pediococcus acidilactici*). Touchdown thermal cycling
222 was conducted using a GeneAmp® PCR System 9700 (Perkin-Elmer, CA, USA),
223 under the following conditions: 94 °C for 10 min, then 30 cycles starting at 94 °C for
224 1 min, 65 °C for 2 min, 72 °C for 3 min (Muyzer et al., 1993). The annealing
225 temperature decreased by 1 °C every second cycle until 55 °C and then remained at

226 55 °C for the remaining 10 cycles. The PCR products were used to obtain DNA
227 fingerprints of the bacterial communities present in the fish intestines on a 40-60%
228 DGGE using a BioRad DGGE system (DCode™ System, Italy) as described
229 elsewhere (Merrifield et al., 2009b). Selected dominant bands were then excised and
230 DNA was eluted in TE buffer at 4 °C overnight before re-PCR. The PCR products
231 were purified using QIAquick PCR Purification Kit (Qiagen) according to
232 manufacturer's instructions and sequenced by GATC laboratories (GATC-biotech
233 laboratories, Germany). In order to obtain the taxonomic classification from the
234 partial 16S rRNA sequences, a BLAST search in GenBank database
235 (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi) was performed. The highest similarities
236 were used to assign the taxonomic description of each sequence.
237

238 *2.7 Statistical analysis*

239 Data were analysed using one-way analysis of variance (ANOVA). Multiple
240 comparisons were performed using Tukey post-hoc test. Differences were
241 considered significant at a value of $P < 0.05$. The statistical analysis was carried out
242 using SPSS for Windows (SPSS Inc., 22.0, Chicago, IL, USA). DGGE banding
243 patterns were transformed into presence/ absence matrices based on band peak
244 intensities (Quantity One® version 4.6.3, Bio-Rad Laboratories, CA, USA). The band
245 intensities were measured (Quantity One® 1-D Analysis Software, Bio-Rad
246 Laboratories Ltd., Hertfordshire, UK). Group differences for the microbiota were
247 calculated using PERMANOVA of Bray-Curtis distances using PRIMER V6 software
248 (PRIMER-E Ltd., Ivybridge, UK) (Anderson et al., 2008).
249

250 **3. Results**

251 *3.1 Growth, feed utilisation and somatic indices*

252 Growth, feed utilisation and somatic indices are presented in Table 2. Tilapia 253 fed the phytase supplemented diet performed significantly better ($P < 0.05$) than 254 those fed control and protease supplemented diets in terms of FBW. Tilapia fed the 255 phytase supplemented diet also displayed better feed utilisation in terms of FCR and 256 PER when compared to tilapia fed the control and protease supplemented diets. The 257 fish fed phytase and carbohydrase supplemented diets have similar growth 258 performance; there was no significant difference ($P < 0.05$) in their FBW and SGR. 259 Good survival was recorded in all the treatments (i.e. ≥ 90%) but higher in phytase 260 and protease treatments. The dietary treatments did not affect ($P > 0.05$) the fish 261 somatic indices assessed.

262

263 *3.2 Whole body composition*

264 The whole body composition of tilapia fed the experimental diets is displayed 265 in Table 3. The body moisture content of tilapia fed the protease supplemented diet 266 was higher ($P < 0.05$) than those fed the control diet. However, there was no 267 difference ($P > 0.05$) in the body moisture contents of tilapia fed the enzymes 268 supplemented diets. The dietary treatment did not affect body lipid, protein or ash 269 levels.

270

271 *3.3 Haemato-immunological parameters*

272 Haematological and immunological parameters are displayed in Table 4. 273 Haematocrit, haemoglobin, leucocyte levels, mean corpuscular haemoglobin, mean 274 corpuscular haemoglobin concentration and serum lysozyme activity were 275 unaffected by the dietary treatments. However, red blood cell levels were higher ($P <$

276 *0.05*) in the blood of tilapia fed the carbohydrase diet compare to fish fed the control
277 diet. There were no differences ($P > 0.05$) in the other haemato-immunological
278 parameters measured.
279
280 *3.4 Intestinal histology*
281 Light and scanning electron microscopy revealed a normal and healthy
282 morphology of the mid-intestines of tilapia fed the experimental diets. The intestines
283 of the fish showed intact epithelial barrier with well organised villi-like mucosal folds,
284 abundant IELs and goblet cells (Figures 1a–1h). The dietary treatments had no
285 significant effects ($P > 0.05$) on the intestinal perimeter ratio, number of goblet cells
286 or IELs (per 100µm) of the fish fed the experimental diets (Table 5). The fish
287 intestines displayed healthy brush border with well organised and tightly packed
288 microvilli revealing no signs of damage (Fig 1i–1l). However, the microvilli of the
289 brush border of tilapia fed control and protease supplemented diets appeared to be
290 less tightly packed (Figures 1i & 1k) compared to those fed phytase and
291 carbohydrase supplemented diets (Figures 1j & 1l). Consequently, the microvilli
292 density of the fish intestines was significantly different among tilapia fed the
293 experimental diets; the microvilli density of tilapia fed the phytase and carbohydrase
294 supplemented diets were significantly higher ($P < 0.05$) than that of tilapia fed the
295 control and protease supplemented diets (Table 5).
296
297 *3.5 Intestinal microbiota*
298 The bacterial community of tilapia fed the experimental diets were analysed
299 by PCR-DGGE. The DGGE banding patterns of the 16S rRNA V3 region from the
300 fish intestinal digesta is displayed in Figure 2 and the taxonomic affiliation of the

301 DGGE bands is displayed in Table 6. A total of eleven OTUs (operational taxonomic
302 units) were selected from the DGGE for sequencing. OTU #9 was detected in all
303 tilapia fed the experimental diets and had 100% sequence alignment to *Clostridium*
304 *ghonii*. OTU #5 was uniquely detected in tilapia fed carbohydrase supplemented diet
305 and was identified as belonging to *Acinetobacter schindleri* (97%). OTU #7 was
306 common in tilapia fed phytase and protease supplemented diets and had 99%
307 sequence alignment to *Arthrobacter russicus.* OTU #8 was common in tilapia fed
308 protease and carbohydrase supplemented diets and had 99% sequence alignment
309 to *Sporosarcina aquimarina.* OTUs #10 and #11 were common in tilapia fed control
310 and phytase supplemented diets and both had 99% sequence alignment to
311 *Austwickia chelonae* and *Intrasporangium calvum,* respectively. OTUs #1 and #3
312 were present in all tilapia fed the exogenous supplemented diets and had 96% and
313 83% sequence alignment to *Aquisphaera giovannonii* and uncultured bacterium
314 clone AMD-A65*,* respectively. OTUs #2, #4 and #6 were common in tilapia fed the
315 control diet and had 93%, 81% and 99% sequence alignment to *Marinobacter*
316 *hydrocarbonoclasticus, Desulforegula conservatrix, and Arthrobacter russicus,*
317 respectively. Firmicutes was the most frequently identified phylum across all the
318 treatments and high frequency of the OTUs from the *Clostridium* genus was also
319 evident. In terms of the number of OTUs, species richness and diversity, no
320 significant differences were observed among the treatments (Table 7). However,
321 high variability in the bacterial community structure was observed among individuals
322 in the same group in all the treatments; the control group showed the greatest
323 differences (Figure 3). The dietary treatments did not affect ($P > 0.05$) the species
324 diversity of PCR-DGGE fingerprints. However, Permanova analysis revealed a

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

327

328 **4. Discussion**

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

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

357 Tilapia fed the carbohydrase supplemented diet had similar growth
358 performance (FBW & SGR) with tilapia fed the control diet which is in agreement
359 with the findings by (Yigit and Olmez, 2011) who reported no benefits on growth
360 when tilapia were fed a carbohydrase supplemented diet. It was hypothesised by the
361 authors that protease supplementation could degrade complex proteins in the diet
362 into usable amino acids and peptides thereby resulting in improved protein
363 digestibility and growth performance. In the current study however, growth
364 performance and nutrient utilisation of fish fed the protease supplemented diet were
365 not significantly different from the fish fed control diet. Contrary to this, Dias et al.
366 (2014) reported a positive effect of protease on tilapia growth performance fed a
367 lower crude protein diet compared to the higher crude protein diet in the present
368 study. It could be inferred from this report that the protease effect is likely to be more
369 pronounced in a low crude protein and low fishmeal diet. The non-effect of protease
370 in a relatively higher crude protein diet could be as a result of non-beneficial effects
371 of digestible protein when the level exceeds the requirement for fish maintenance
372 and growth.

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

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

386 To the authors' knowledge, this is the first study investigating the effect of
387 exogenous enzymes (phytase, protease and carbohydrase) on the intestinal
388 microbiota of tilapia in a feeding trial. Previous studies have demonstrated that
389 different feed additives such as antibiotics (He et al., 2010), probiotics (He et al.,
390 2013; Standen et al., 2013; Standen et al., 2015) and prebiotics (Qin et al., 2014)
391 can modulate the gut microbiota in tilapia. Zhou et al. (2013) reported significant
392 changes in bacteria species and density of the intestinal microbiota of grass carp
393 (*Ctenopharyngodon idella*) fed cellulase supplemented duckweed-based diets. In the
394 present study, the predominant allochthonous bacterial species in the intestine of
395 tilapia was *Clostridium ghonii,* which was the only phylotype found in all the
396 individual tilapia regardless of the dietary treatment. Other authors have also found
397 members of Family *Clostridiaceae* in tilapia's intestine suggesting that members of
398 this family may be adapted to play an important role in the tilapia gut system (Zhou
399 et al., 2011). There were some phylotypes that were only present in the intestine of

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

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

424 microbiota and if these modulations contribute towards the improved growth
425 performance of the host.
426

427 **Acknowledgements**

428 The authors are grateful to the Commonwealth Scholarship Commission in the UK
429 and the School of Biological Sciences, Plymouth University for funding. The authors
430 also thank DSM Nutritional Products for supplying the exogenous enzymes. Ben
431 Eynon, Matthew Emery, Glenn Harper and peers in the Aquatic Animal Nutrition and
432 Health Research Group of Plymouth University are very much appreciated for
433 technical supports.

434

435 **References**

436 Adeola, O., Cowieson, A., 2011. Board-invited review: opportunities and challenges
437 in using exogenous enzymes to improve nonruminant animal production. J.
438 Anim. Sci. 89, 3189-3218.
439 Anderson, M., Gorley, R., Clarke, K.P., 2008. for PRIMER: Guide to software and
440 statistical methods. PRIMER-E, Plymouth, UK.
441 AOAC, 1995. Official Methods of Analysis, Association of Official Analytical Chemists,
442 Arlington, VA.
443 Bedford, M., Cowieson, A., 2012. Exogenous enzymes and their effects on intestinal
444 microbiology. Anim. Feed Sci. Technol. 173, 76-85.
445 Cao, L., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z., Li, D.,
446 2007. Application of microbial phytase in fish feed. Enzyme Microb. Technol.
447 40, 497-507.
448 Cao, L., Yang, Y., Wang, W., Yakupitiyage, A., Yuan, D., Diana, J., 2008. Effects of
449 pretreatment with microbial phytase on phosphorous utilization and growth
450 performance of Nile tilapia (Oreochromis niloticus). Aquacult. Nutr. 14, 99-109.
451 Castillo, S., Gatlin, D.M., 2015. Dietary supplementation of exogenous carbohydrase
452 enzymes in fish nutrition: a review. Aquaculture 435, 286-292.
453 Dias, J., Rema, P., Nunes, C., Vogel, K., Verlhac, V., 2014. Efficacy of dietary
454 protease supplementation on growth performance and nutrient digestibility
455 and utilization of juvenile Nile tilapia fed low fishmeal diets with variable crude
456 protein level, FeedInfo News.
457 Dimitroglou, A., Merrifield, D., Moate, R., Davies, S., Spring, P., Sweetman, J.,
458 Bradley, G., 2009. Dietary mannan oligosaccharide supplementation
459 modulates intestinal microbial ecology and improves gut morphology of
460 rainbow trout,(Walbaum). J. Anim. Sci. 87, 3226-3234.
461 Dimitroglou, A., Merrifield, D.L., Carnevali, O., Picchietti, S., Avella, M., Daniels, C.,
462 Güroy, D., Davies, S.J., 2011. Microbial manipulations to improve fish health
463 and production–a Mediterranean perspective. Fish Shellfish Immunol. 30, 1-
464 16.
465 Dimitroglou, A., Merrifield, D.L., Spring, P., Sweetman, J., Moate, R., Davies, S.J.,
466 2010. Effects of mannan oligosaccharide (MOS) supplementation on growth
467 performance, feed utilisation, intestinal histology and gut microbiota of
468 gilthead sea bream (Sparus aurata). Aquaculture 300, 182-188.
469 FAO, 2011. Tilapia market report. Globefish, Food and Agriculture Organization of
470 the United Nations, Rome.
471 FAO, 2014. The State of World Fisheries and Aquaculture. Opportunities and
472 challenges, Food and Agriculture Organization of the United Nations, Rome.
473 Ferguson, R., Merrifield, D.L., Harper, G.M., Rawling, M.D., Mustafa, S., Picchietti,
474 S., Balcàzar, J.L., Davies, S.J., 2010. The effect of Pediococcus acidilactici on
475 the gut microbiota and immune status of on-growing red tilapia (Oreochromis
476 niloticus). J. Appl. Microbiol. 109, 851-862.
477 Francis, G., Makkar, H.P., Becker, K., 2001. Antinutritional factors present in plant-
478 derived alternate fish feed ingredients and their effects in fish. Aquaculture
479 199, 197-227.
480 He, S., Zhang, Y., Xu, L., Yang, Y., Marubashi, T., Zhou, Z., Yao, B., 2013. Effects of
481 dietary Bacillus subtilis C-3102 on the production, intestinal cytokine
482 expression and autochthonous bacteria of hybrid tilapia Oreochromis
483 niloticus♀× Oreochromis aureus♂. Aquaculture 412, 125-130.

484 He, S., Zhou, Z., Liu, Y., Cao, Y., Meng, K., Shi, P., Yao, B., Ringø, E., 2010. Effects
485 of the antibiotic growth promoters flavomycin and florfenicol on the
486 autochthonous intestinal microbiota of hybrid tilapia (Oreochromis niloticus♀×
487 O. aureus♂). Arch. Microbiol. 192, 985-994.
488 Jiang, N., Tan, N.S., Ho, B., Ding, J.L., 2007. Respiratory protein–generated reactive
489 oxygen species as an antimicrobial strategy. Nat. Immunol. 8, 1114-1122.
490 Jiang, T.T., Feng, L., Liu, Y., Jiang, W.D., Jiang, J., Li, S.H., Tang, L., Kuang, S.Y.,
491 Zhou, X.Q., 2014. Effects of exogenous xylanase supplementation in plant
492 protein-enriched diets on growth performance, intestinal enzyme activities and
493 microflora of juvenile Jian carp (Cyprinus carpio var. Jian). Aquacult. Nutr. 20,
494 632-645.
495 Khattab, R., Arntfield, S., 2009. Nutritional quality of legume seeds as affected by
496 some physical treatments 2. Antinutritional factors. LWT-Food Science and
497 Technology 42, 1113-1118.
498 Kiarie, E., Nyachoti, C., Vitti, D., Kebreab, E., 2010. Bioavailability of Calcium and
499 Phosphorus in Feedstuffs for Farm Animals. Phosphorus and Calcium
500 Utilization and Requirements in Farm Animals, 76.
501 Kiarie, E., Romero, L.F., Nyachoti, C.M., 2013. The role of added feed enzymes in
502 promoting gut health in swine and poultry. Nutrition research reviews 26, 71-
503 88.
504 Kumar, V., Sinha, A., Makkar, H., De Boeck, G., Becker, K., 2012. Phytate and
505 phytase in fish nutrition. J. Anim. Physiol. Anim. Nutr. 96, 335-364.
506 Merrifield, D., Dimitroglou, A., Bradley, G., Baker, R., Davies, S., 2009a. Soybean
507 meal alters autochthonous microbial populations, microvilli morphology and
508 compromises intestinal enterocyte integrity of rainbow trout, Oncorhynchus
509 mykiss (Walbaum). J. Fish Dis. 32, 755-766.
510 Merrifield, D.L., Burnard, D., Bradley, G., Davies, S.J., Baker, R., 2009b. Microbial
511 community diversity associated with the intestinal mucosa of farmed rainbow
512 trout (Oncoryhnchus mykiss Walbaum). Aquacult. Res. 40, 1064-1072.
513 Merrifield, D.L., Carnevali, O., 2014. Probiotic modulation of the gut microbiota of fish.
514 Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics, 185-222.
515 Muyzer, G., De Waal, E.C., Uitterlinden, A.G., 1993. Profiling of complex microbial
516 populations by denaturing gradient gel electrophoresis analysis of polymerase
517 chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol.
518 59, 695-700.
519 NRC, 2011. Nutrient Requirements of Fish and Shrimp, The National Academies
520 Press, Washington DC.
521 Nwanna, L., 2007. Effect of dietary phytase on growth, enzyme activities and
522 phosphorus load of Nile tilapia (Oreochromis niloticus). Journal of Engineering
523 and Applied Sciences 2, 972-976.
524 Olli, J.J., Hjelmeland, K., Krogdahl, Å., 1994. Soybean trypsin inhibitors in diets for
525 Atlantic salmon (Salmo salar, L): effects on nutrient digestibilities and trypsin
526 in pyloric caeca homogenate and intestinal content. Comparative
527 Biochemistry and Physiology Part A: Physiology 109, 923-928.
528 Portz, L., Liebert, F., 2004. Growth, nutrient utilization and parameters of mineral
529 metabolism in Nile tilapia Oreochromis niloticus (Linnaeus, 1758) fed plant-
530 based diets with graded levels of microbial phytase. J. Anim. Physiol. Anim.
531 Nutr. 88, 311-320.
532 Qin, C., Zhang, Y., Liu, W., Xu, L., Yang, Y., Zhou, Z., 2014. Effects of chito-
533 oligosaccharides supplementation on growth performance, intestinal cytokine

534 expression, autochthonous gut bacteria and disease resistance in hybrid
535 tilapia Oreochromis niloticus♀× Oreochromis aureus♂. Fish Shellfish Immunol.
536 40, 267-274.
537 Rawling, M.D., Merrifield, D.L., Davies, S.J., 2009. Preliminary assessment of dietary
538 supplementation of Sangrovit® on red tilapia (Oreochromis niloticus) growth
539 performance and health. Aquaculture 294, 118-122.
540 Standen, B., Rawling, M., Davies, S., Castex, M., Foey, A., Gioacchini, G., Carnevali,
541 O., Merrifield, D., 2013. Probiotic Pediococcus acidilactici modulates both
542 localised intestinal-and peripheral-immunity in tilapia (Oreochromis niloticus).
543 Fish Shellfish Immunol. 35, 1097-1104.
544 Standen, B., Rodiles, A., Peggs, D., Davies, S., Santos, G., Merrifield, D., 2015.
545 Modulation of the intestinal microbiota and morphology of tilapia, Oreochromis
546 niloticus, following the application of a multi-species probiotic. Appl. Microbiol.
547 Biotechnol. 99, 8403-8417.
548 Suhairin, A., Manap, A., Yazid, M., Hussin, M., Shobirin, A., Mustafa, S., 2010.
549 Phytase: application in food industry. International Food Research Journal 17,
550 13-21.
551 Wang, M., Lu, M., 2015. Tilapia polyculture: a global review. Aquacult. Res.
552 Yigit, N., Olmez, M., 2011. Effects of cellulase addition to canola meal in tilapia
553 (Oreochromis niloticus L.) diets. Aquacult. Nutr. 17, e494-e500.
554 Zhou, Y., Yuan, X., Liang, X.-F., Fang, L., Li, J., Guo, X., Bai, X., He, S., 2013.
555 Enhancement of growth and intestinal flora in grass carp: the effect of
556 exogenous cellulase. Aquaculture 416, 1-7.
557 Zhou, Z., He, S., Liu, Y., Cao, Y., Meng, K., Yao, B., Ringø, E., Yoon, I., 2011. Gut
558 microbial status induced by antibiotic growth promoter alters the prebiotic
559 effects of dietary DVAQUA® on Aeromonas hydrophila-infected tilapia:
560 Production, intestinal bacterial community and non-specific immunity. Vet.
561 Microbiol. 149, 399-405.
562 Zijlstra, R., Owusu-Asiedu, A., Simmins, P., 2010. Future of NSP-degrading
563 enzymes to improve nutrient utilization of co-products and gut health in pigs.
564 Livestock Science 134, 255-257.

565
566

567 **Table 1** Formulation and composition of the experimental diets
568

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

569 [a]Hamlet HP100, Hamlet Protein, Denmark
570 [b]Soya UK
571 [c]Sigma- Aldrich Ltd., UK
572 [d]Herring meal LT94 – United Fish Products Ltd., Aberdeen, UK
573 [e]Roquette Frêres, France
574 [f] Premier nutrition vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit
575 D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate)
576 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorus 5.2 g kg^{-1}
577 [g] RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products
578 [h] RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products
579 [i] ROXAZYME® G2 (contains 2700U g^{-1} xylanase, 700U g^{-1} β-glucanase and 800U g^{-1}
580 cellulose) from DSM Nutritional Products
581 [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

596 **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

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

598

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: $H' = -\Sigma (pi(\ln pi))$

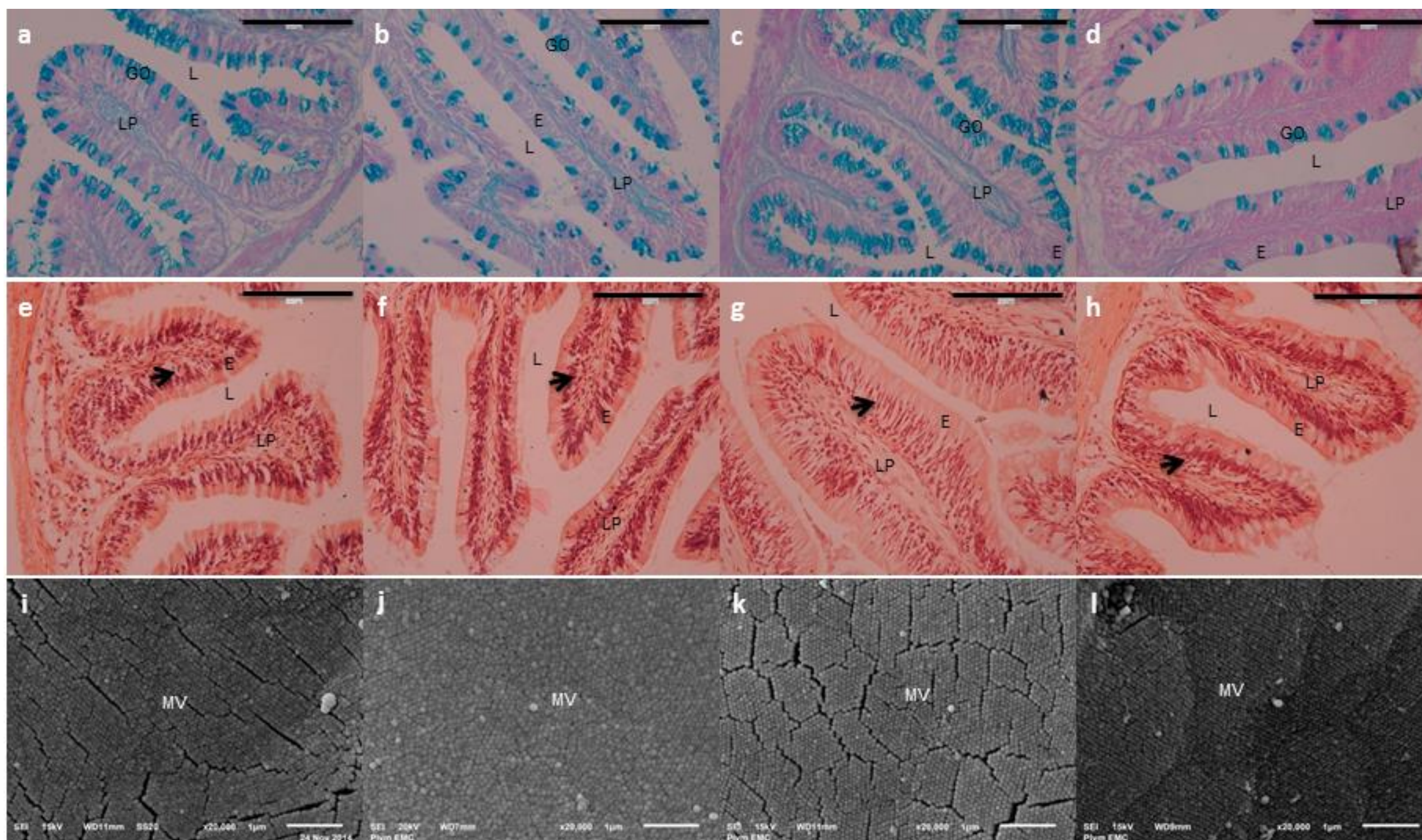
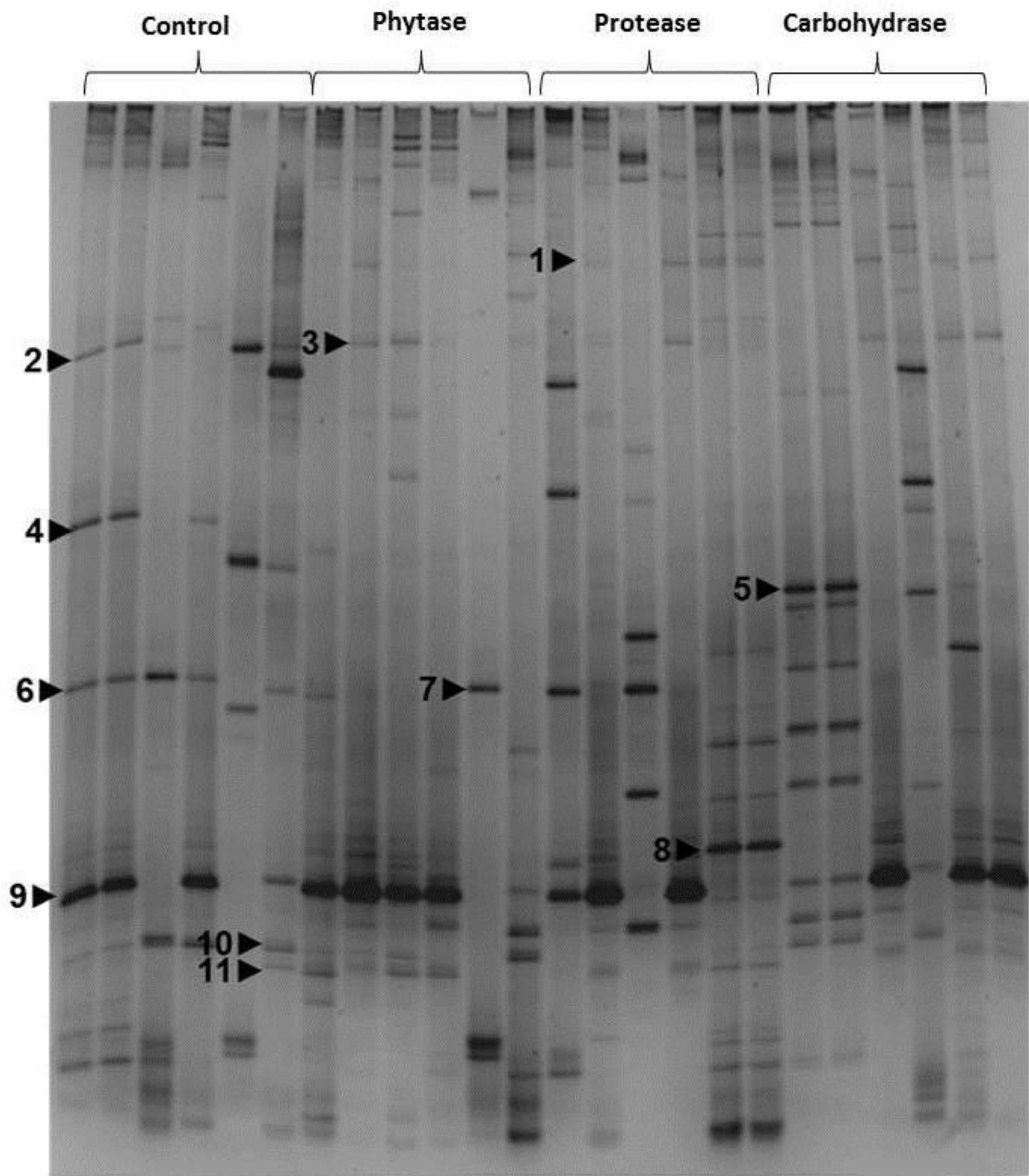


Figure 1 Light (a-h) and scanning electron (i-l) micrographs of the mid-intestine of tilapia fed the control (a, e and i), phytase (b, f and j), protease (c, g and k) and carbohydrase (d, h and l) diets. Goblet cells (GO) are filled with abundant acidic mucins (blue; a-d) in all treatments and abundant IELs (arrows) are present in the epithelia. Abbreviations are E enterocytes, LP lamina propria, L 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) or 1 µm (i-l).



615

616 **Figure 2** 40 - 60% DGGE banding patterns of V3 region of 16S rRNA fragments
617 from tilapia distal intestinal digesta.

618

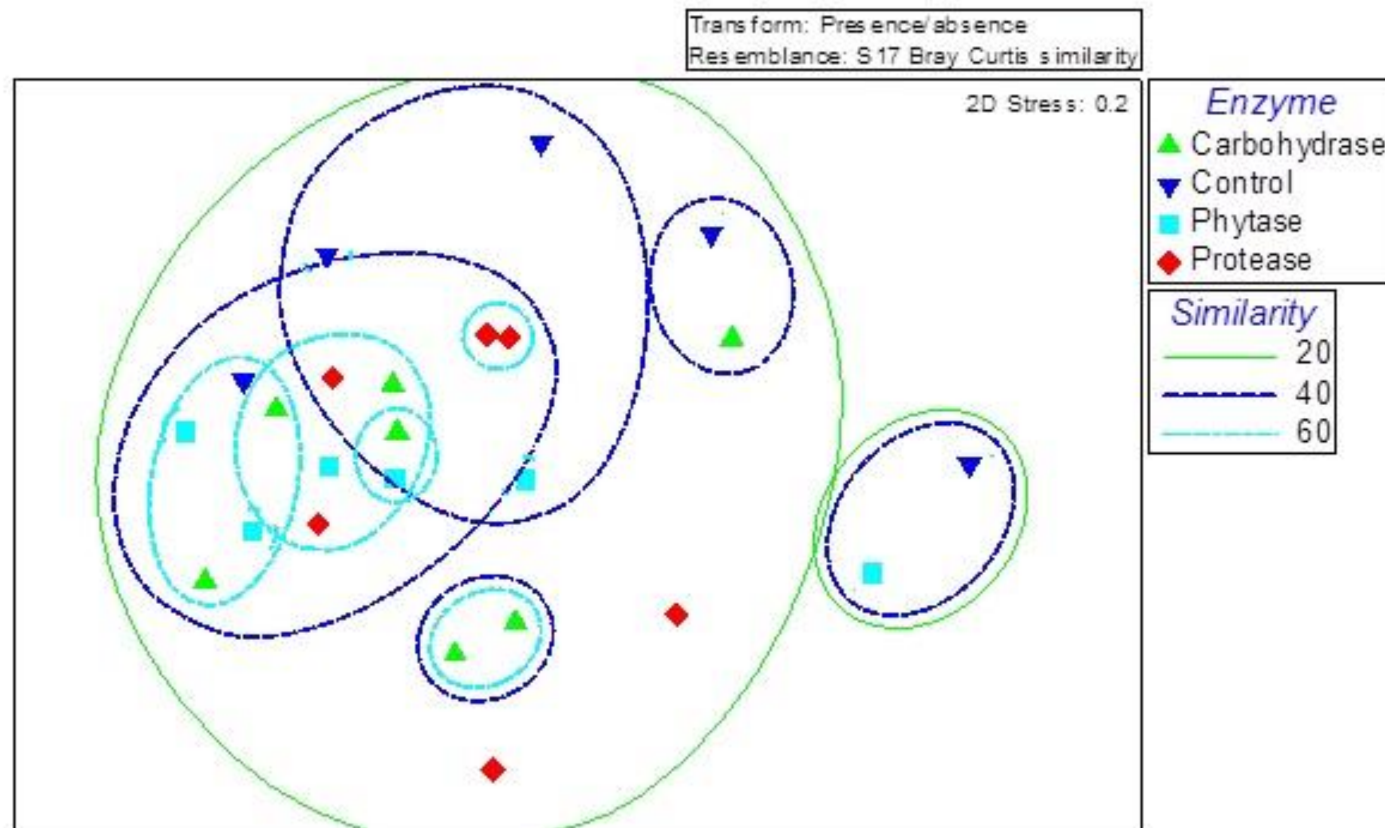
619

620

621

622

623



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.