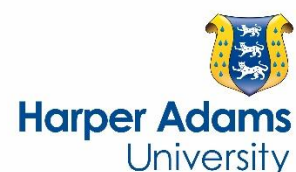


Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome

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1 **Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis***
2 ***niloticus*) growth, intestinal morphology and microbiome**

3
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13
14
15 **Abstract**

16 A study was carried out to investigate the combined effect of exogenous enzymes and
17 probiotic supplementation on tilapia growth, intestinal morphology and microbiome
18 composition. Tilapia (34.56 ± 0.05 g) were fed one of four diets (35% protein, 5% lipid); one
19 of which was a control and the remaining three diets were supplemented with either enzymes
20 (containing phytase, protease and xylanase), probiotic (containing *Bacillus subtilis*, *B.*
21 *licheniformis* and *B. pumilus*) or enz-pro (the combination of the enzymes and probiotic).
22 Tilapia fed diet supplemented with enz-pro performed better ($P < 0.05$) than tilapia fed the
23 control and probiotic supplemented diets in terms of final body weight (FBW), specific
24 growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). The
25 dietary treatments did not affect somatic indices. The serum lysozyme activity was
26 significantly higher ($P < 0.05$) in tilapia fed the probiotic supplemented diet than of those fed
27 the remaining experimental diets. The intestinal perimeter ratio was higher ($P < 0.05$) in
28 tilapia fed enz-pro supplemented diet when compared to those fed with the control and
29 probiotic supplemented diets. Goblet cells abundance, microvilli diameter and total

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30 enterocyte absorptive surface was higher ($P < 0.05$) in tilapia fed diet supplemented with
31 enz-pro than those fed the control diet. High-throughput sequencing revealed that majority of
32 reads derived from the tilapia digesta belonged to members of Fusobacteria (predominantly
33 *Cetobacterium*) distantly followed by Proteobacteria and Firmicutes. The alpha and beta
34 diversities did not differ among dietary treatments indicating that the overall microbial
35 community was not modified to a large extent by dietary treatment. In conclusion,
36 supplementation of the diet with a combination of enzymes and probiotic is capable of
37 improving tilapia growth and intestinal morphology without deleterious effect on the
38 intestinal microbial composition.

39

40 **Keywords:** Enzymes, probiotic, histology, microbiome, high-throughput sequencing, tilapia

41 **1.0 Introduction**

42 The growth of aquaculture, the world's fastest growing food production sector, is linked to
43 population increase and consequently the intensification and diversification of aquaculture
44 operations (Msangi et al., 2013). The rearing technologies for the intensive operations in
45 aquaculture under poor management can be accompanied by sub-optimum environmental
46 conditions as a result of overcrowding and overfeeding. These conditions may be stressful for
47 fish, leading to decreased performance and subsequently compromised immune responses
48 which leave fish prone to infection and disease by opportunistic pathogens. However, with
49 the need to meet global animal protein demand and the growing pressure on fish farmers to
50 reduce production cost without necessarily transferring the cost to the consumers, the
51 stressful conditions associated with the intensive aquaculture operation is likely to continue in
52 many parts of the world. The growing concept of immune-nutrition (production of high
53 quality feed with optimal growth and immune boosting effects) could be of benefit to
54 intensive aquaculture operations (Nakagawa et al., 2007, Kiron, 2012).

55 The gastro-intestinal (GI) microbiota of fish has been reported to play a key role in nutrition
56 and immunity. According to Nayak (2010), GI microbiota are involved in major nutritional
57 functions which include digestion, nutrient utilisation and the production of specific amino
58 acids, enzymes, short-chain fatty acids, vitamins and mineral availability. The nutritional role
59 of GI microbiota includes the production of vitamins and the secretion of digestive enzymes
60 that promote nutrient digestion as well as synthesise nutrients and metabolites required by
61 fish (Okutani et al., 1967, Saha et al., 2006, Li et al., 2010, Liu et al., 2016). In addition, GI
62 microbiota are capable of influencing immune status, disease resistance, survival, feed
63 utilisation and may have a role in preventing pathogens from colonising the host (Denev et al.,
64 2009, Ringø et al., 2015). Apart from nutrition and immunological effects, fish GI microbiota

65 have important functions in host metabolism, mucosal development and promote gut
66 maturation (Bates et al., 2006, Rawls et al., 2004, Round and Mazmanian, 2009).

67 It is well established that GI microbial communities are sensitive to rearing environment,
68 seasonal and diet changes including the supplementation with probiotic (Dimitroglou et al.,
69 2011, Merrifield et al., 2010, Romero et al., 2014) and exogenous digestive enzymes
70 (Bedford and Cowieson, 2012, Geraylou et al., 2012, Zhou et al., 2013, Jiang et al., 2014,
71 Adeoye et al., 2016, Hu et al., 2016). Research into the use of exogenous digestive enzyme
72 and probiotic supplements is increasing since aquafeed manufacturers are increasingly
73 interested in producing 'functional and environmentally friendly aquafeeds'. The potential
74 effects of exogenous digestive enzymes (Kumar et al., 2012, Castillo and Gatlin, 2015,
75 Lemos and Tacon, 2016) and probiotic (Pérez - Sánchez et al., 2014) on fish have been
76 reviewed as individual supplement. To the authors' understanding, there is no previous report
77 on combined used of exogenous digestive enzymes and probiotic as supplement in fish.
78 However, the combined supplementation of exogenous enzymes and probiotic could result in
79 a complimentary mode of actions: ability to produce fibre-degrading enzymes by probiotic
80 may complement endogenous enzyme activity. On the other hand, exogenous digestive
81 enzymes may increase availability of suitable substrate for probiotic as well as promote the
82 growth of other beneficial bacteria (GI microbiota).

83 Given the potential complimentary mode of actions of exogenous digestive enzymes and
84 probiotic, the two products could improve the growth performance and health status of
85 farmed fish when fed diets supplemented with both the enzymes and probiotic as a cocktail;
86 Nile tilapia (*Nile tilapia*) is an important freshwater fish species of considerable economic
87 value globally. Therefore, the objective of this study was to evaluate the combined effects of
88 exogenous digestive enzymes and probiotic on growth, intestinal morphology and
89 microbiome composition of Nile tilapia.

90 2.0 Materials and methods

91 2.1 *Experimental design and diets preparation*

92 All experimental work involving fish was in accordance with the principles of the Animals
93 (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

94 The trial was conducted in a flow – through aquaculture system in King Mongkut’s Institute
95 of Technology Ladkrabang - Thailand. The flow – through system contains 12 square
96 concrete tanks (508 L capacity each) and were supplied with freshwater sourced from a local
97 river system. Three hundred and sixty all male Nile tilapia (*Oreochromis niloticus*) of mean
98 weight 34.56 ± 0.05 g obtained from Charoen Pokphand farm in Thailand were randomly
99 distributed (30 fish per tank) into the 12 tanks after two weeks of acclimatization. The
100 photoperiod and water temperature (30.34 ± 0.15 °C) was maintained at ambient condition.
101 The water pH (6.20 ± 0.22) and dissolved oxygen levels (>5.0 mg L⁻¹) were monitored daily
102 using a HQ40d pH meter and dissolved oxygen multi-parameter meter (HACH Company,
103 Loveland, USA). NH₃ (0.304 ± 0.08 mg L⁻¹), NO₂ (0.016 ± 0.002 mg L⁻¹) and NO₃ (1.46 ± 0.19
104 mg L⁻¹) were also monitored on a weekly basis using a nutrient analyser (SEAL AQ2
105 Analyser, Hampshire, UK). A constant water flow of 4.9 L min⁻¹ (per tank) was used during
106 the experiment to maintain the water quality and ensure optimum conditions for the fish.

107 A commercial diet (No. 461; 35% protein, 5% lipid) was obtained from INTEQC Feed Co.
108 Ltd., Thailand and was used as basal formulation. The commercial diet was ground in a
109 blender to powder and sieved to remove large particles. An enzyme cocktail (containing
110 phytase, protease and xylanase), Sanolife PRO-F (a mixture of *Bacillus subtilis*, *B.*
111 *licheniformis* and *B. pumilus*) and a combination of the enzyme cocktail and Sanolife PRO-F
112 were added to the diets separately as stated in Table 1. The diets were coded as control (zero
113 supplementation), enzymes (phytase, protease and xylanase supplementation), probiotic

114 (probiotic supplementation) and enz-pro (enzymes and probiotic supplementation as a
115 cocktail). The diets were mixed thoroughly for 15 min to ensure homogeneity. Warm water
116 was added to form a consistency suitable for subsequent cold press extrusion. Afterwards, the
117 diets were dried in an air convection oven set at 45 °C for 24 h. The basal diet served as the
118 control and was prepared in the same way as those supplemented with the enzymes cocktail
119 and probiotic, with the exception of the supplementation. Tilapia were fed the experimental
120 diets for seven weeks at 3 % biomass day⁻¹ in three equal rations. Daily feed was adjusted on
121 a weekly basis by batch weighing following a 24 h deprivation period.

122 2.2 *Growth performance, feed utilisation and somatic indices*

123 Growth performance, feed utilisation and somatic indices were assessed by final body weight
124 (FBW), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio
125 (PER), hepatosomatic index (HSI), viscero-somatic index (VSI) and condition factor (K),
126 Calculations were carried out using the following formulae:

127 $SGR = 100 ((\ln FBW - \ln IBW)/T)$, where FBW = final body weight (g) and IBW = initial
128 body weight (g)

129 $FCR = FI/WG$, where FI = feed intake (g) and WG = wet weight gain (g)

130 $PER = WG/PI$, where WG = wet weight gain (g) and PI = protein ingested (g),

131 $K = (100 \times FW)/FL^3$, where FL = FL = final length (cm)

132 $HSI = 100 (LW/ FBW)$, where LW = liver weight (g) and FBW = final body weight (g)

133 $VSI = 100 (VW/ FBW)$, where VW = visceral weight (g)

134 All fish were euthanized with buffered tricaine methanesulfonate, MS222 (Pharmaq Ltd.
135 Hampshire, UK) at a concentration of 200 mg L⁻¹ followed by destruction of the brain prior
136 to sampling. For proximate composition analysis (AOAC, 1995), at the onset of the trial 12

137 fish were pooled to constitute three samples; at the end of the trial, three fish per tank were
138 sampled. The fish were also used to record viscera weight and whole body weight in order to
139 calculate the HSI and VSI.

140 2.3 *Haemato – immunological parameters*

141 At the end of the feeding trial, blood from three fish per tank (n = 9) was taken from the
142 caudal arch using a 25 gauge needle and a 1 mL syringe after fish were anaesthetized with
143 MS222 (Pharmaq Ltd. Hampshire, UK) at 150 mg L⁻¹. Blood smears were prepared for
144 determination of differential leucocyte counts and additional blood was left to clot for a
145 period of 12 h (at 4°C) to isolate serum. Serum was isolated by centrifugation at 3600 g for 5
146 min and was stored at -80 °C until further analysis. Haematocrit (measured and read as %
147 packed cell volume; PCV), haemoglobin, red blood cells (RBC), serum lysozyme activity,
148 white blood cells (WBC) and differential leucocyte proportions were determined according to
149 standard methods as described by Rawling et al. (2009).

150 2.4 *Intestinal histology*

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

161 microscopy (SEM) and transmission electron microscopy (TEM), samples were washed in 1 %
162 S-carboxymethyl-L-cysteine for 30 seconds (SEM only) to remove mucus before fixing in
163 2.5 % glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2). samples were processed as
164 described elsewhere (Dimitroglou et al., 2009) and screened with a JSM 6610 LV (Jeol,
165 Tokyo, Japan) SEM or JEN 1400 (Jeol, Tokyo, Japan) TEM. The SEM images were analysed
166 to assess microvilli count per μm^2 (MCVT) and enterocyte apical area (EAA), μm^2 . The
167 TEM images were analysed for microvilli length and diameter. All images were analysed
168 with ImageJ version 1.47 (National Institute of Health, USA).

169 Enterocyte total absorptive surface (ETAS), μm^2 was calculated according to the following:

$$170 \quad \text{ETAS} = ((2\pi \times \frac{1}{2} \text{MVD} \times \text{MVL}) + (\pi \times \frac{1}{2} \text{MVD}^2)) \times \text{MVCT} \times \text{EAA}$$

171 Where ETAS = enterocyte total absorptive surface (μm^2); π = pie constant = 22/7; MVD =
172 microvilli diameter (μm); MVL = microvilli length (μm); MVCT = microvilli count (No.
173 / μm^2); and EAA = enterocyte apical area.

174 2.5 *Intestinal microbiology*

175 The GI tract was aseptically removed and faecal matter from the mid-intestine was isolated
176 and processed on an individual fish basis. DNA was extracted from 100 mg faecal matter
177 after lysozyme (50 mg mL⁻¹ in TE buffer) incubation for 30 min at 37 °C using PowerFecal®
178 DNA Isolation Kit according to the manufacturer's instructions.

179 2.5.1 High-throughput sequencing analysis

180 DNA extractions from the faecal matter were prepared for high-throughput sequencing as
181 described by Standen et al. (2015). In brief, PCR amplification of the 16S rRNA V1-V2
182 region was conducted using primers 27F (5' -AGA GTT TGA TCM TGG CTC AG-3')
183 and 338R (5' -GCW GCC WCC CGT AGG WGT-3'). Each PCR contain 0.5 μL primer

184 27F and 338R (50pmol μL^{-1} ; Eurofins MWG, Germany), 25 μL MyTaqTM Red Mix
185 (Bioline), 22 μL molecular grade water (Ambion) and 2 μL DNA template. Thermal cycling
186 was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the
187 following conditions: initial denaturation at 94 °C for 7 minutes, then 10 cycles at 94 °C for
188 30 seconds, touchdown of 1 °C per cycle from 62-53 °C for 30 seconds and 72 °C for 30
189 seconds. Furthermore, 20 cycles were performed at 94 °C for 30 seconds, 53 °C for 30
190 seconds and 72 °C for 30 seconds before a final extension for 7 minutes at 72 °C. The quality
191 of the PCR products was checked using agarose gel electrophoresis. PCR products were
192 purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit[®] 2.0
193 Fluorometer (Invitrogen). Before sequencing, the amplicons were assessed for fragment
194 concentration using an Ion Library Quantitation Kit (Life Technologies TM, USA), the
195 concentrations were then adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles
196 using Ion PGM Template OT2 400 kit (Life TechnologiesTM, USA) according to the
197 manufacturer's instructions. Multiplexed sequencing was conducted using Ion Xpress
198 Barcode Adapters (Life TechnologiesTM) and a 318TM chip (Life TechnologiesTM) on an Ion
199 Torrent Personal Genome Machine (Life TechnologiesTM). The sequences were binned by
200 sample and filtered within the PGM software to remove low quality reads. Data were
201 exported as FastQ files.

202 Phylogenetic analyses were performed after the removal of reads with low quality scores (Q
203 < 20) with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and
204 sorted by sequence similarity into a single fasta file, denoised and analysed using the QIIME
205 1.8.0 pipeline (Caporaso et al., 2010b). The USEARCH quality filter pipeline (Edgar, 2010)
206 was used to filter out putative chimeras and noisy sequences and carry out OTU picking on
207 the remaining sequences. The taxonomic affiliation of each OTU was determined based on
208 the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang et al., 2007)

209 clustering the sequences at 95 % similarity with a 0.80 confidence threshold and a minimum
210 sequence length of 150 base pairs. Non-chimeric OTUs were identified with a minimum
211 pairwise identity of 95 %, and representative sequences from the OTUs were aligned using
212 PyNAST (Caporaso et al., 2010a). To estimate bacterial diversity, the number of OTUs
213 present in the samples was determined and a rarefaction analysis was performed by plotting
214 the number of observed OTUs against the number of sequences. Good's coverage, Shannon-
215 Wiener (diversity) and Chao1 (richness) indices were calculated. The similarities between the
216 microbiota compositions of the intestinal samples were compared using weighted principal
217 coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean
218 (UPGMA).

219 2.7 *Statistical analysis*

220 All data are presented as mean \pm standard deviation. Statistical analysis (except high-
221 throughput sequencing) was carried out using SPSS for Windows (SPSS Inc., 22.0, Chicago,
222 IL, USA). Data were checked for normality and equality of variance using Kolmogorov-
223 Smirnov and Bartlett's test, respectively. Where normal assumptions were met, data were
224 analysed using one-way analysis of variance (ANOVA) followed by a post-hoc Duncan test
225 to determine significant differences. Where data violated these conditions after log
226 transformation, a Kruskal- Wallis test was used. Differences between treatments were then
227 determined using a Mann-Whitney U-test. For high-throughput sequence data, a Kruskal-
228 Wallis test was performed followed by pairwise comparison to compare alpha diversity
229 metrics, and Vegan and ape packages of R were used to analyse the beta diversity of the
230 groups. STAMP v2.1.3 and PRIMER V7 software (PRIMER-E Ltd., Ivybridge, UK) were
231 used to distinguish differences at each taxonomic level for high-throughput sequence data. In
232 all cases significance was accepted at $P < 0.05$.

233 3.0 Results

234 3.1 *Growth performance, feed utilisation and somatic indices*

235 Growth performance and feed utilisation was assessed using tilapia FBW, SGR, FCR and
236 PER (Table 2). Tilapia fed the diet supplemented with enz-pro performed better ($P < 0.05$)
237 than tilapia fed the control and probiotic supplemented diets in term of FBW, SGR, FCR and
238 PER. However, there was no difference ($P > 0.05$) in the performance of tilapia fed the diet
239 supplemented with the enzymes and those fed diet supplemented with enz-pro in terms of
240 FBW, SGR and FCR. The dietary treatment did not have a significant effect on the tilapia
241 somatic indices. A 100% survival was recorded in all the treatments.

242 3.2 *Haemato – immunological parameters*

243 The haemato-immunological parameters of tilapia fed the experimental diets are displayed in
244 Table 3. Serum lysozyme activity was significantly higher ($P < 0.05$) in tilapia fed the
245 probiotic supplemented diet compared to serum lysozyme activity in tilapia fed the control
246 and enz-pro treatments. No differences were observed between treatments in any other
247 haematological parameter measured.

248 3.3 *Intestinal histology*

249 The mid-intestine of tilapia fed each of the experimental diets was examined by light
250 microscopy (Figure 1), scanning and transmission electron microscopy (Figure 2). Tilapia
251 from all treatments showed intact epithelial barriers with extensive mucosal folds extending
252 into the lumen. Each fold consisted of simple lamina propria with abundant IELs and goblet
253 cells (Figure 1). Tilapia fed the diet supplemented with enz-pro had significantly higher
254 perimeter ratio and microvilli count (density) compared to tilapia fed probiotic supplemented
255 and control diets (Table 4). Goblet cells abundance was significantly higher ($P < 0.05$) in
256 tilapia fed the diet supplemented with enz-pro than those fed the control diet. Microvilli

257 diameter of tilapia fed a diet supplemented with enz-pro was larger ($P < 0.05$) than tilapia fed
258 the control diet. This translated to higher ($P < 0.05$) enterocyte absorptive area in tilapia fed
259 diets supplemented with enzymes and a combination of both enzymes and probiotic than
260 tilapia fed with the control diet.

261 3.4 *Intestinal microbiology*

262 A total of 536,602 sequence reads from the tilapia digesta were retained after trimming; after
263 removing low quality reads, 24,521±14,451, 25,588±12,901, 32,708±10,388 and
264 24,503±12,255 sequences for control, enzymes, probiotic and enz-pro treatments,
265 respectively, were used for downstream analyses. Good's coverage rarefaction curves for the
266 treatments reached a plateau close to 1 (0.9994 – 0.9996) (Figure 1a and Table 5), an
267 indication that sufficient coverage was achieved and that the OTUs detected in the samples
268 are representative of the sampled population.

269 The majority of reads derived from the tilapia digesta belonged to members of Fusobacteria (>
270 89%) distantly followed by Proteobacteria (> 7%) and Firmicutes (> 0.4%) (Figure 3c).
271 Table 6 shows the most abundant genera in tilapia digesta. *Cetobacterium*, *Aquaspirillum*,
272 *Edwardsiella* and *Plesiomonas* as well as unknown genera from the order *Clostridiales*,
273 family *Clostridiaceae*, class *Gammaproteobacteria* and order *Aeromonadales* were present in
274 all treatments with *Cetobacterium* being dominant (> 84%) in all treatments. *Cetobacterium*
275 accounted for 92.1%, 89.3%, 84.2% and 91% 16S rRNA reads in tilapia fed the control,
276 enzymes, probiotic and enz-pro diets, respectively. Unknown genera from the families
277 *Leuconostocaceae* and *Methylocystaceae* were present in the control, enzymes and probiotic
278 treatments but absent in the enz-pro treatment. *Weissella* and an unknown genus from the
279 family *Methylocystaceae* were present in the enzymes and probiotic treatments. *Balneimonas*
280 was present in enzymes and enz-pro treatments. An unknown genus from the class

281 *Betaproteobacteria* was also present in the control, probiotic and enz-pro treatments.
282 However, *Corynebacterium*, *Bacillus*, *Staphylococcus* and *Rhodobacter* were only detected in
283 probiotic treatment.

284 The alpha diversity parameters are presented in Table 5. There was no significant difference
285 between the treatments for the alpha diversity metrics assessed. Figure 3b shows the beta
286 diversity of the digesta through PCoA plots (based on Bray-Curtis dissimilarity matrix). The
287 PCoA plot shows a spatial differentiation among the treatments.

288 **4.0 Discussion**

289 The previous reports on the use of exogenous digestive enzymes (Cao et al., 2007, Kumar et
290 al., 2012, Castillo and Gatlin, 2015, Lemos and Tacon, 2016) and probiotic (Pandiyan et al.,
291 2013, Pérez - Sánchez et al., 2014) as individual supplement in fish diet abounds. However,
292 to the authors' knowledge no research has been conducted previously on the combined
293 effects of exogenous digestive enzymes and probiotic on growth, intestinal morphology and
294 microbiome of Nile tilapia. In this study, Nile tilapia were fed diet supplemented with
295 enzymes, probiotic or a combination of both the enzymes and probiotic. Given the potential
296 complimentary modes of actions of exogenous digestive enzymes and probiotic, the two
297 products (when used in combination) could offer more benefits than when used alone. This is
298 confirmed in this study with improved growth performance in terms of FBW, SGR, FCR and
299 PER observed in tilapia fed diet supplemented with enz-pro a combination of enzymes and
300 probiotic. The enhanced growth performance could be attributed to the ability of probiotic to
301 produce fibre-degrading enzymes that may complement endogenous enzyme activity for
302 digestion in fish (Roy et al., 2009, Ray et al., 2010, Ray et al., 2012) as well as the external
303 exogenous enzyme capacity to increase the availability of suitable substrates for probiotic
304 action (Bedford and Cowieson, 2012). In addition, the enzymes could positively affect the gut

305 microbiota through improved digestibility and enhanced nutrient absorption and assimilation.
306 The indigestible NSPs and trypsin inhibitors that appear to induce necrotic enteritis in certain
307 fish species are well known substrates for xylanase and protease enzymes respectively.
308 Furthermore, xylanase may increase the digestion of NSPs (e.g. arabinoxylans) which could
309 provide substrates for utilisation by gut bacteria (Bedford, 2000).

310 The use of enzymes and probiotic as individual supplements in this study did not have
311 significant effects on the growth performance of tilapia. This is somewhat contrary to the
312 results of Hlophe - Ginindza et al. (2015) who observed significantly improved growth
313 performance in tilapia (*Oreochromis mossambicus*) when an exogenous enzyme cocktail,
314 Natuzyme[®] (containing protease, lipase, α -amylase, cellulase, amyloglucosidase, β -glucanase,
315 pentosanase, hemicellulose, xylanase, pectinase, acid phosphatase and acid phytase) was
316 added to a plant-based diet. The inconsistency in the findings may be due to lower application
317 dosage of enzymes (75 mg kg⁻¹ phytase, 300 mg kg⁻¹ protease and 250 mg kg⁻¹ xylanase)
318 used in the current study compared to 500 mg kg⁻¹ used by Hlophe - Ginindza et al. (2015),
319 in addition to the broader diversity of enzymes in Natuzyme[®] or the different tilapia species.
320 On the other hand, the lack of effect on tilapia growth fed probiotic supplemented diet in the
321 current study is similar to the findings of Ng et al. (2014) who reported that dietary probiotic
322 (*B. subtilis*, *B. licheniformis* or *Pediococcus sp.*) had no effect on growth or feeding
323 efficiencies of tilapia. Shelby et al. (2006) also observed a non-effect of dietary *Enterococcus*
324 *faecium* and *Pediococcus acidilactici* or mixtures of *B. subtilis* and *B. licheniformis* on
325 growth of tilapia. However, *B. subtilis* when used solely as a dietary supplement was reported
326 to be an effective growth promoter in tilapia (Aly et al., 2008), yellow croaker, *Larimichthys*
327 *crocea* (Ai et al., 2011) and rohu, *Labeo rohita* (Nayak and Mukherjee, 2011).

328 The improvement in intestinal morphology in the current study could be the result of
329 complimentary changes to meet the increased rates of digestion and absorption after exposure
330 to the diets. In this study, tilapia fed the diet supplemented with probiotic and enzymes
331 presented a higher perimeter ratio, microvilli count (density) and larger diameter which
332 translated to increased enterocyte absorptive area and subsequently resulted in the improved
333 growth performance when compared with tilapia fed the control diet. This could be attributed
334 to the combined effect of enzymes and probiotic to confer a superior beneficial effect than
335 when used alone. However, there was no significant difference between intestinal histology
336 of tilapia fed the control and probiotic supplemented diets. This is contrary to Standen et al.
337 (2015) who reported increased population of IELs, a higher absorptive surface area index and
338 higher microvilli density in the intestine of tilapia fed a diet supplemented with AquaStar®
339 Growout, a multi-species probiotic containing *Lactobacillus reuteri*, *Bacillus subtilis*,
340 *Enterococcus faecium* and *Pediococcus acidilactici*. This difference could be attributed to
341 different probiotic composition as well as application dosage which is 20 mg kg⁻¹ in the
342 present study compared to 5 g kg⁻¹ used by Standen et al. (2015).

343 In this study, the dietary treatment did not have significant effect on the tilapia
344 haematological parameters. Emadinia et al. (2014) also reported that supplementation of
345 poultry diets with an enzyme cocktail (xylanase, β-glucanase, cellulase, pectinase, phytase,
346 protease, lipase, and α-amylase) had no effects on haemato-immunological parameters.
347 However, in the present study the serum lysozyme activity was significantly higher in tilapia
348 fed the probiotic supplemented diet compared to those fed the control and enz-pro
349 supplemented diets respectively. This is similar to the findings of Mandiki et al. (2011) who
350 reported that dietary *Bacillus* probiotic have a stimulating effect on lysozyme activity in
351 Eurasian perch, *Perca fluviatilis*. Standen et al. (2013) also reported that dietary probiotic are
352 able to stimulate innate immune response in tilapia.

353 Gut microbiota may function to prevent pathogens from colonization of the intestinal tract.
354 The importance of commensal gut microbiota is highly important for normal functioning of
355 the immune apparatus of the GI tract in fish (Rawls et al., 2004, Pérez et al., 2010, Ringø et
356 al., 2015). The population size and composition of intestinal microbiota could influence the
357 extent of nutrient digestion and absorption by the host (Merrifield et al., 2010, Dimitroglou
358 et al., 2011, Bedford and Cowieson, 2012, Ray et al., 2012). In addition, GI microbiota are
359 understood to influence disease resistance, development, survival and feed utilisation (Denev
360 et al., 2009). Jiang et al. (2014) reported that dietary supplementation of xylanase affected the
361 abundance of *Lactobacillus*, *Escherichia coli* and *Aeromonas* in the intestine of juvenile Jian
362 carp. The intestinal microbiota of grass carp fed dietary cellulase changed in respect to
363 bacteria species and density (Zhou et al., 2013). Adeoye et al. (2016) also reported alteration
364 in the intestinal bacterial community profile of tilapia fed carbohydrase supplemented diet.
365 Similarly, several studies have reported the modulating effect of probiotic on fish GI
366 microbiota (Dimitroglou et al., 2011, Pandiyan et al., 2013, Pérez - Sánchez et al., 2014,
367 Standen et al., 2015). However, in the present study exogenous enzymes and probiotic did not
368 modify to a large extent microbial community of tilapia fed the experimental diets.
369 Regardless of the dietary treatments, certain OTUs such as *Clostridiales*, *Cetobacterium*,
370 *Aquaspirillum*, *Gammaproteobacteria*, *Aeromonadales*, *Edwardsiella* and *Plesiomonas* were
371 found in the intestinal tract of tilapia, forming core microbiome. This is similar to findings by
372 Larsen et al. (2014) who reported dominance of genus *Cetobacterium* in the gut of warm
373 water fish species. Similarly, shared core gut microbiota was observed in zebrafish
374 irrespective of geographical locations (Roeselers et al., 2011). Wong et al. (2013) also
375 reported core intestinal microbiota in rainbow trout being resistant to variation in diet and
376 rearing density. Similarly, the tilapia microbiome was quite stable and resistant to potential
377 changes in community abundance and diversity in response to the dietary supplements used

378 in this study. However, the functionality of the microbiome may have been altered and this
379 may have contributed towards the improved performance of the tilapia fed the enzymes and
380 probiotic cocktail. Future studies should include metagenomics and metatranscriptomics of
381 the gut microbiome to investigate this hypothesis.

382 In conclusion, supplementation of tilapia diets with a combination of enzymes and probiotic
383 is capable of improving tilapia growth and intestinal histology without deleterious effect on
384 the fish health or intestinal microbiota. It is pertinent therefore to consider these finding for
385 the future development of diets specific for tilapia under a variety of culture conditions and
386 stages of growth from fry to fingerlings and on-growing to production (harvest) size.

387

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566

568 **Table 1.** Dietary formulation and proximate composition (g kg⁻¹) of experimental diets

	Control	Enzymes	Probiotics	Enz-pro
Commercial feed ^a	1000	999.94	999.98	998.92
Phytase ^b (mg)	0	7.5	0	7.5
Protease ^c (mg)	0	30	0	30
Xylanase ^d (mg)	0	25	0	25
Probiotics ^e (mg)	0	0	20	20
Total	1000	1000	1000	1000
<i>Proximate composition (% as fed basis)</i>				
Moisture	8.03±0.04	6.87±0.14	8.06±0.06	6.63±0.09
Protein	34.32±0.28	34.78±0.09	34.43±0.13	34.56±0.08
Lipid	5.49±0.04	5.33±0.10	5.38±0.70	5.22±0.08
Ash	13.13±0.11	13.13±0.17	13.16±0.04	13.4±0.04
Energy (MJ kg ⁻¹)	17.06±0.00	17.56±0.1	17.31±0.4	17.66±2.1
Fibre	3.65±0.06	3.15±0.12	3.15±0.07	3.21±0.05

569 ^aNo. 461, INTEQC Feed Co Ltd., Thailand570 ^bRONOZYME[®] Hiphos (contains 10,000FYT g⁻¹) from DSM Nutritional Products571 ^cRONOZYME[®] ProAct (contains 75,000 PROT g⁻¹) from DSM Nutritional Products572 ^dRONOZYME[®] WX (contains 1000 FXU g⁻¹) from DSM Nutritional Products573 ^eSanolife PRO-F (contains 1 x 10¹⁰ CFU g⁻¹ *B. subtilis*, *B. licheniformis* and *B. pumilus*) from

574 INVE Aquaculture

575

576 **Table 2.** Growth performance, feed utilisation and somatic indices of tilapia fed the
 577 experimental diets

	Control	Enzymes	Probiotics	Enz-pro
IBW (g fish ⁻¹)	34.5±0.18	34.54±0.05	34.6±0.13	34.61±0.29
FBW (g fish ⁻¹)	138.04±2.44 ^a	139.49±2.83 ^{ab}	136.61±1.34 ^a	143.42±3.06 ^b
SGR (% day ⁻¹)	3.30±0.05 ^a	3.32±0.04 ^{ab}	3.27±0.02 ^a	3.38±0.04 ^b
FI (g fish ⁻¹)	92.24±0.92	92.83±1.22	92.35±0.27	93.00±1.39
FCR	0.94±0.02 ^a	0.93±0.02 ^{ab}	0.96±0.02 ^a	0.9±0.01 ^b
PER	2.49±0.06 ^{ab}	2.53±0.06 ^b	2.42±0.05 ^a	2.63±0.02 ^c
HSI	3.19±0.23	3.18±0.26	2.86±0.46	3.10±0.02
VSI	21.72±0.66	21.44±2.96	23.40±1.31	21.83±1.61
K-factor	2.11±0.08	2.06±0.05	2.10±0.07	2.06±0.04
Survival (%)	100	100	100	100

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

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586 **Table 3.** Haemato – immunological parameters of tilapia fed the experimental diets

	Control	Enzymes	Probiotics	Enz-pro
Haematocrit, (%PCV)	40.11±3.34	39.11±1.35	41.67±3.48	39.66±1.53
Haemoglobin, (g dL ⁻¹)	11.35±1.21	10.66±0.91	11.93±2.50	11.33±0.22
RBC (10 ⁶ μL ⁻¹)	1.74±0.10	2.02±0.47	1.92±0.32	1.87±0.09
RBC (10 ³ μL ⁻¹)	20.28±1.34	20.37±4.00	20.59±0.08	20.64±2.82
MCV (fL)	232.53±12.95	207.97±36.80	223.30±34.69	213.04±12.66
MCH (pg)	66.10±4.60	56.25±6.66	62.76±7.18	61.00±4.19
MCHC (g dL ⁻¹)	28.29±1.59	27.25±1.59	28.75±3.98	28.62±0.97
Lymphocytes (%)	90.43±2.57	91.40±2.38	91.77±1.30	89.43±3.54
Monocytes (%)	5.14±1.87	4.26±2.06	3.94±0.54	5.74±1.97
Granulocytes (%)	4.42±0.70	4.34±0.33	4.29±0.76	4.83±1.62
Serum lysozyme (U)	115.31±22.87 ^a	154.21±24.93 ^{ab}	170.39±22.98 ^b	127.97±6.43 ^a

587 Figures in each row with different superscript are significantly different ($P < 0.05$).

588 RBC, red blood cells; WBC, leucocytes; MCV, mean corpuscular volume (haematocrit
589 (%PCV) x 10)/RBC 10⁶ μL⁻¹); MCH, mean corpuscular haemoglobin (haemoglobin (g dL⁻¹)
590 x 10)/RBC (10⁶ μL⁻¹); MCHC, mean corpuscular haemoglobin concentration (haemoglobin
591 (g dL⁻¹) x 100)/haematocrit (%PCV); %, mean percentage of total leucocytes; U, lysozyme
592 activity mL⁻¹ min⁻¹

593

594 **Table 4.** Intestinal histology of tilapia fed the experimental diets

	Control	Enzymes	Probiotics	Enz-pro
Perimeter ratio	5.30±0.7 ^a	5.84±0.4 ^{ab}	5.22±0.5 ^a	6.72±0.8 ^b
Goblet cells (per 100µm)	3.85±0.6 ^a	4.66±0.6 ^{ab}	4.55±0.6 ^{ab}	5.11±0.2 ^b
IELs (per 100µm)	29.16±5	29.48±2	29.85±5	28.68±4
Microvilli count (per µm ²)	91.82±4 ^a	110.30±2.2 ^{bc}	103.75±5.9 ^b	115.17±6.5 ^c
Enterocyte apical area (µm ²)	11.30±1.3	12.39±1.4	12.06±1	12.47±2.1
Microvilli length (µm)	1.24±0.04	1.35±0.03	1.32±0.2	1.27±0.04
Microvilli diameter (µm)	0.117±0.01 ^a	0.123±0.01 ^{ab}	0.123±0.01 ^{ab}	0.130 ^b
ETAS(µm ²)	499.9±82 ^a	762.17±85 ^b	674.55±145 ^{ab}	773.7±151 ^b

595 Values with different superscripts indicate significant differences ($P < 0.05$). IELs,

596 Intraepithelial leucocytes; ETAS = enterocyte total absorptive surface (µm²).

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598

599 **Table 5.** Number of reads, reads assigned to OTUs, Good's coverage and alpha diversity indices of allochthonous intestinal microbiota
 600 composition between control, enzymes, probiotics and enz-pro treatments after 7 weeks of experimental feeding

	Reads (pre-trimming)	Reads assigned (post trimming)	Good's coverage	Observed species	Shanon's diversity index	Chao1 (Richness) Index
Control	41,748±22,108	24,521±14,451	0.9994±0.0001	75.90±9.54	2.82±0.10	92.00±11.19
Enzymes	42,898±20,096	25,588±12,901	0.9995±0.0007	75.18±14.54	2.78±0.14	88.77±12.04
Probiotics	57,638±15,492	32,708±10,388	0.9996±0.0002	76.95±17.94	3.20±0.60	87.28±16.15
Enz-pro	40,244±18,342	24,503±12,255	0.9994±0.0001	72.12±7.10	2.94±0.25	88.04±8.18

601 There were no significant differences between the treatments

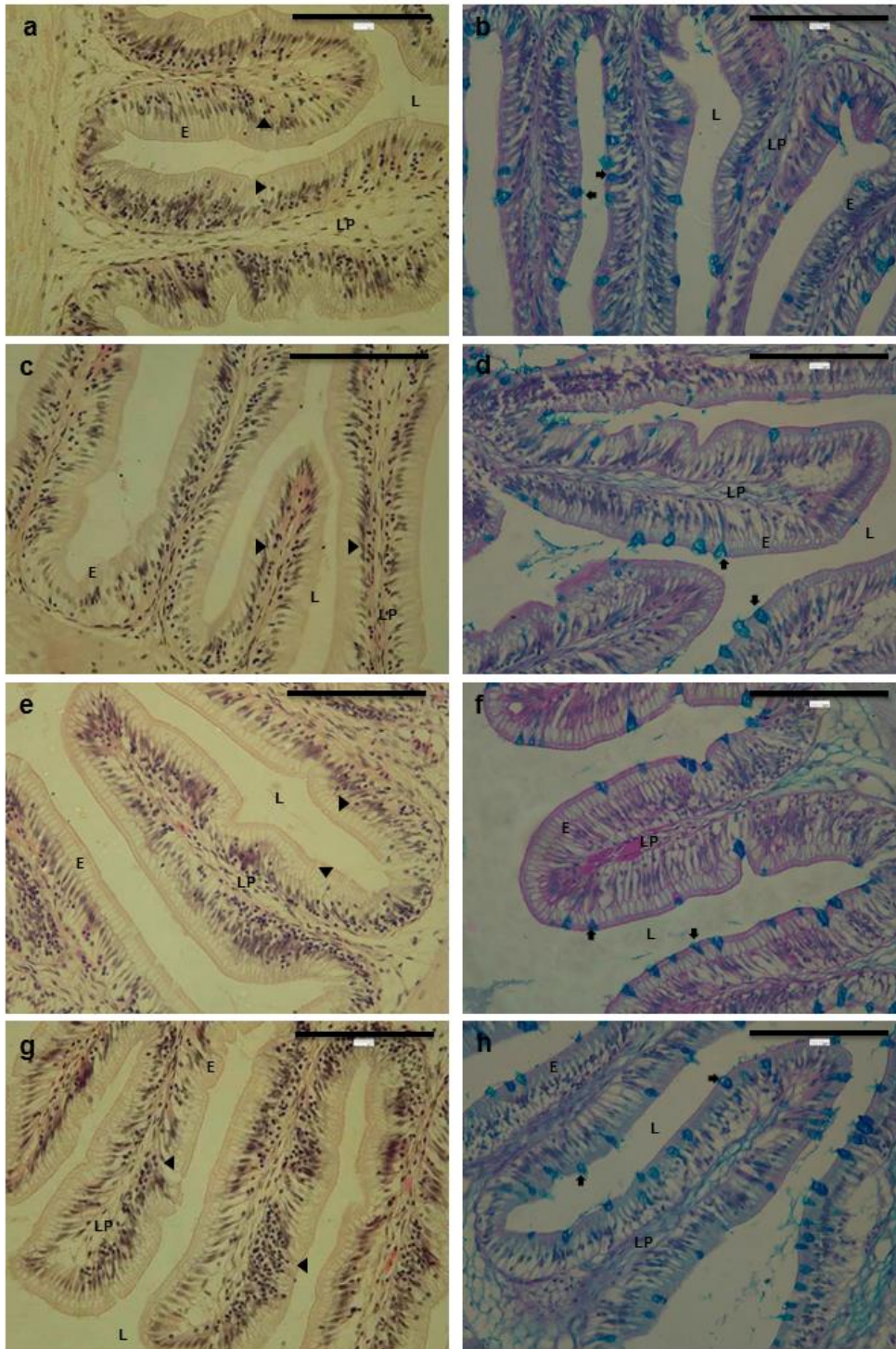
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604 **Table 6.** Abundance of the OTUs present in digesta samples (expressed as %). General level
 605 identification is presented where possible

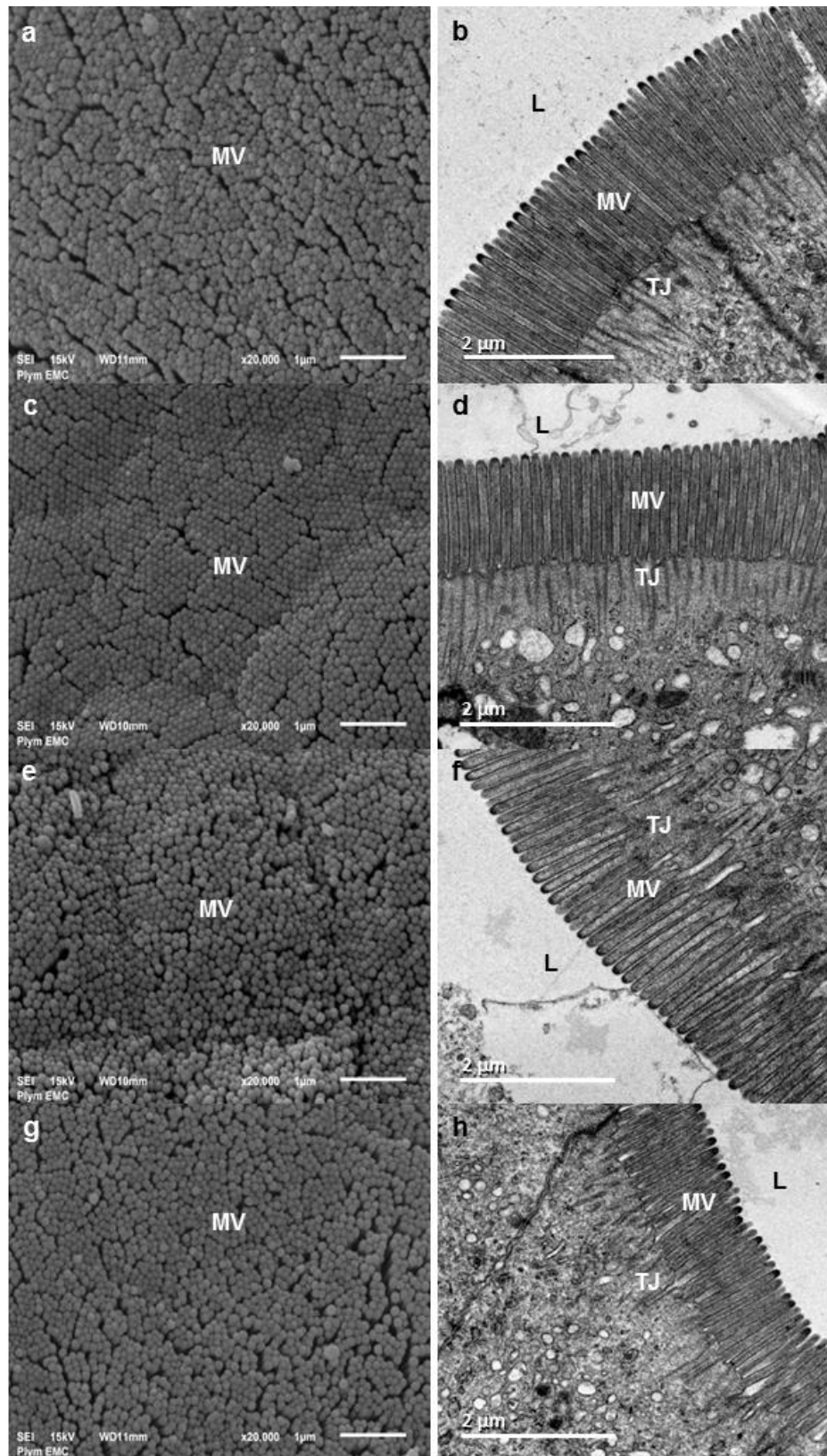
OTU	Control	Enzymes	Probiotics	Enz-Pro
<i>Cetobacterium</i>	92.1±3.8	89.3±4.8	84.21±4.3	91.0±3.4
<i>Plesiomonas</i>	4.0±2.5	7.7±4.4	5.6±1.9	4.0±2.2
Unknown genus from order <i>Aeromonadales</i>	2.4±2.4	1.0±0.5	3.1±2.4	2.7±2.4
<i>Aquaspirillum</i>	0.9±0.4	0.4±0.3	1.2±1.3	0.7±0.7
Unknown genus from family <i>Leuconostocaceae</i>	0.1±0.1	0.2±0.3	1.5±2.9	0.0±0.0
Unknown genus from family <i>Leuconostocaceae</i>	0.1±0.2	0.2±0.3	2.0±3.9	0.0±0.0
<i>Edwardsiella</i>	0.2±0.1	0.6±0.7	1.2±1.4	0.3±0.1
Unknown genus from order <i>Clostridiales</i>	0.1±0.1	0.2±0.1	0.1±0.0	0.1±0.1
Unknown genus from family <i>Clostridiaceae</i>	0.1±0.1	0.1±0.1	0.1±0.1	0.1±0.1
Unknown genus from class <i>Gammaproteobacteria</i>	0.1±0.0	0.1±0.1	0.3±0.2	0.1±0.1
Unknown genus from class <i>Betaproteobacteria</i>	0.2±0.3	0.0±0.0	0.6±1.2	0.1±0.0
<i>Weissella</i>	0.0±0.0	0.1±0.2	0.7±1.4	0.0±0.0
Unknown genus from family <i>Methylocystaceae</i>	0.1±0.1	0.2±0.4	0.3±0.6	0.0±0.0
<i>Balneimonas</i>	0.0±0.0	0.1±0.1	0.0±0.0	0.6±1.2
Unknown genus from family <i>Methylocystaceae</i>	0.0±0.0	0.1±0.1	0.2±0.3	0.0±0.0
<i>Rhodobacter</i>	0.0±0.0	0.0±0.0	0.4±0.9	0.0±0.0
<i>Leuconostoc</i>	0.0±0.0	0.1±0.1	0.1±0.2	0.0±0.0
<i>Staphylococcus</i>	0.0±0.0	0.0±0.0	0.2±0.4	0.0±0.0
<i>Corynebacterium</i>	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0
<i>Bacillus</i>	0.0±0.0	0.0±0.0	0.1±0.2	0.0±0.0

606 There was no significant difference across the treatments



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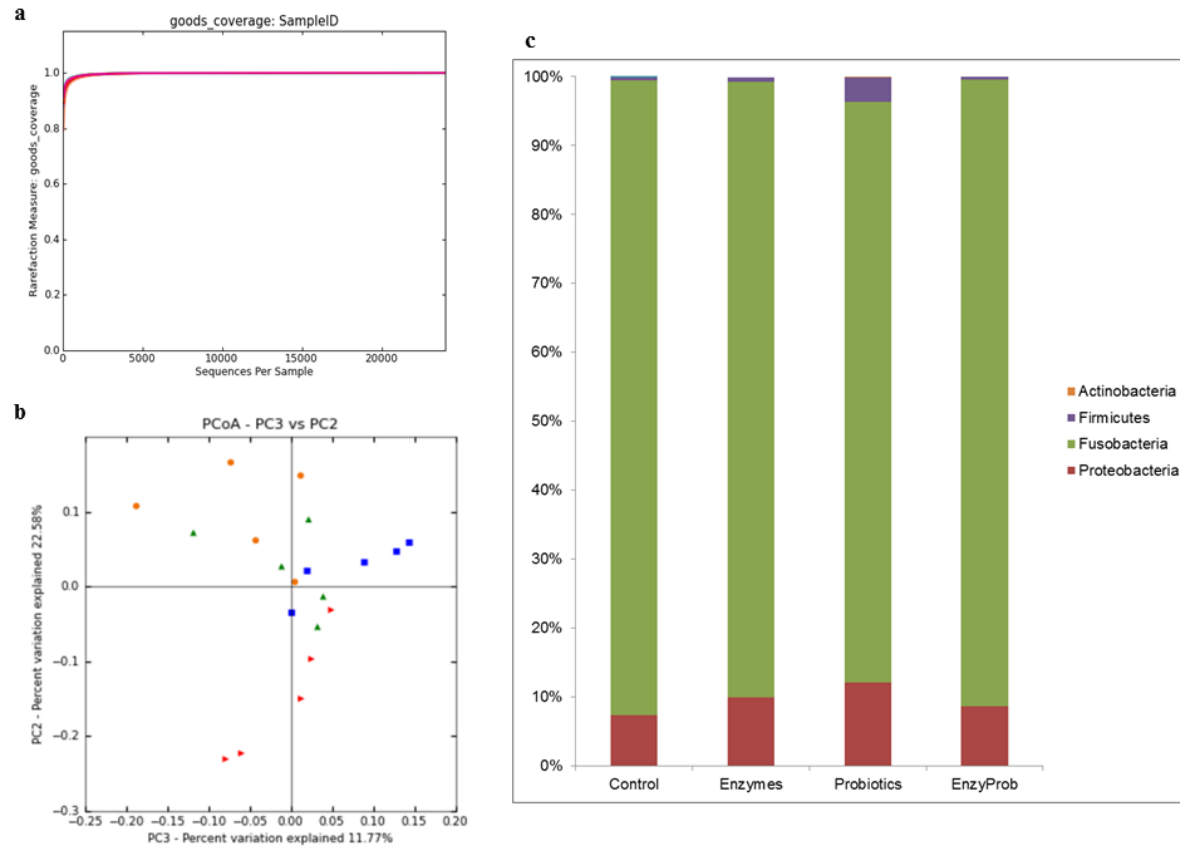
609 **Figure 1.** Light micrograph of the mid-intestine of tilapia fed control (a & b), enzymes (c &
 610 d), probiotics (e & f) and enz-pro (g & h) diets. Goblet cells (arrows) and abundant IELs
 611 (arrowheads) are present in the epithelia. Abbreviations are E enterocytes, LP lamina propria
 612 and L lumen. Light microscopy staining: [a, c, e & g] H & E; [b, d, f & h] Alcian Blue-PAS.
 613 Scale bars = 100 μ m.



614

615 **Figure 2.** Scanning electron (a, c, e & g) and transmission electron (b, d, f & h) micrographs
 616 of the mid-intestine of tilapia fed control (a & b), enzymes (c & d), probiotics (e & f) and
 617 enz-pro (g & h) diets. Abbreviations are L lumen, TJ tight junction, MV microvilli. Scale
 618 bars = 1 µm (a, c, e & g), 2 µm (b, d, f & h).

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620

621 **Figure 3.** 16S rRNA V1-V2 high-throughput sequencing libraries of digesta from the tilapia intestine. (a) Good's coverage rarefaction curves of
622 the tilapia digesta; (b) PCoA plots using Bray-Curtis dissimilarity matrix where data points represent samples from tilapia fed a control diet (red
623 triangles), enzymes diet (blue squares), probiotic diet (green triangles) and enz-pro diet (orange circles); and (c) proportion of 16S rRNA reads
624 from the tilapia digesta by dietary treatment assigned at the phylum level.