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

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DOI: http://dx.doi.org/10.1016/j.aquaculture.2016.05.028

Adeoye, A.A., Yomla, R., Jaramillo-Torres, Rodiles, A., Merrifield, D.L. and Davies, S.J.. 2016. Combined effects of exogenous enzymes and probiotic on Nile tilapia (*Oreochromis niloticus*) growth, intestinal morphology and microbiome. *Aquaculture,* 463, pp.61-70.

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

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Abstract

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

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

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

1.0 Introduction

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

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

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

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

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

2.0 Materials and methods

2.1 *Experimental design and diets preparation*

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

The trial was conducted in a flow – through aquaculture system in King Mongkut's Institute of Technology Ladkrabang - Thailand. The flow – through system contains 12 square concrete tanks (508 L capacity each) and were supplied with freshwater sourced from a local river system. Three hundred and sixty all male Nile tilapia (*Oreochromis niloticus*) of mean weight 34.56 ± 0.05 g obtained from Charoen Pokphand farm in Thailand were randomly distributed (30 fish per tank) into the 12 tanks after two weeks of acclimatization. The photoperiod and water temperature (30.34±0.15 °C) was maintained at ambient condition. The water pH (6.20±0.22) and dissolved oxygen levels (>5.0 mg L^{-1}) were monitored daily using a HQ40d pH meter and dissolved oxygen multi-parameter meter (HACH Company, Loveland, USA). NH_3 (0.304±0.08 mg L^{-1}), NO_2 (0.016±0.002 mg L^{-1}) and NO_3 (1.46±0.19 mg L^{-1}) were also monitored on a weekly basis using a nutrient analyser (SEAL AQ2 Analyser, Hampshire, UK). A constant water flow of 4.9 L min^{-1} (per tank) was used during the experiment to maintain the water quality and ensure optimum conditions for the fish.

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

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

2.2 *Growth performance, feed utilisation and somatic indices*

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

SGR = 100 ((ln FBW – ln IBW)/T), where FBW = final body weight (g) and IBW = initial body weight (g)

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

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

K = (100 x FW)/ FL^3, where FL = FL = final length (cm)

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

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

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

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

2.3 *Haemato – immunological parameters*

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

2.4 *Intestinal histology*

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

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 $$ETAS = ((2\pi \times ½\ MVD \times MVL) + (\pi \times ½\ MVD^2)) \times MVCT \times EAA$$

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

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

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

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

2.7 *Statistical analysis*

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

3.0 Results

3.1 *Growth performance, feed utilisation and somatic indices*

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

3.2 *Haemato – immunological parameters*

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

3.3 *Intestinal histology*

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

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

3.4 *Intestinal microbiology*

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

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

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

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

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

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

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

Acknowledgment

The authors are grateful to the Commonwealth Scholarship Commission in the UK and the School of Biological Sciences, Plymouth University for funding. The authors also thank DSM Nutritional Products and INVE Aquaculture for supplying the exogenous enzymes and probiotic respectively. Members of staff at Faculty of Agriculture, King Mongkut's Institute of Technology Ladkrabang, Thailand as well as peers in the Aquatic Animal Nutrition and Health Research Group of Plymouth University are very much appreciated for technical supports.

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Tables

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
Proximate composition (% as fed basis)				
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

[a]No. 461, INTEQC Feed Co Ltd., Thailand

[b]RONOZYME® Hiphos (contains 10,000FYT g^{-1}) from DSM Nutritional Products

[c]RONOZYME® ProAct (contains 75,000 PROT g^{-1}) from DSM Nutritional Products

[d]RONOZYME® WX (contains 1000 FXU g^{-1}) from DSM Nutritional Products

[e]Sanolife PRO-F (contains 1 x 10^{10} CFU g^{-1} *B. subtilis, B. licheniformis* and *B. pumilus*) from INVE Aquaculture

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}

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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]

Values with different superscripts indicate significant differences ($P < 0.05$). IELs, Intraepithelial leucocytes; ETAS = enterocyte total absorptive surface (μm^2).

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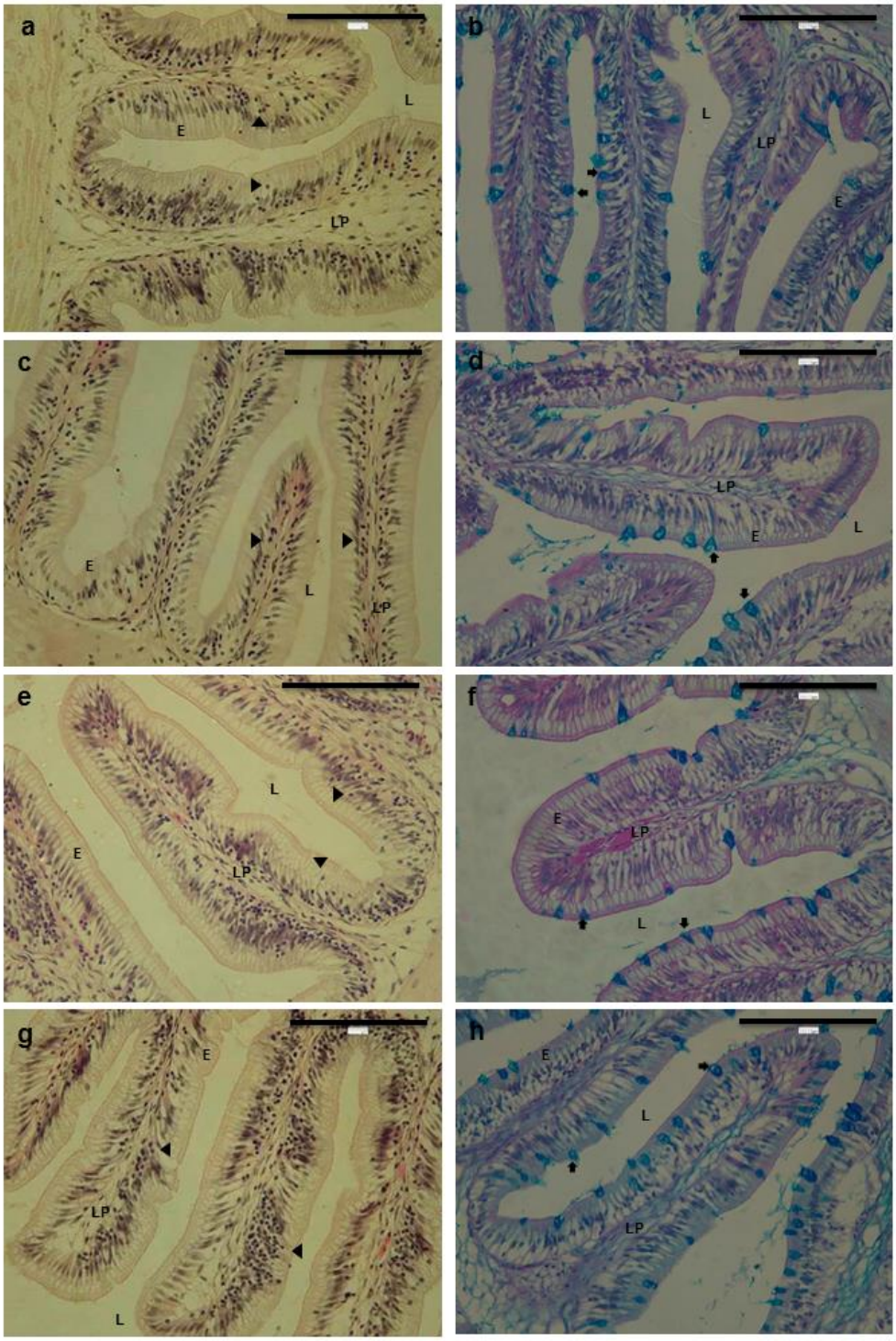
604 **Table 6.** Abundance of the OTUs present in digesta samples (expressed as %). General level
605 identification is presented where possible

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

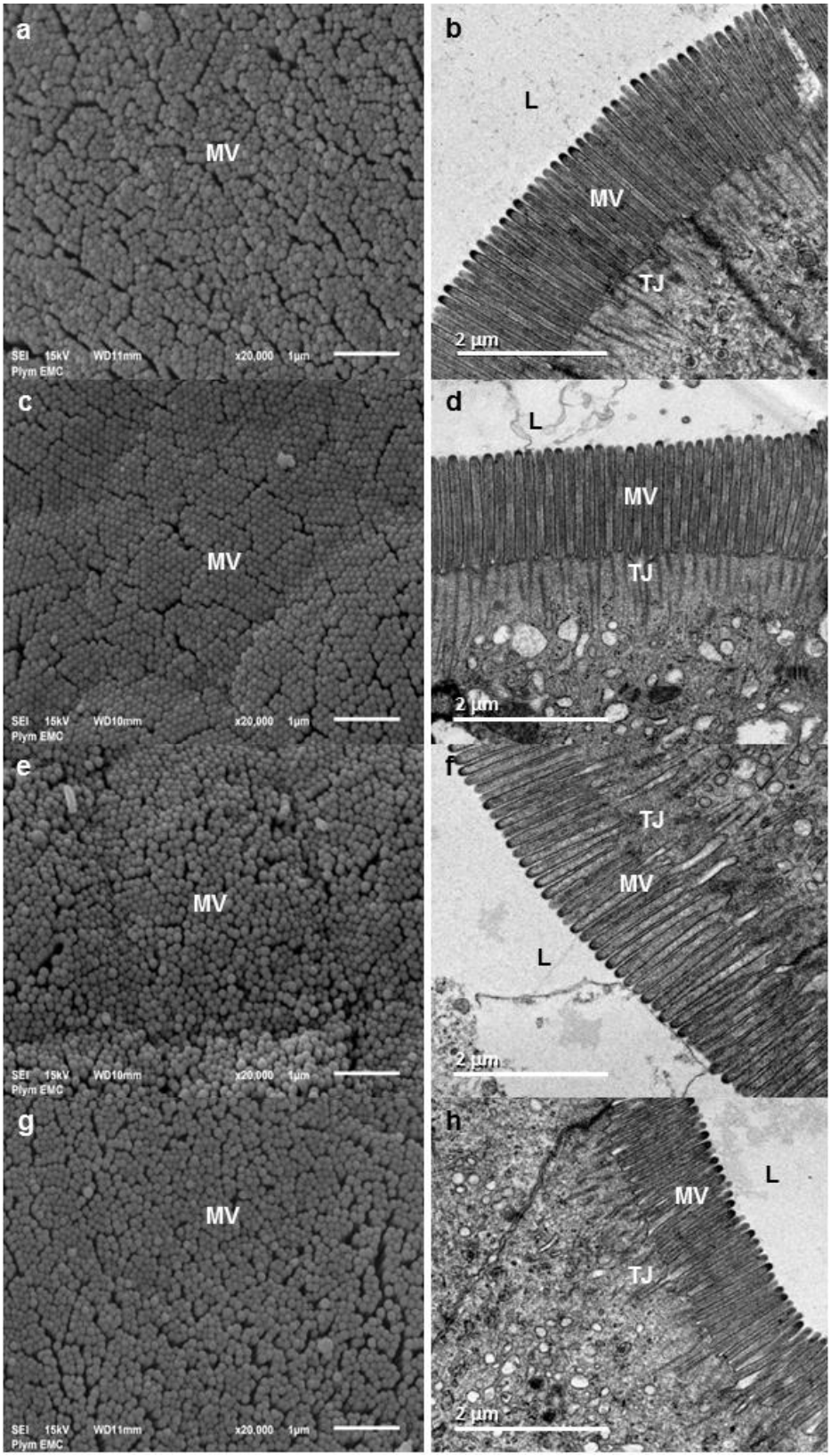
606 There was no significant difference across the treatments

607 **Figures**



608

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 µm.



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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).

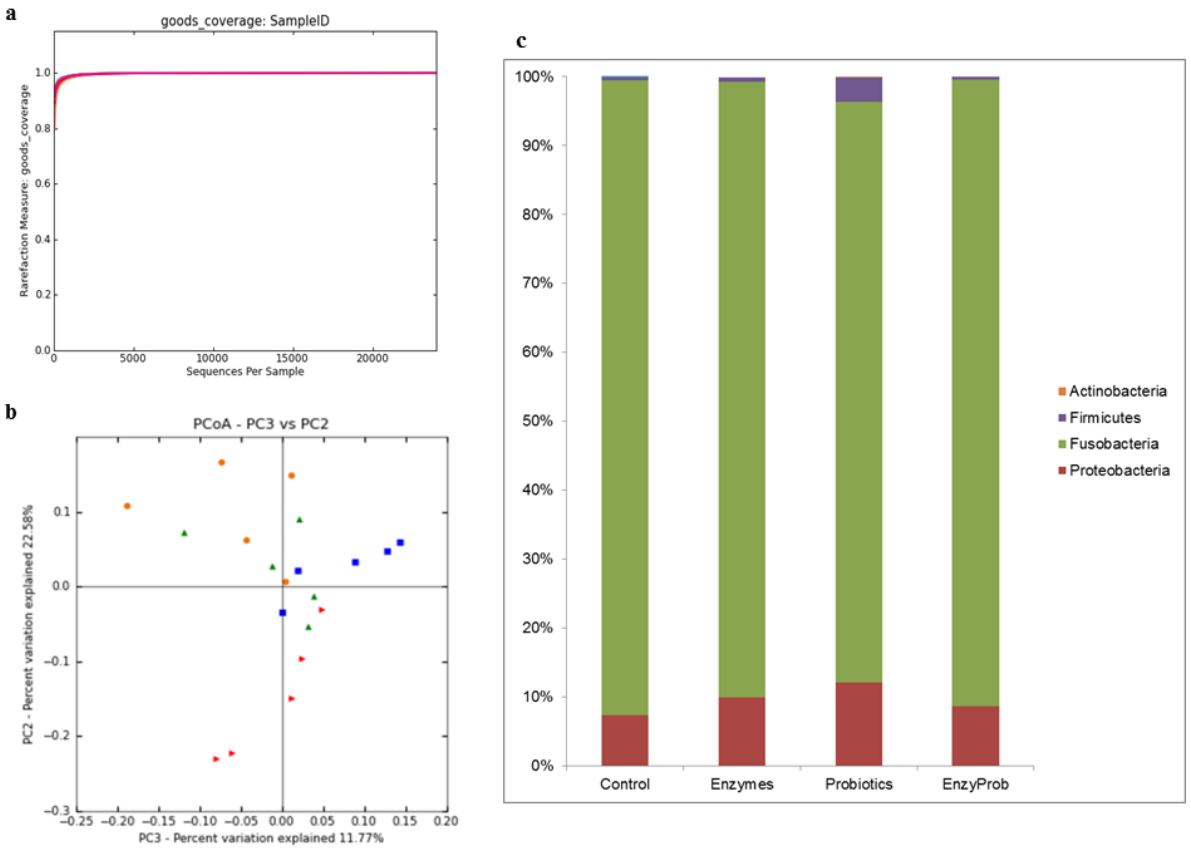

Figure 3. 16S rRNA V1-V2 high-throughput sequencing libraries of digesta from the tilapia intestine. (**a**) Good's coverage rarefaction curves of the tilapia digesta; (**b**) PCoA plots using Bray-Curtis dissimilarity matrix where data points represent samples from tilapia fed a control diet (red triangles),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