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Merrifield,

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

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4 Ayodeji A. Adeoye^{a*}, Rungtawan Yomla^a, Alexander Jaramillo-Torres^a, Ana Rodiles^a, Daniel
5 L. Merrifield^a and Simon J. Davies^b

6 ^aSchool of Biological Sciences, Plymouth University, UK

7 ^bDepartment of Animal Production, Welfare and Veterinary Sciences, Harper Adams
8 University, UK
9

10 **Abstract**

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

* Corresponding author: Ayodeji Adeoye, School of Biological Sciences, Plymouth University, Plymouth - UK, PL4 8AA. E-mail address: ayodeji.adeoye@plymouth.ac.uk. Tel: +44 1752 584684

28 *Cetobacterium*) distantly followed by Proteobacteria and Firmicutes. The alpha and beta
29 diversities did not differ among dietary treatments indicating that the overall microbial
30 community was not modified to a large extent by dietary treatment. In conclusion,
31 supplementation of the diet with a combination of enzymes and probiotic is capable of
32 improving tilapia growth and intestinal morphology without deleterious effect on the
33 intestinal microbial composition.

34

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

36 1.0 Introduction

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

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

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

62 It is well established that GI microbial communities are sensitive to rearing environment,
63 seasonal and diet changes including the supplementation with probiotic (Dimitroglou et al.,
64 2011, Merrifield et al., 2010, Romero et al., 2014) and exogenous digestive enzymes
65 (Bedford and Cowieson, 2012, Geraylou et al., 2012, Zhou et al., 2013, Jiang et al., 2014,
66 Adeoye et al., 2016, Hu et al., 2016). Research into the use of exogenous digestive enzyme
67 and probiotic supplements is increasing since aquafeed manufacturers are increasingly
68 interested in producing 'functional and environmentally friendly aquafeeds'. The potential
69 effects of exogenous digestive enzymes (Kumar et al., 2012, Castillo and Gatlin, 2015,
70 Lemos and Tacon, 2016) and probiotic (Pérez - Sánchez et al., 2014) on fish have been
71 reviewed as individual supplement. To the authors' understanding, there is no previous report
72 on combined used of exogenous digestive enzymes and probiotic as supplement in fish.
73 However, the combined supplementation of exogenous enzymes and probiotic could result in
74 a complimentary mode of actions: ability to produce fibre-degrading enzymes by probiotic
75 may complement endogenous enzyme activity. On the other hand, exogenous digestive
76 enzymes may increase availability of suitable substrate for probiotic as well as promote the
77 growth of other beneficial bacteria (GI microbiota).

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

85 2.0 Materials and methods

86 2.1 Experimental design and diets preparation

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

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

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

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

117 2.2 *Growth performance, feed utilisation and somatic indices*

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

122 $SGR = 100 ((\ln FBW - \ln IBW)/T)$, where FBW = final body weight (g) and IBW = initial
123 body weight (g)

124 $FCR = FI/WG$, where FI = feed intake (g) and WG = wet weight gain (g)

125 $PER = WG/PI$, where WG = wet weight gain (g) and PI = protein ingested (g),

126 $K = (100 \times FW)/FL^3$, where FL = FL = final length (cm)

127 $HSI = 100 (LW/ FBW)$, where LW = liver weight (g) and FBW = final body weight (g)

128 $VSI = 100 (VW/ FBW)$, where VW = visceral weight (g)

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

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

135 2.3 *Haemato – immunological parameters*

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

145 2.4 *Intestinal histology*

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

156 microscopy (SEM) and transmission electron microscopy (TEM), samples were washed in 1 %
157 S-carboxymethyl-L-cysteine for 30 seconds (SEM only) to remove mucus before fixing in
158 2.5 % glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2). samples were processed as
159 described elsewhere (Dimitroglou et al., 2009) and screened with a JSM 6610 LV (Jeol,
160 Tokyo, Japan) SEM or JEN 1400 (Jeol, Tokyo, Japan) TEM. The SEM images were analysed
161 to assess microvilli count per μm^2 (MCVT) and enterocyte apical area (EAA), μm^2 . The
162 TEM images were analysed for microvilli length and diameter. All images were analysed
163 with ImageJ version 1.47 (National Institute of Health, USA).

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

165
$$\text{ETAS} = ((2\pi \times \frac{1}{2} \text{MVD} \times \text{MVL}) + (\pi \times \frac{1}{2} \text{MVD}^2)) \times \text{MVCT} \times \text{EAA}$$

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

169 2.5 *Intestinal microbiology*

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

174 2.5.1 High-throughput sequencing analysis

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

179 27F and 338R (50pmol μL^{-1} ; Eurofins MWG, Germany), 25 μL MyTaqTM Red Mix
180 (Bioline), 22 μL molecular grade water (Ambion) and 2 μL DNA template. Thermal cycling
181 was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the
182 following conditions: initial denaturation at 94 °C for 7 minutes, then 10 cycles at 94 °C for
183 30 seconds, touchdown of 1 °C per cycle from 62-53 °C for 30 seconds and 72 °C for 30
184 seconds. Furthermore, 20 cycles were performed at 94 °C for 30 seconds, 53 °C for 30
185 seconds and 72 °C for 30 seconds before a final extension for 7 minutes at 72 °C. The quality
186 of the PCR products was checked using agarose gel electrophoresis. PCR products were
187 purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit[®] 2.0
188 Fluorometer (Invitrogen). Before sequencing, the amplicons were assessed for fragment
189 concentration using an Ion Library Quantitation Kit (Life Technologies TM, USA), the
190 concentrations were then adjusted to 26 pM. Amplicons were attached to Ion Sphere Particles
191 using Ion PGM Template OT2 400 kit (Life TechnologiesTM, USA) according to the
192 manufacturer's instructions. Multiplexed sequencing was conducted using Ion Xpress
193 Barcode Adapters (Life TechnologiesTM) and a 318TM chip (Life TechnologiesTM) on an Ion
194 Torrent Personal Genome Machine (Life TechnologiesTM). The sequences were binned by
195 sample and filtered within the PGM software to remove low quality reads. Data were
196 exported as FastQ files.

197 Phylogenetic analyses were performed after the removal of reads with low quality scores (Q
198 < 20) with FASTX-Toolkit (Hannon Laboratory, USA). Sequences were concatenated and
199 sorted by sequence similarity into a single fasta file, denoised and analysed using the QIIME
200 1.8.0 pipeline (Caporaso et al., 2010b). The USEARCH quality filter pipeline (Edgar, 2010)
201 was used to filter out putative chimeras and noisy sequences and carry out OTU picking on
202 the remaining sequences. The taxonomic affiliation of each OTU was determined based on
203 the Greengenes database (DeSantis et al., 2006) using the RDP classifier (Wang et al., 2007)

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

214 2.7 *Statistical analysis*

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

228 3.0 Results

229 3.1 *Growth performance, feed utilisation and somatic indices*

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

237 3.2 *Haemato – immunological parameters*

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

243 3.3 *Intestinal histology*

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

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

256 3.4 *Intestinal microbiology*

257 A total of 536,602 sequence reads from the tilapia digesta were retained after trimming; after
258 removing low quality reads, 24,521±14,451, 25,588±12,901, 32,708±10,388 and
259 24,503±12,255 sequences for control, enzymes, probiotic and enz-pro treatments,
260 respectively, were used for downstream analyses. Good's coverage rarefaction curves for the
261 treatments reached a plateau close to 1 (0.9994 – 0.9996) (Figure 3a and Table 5), an
262 indication that sufficient coverage was achieved and that the OTUs detected in the samples
263 are representative of the sampled population.

264 The majority of reads derived from the tilapia digesta belonged to members of Fusobacteria (>
265 89%) distantly followed by Proteobacteria (> 7%) and Firmicutes (> 0.4%) (Figure 3c).
266 Table 6 shows the most abundant genera in tilapia digesta. *Cetobacterium*, *Aquaspirillum*,
267 *Edwardsiella* and *Plesiomonas* as well as unknown genera from the order *Clostridiales*,
268 family *Clostridiaceae*, class *Gammaproteobacteria* and order *Aeromonadales* were present in
269 all treatments with *Cetobacterium* being dominant (> 84%) in all treatments. *Cetobacterium*
270 accounted for 92.1%, 89.3%, 84.2% and 91% 16S rRNA reads in tilapia fed the control,
271 enzymes, probiotic and enz-pro diets, respectively. Unknown genera from the families
272 *Leuconostocaceae* and *Methylocystaceae* were present in the control, enzymes and probiotic
273 treatments but absent in the enz-pro treatment. *Weissella* and an unknown genus from the
274 family *Methylocystaceae* were present in the enzymes and probiotic treatments. *Balneimonas*
275 was present in enzymes and enz-pro treatments. An unknown genus from the class

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

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

283 **4.0 Discussion**

284 The previous reports on the use of exogenous digestive enzymes (Cao et al., 2007, Kumar et
285 al., 2012, Castillo and Gatlin, 2015, Lemos and Tacon, 2016) and probiotic (Pandiyan et al.,
286 2013, Pérez - Sánchez et al., 2014) as individual supplement in fish diet abounds. However,
287 to the authors' knowledge no research has been conducted previously on the combined
288 effects of exogenous digestive enzymes and probiotic on growth, intestinal morphology and
289 microbiome of Nile tilapia. In this study, Nile tilapia were fed diets supplemented with
290 enzymes, probiotic and a combination of both the enzymes and probiotic. Given the potential
291 complimentary modes of actions of exogenous digestive enzymes and probiotic, the two
292 products (when used in combination) could offer more benefits than when used alone. This is
293 confirmed in this study with improved growth performance in terms of FBW, SGR, FCR and
294 PER observed in tilapia fed diet supplemented with enz-pro a combination of enzymes and
295 probiotic. The enhanced growth performance could be attributed to the ability of probiotic to
296 produce fibre-degrading enzymes that may complement endogenous enzyme activity for
297 digestion in fish (Roy et al., 2009, Ray et al., 2010, Ray et al., 2012) as well as the external
298 exogenous enzyme capacity to increase the availability of suitable substrates for probiotic
299 action (Bedford and Cowieson, 2012). In addition, the enzymes could positively affect the gut

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

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

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

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

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

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

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

382

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391

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561

562 **Tables**563 **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

564 ^aNo. 461, INTEQC Feed Co Ltd., Thailand565 ^bRONOZYME[®] Hiphos (contains 10,000FYT g⁻¹) from DSM Nutritional Products566 ^cRONOZYME[®] ProAct (contains 75,000 PROT g⁻¹) from DSM Nutritional Products567 ^dRONOZYME[®] WX (contains 1000 FXU g⁻¹) from DSM Nutritional Products568 ^eSanolife PRO-F (contains 1 x 10¹⁰ CFU g⁻¹ *B. subtilis*, *B. licheniformis* and *B. pumilus*) from

569 INVE Aquaculture

570

571 **Table 2.** Growth performance, feed utilisation and somatic indices of tilapia fed the
 572 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

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

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581 **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

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

583 RBC, red blood cells; WBC, leucocytes; MCV, mean corpuscular volume (haematocrit
584 (%PCV) x 10)/RBC 10⁶ μL⁻¹); MCH, mean corpuscular haemoglobin (haemoglobin (g dL⁻¹)
585 x 10)/RBC (10⁶ μL⁻¹); MCHC, mean corpuscular haemoglobin concentration (haemoglobin
586 (g dL⁻¹) x 100)/haematocrit (%PCV); %, mean percentage of total leucocytes; U, lysozyme
587 activity mL⁻¹ min⁻¹

588

589 **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

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

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

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593

594 **Table 5.** Number of reads, reads assigned to OTUs, Good's coverage and alpha diversity indices of allochthonous intestinal microbiota
 595 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 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

596 There were no significant differences between the treatments

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599 **Table 6.** Abundance of the OTUs present in digesta samples (expressed as %). General level
 600 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

601 There was no significant difference across the treatments

602 **Figure legends**

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

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

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

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