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Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*

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Abstract

The growth performance, immunological status, intestinal morphology and microbiology of tilapia, *Oreochromis niloticus*, were investigated after dietary administration of the commercial probiotic AquaStar® Growout. Tilapia (29.02 ± 0.33g) were split into five treatments; control (CON), 1.5g kg^{-1} probiotic (PRO-1.5), 3g kg^{-1} probiotic (PRO-3), pulsed probiotic feeding (PRO-PULSE) or an initial probiotic feed followed by control feeding (PRO-INI). After six weeks of experimental feeding, fish fed PRO-3 displayed significantly higher final weight, weight gain and SGR compared to the CON or PRO-INI treatments. Supplementation of the probiotic at this dose induced an up-regulation of intestinal caspase-3, PCNA and HSP70 mRNA levels compared to the CON fed fish. Immuno-modulatory

pathways were also affected; significantly higher expression of TLR2, pro-inflammatory genes TNFα and IL-1β, and anti-inflammatory genes IL-10 and TGFβ suggest that the probiotic may potentiate a higher state of mucosal tolerance and immuno-readiness. Histological appraisal revealed significantly higher numbers of intraepithelial leucocytes in the intestine of PRO-3 fed fish compared with treatments CON, PRO-PULSE and PRO-INI but not PRO-1.5. Additionally, fish receiving PRO-3 had a significantly higher abundance of goblet cells in their mid-intestine when compared with fish from all other treatments. Together, these data suggest that continuous provision of AquaStar® Growout at 3g kg^{-1} can improve tilapia growth and elevate the intestinal immunological status of the host.

Keywords: probiotic; tilapia; fish; immunity; gene expression; growth performance; histology, intestinal microbiology.

1 Introduction

Aquaculture continues to be the fastest growing animal protein production industry [1]. As a result of extensive research, probiotics are becoming an increasingly popular choice as a prophylactic approach to avoid disease and improve health and production of farmed fish, including tilapia.

A number of probiotic investigations have focused on growth benefits in tilapia, with many studies reporting positive results after probiotic supplementation [2-8]. As well as strong growth performance, it is important that fish are healthy and capable of mounting an effective immune response when exposed to pathogens. The gastrointestinal (GI) tract plays an important role in the mucosal barrier function. Not only does it serve as a physico-chemical

barrier against invading pathogens, there are also tolerance mechanisms in place which allow the residence of commensal and mutualistic microbes [9]. Probiotics can have beneficial effects on the gut-associated lymphoid tissues (GALT). These benefits can manifest themselves within the intestine by means of reinforcing barrier defences by elevating populations of intra epithelial leucocytes and goblet cells and also by inducing the expression of pro-inflammatory cytokines (e.g. TNFα, IL-1β), thus maintaining the capacity of recognising and responding to pathogens, and regulatory cytokines (e.g. TGFβ, IL-10) for the maintenance of mucosal tolerance [10-15]. These cytokines are the end products of complex molecular pathways which are initiated by toll-like receptors (TLR's) recognising their corresponding microbe associated molecular pattern (MAMP) [16]. Probiotic supplementation can up-regulate the expression of intestinal TLR3 in Atlantic salmon, *Salmo salar*, and intestinal TLR2 and TLR5 in grouper, *Epinephelus coioides*, with a corresponding induction of intestinal IL-1β, TNFα, IL-8 and TGFβ expression [17, 18].

Currently, there are multiple probiotic formulations commercially available. It is essential that probiotic candidates are evaluated for efficacy and the dosage and feeding regime should be optimised [19]. The current investigation aimed to evaluate multiple doses and feeding regimes of a commercially available multi-species probiotic, AquaStar® Growout (a mix of *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici* at 1×10^9 CFU g^{-1}), on tilapia growth performance, intestinal integrity, intestinal microbiology and intestinal immunity.

2 *Materials and methods*

2.1 *Experimental design and dietary preparation*

All experimental work involving fish was conducted under the Home Office project licence PPL30/2644 and was in accordance with the Animals (Scientific Procedures) Act 1986 and the Plymouth University Ethical Committee.

Three iso-nitrogenous and iso-lipidic diets were formulated using Feedsoft Professional® according to the known requirements of tilapia [20] (Table 1). Dry ingredients were mixed in small batches to ensure a homogenous mix before adding the oil and warm water in a Hobart food mixer (Hobart Food equipment, Australia) to form a consistency suitable for cold press extrusion (PTM P6 extruder, Plymouth, UK) to produce 2mm pellets. The lyophilised probiotic (AquaStar® Growout; Biomin GmbH) was added at the expense of corn starch and the basal diet void of the probiotic served as a control diet. Diets were dried for 24 hours in an air convection oven set to 44°C, broken up by hand and stored in refrigerated air tight containers prior to use. The dietary proximate composition was analysed using AOAC protocols [21] (Table 1). Probiotic viability was checked using selective media (de Man, Rogosa and Sharpe (MRS) media, *Bacillus* selective agar and Slanetz and Bartley media for *Lactobacillus*/*Pediococcus*, *Bacillus* and *Enterococcus* spp., respectively) by spread plating 10-fold serial dilutions and counting statistically viable plates (i.e. 20-200 colonies). Fresh diets were produced at the trial midpoint to ensure high probiotic viability.

Nile tilapia, *Oreochromis niloticus*, (Fishgen Ltd., Swansea, UK), were transferred to the Aquaculture and Fish Nutrition Research Aquarium, Plymouth University, UK where they were allowed six weeks of acclimation. Five hundred tilapia were randomly distributed to ten 150L fibreglass tanks (50 fish per tank; average weight = 29.02 ± 0.33g; n = 2). Treatments were as follows; control (basal diet void of AquaStar® Growout), low probiotic dose

(continuous feeding of the basal diet supplemented with AquaStar® Growout at 1.5g kg^{-1}), high probiotic dose (continuous feeding of the basal diet supplemented with AquaStar® Growout at 3 g kg^{-1}), probiotic pulse feeding (alternating weekly between AquaStar® Growout feeding at 1.5g kg^{-1} and control feeding) and lastly initial probiotic feeding (first two weeks AquaStar® Growout feeding at 1.5g kg^{-1} followed by remainder of the trial on the control diet). Diet codes were assigned for ease of analysis (Table 2). Fish were fed experimental diets for six weeks at a rate of 1- 5% biomass per day in four equal rations (all treatments received the same % input each day); higher feeding rates were provided at the beginning of the trial but this was decreased incrementally during the trial as fish grew larger and their appetite decreased. Daily feed was adjusted on a weekly basis by batch weighing following a 24 hour starvation period. Fish were held at 28 ± 1°C with a 12:12 h light: dark photoperiod. Water quality was monitored daily and maintained at pH = 6.5 ± 0.5 (adjusted with $NaHCO_3$ as necessary) and dissolved oxygen > 6.0 mg l^{-1}. Ammonium, nitrite and nitrate levels were monitored weekly (0.08 ± 0.02, 0.15 ± 0.05 and 18.30 ± 3.30 mg l^{-1}, respectively) and regular water changes prevented the accumulation of these compounds as well as preventing background build-up of probiotics.

2.2 Growth performance and carcass composition

Growth performance and feed utilisation were assessed by net weight gain (NWG), feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR) and protein efficiency ratio (PER). Calculations were carried out using the following formulae: NWG = FW - IW; SGR = 100 ((ln FW - ln IW)/T); FCR = FI/WG; PER = WG/PI, where FW = final weight (g), IW = initial weight (g), T = duration of feeding (days), WG = wet weight gain (g), FI = feed intake (g) and PI = protein ingested (g). At the end of the trial four fish per tank were pooled

into two samples (thus $n = 4$) to determine final carcass composition. Proximate composition analysis was conducted according to AOAC protocols [21].

2.3 RT-PCR

The mid-intestine was sampled from four fish per tank ($n = 8$) for gene expression of caspase-3, PCNA, HSP70, TLR2, TGFβ, IL-10, TNFα and IL-1β after six weeks. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol as described in Rawling *et al*. [22] with the addition of an extra isopropanol step. RNA concentration and purity was measured spectophotometrically (NanoDrop Technologies) and RNA integrity was checked by running each sample on a 1% agarose gel. Any samples with DNA contamination were cleaned using RNeasy MiniElute Cleanup Kit (Qiagen). RNA samples were subsequently stored at -80°C until use.

A total concentration of 1 µg of RNA was used for cDNA synthesis using iScript cDNA Synthesis Kit (BioRad) according to manufacturer's instructions. Primer efficiencies were determined using serial 1/10 dilutions of pooled cDNA and resulting plots of Ct versus the logarithmic cDNA input, using the equation $E = 10^{(-1/slope)}$. Primer sequences and efficiencies are reported in Table 3. PCR reactions were run in duplicate (total reaction volume = 7.5 µl) were set on a 384-well plate and each reaction consisted of 2 µl of cDNA (1/10 dilution), 3.75 µl of 2X concentrated SYBR Green Supermix (Biorad), 0.225 µl of each forward and reverse primers (0.3 µM) and 1.3 µl of DEPC treated water (Ambion). All quality control measures and RT-reactions were carried out according to the MIQE guidelines [23]. The thermal profile for all reactions were 10 min at 95°C and then 40 cycles of 15s at 95°C and 60s at 60°C. Fluorescence monitoring occurred at the end of each cycle and melt curve analyses were performed in all cases to check for a single peak. GAPDH, β-actin and EF1-α

were all assessed as reference genes. Reference genes were imported into GeNorm (v 3.4, Center for Medical Research, Ghent University, Belgium) to assess the optimal number, and choices of reference genes. Experimental treatments were each compared to the control and analysed using the relative expression software tool (REST©) [24] and reported as fold change.

2.4 Intestinal histology

Four tilapia per tank were sampled at week six (n = 8) for histological appraisal of the mid-intestine. Tissue samples were fixed in 10% formalin and transferred to 70% ethanol after 48 hours. Samples were then dehydrated in graded ethanol concentrations prior to embedding in paraffin wax. In each specimen, multiple sections (5µm) were stained with haematoxylin and eosin (H & E) and Alcian Blue-PAS to assess the intestinal perimeter ratio (arbitrary units; AU) after Dimitroglou *et al*. [25], intraepithelial leucocytes (IEL's) levels and goblet cell abundance in the epithelium. IEL's and goblet cells were counted across a standardized distance of 100 µm and then calculated by averaging the cell numbers from all samples within each treatment. All light microscopy images were analysed with Image J 1.46r (National Institute of Health, USA).

2.5 Intestinal microbiological analyses

After the experimental period, four fish per tank were euthanized by overdose (300mg l^{-1}) of tricaine methane sulphonate (MS222; Pharmaq, Fordingbridge, UK). The GI tract was aseptically removed in its entirety. Faecal matter from the mid-intestine was isolated, and pooled between two fish (thus n = 4 per treatment) to assess allochthonous bacterial

populations. Intestinal samples were either used immediately for culture based analysis or stored at -20°C for culture independent analysis.

2.5.1 Culture dependent analysis

Samples were serially diluted with PBS and 20µl was spotted onto duplicate MRS agar, Slanetz and Bartley and *Bacillus* selective media using the Miles and Misra method [26] to assess the allochthonous presumptive probiotic bacterial populations. Tryptone soya agar (TSA) was used to determine the total aerobic heterotrophic bacterial populations. Plates were incubated for 48 hours at 28°C and colony forming units (CFU g^{-1}) were calculated by counting colonies from statistically viable plates (between 3-30 colonies). Representative subsets of the presumptive probiotics were identified by using 16S rRNA gene sequence analysis using the protocol described in Ferguson et al. [27].

2.5.2 Culture independent analysis

At week six, digesta samples (n = 4) were used for culture independent analyses. DNA was extracted using the QIAamp Stool Mini Kit (Qiagen) with a lysozyme pre-treatment (50 mg mL^{-1} in TE buffer for 30 min at 37°C) and a phenol-chloroform clean up, as described [28]. PCR amplification of the 16S rRNA V3 region was conducted using the reverse primer P2 and the forward primer P3 [29]. A 40-60% DGGE was performed, and presumptive probiotic bands extracted, using a DCode Universal Mutation Detection System (Bio-Rad laboratories, Italy) according to Merrifield et al [30]. The presumptive probiotic nucleotide sequences were submitted to a BLAST search to retrieve the closest known alignment identities.

2.6 Statistical analyses

All data are presented as means ± standard deviation. All data were checked for normality and analysed using ANOVA with *post-hoc* Tukey's HSD test (Statgraphics Centurion XVI, Warrenton, VA, USA). Where data were not normally distributed, data were analysed using a Kruskal- Wallis test with *post-hoc* Mann-Whitney U-tests. RT-PCR data were analysed using REST© 2009 (Qiagen, Hilden, Germany). DGGE banding patterns were transformed into presence/ absence matrices based on band peak intensities (Quantity One® version 4.6.3, Bio-Rad Laboratories, CA, USA). Band intensities were measured (Quantity One® 1-D Analysis Software, Bio-Rad Laboratories Ltd., Hertfordshire, UK), and analysed using Primer V6 software (PRIMER-E Ltd, Ivybridge, UK). In all cases, significance was accepted at $P < 0.05$.

3 Results

3.1 Growth performance and carcass composition

Growth performance was assessed by means of routine growth and feed utilisation parameters after six weeks of feeding experimental feeding (Table 4). Tilapia fed the PRO-3 diet displayed the best growth performance. In this treatment the final weight, weight gain and SGR were significantly higher when compared to either CON or PRO-INI (P = 0.019, 0.014 and 0.021, respectively). However, they did not significantly differ from treatments PRO-1.5 or PRO-PULSE. No differences in feed intake, PER or FCR were observed between any treatment (P = 0.054, 0.190 and 0.237, respectively). Additionally, there were no significant differences in carcass proximal composition (Table 4).

3.2 RT-PCR

Relative intestinal gene expression of caspase-3, PCNA, HSP70, TLR2, TNFα, IL-1β, IL-10 and TGFβ were analysed. The largest fold change was observed in caspase-3 mRNA levels which were up-regulated approximately seven fold in PRO-3 when compared to the control group (P = 0.001). The gene expression of PCNA and HSP70 were six and three and half times higher in PRO-3, respectively, when compared to the control treatment (P < 0.001 and 0.028 respectively; Figure 1).

Further changes were observed for the immunity related genes (Figure 2). TLR2 mRNA expression was significantly up-regulated, more than four fold, in PRO-3 when compared to the control treatment (P = 0.004). The pro-inflammatory cytokine genes TNFα and IL-1β were up-regulated three and five times, respectively, in the intestine of the PRO-3 fed fish compared to the CON fed fish (P = 0.028 and 0.003, respectively). Furthermore, tolerogenic cytokine IL-10 and TGFβ mRNA levels were also up-regulated by approximately five and six fold, respectively, in PRO-3 when compared to the control treatment (P = 0.005 and 0.003, respectively).

There were no significant changes in gene expression between any of the investigated genes between treatments PRO-1.5, PR0-PULSE and PRO-INI when compared to the control treatment (P > 0.05).

3.3 Intestinal histology

Light microscopy was used to examine the perimeter ratio, IEL and goblet cell levels from the mid-intestine (Table 5). Fish from all dietary treatments had an intact epithelial barrier with extensive mucosal folds, abundant IEL's and numerous goblet cells. Tilapia in different

234 treatments showed altered perimeter ratios (P = 0.007). The highest perimeter ratio was
235 recorded in PRO-INI which was significantly higher than PRO-1.5 but not CON, PRO-3 or
236 PRO-PULSE. Perimeter ratio in PRO-3 was also significantly higher when compared to the
237 lower probiotic dose, PRO-1.5. However, perimeter ratio remained unchanged between
238 treatments PRO-1.5, CON, and PRO-PULSE. IEL and goblet cell abundance remained
239 unchanged by dietary treatment in groups CON, PRO-1.5, PRO-PULSE and PRO-INI.
240 However, IEL levels were significantly elevated in PRO-3 when compared to treatments
241 CON, PRO-PULSE and PRO-INI (P < 0.05) but not PRO-1.5. PRO-3 also contained
242 significantly larger populations of goblet cell when compared to all other treatments (P <
243 0.001; Table 5).

244

245 *3.4 Culture dependent analysis*

246 The effect of AquaStar® Growout treatment on the aerobic heterotrophic bacteria was
247 determined using culture based methods (Table 6). No significant differences were observed
248 in TVC levels between the treatments with allochthonous levels approximately log 6 CFU g^{-1}
249 for each treatment (P = 0.993). The highest LAB levels were observed in the digesta of PRO-
250 3 fed tilapia, these were significantly higher than of CON and PRO-INI (P = 0.006).
251 Similarly, PRO-3 resulted in the highest *Bacillus* levels which were significantly higher than
252 those found in PRO-PULSE but not in other treatments (P = 0.026). LAB and *Bacillus*
253 populations were not different in treatments CON, PRO-1.5, PRO-PULSE and PRO-INI.
254 Furthermore, enterococci levels were significantly higher in PRO-3 when compared to CON,
255 PRO-PULSE and PRO-INI. Despite being numerically higher, they were not different to
256 enterococci levels recovered in PRO-1.5 digesta. Representative subsets of the presumptive
257 probiotics were confirmed as the probiotics by 16S rRNA gene sequence analysis.

3.5 DGGE

The influence of dietary AquaStar® Growout on the intestinal microbial diversity in tilapia was investigated using DGGE after six weeks of feeding experimental diets. Presumptive probiotic bands were identified by migration to the same position as known *B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici* samples. These bands were also isolated from DGGE gels and subsequent sequencing confirmed the presence of all four probiotic species from AquaStar® Growout fingerprints; these were not detected in control sample fingerprints. Table 7 displays the microbial ecological parameters derived from the DGGE fingerprints. There were no significant differences between treatments with regards to number of OTU's (*N*), species richness or diversity indices (P = 0.083, 0.086 and 0.102, respectively). Replicates from CON and PRO-PULSE showed the highest similarity percentage (SIMPER), this was significantly higher than replicates in PRO-1.5 but not those in PRO-3 or PRO-INI. Apart from PRO-1.5, all other treatments displayed no differences with regards to SIMPER analyses. ANOSIM revealed that the microbial communities within PRO-3 fed tilapia were significantly dissimilar to CON, PRO-PULSE and PRO-INI (53.35%, 58.25% and 58.10% dissimilar, respectively; P = 0.03) but not PRO-1.5 (34.54% dissimilar; P = 0.23). Additionally, the microbial community within PRO-1.5 was significantly dissimilar to the microbial community within the intestine of PRO-PULSE (40.33% dissimilar; P = 0.03). This can be visualised in Figure 3 where there is a clustering effect of the communities from the PRO-3 replicates. Replicates from treatments CON, PRO-PULSE and PRO-INI showed loose clustering with level of overlap between these three treatments. Two out of four replicates from PRO-1.5 show high similarity to those from PRO-3, whereas the remaining two replicates are more similar to the other treatments (Figure 3).

4 Discussion

The administration of AquaStar® Growout at 3g kg^{-1} for six weeks resulted in improved growth performance when compared to treatments CON or PRO-INI. AquaStar® Hatchery (which contains a higher concentration of the same probiotic strains as AquaStar® Growout) has previously been reported to improve growth performance of rainbow trout (*Oncorhynchus mykiss*) [31]. Although there is no data regarding the growth promoting effects of AquaStar® Growout in tilapia, dietary provision of *Bacillus* spp., *Enterococcus* spp. and *Lactobacillus* spp., either singularly or in combination with other species have been reported to improve tilapia growth performance indicators [2, 4-8, 32-35]. The mechanisms which underpin these improvements are only partly described. Previous work on tilapia suggests that Aquastar® Growout may increase the intestinal absorptive surface area by improving the microvilli density and microvilli length [36]. Probiotics may also be important in the production of digestive enzymes. Essa *et al*. [6] reported elevated intestinal amylase, protease and lipase activities in tilapia supplemented with *B. subtilis* and/ or *L. rhamnosus* and elevated intestinal protease activity in fish supplemented with *S. cerevisae*.

Heat shock proteins have important roles in protein metabolism, protein folding, protein chaperoning, mediating the repair and degradation of damaged proteins and are also involved in generating an immune response [38]. Furthermore it has also been proposed that heat shock proteins play important roles in the long term adaptation of animals to their environments through genetic mechanisms [39]. Fish exhibiting higher HSP70 expression may therefore be more able to generate an efficient immune response and also be more tolerant to a wider range of environmental conditions. In the present study gene expression analyses were used to elucidate the effect of the probiotic treatment on the mid-intestine at the molecular level. Many authors have reported lower expression of HSP70 after probiotic administration in fish [40-42] including tilapia [11]. Here, intestinal HSP70 gene expression

showed the opposite trend as it was significantly higher in PRO-3 when compared to the control. Using an *ex vivo* approach, Ren et al. [13] demonstrated that exposure to *Aeromonas hydrophila* did not cause an upregulation of HSP70 in the anterior or posterior intestine of tilapia. Conversely, the addition of *Lactobacillus plantarum*, as well as a mix of *A. hydrophila* and *L. plantarum* to the intestinal sac caused an upregulation of HSP70 [13]. Similar results were reported by Liu et al. [12] after the feeding hybrid tilapia, *O. niloticus* x *Oreochromis aureus*, diets supplemented with two *Lactobacillus* species. From their studies it was also evident that there appears to be a dosage, as well as temporal effect. For example, after 10 days of feeding on the probiotic diet, intestinal HSP70 was significantly up-regulated, down-regulated after 20 days and not different after 35 days when compared to the control treatment.

Caspase-3 and PCNA gene expression were both significantly up-regulated in PRO-3 when compared with the control group. Caspase-3 is part of the cysteine-aspartic acid protease family where it is activated by initiator caspases-8 or 9 resulting in programmed cell death (apoptosis). On the other hand, PCNA (proliferating cell nuclear antigen) is a marker for cell proliferation and is crucial for cellular and DNA replication. Organised apoptosis is essential for the health of the host since it results in the elimination of dangerous or damaged cells without causing an inflammatory response or tissue damage [43]. Since the GI tract is one of the key sites of interaction with the external environment [44] the intestine could be exposed to a number of opportunistic pathogens or chemical contaminants, especially in aquaculture where high stocking densities and water quality can be problematic. Therefore, both an elevated proliferative and apoptotic capacity is likely to be beneficial to the host.

The gut associated lymphoid tissue (GALT) in fish differs from their mammalian counterparts since fish lack Peyer's patches and mesenteric lymph nodes. Teleosts possess a more diffusely organised GALT which provides a physical, chemical and cellular barrier to

pathogenic invasion [9]. Similar to mammalian models, immune and epithelial cells within the GALT of fish express pattern recognition receptors (PRR's) including toll-like receptors (TLR's), which are sensitive to a number of pathogen associated molecular patterns (PAMP's). Upon ligation, a cascade effect is initiated through a series of adaptor proteins and transcription factors resulting in the transcription of important immune molecules such as cytokines, chemokines and defensins [9].

TLR2 gene expression was up-regulated in PRO-3 when compared with the control treatment. TLR2 is ligated by lipoteichoic acid (LTA), which is a major constituent in the cell wall of Gram-positive bacteria [45], such as those present in AquaStar® Growout. This up-regulation, induced by Gram-positive probiotics might be of particular importance because tilapia are susceptible to a number of Gram-positive infections, in particular *Streptococcus iniae* and *Streptococcus agalactiae*. Indeed, TLR2 was up-regulated in Mrigal carp (*Cirrhinus mrigala*) following *Streptococcus uberis* infection as well as *A. hydrophila* infection [46], another destructive pathogen in tilapia culture. It has been demonstrated that TLR's may have important roles to play in the probiotic modulation of the innate immune system in other fish species [17, 18]. Sun et al. [18] reported an upregulation in both TLR2 and TLR5 in grouper (*Epinephelus coioides*) after *Psychrobacter* sp. supplementation. Furthermore, the authors demonstrated a higher expression of pro-inflammatory genes IL-1β and IL-8, and the anti-inflammatory gene TGFβ after probiotic supplementation. The present study also reports higher gene expression of both pro-inflammatory cytokines (TNFα and IL-1β) and anti-inflammatory cytokines (IL-10 and TGFβ) after probiotic administration at 3g kg^{-1} when compared to the control treatment. Here, despite the up-regulation of pro-inflammatory cytokines, there was no evidence of inflammation from histology examinations. It is possible that this was balanced by the up-regulation of anti-inflammatory cytokine gene expressions. Other authors have reported higher expression of pro-inflammatory cytokines in tilapia after

probiotic feeding [10-15]. It is postulated that the induction of pro-inflammatory cytokines improves immune readiness of the host. In support of this, disease resistance studies in tilapia have demonstrated that probiotics are able to increase the expression of TNFα and IL-1β and consequently the tilapia survival levels were significantly higher when exposed to *A. hydrophila* [12, 15].

The current study also demonstrated that the probiotics also have anti-inflammatory signalling effects, by inducing the up-regulation of TGFβ and IL-10. Naturally, anti-inflammatory cytokines will have an immune-suppressive effect on the host; this could be indicative of a tolerance mechanism where the host does not interpret the probiotic as a threat. This has been demonstrated in other fish studies where TGFβ was up-regulated after probiotic administration [11, 12]. To the authors knowledge this is the first study to demonstrate probiotic modulation of IL-10 in the intestine of tilapia after probiotic feeding. However, similar results have been reported in rainbow trout after *L. plantarum* supplementation [47].

Histological analyses revealed significantly larger populations of IEL's in the mid-intestine of tilapia in PRO-3 when compared to treatments CON, PRO-PULSE or PRO-INI. Similar results have been obtained in other studies using tilapia fed diets supplemented with either *P. acidilactici* or *Lactobacillus rhamnosus* for six weeks and 30 days, respectively [10, 14]. Probiotic administration has led to increased IEL abundance in other commercially important fish species including European sea bass (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*) [48, 49]. Whilst the type of IEL cannot be eluded to in this study, Picchietti et al. [49] characterised elevated T-cells and acidophilic granulocytes in the posterior intestine of European sea bass. These data suggest that probiotics not only act upon the innate immune system in fish, but may have important roles to play through adaptive immunity mechanisms too.

Whilst all fish displayed abundant goblet cells within the intestine, there were significantly larger populations in the mid-intestine of tilapia fed PRO-3 when compared to all other treatments. Intestinal mucus is vital to the defensive barrier, both physically and chemically, since it functions to trap and remove pathogens, preventing their attachment to the epithelia. Dietary applications of *L. rhamnosus* and *P. acidilactici* have also been reported to increase the number of goblet cells in the tilapia intestine [14, 50].

This study was successful in recovering each probiotic species from tilapia digesta, a requirement which is essential for any probiotic candidate. Furthermore, probiotic supplementation was capable of modulating the composition of intestinal microbiota. This supports a previous study which also reported the detection of these probiotic species, and modulation of the intestinal microbiota, of tilapia using DGGE and high-throughput sequencing [36].

In conclusion, under the current experimental conditions, the continuous supplementation of AquaStar® Growout at 3g kg^{-1} can improve growth performance and elevate the intestinal immunological status in tilapia. The probiotic may act to augment mucosal tolerance mechanisms whilst creating a state of immune readiness, improved barrier function through the increase the number of goblet cells and IELs in the intestine, which may ultimately retard pathogen infection and translocation. Future studies should assess these using challenge trials.

Conflict of interest

The authors declare that there are no conflicts of interest that could have direct or potential influence or impart bias on the work.

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Figure 1: Relative mid-intestinal gene expression of caspase-3 and PCNA and HSP70 after six weeks of feeding experimental diets. Values are reported in fold change when compared against the expression in the control treatment (set to 1.0). Asterisks highlight significant differences ($P < 0.05$) when compared to the control treatment.

Figure 2: Relative gene expression of mid-intestinal TLR2 receptor (A) pro-inflammatory cytokines TNFα (B) and IL-1β (C) and anti-inflammatory cytokines TGFβ (D) and IL-10 (E) and after six weeks of feeding experimental diets. Values are reported in fold change when compared against the expression in the control treatment (set to 1.0). Asterisks highlight significant differences ($P < 0.05$) when compared to the control treatment.

Figure 3: nMDS plot showing similarity of the intestinal allochthonous microbiota of each treatment after six weeks of feeding experimental diets. Lines represent different levels of similarity.

Table 1: Dietary formulation and chemical composition (%).

	Basal	1.5g kg^{-1}	3g kg^{-1}
Fishmeal[a]	10.00	10.00	10.00
Soyabean meal[b]	33.89	33.89	33.89
Corn Starch[c]	31.90	31.75	31.60
Lysamine pea protein[d]	5.00	5.00	5.00
Glutalys[d]	10.00	10.00	10.00
Fish oil	3.75	3.75	3.75
Corn oil	4.00	4.00	4.00
Vitamin& mineral premix[f]	0.50	0.50	0.50
CMC-binder[c]	0.50	0.50	0.50
Methionine[c]	0.36	0.36	0.36
AquaStar® Growout[g]	0.00	0.15	0.30
Proximate composition (% as fed basis)			
Moisture	7.16 ± 0.03	5.89 ± 0.09	8.23 ± 0.19
Crude protein	37.57 ± 0.16	38.08 ± 0.30	37.03 ± 0.13
Lipid	10.09 ± 0.03	10.61 ± 0.24	10.41 ± 0.09
Ash	4.29 ± 0.04	4.25 ± 0.07	4.20 ± 0.01
Energy (MJ kg^{-1})	19.72 ± 0.05	19.57 ± 0.40	18.97 ± 0.19

[a] Herring meal LT92 – United Fish Products Ltd., Aberdeen, UK.
[b] Hamlet HP100, Denmark.
[c] Sigma- Aldrich Ltd., UK.
[d] Roquette Frêres, France.
[e] Natural wheat bran, Holland & Barrett, UK.
[f] Premier nutrition vitamin/mineral premix contains: 121 g kg^{-1} calcium, Vit A 1.0 µg kg^{-1}, Vit D3 0.1 µg kg^{-1}, Vit E (as alpha tocopherol acetate) 7.0 g kg^{-1}, Copper (as cupric sulphate) 250 mg kg^{-1}, Magnesium 15.6 g kg^{-1}, Phosphorous 5.2 g kg^{-1}.
[g] Biomin Holding GmbH, Industriestrasse 21, 3130 Herzogenburg, Austria.

444 **Table 2:** Dietary codes used throughout the research article.

Dietary code	Diet
CON	Continuous feeding of basal diet (without probiotic)
PRO-1.5	Continuous feeding of the basal diet supplemented with AquaStar® Growout at 1.5g kg^{-1}
PRO-3	Continuous feeding of the basal diet supplemented with AquaStar® Growout at 3g kg^{-1}
PRO-PULSE	Alternating weekly between AquaStar® Growout feeding at 1.5g kg^{-1} and the basal diet
PRO-INI	Initial two weeks AquaStar® Growout feeding at 1.5g kg^{-1} followed by remainder of the trial on the basal diet

445 **Table 3:** Primer sequences used for RT-PCR

Gene	Forward 5' - 3'	Reverse 5' – 3'	Amplicon size	Tm (°C)	E-value	GenBank number
β-actin	TGACCTCACAGACTACCTCATG	TGATGTCACGCACGATTTCC	89	58.8	2.1	KJ126772.1
GAPDH	CCGATGTGTCAGTGGTGGAT	GCCTTCTTGACGGCTTCCTT	82	59.4	2.0	JN381952.1
EF1α	TGATCTACAAGTGCGGAGGAA	GGAGCCCTTTCCCATCTCA	80	58.4	2.0	AB075952.1
Caspase-3	GGCTCTTCGTCTGCTTCTGT	GGGAAATCGAGGCGGTATCT	80	59.4	2.1	GQ421464.1
PCNA	CCCTGGTGGTGGAGTACAAG	AGAAGCCTCCTCATCGATCTTC	80	60.9	2.0	XM_003451046.2
HSP70	ACCCAGACCTTCACCACCTA	GTCCTTGGTCATGGCTCTCT	84	59.4	2.0	FJ213839.1
TLR2	GCAGTGCCTTGAGTCTTGATC	ACCGTGGAGATCGAGAACCT	101	59.6	2.1	XM_005460165
TNFα	CCAGAAGCACTAAAGGCGAAGA	CCTTGGCTTTGCTGCTGATC	82	59.9	2.0	AY428948.1
IL-1β	TGGTGACTCTCCTGGTCTGA	GCACAACTTTATCGGCTTCCA	86	58.7	2.1	XM_005457887.1
TGFβ	GTTTGAACTTCGGCGGTACTG	TCCTGCTCATAGTCCCAGAGA	80	59.8	2.1	XM_003459454.2
IL-10	CTGCTAGATCAGTCCGTCGAA	GCAGAACCGTGTCCAGGTAA	94	59.6	2.1	XM_003441366.2

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448 **Table 4:** Growth performance and final carcass composition of tilapia after six weeks of feeding on experimental diets.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Initial weight (g fish^{-1})	29.42 ± 0.37	28.66 ± 0.25	29.10 ± 0.59	28.94 ± 0.03	29.42 ± 0.08
Average weight (g fish^{-1})	68.20 ± 0.63^{a}	68.83 ± 0.39^{ab}	71.74 ± 0.83^{b}	68.81 ± 0.04^{ab}	67.57 ± 1.34^{a}
Weight gain (g fish^{-1})	38.78 ± 0.10^{a}	40.17 ± 0.13^{ab}	42.64 ± 0.23^{b}	39.87 ± 0.06^{ab}	38.15 ± 1.42^{a}
Feed intake (g fish^{-1})	53.46 ± 1.23	55.39 ± 0.57	56.42 ± 0.70	55.42 ± 0.05	54.91 ± 0.04
PER	1.47 ± 0.15	1.47 ± 0.02	1.59 ± 0.02	1.44 ± 0.00	1.34 ± 0.10
FCR (g g^{-1})	1.38 ± 0.07	1.38 ± 0.01	1.33 ± 0.01	1.39 ± 0.00	1.44 ± 0.06
SGR (% day^{-1})	2.48 ± 0.06^{a}	2.58 ± 0.01^{ab}	2.66 ± 0.02^{b}	2.55 ± 0.01^{ab}	2.45 ± 0.06^{a}
Carcass proximate composition (%)					
Moisture	68.75 ± 0.44	68.97 ± 0.78	69.41 ± 0.89	69.81 ± 1.14	68.72 ± 0.59
Ash*	9.88 ± 0.37	10.17 ± 0.49	9.67 ± 0.31	10.52 ± 0.74	10.20 ± 0.08
Lipid*	34.68 ± 0.53	32.42 ± 0.78	34.94 ± 1.79	32.67 ± 1.68	33.78 ± 0.73
Protein*	52.03 ± 0.42	53.41 ± 0.52	52.48 ± 1.50	54.43 ± 1.32	52.90 ± 1.38
Energy* (MJ kg^{-1})	24.67 ± 0.17	24.39 ± 0.52	24.72 ± 0.53	24.56 ± 0.42	25.05 ± 0.19

449 * Parameters reported as percentage of dry weight matter.
450 [a, b] Different superscripts indicate a significant difference ($P < 0.05$).

Table 5: Histological data from the mid-intestine of tilapia fed control and AquaStar® Growout supplemented diets after six weeks of feeding on experimental diets.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Perimeter ratio (AU)	2.57 ± 0.58^{ab}	2.03 ± 0.29^{a}	3.16 ± 0.86^{b}	2.94 ± 0.47^{ab}	3.68 ± 0.72^{b}
IEL's (per 100 µm)	34.04 ± 4.41^{a}	37.39 ± 3.60^{ab}	41.63 ± 2.66^{b}	34.85 ± 2.99^{a}	31.95 ± 1.61^{a}
Goblet cells (per 100 µm)	4.96 ± 1.53^{a}	4.95 ± 0.91^{a}	8.56 ± 0.82^{b}	5.18 ± 0.64^{a}	5.58 ± 1.33^{a}

[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

461 **Table 6:** Allochthonous TVC, LAB, enterococci and *Bacillus* spp. (log CFU g^{-1}) in the
462 intestinal tract of tilapia after six weeks of feeding on experimental diets.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
TVC	5.89 ± 0.59	5.92 ± 0.27	5.94 ± 0.28	6.01 ± 0.53	6.05 ± 0.51
LAB	1.08 ± 1.34^{a}	3.30 ± 1.86^{ab}	5.39 ± 0.83^{b}	2.45 ± 2.18^{ab}	n.d^{a}
Bacillus spp.	4.30 ± 0.25^{ab}	4.57 ± 0.22^{ab}	5.18 ± 0.58^{b}	3.87 ± 0.43^{a}	4.10 ± 0.45^{ab}
Enterococci	n.d^{a}	3.13 ± 1.72^{bc}	5.03 ± 0.99^{c}	0.94 ± 1.12^{ab}	n.d^{a}

463 n.d = not detected
464 $^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

Table 7: Microbial community analysis of the intestinal allochthonous bacterial populations of tilapia from DGGE fingerprints after six weeks of feeding on experimental diets.

	Microbial ecological parameters				Similarity (ANOSIM)		
	N	Richness	Diversity	SIMPER (%)	*R*- value	*P*- value	Dissimilarity (%)
CON	17.75 ± 1.64	5.82 ± 0.39	2.87 ± 0.10	84.14 ± 7.35[a]			
PRO-1.5	15.25 ± 4.87	5.19 ± 1.19	2.67 ± 0.34	62.54 ± 15.42[b]			
PRO-3	13.00 ± 1.00	4.68 ± 0.25	2.56 ± 0.08	78.36 ± 8.88[ab]			
PRO-PULSE	19.25 ± 2.49	6.16 ± 0.57	2.95 ± 1.13	82.42 ± 4.37[a]			
PRO-INI	15.00 ± 1.41	5.17 ± 0.34	2.70 ± 0.09	72.81 ± 12.24[ab]			
Pairwise comparisons							
CON vs PRO-1.5					0.27	0.09	35.11
CON vs PRO-3					1.00	0.03	53.35
CON vs PRO-PULSE					0.37	0.06	20.84
CON vs PRO-INI					0.17	0.11	23.64
PRO-1.5 vs PRO-3					0.15	0.23	34.54
PRO-1.5 vs PRO-PULSE					0.47	0.03	40.33
PRO-1.5 vs PRO-INI					0.44	0.06	42.48
PRO-3 vs PRO-PULSE					1.00	0.03	58.25
PRO-3 vs PRO-INI					0.98	0.03	58.10
PRO-PULSE vs PRO-INI					0.08	0.37	22.14

N = number of operational taxonomic units; Richness = Margalef species richness; Diversity = Shannon's diversity index; SIMPER = similarity percentage within group replicates.
[a, b] Different superscripts indicate a significant difference ($P < 0.05$).

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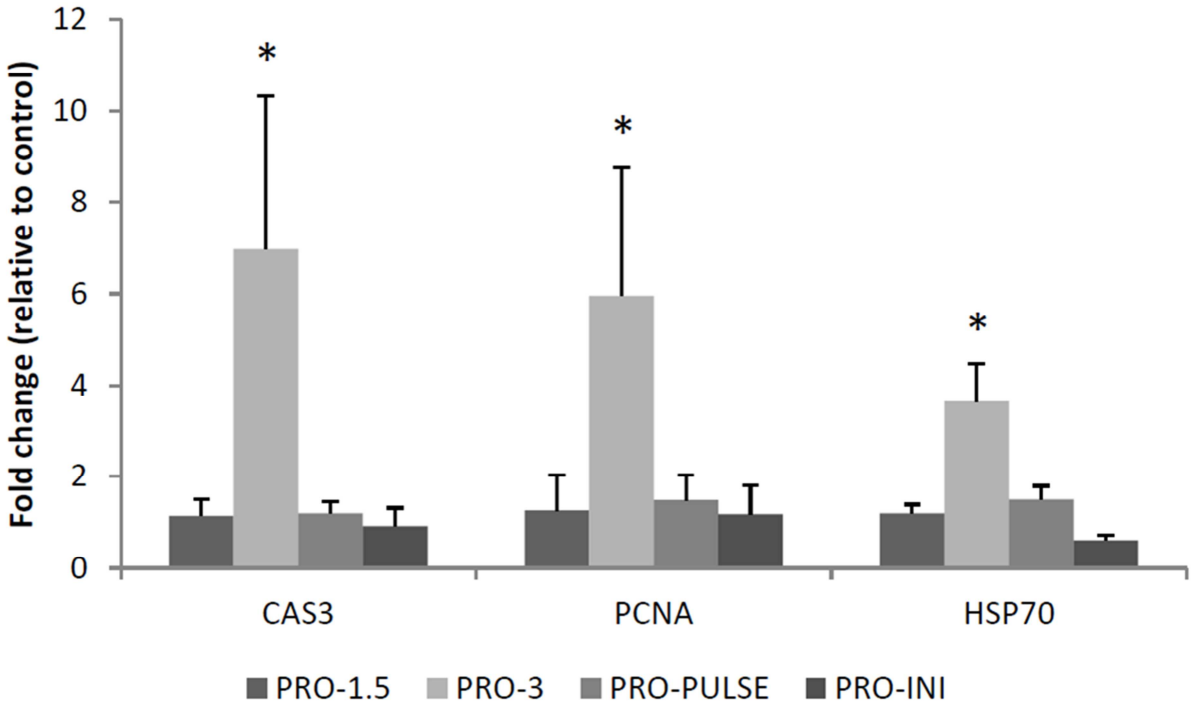

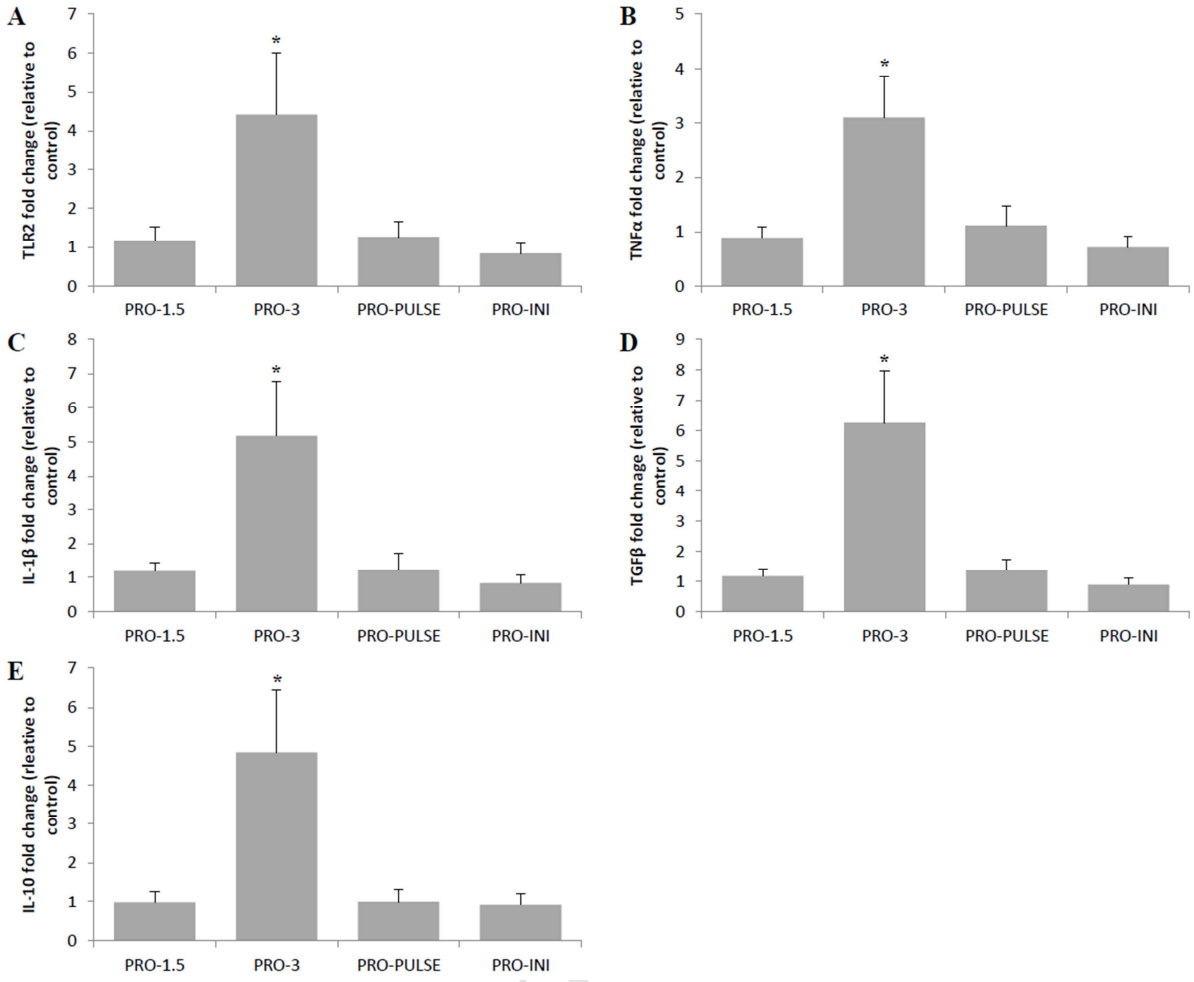
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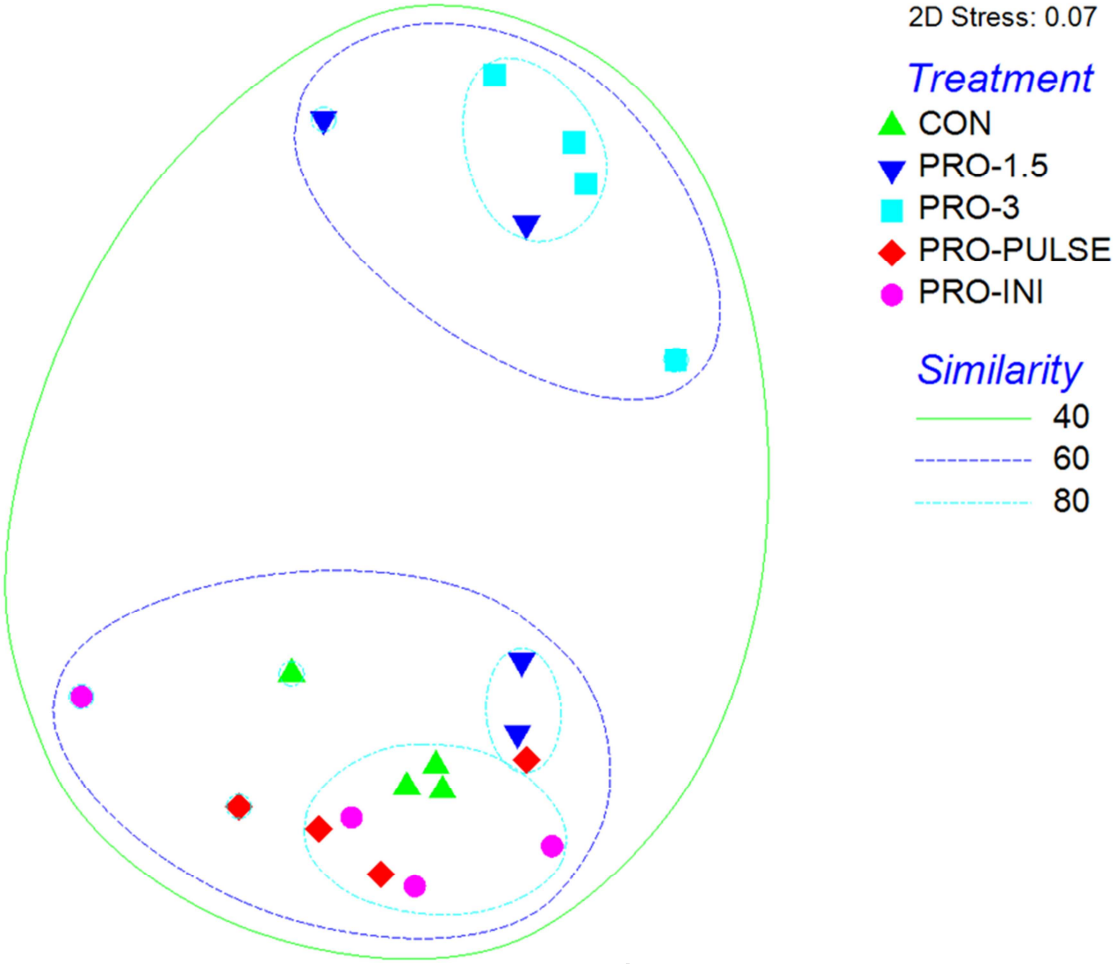
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Highlights for manuscript “Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*”

- AquaStar® Growout improves the growth performance of juvenile tilapia.
- AquaStar® Growout can augment mucosal tolerance.
- AquaStar® Growout improves immune readiness.