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BIOTECHNOLOGICAL PRODUCTS AND PROCESS ENGINEERING

Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*, following the application of a multi-species probiotic

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Abstract The intestinal microbiota and morphology of tilapia (*Oreochromis niloticus*) were investigated after the application of a multi-species probiotic containing *Lactobacillus reuteri*, *Bacillus subtilis*, *Enterococcus faecium* and *Pediococcus acidilactici* (AquaStar® Growout). Tilapia (55.03 ± 0.44 g) were fed either a control diet or a probiotic diet (control diet supplemented with AquaStar® Growout at 5 g kg^{-1}). After four and eight weeks, culture-dependent analysis showed higher levels of lactic acid bacteria (LAB), enterococci and *Bacillus* spp. in the mucosa and digesta of fish fed AquaStar® Growout. At week four, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) revealed a higher similarity within the probiotic fed replicates than replicates of the control group; after eight weeks, the compositional dissimilarity of the microbiome profiles between the groups was greater than the dissimilarities within each group ($P < 0.05$). High-throughput sequencing revealed that the probiotic treatment significantly reduced the number of operational taxonomic units and species richness in the digesta. Significantly higher proportions of reads belonging to *Proteobacteria* and *Cyanobacteria* were detected in the control group whereas the probiotic-fed fish displayed a significantly higher abundance of reads assigned to the *Firmicutes* (which accounted for >99 % of reads). *Bacillus*, *Cetobacterium* and *Mycobacterium* were the dominant genera in the digesta of control fish whereas *Bacillus*, *Enterococcus* and *Pediococcus* were the largest constituents in probiotic-fed fish. The addition of AquaStar® Growout to tilapia diets led to increased populations of intraepithelial leucocytes, a higher absorptive surface area index and higher microvilli density in the intestine. These data suggest that AquaStar® Growout can modulate both the intestinal microbiota and morphology of tilapia.

Keywords Probiotic · Intestinal microbiota · High-throughput sequencing · Intraepithelial leucocyte (IEL) · Microscopy · Tilapia

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Introduction

In the context of aquaculture, a probiotic can be considered as a microbial cell provided via the diet or rearing water that benefits the fish host, fish farmer or fish consumer which is in part achieved by improving the microbial balance of the fish (Merrifield et al. 2010a). Considering that a probiotic exerts its benefits via the modulation of the microbiome, there is a paucity of comprehensive data detailing these changes in fish. This is essential information given that the intestinal microbiomes of fish are diverse and complex communities primarily consisting of bacteria and, to a lesser extent, yeasts, *Archaea*, viruses and protists (Romero et al. 2014). As with mammals, the intestinal microbiota of fish have important functions in host metabolism, mucosal development and maturation, nutrition, immunity and disease resistance (Rawls et al. 2004; Bates et al. 2006; Round and Mazmanian 2009).

Tilapia production is the most widespread aquaculture type in the world (FAO 2014). However, it is hampered from disease outbreaks, particularly from *Streptococcus iniae*, *Aeromonas hydrophila* and *Edwardsiella tarda*. Traditionally, these have been controlled by antibiotics, but their abuse has resulted in the evolution of antibiotic resistance (Defoirdt et al. 2011). As such, probiotics have been incorporated into many tilapia production systems. Over the past two decades, a plethora of scientific investigations have focused on testing the efficacy of probiotics on tilapia. Most research concerning probiotic supplementation in tilapia has focused on growth and immunostimulation with less attention on intestinal microbiology. Of the 187 finfish probiotic studies discussed in recent reviews (Carnevali et al. 2014; Lauzon et al. 2014; Merrifield and Carnevali 2014), only 74 (40 %) investigated aspects of the gut microbiota. In tilapia, only 26 % (8 from 31) of the studies investigated the intestinal microbiota. This minority of studies primarily used culture-based approaches to enumerate probiont levels and, to a lesser extent, total cultivable communities. More recently, polymerase chain reaction denaturing gradient gel electrophoresis (PCR-DGGE) has been used to assess the impact of a limited number of probiotics on the tilapia intestinal microecology (Zhou et al. 2009; Ferguson et al. 2010; Liu et al. 2013). This limited understanding of the changes in the gut microbiota prevents a full depiction of the mechanisms of action of probiotics in fish and ultimately prevents the optimisation of probiotic application strategies.

Therefore, the aim of this study was to assess the effects of AquaStar[®] Growout (Biomin Holding GmbH, Austria), a lyophilised probiotic mixture containing *Bacillus subtilis*, *Enterococcus faecium*, *Lactobacillus reuteri* and *Pediococcus acidilactici*, on the gastrointestinal (GI) microbiota of tilapia using a multidisciplinary approach, including high-throughput sequencing. In addition, the impact of the probiotic, and the potentially modulated microbial community, on the host intestinal morphology was assessed.

Materials and methods

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.

Experimental design and diet preparation

Nile tilapia, *Oreochromis niloticus*, were transferred to the Aquaculture and Fish Nutrition Research Aquarium (Plymouth University, UK) where they were allowed 6 weeks of acclimation. Three hundred and twenty tilapia were randomly distributed to eight 150-l fibreglass tanks (40 fish per

tank; average weight = 55.03 ± 0.44 g). Fish were fed experimental diets for eight weeks at a rate of 1–3 % of biomass per day in four equal rations (both 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-h 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 (0.07 ± 0.02, 0.04 ± 0.02 and 16.20 ± 2.30 mg l^{-1}, respectively), and regular water changes prevented the build-up of these compounds as well as preventing background build-up of probiotics.

Two iso-nitrogenous and iso-lipidic diets were formulated using Feedsoft Professional[®] according to the known requirements of tilapia (NRC 2011) (Table 1). Dry ingredients were mixed in small batches to ensure a homogenous mix before

Table 1 Dietary formulation and chemical composition (%)

	Control	Probiotic
Fishmeal[a]	5.00	5.00
Soyabean meal[b]	37.26	37.26
Corn starch[c]	24.28	23.78
Lysamine pea protein[d]	5.00	5.00
Glutalys[d]	10.00	10.00
Wheat bran[e]	10.00	10.00
Fish oil	4.50	4.50
Corn oil	2.95	2.95
Vitamin & mineral premix[f]	0.50	0.50
CMC-binder[c]	0.50	0.50
AquaStar[®] Growout[g]	0.00	0.50
Proximate composition (% as fed basis)		
Dry matter	92.89	92.10
Crude protein	35.74	35.88
Lipid	10.06	9.82
Ash	4.19	4.22
Energy (MJ kg^{-1})	20.06	20.00

[a] Herring meal LT92—United Fish Products Ltd., Aberdeen, UK

[b] De-hulled, solvent extracted soybean meal, 48% protein (Sourced from BioMar, 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}

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adding the oil and warm (40 °C) 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 3-mm pellets. The lyophilised probiotic (AquaStar® Growout; Biomin Holding GmbH, Austria) was added at 5 g kg^{-1} (as recommended by the manufacturer) at the expense of corn starch and the basal diet devoid of the probiotic served as a control diet. Diets were dried for 24 h in an air convection oven set to 44 °C, broken up by hand and stored in airtight containers at 4 °C until use. The proximate composition of the diets was analysed using AOAC protocols (1995) (Table 1). Probiotic viability was checked by spread plating tenfold serial dilutions and counting statistically viable plates (i.e. 20–200 colonies), using selective media (de Man, Rogosa and Sharpe (MRS) media for LAB, *Bacillus* selective agar for *Bacillus* spp. and Slanetz and Bartley media for *Enterococcus* spp.; Oxoid, Basingstoke, UK). Fresh diets were produced at the trial midpoint to ensure high probiotic viability.

Fish were sampled, as described in detail in the following sections, after four and eight weeks of feeding on the experimental diets. The weight of fish sampled (week four: control = 89.12 ± 20.87 g and probiotic = 85.29 ± 20.29 g; week eight: control = 162.28 ± 65.30 g and probiotic = 167.01 ± 56.94 g) were not significantly different at either time point. Tilapia survival rates during the feeding trial were >99 %.

Intestinal microbiological analyses

At weeks four and eight, two fish per tank were euthanized by overdose (300 mg l^{-1}) of tricaine methane sulphonate (MS222; Pharmaq, Fordingbridge, UK). The GI tract was aseptically removed, and faecal matter from the mid-intestine was isolated and pooled by tank (thus $n = 4$ per treatment) to assess allochthonous populations. Mid-intestine mucosa samples were removed aseptically, washed with sterile phosphate-buffered saline (PBS, Sigma Aldrich, UK), homogenised and processed on an individual fish basis; thus, $n = 8$. Intestinal samples were either used immediately for culture-based analysis or stored at −20 °C for culture-independent analysis.

Culture-dependent analysis

Samples were serially diluted with PBS, and 20 μl was spotted onto duplicate MRS agar, Slanetz and Bartley and *Bacillus* selective media using the Miles and Misra method (Miles et al. 1938) to assess autochthonous and allochthonous presumptive probiotic bacterial populations. Tryptone soya agar (TSA) was used to determine the total aerobic heterotrophic bacterial populations. Plates were incubated for 72 h at 28 °C, and colony forming units (CFU g^{-1}) were calculated by counting colonies from statistically viable plates (between 3 and 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. (2010).

Culture-independent analysis

At weeks four and eight, digesta samples from two fish per tank were pooled and used for culture-independent analyses ($n = 4$). DNA was extracted using the QIAamp Stool Mini Kit (Qiagen) with a lysozyme pretreatment (50 mg mL^{-1} in TE buffer for 30 min at 37 °C) and a phenol-chloroform clean up, as described elsewhere (Al-Hisnawi et al. 2014).

PCR-DGGE

PCR amplification of the 16S rRNA V3 region was conducted using the reverse primer P2 and the forward primer P3 (Muyzer et al. 1993). 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 (2010b). The presumptive probiotic nucleotide sequences were further identified using BLAST in the NCBI nucleotide collection database.

High-throughput sequencing analysis

DNA extractions from week eight digesta samples were used for high-throughput sequencing using primers 338R (5′-GCW GCC WCC CGT AGG WGT-3′) and 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′). PCR products were purified (QIAquick PCR Purification Kit; Qiagen) and quantified using a Qubit® 2.0 Fluorometer (Invitrogen). Prior to sequencing, the amplicons were assessed for fragment concentration using an Ion Library Quantitation Kit (Life Technologies TM, USA), then concentrations were 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™). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files and deposited in MG-RAST under the accession numbers 4,621, 988.3–4,621,995.3.

All phylogenetic analyses were performed after the removal of 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 operational taxonomic

unit (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 300 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). Representative sequences belonging to probiotic genera, for both probiotic and control treatments (if applicable) were further identified using the NCBI nucleotide collection database BLAST.

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. Additionally, 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).

Persistence of the probiotics after reverting to non-supplemented diet

After reverting the probiotic-treated fish to the control diet at the end of the trial (eight weeks), two fish were sampled on days 3, 6, 9 and 18 post cessation of probiotic feeding to assess probiotic persistence within the intestine by PCR-DGGE analysis. Presumptive probiotic bands were excised for sequence analysis as described previously.

Intestinal histology

At weeks four and eight, two fish per tank were sampled for histological appraisal of the mid-intestine. For light microscopy, the tissue samples were fixed in 10 % formalin and transferred to 70 % ethanol after 24 h. Samples were then dehydrated in graded ethanol concentrations prior to embedding in paraffin wax. In each specimen, multiple sections (5 μm) were stained with haematoxylin and eosin (H & E) and Alcian Blue-PAS to assess the mucosal fold length, intestinal perimeter ratio (arbitrary units; AU), intraepithelial leucocyte (IELs) levels and goblet cell abundance in the epithelium. IELs and goblet cells were counted across a standardised distance of 100 μm and then calculated by averaging the cell numbers from all samples within each treatment.

After eight weeks, the mid-intestines from two fish per tank were sampled for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Samples (ca. 2 mm) were washed in 1 % S-carboxymethyl-L-cysteine for 30 s (SEM only) to remove any mucus before fixing in 2.5 % glutaraldehyde in sodium cacodylate buffer (0.1 M pH 7.2).

Samples were processed as described elsewhere (Dimitroglou et al. 2009) and screened with a JSM 6610 LV (Jeol, Tokyo, Japan) SEM or JEN 1400 (Jeol, Tokyo, Japan) TEM. All electron micrographs were analysed with Image J 1.46r (National Institute of Health, USA) to determine microvilli length and density, as described elsewhere (Dimitroglou et al. 2009).

An absorptive surface area index (ASI) was calculated according to the following: ASI (AU) = microvilli length (μm) × microvilli density (AU) × intestinal perimeter ratio (AU).

Statistical analyses

All data are presented as mean ± standard deviation. PCR-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). All data were checked for normality using a Kolmogorov-Smirnov test and analysed using a t-test. Where data were not normally distributed, data were analysed using a Kruskal-Wallis test (Statgraphics Centurion XVI, Warrenton, VA, USA). High-throughput sequencing data were uploaded to Stamp v2.0.8, and t-tests were used to distinguish differences at each taxonomic level. In all cases, significance was accepted at $P < 0.05$.

Results

Culture-dependent analysis

The effect of AquaStar® Growout on the heterotrophic intestinal bacteria was determined using culture-based methods. Total viable bacteria, lactic acid bacteria (LAB), enterococci and *Bacillus* spp. counts in tilapia intestines were enumerated on TSA, MRS, Slanetz & Bartley and *Bacillus* spp. selective media, respectively. Table 2 displays the allochthonous and autochthonous total viable cell counts (TVC) at weeks four and eight. No significant differences were observed in TVC levels between the treatments at either time points, with allochthonous levels fluctuating around log 7 CFU g^{-1} and autochthonous levels slightly lower, fluctuating around log 5–6 CFU g^{-1}. LAB, *Bacillus* spp. and enterococci levels were all significantly higher in the digesta and mucosa of tilapia fed probiotic-supplemented diets at both time points ($P < 0.05$). The highest LAB levels were recorded at week eight in the digesta of the probiotic fed fish (log 6.41 CFU g^{-1}). Subsets of these isolates were confirmed as the probiotics administered by 16S rRNA sequence analysis and by migration to the same

Table 2 Total viable counts (log CFU g^{-1}) of autochthonous (M) and allochthonous (D) heterotrophic aerobic bacteria, LAB, enterococci and *Bacillus* spp. in the GI tract of tilapia fed experimental diets after four and eight weeks

	Week	Region	Control	Probiotic	P value
TVC	4	D	5.34 ± 1.90	7.47 ± 0.67	0.14
		M	5.85 ± 1.02	6.30 ± 0.48	0.35
	8	D	7.36 ± 0.61	6.93 ± 0.53	0.39
		M	4.93 ± 0.65	4.78 ± 0.43	0.63
LAB	4	D	3.34 ± 0.37^a	5.91 ± 0.98^b	0.03
		M	2.80 ± 0.20^a	4.64 ± 1.45^b	0.02
	8	D	3.10 ± 0.69^a	6.41 ± 0.73^b	0.04
		M	$n.d^a$	4.43 ± 0.86^b	<0.01
Bacillus spp.	4	D	$-^c$	$-^c$	$-^c$
		M	$-^c$	$-^c$	$-^c$
	8	D	2.66 ± 0.77^a	6.39 ± 0.45^b	0.03
		M	2.00 ± 0.00^a	4.91 ± 0.86^b	<0.01
Enterococci	4	D	2.77 ± 0.13^a	5.09 ± 1.51^b	0.04
		M	$n.d^a$	4.27 ± 1.59^b	0.01
	8	D	$n.d^a$	6.28 ± 0.62^b	0.02
		M	$n.d^a$	4.45 ± 0.73^b	<0.01

n.d. not detected

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

[c] No data available due to overgrowth on the plates

position as known *B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici* samples in a PCR-DGGE.

PCR-DGGE

The influence of dietary AquaStar® Growout on the intestinal microbial diversity in tilapia was investigated using PCR-DGGE at weeks four and eight. PCR-DGGE analysis revealed complex microbial communities in both treatments with samples containing 25–35 OTUs (Fig. 1). Presumed probiotic bands were isolated from PCR-DGGE gels at both time points, and sequencing revealed that the nearest neighbour for all bands were the respective probiotic species; these were not detected in control sample fingerprints (Table S1). Table 3 displays the microbial ecological parameters derived from the PCR-DGGE fingerprints from weeks four and eight. At week four, within group replicates similarity percentage analyses (SIMPER) revealed a significantly higher percentage similarity among replicates from the probiotic treatment when compared with the control replicates (75.10 ± 7.80 vs. 51.91 ± 2.88, respectively) ($P = 0.02$). This was not the case at week eight. The number of OTUs, species richness, evenness and diversity remained unaffected at weeks four and eight. Analysis of similarities (ANOSIM) showed that the compositional dissimilarity between the groups (61.51 %) was greater than those within each group at week eight ($R = 0.89$ and $P = 0.03$).

High-throughput sequencing analysis

A total of 1,609,610 sequence reads were obtained from the Ion Torrent® PGM; after removing low quality reads 68,161 ± 2701 and 38,444 ± 4135 sequences were obtained for the probiotic and control fish, respectively, and used for downstream analyses. Good's coverage estimators for both treatments were >0.99 indicating that sufficient sequencing coverage was achieved and that the OTUs detected in the samples are representative of the sampled population (Table 4). Rarefaction curves approached the saturation phase in both treatments at approx. 30,000–40,000 sequence reads, although the plateau was higher for those samples belonging to the control group (Fig. 2a). Consequently, there was a significantly higher number of OTUs and species richness (Chao1) in the control group when compared to probiotic-fed fish (Table 4). The PCoA plot demonstrates a clear separation between each treatment (Fig. 2b) suggesting that there is clear dissimilarity between the intestine microbiota of fish fed control diets compared with fish fed a probiotic supplemented diet. This is supported by the UPGMA which shows clear differentiation between control and AquaStar® Growout replicates, with replicates clustering by treatment (Fig. 2c). Figure 2d illustrates that 40 genera were present (i.e. accounting for >0.01 % of the reads) in control samples which were not present in probiotic samples. Twenty-nine genera were common to samples in both control and probiotic treatments.

Figure 3 shows the major bacterial constituents in the digesta of fish fed either a control or AquaStar® Growout-based diet identified to phyla and genera levels. *Firmicutes* accounted for >99 % of 16S reads in probiotic-fed fish. *Firmicutes* were also the dominant phyla in the digesta of control fish although their presence was significantly lower (44.80 % of reads; $P = 0.01$). *Proteobacteria* and *Cyanobacteria* reads were significantly higher in control samples (8.50 and 25.11 %, respectively) than in the probiotic samples (0.36 %; $P = 0.03$ and 0.18 %; $P = 0.05$, respectively). *Actinobacteria*, *Bacteroidetes*, *Fusobacteria*, *Nitrospirae*, *Spirochaetes* and the phylum TM6 were also present in both treatments although their relative abundances were lower and not significantly different between treatments.

The relative abundance of reads assigned to *Enterococcus* was significantly ($P \leq 0.01$) higher in the probiotic-fed fish when compared to control fish (52.50 vs. 1.35 %, respectively). Reads belonging to *Burkholderia*, *Leuconostoc*, *Acinetobacter*, *Legionella*, *Lactobacillus*, *Corynebacterium*, *Weisella*, *Sphingomonas*, *Rhodococcus* and *Hyphomicrobium* were all significantly more abundant in the control samples when compared to the probiotic-fed fish ($P < 0.05$). In the AquaStar® Growout-fed fish, after *Enterococcus*, the next most abundant genera were *Bacillus* (45.94 %) and *Pediococcus* (0.44 %). *Lactobacillus* reads accounted for 0.08 % of sequences in probiotic samples.
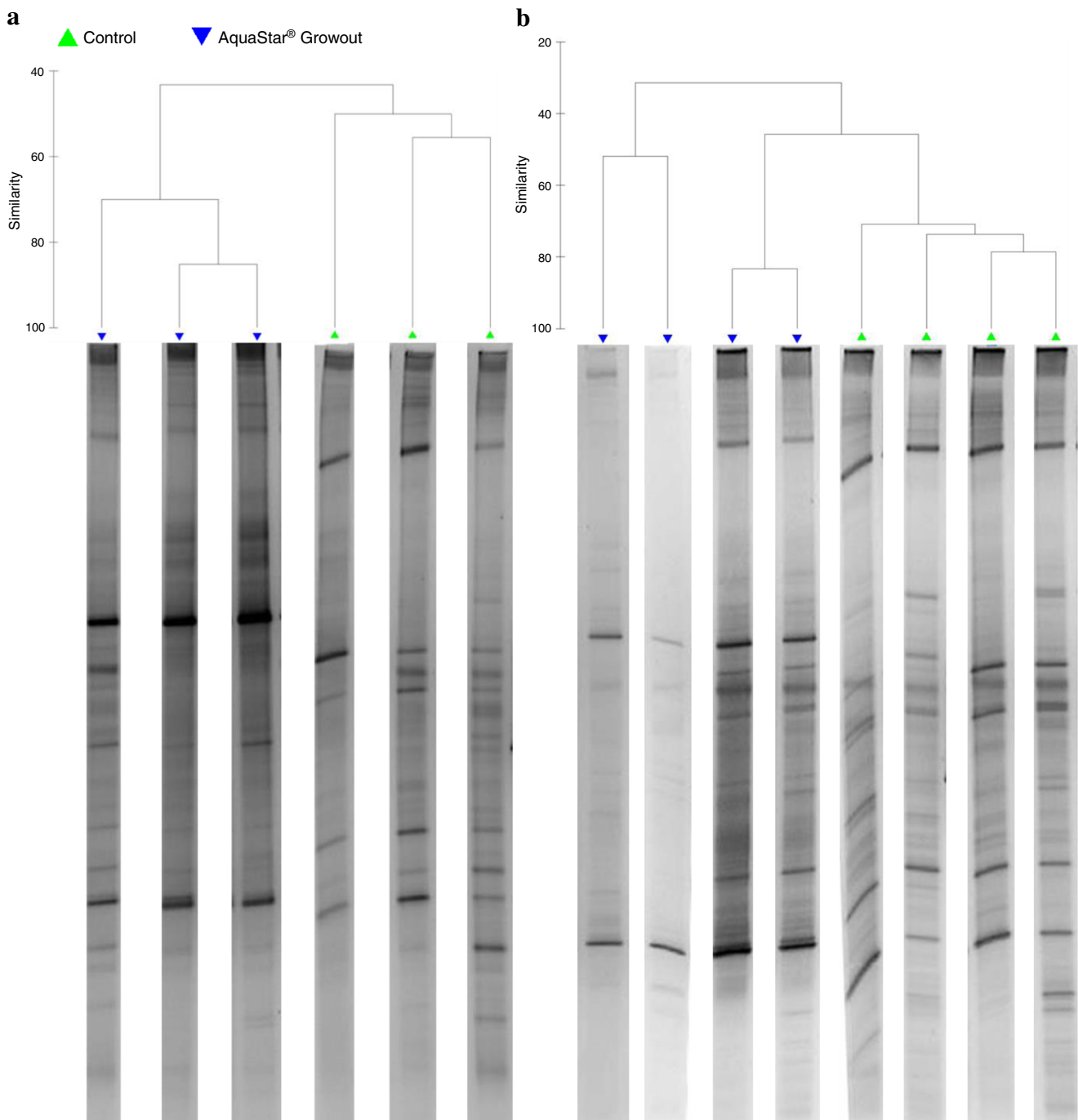
Fig. 1 Dendrograms representing the relatedness of the microbial communities in the digesta of tilapia after 4 weeks (**a**) and 8 weeks (**b**) of feeding with a control diet (*green triangles*) and probiotic diet (*blue triangles*). PCR-DGGE fingerprints below represent amplified products from the V3 region of the samples which correspond to those used in the dendrogram

Bacillus (34 %) also comprised a large component of the allochthonous microbiota in control fish, and low levels of *Enterococcus* (1.35 %), *Lactobacillus* (1.04 %) and *Pediococcus* (0.15 %) sequence reads were also present. BLAST searches using representative sequences belonging to each of these genera confirmed the presence of *P. acidilactici*, *B. subtilis* and *L. reuteri* in AquaStar® Growout samples; however, these species were not present

in the control samples. In the control fish, the *Bacillus* spp. were identified as *B. megaterium* and *B. aquimaris*, *Pediococcus* spp. as *P. pentasaceus*, and *Lactobacillus* reads were predominantly *L. aviarius*. *Enterococcus* reads in both treatment groups were identified as *E. faecium*.

Cetobacterium (accounting for 13.80 % of the reads) and *Mycobacterium* (5.27 %) were also present in the control group; however, they were found at lower levels in the

Table 3 Microbial community analysis of the allochthonous intestinal bacterial populations of tilapia from PCR-DGGE fingerprints after four and eight weeks of experimental feeding

	Microbial ecological parameters					Similarity (ANOSIM)		
	N	Richness	Evenness	Diversity	SIMPER (%)	R value	P value	Dissimilarity (%)
Week 4								
Control	24.67 ± 4.19	2.37 ± 0.38	0.97 ± 0.01	3.10 ± 0.20	51.91 ± 2.88[a]			
AquaStar® Growout	28.33 ± 0.47	2.61 ± 0.03	0.96 ± 0.00	3.22 ± 0.02	75.10 ± 7.80[b]			
Week 8								
Control	32.25 ± 2.38	2.89 ± 0.18	0.99 ± 0.00	3.43 ± 0.07	73.10 ± 3.60			
AquaStar® Growout	31.33 ± 4.11	2.80 ± 0.29	0.97 ± 0.01	3.32 ± 0.17	57.63 ± 18.25			
Pairwise comparisons								
Control vs. AquaStar® Growout (week 4)						0.78	0.10	56.77
Control vs. AquaStar® Growout (week 8)						0.89	0.03	61.51

N = number of operational taxonomic units; *Richness* = Margalef species richness; *Evenness* = Pielou's evenness; *Diversity* = Shannon's diversity index; *SIMPER* = similarity percentage within group replicates

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

probiotic treatment (0.02 and 0.31 % respectively; $P > 0.05$). *Streptococcus* was also found at 0.48 % in control samples but was not detected in three out of four replicates of the AquaStar® Growout treatment (present at 0.01 % in the fourth replicate).

Persistence of probiotics after reverting to the control diet

The persistence of each probiotic in the intestine of the AquaStar® Growout-fed fish was assessed by PCR-DGGE analysis on 3, 6, 9 and 18 days post reverting to the control diet (Fig. 4). *E. faecium* was present 6 days after reverting to control diets, although bands were only visible in two of the four replicates. Bands representing amplicons from *P. acidilactici*, *L. reuteri* and *B. subtilis* all showed signs of decreasing intensity but were still present after 18 days of reverting to the control diet (Fig. 4). Sequence analysis confirmed that these OTUs were the respective probiotic species (Table S1). The number of OTUs, species richness, species evenness and diversity of microbial communities were altered after reverting to the control diet; these parameters followed the same pattern, initially

decreasing from day 0 to day 6, before increasing at day 9 where they were at their highest post cessation of probiotic feeding, before decreasing again on day 18 (Fig. 4).

Intestinal histology

Light microscopy was used to examine the mid-intestine of fish fed either the control diet or AquaStar® Growout-supplemented diet. Fish from both dietary treatments had an intact epithelial barrier with extensive mucosal folds, abundant IELs and numerous goblet cells (Fig. 5). At week four, there were no differences between the control and probiotic group when measuring fold length, perimeter ratio, IEL and goblet cell abundance (Table 5). At week eight, the perimeter ratio was lower in the control group compared to the probiotic treatment (5.36 ± 1.24 vs. 6.48 ± 0.74, respectively), and statistical analysis suggests that this was approaching significance ($P = 0.09$). After eight weeks of experimental feeding, IEL abundance was significantly elevated in the AquaStar® Growout treatment (40.01 ± 4.46 per 100 μm) when compared to the control treatment (32.68 ± 4.81 per 100 μm;

Table 4 Number of reads assigned to OTUs and diversity/richness indices of allochthonous intestinal microbiota composition of fish fed a control diet or probiotic diet after 8 weeks of experimental feeding

Treatment	Raw 16S rRNA reads	Reads >20 Phred score	Reads assigned (after USEARCH function)	Good's coverage	Number of OTUs	Shannon's diversity index	Chao1 index
Control	244,815 ± 46,578	166,352 ± 38,556	38,444 ± 4135	1.000 ± 0.00	129.49 ± 10.44[a]	4.04 ± 0.71	136.08 ± 10.74[a]
AquaStar® Growout	157,588 ± 8518	108,880 ± 5108	68,161 ± 2701	0.999 ± 0.00	90.16 ± 10.66[b]	3.87 ± 0.07	114.29 ± 9.87[b]

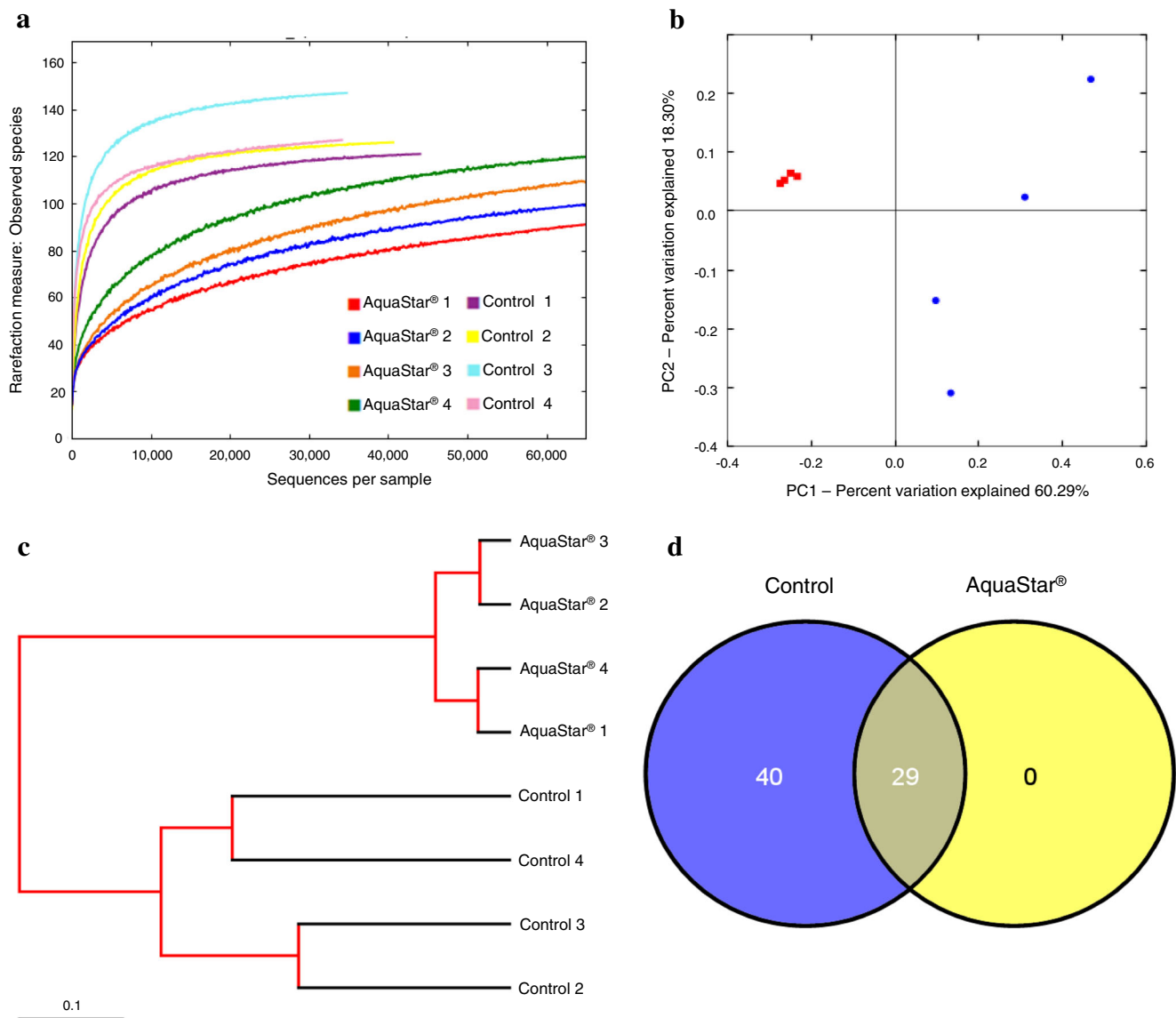[a, b] Different superscripts indicate a significant difference ($P < 0.05$)

Fig. 2 Bacterial community composition and relatedness in the digesta of tilapia fed either a control or probiotic supplemented diet for 8 weeks. **a** Comparison of rarefaction curves between allochthonous intestinal microbiota composition between fish fed a control diet or probiotic diet. **b** PCoA plots where data points represent samples from fish fed a control diet (*blue circles*) and probiotic diet (*red squares*). **c** UPGMA showing hierarchical clustering of intestinal microbiota from each treatment. Bootstrap values are indicated by *red* branches (75–100 %). **d** Venn diagram showing the number of genera (accounting for >0.01 % reads) exclusively assigned to control replicates, probiotic replicates and genera which are common in both treatments

$P = 0.02$). Mucosal fold length and goblet cell numbers remained unaffected by dietary treatment (Table 5).

Microvilli density and length were analysed by SEM and TEM, respectively, at eight weeks only. Fish in both treatments appeared to have a healthy brush border with organised, closely packed microvilli showing no signs of intracellular gaps or necrotic enterocytes. The microvilli density in the intestine of the AquaStar® Growout-fed fish (4.58 ± 0.69) was significantly higher than the control-fed fish (3.49 ± 0.75; $P < 0.05$) (Table 5). Numerical increases ($P = 0.08$) in microvilli length (probiotic = 1.37 ± 0.19 μm vs. control group = 1.19 ± 0.14 μm) and the perimeter ratio ($P = 0.09$), combined with a significant increase in microvilli density, resulted in a significant ($P = 0.01$) increase in the absorptive surface area index (ASI) of the AquaStar® Growout-fed fish (40.84 ± 5.17) compared to those receiving the control diet (22.07 ± 3.85) (Table 5).

Discussion

The intestinal microbiomes of fish are complex communities which have been demonstrated to impact host health, mucosal development and differentiation, metabolism, nutrition and

Fig. 3 Comparison of allochthonous intestinal microbiota composition between fish fed a control or probiotic diet after 8 weeks of experimental feeding. *Heatmap* shows bacterial OTUs assigned at the phylum level and *bars* show OTUs assigned at the genus level (showing genera accounting for >0.25 % of total reads)
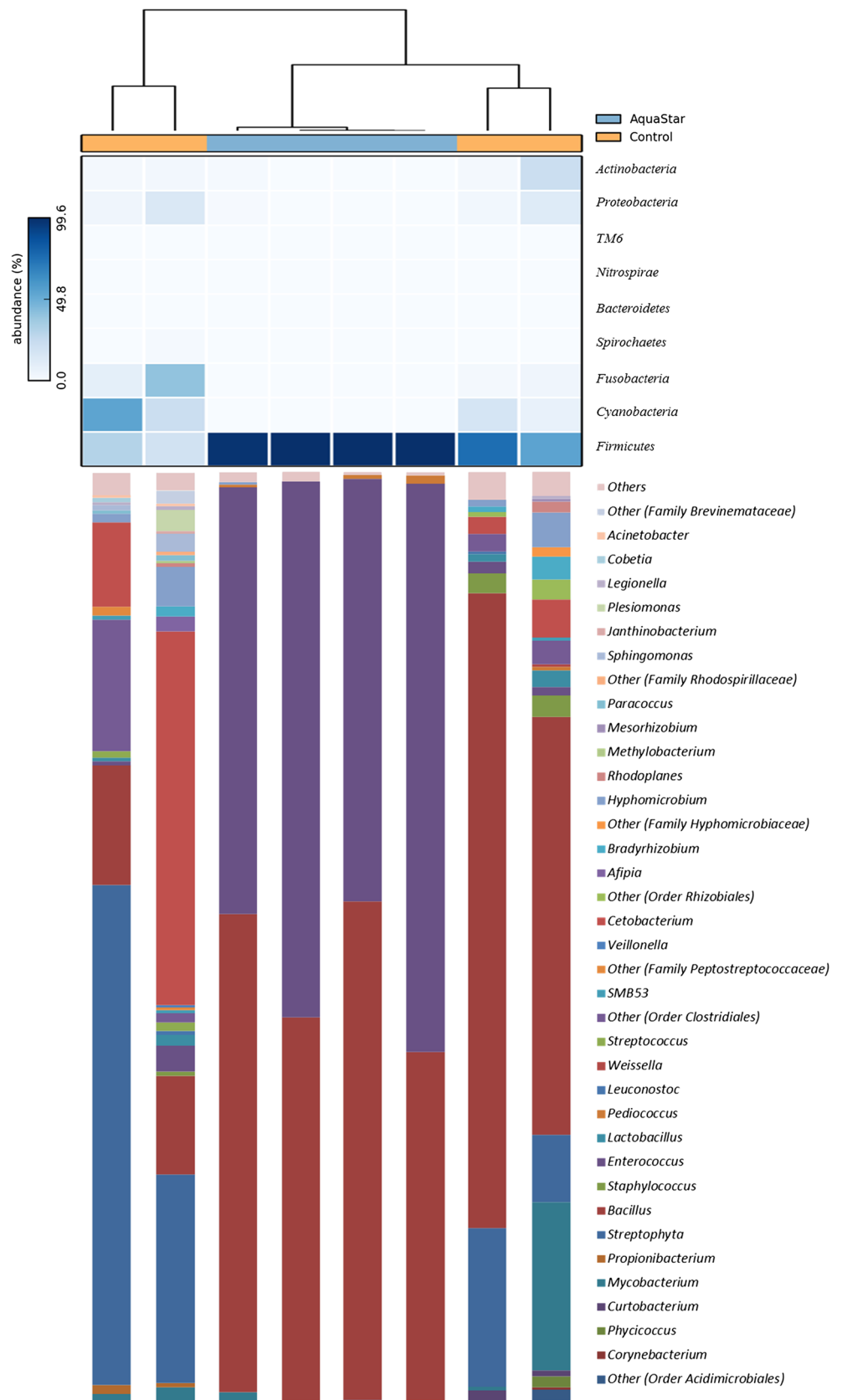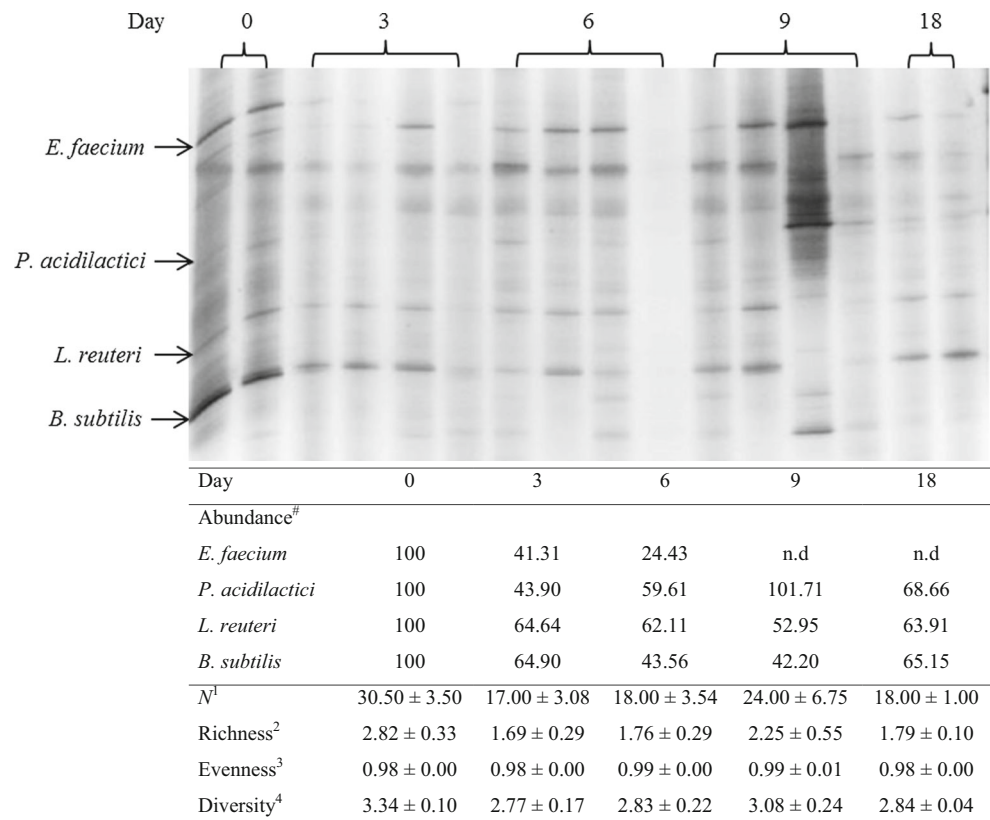
Fig. 4 PCR-DGGE fingerprints showing the persistence of the probiotic bacteria within the intestinal tract of tilapia, after reverting to the control diet. *Numbers above lanes* indicate day post cessation of probiotic provision. Each DGGE lane represents a pooled sample from two fish. The *table* shows microbial diversity and percentage band intensity (of *E. faecium*, *P. acidilactici*, *L. reuteri* and *B. subtilis*)

Day	0	3	6	9	18
Abundance[#]					
E. faecium	100	41.31	24.43	n.d	n.d
P. acidilactici	100	43.90	59.61	101.71	68.66
L. reuteri	100	64.64	62.11	52.95	63.91
B. subtilis	100	64.90	43.56	42.20	65.15
N[1]	30.50 ± 3.50	17.00 ± 3.08	18.00 ± 3.54	24.00 ± 6.75	18.00 ± 1.00
Richness[2]	2.82 ± 0.33	1.69 ± 0.29	1.76 ± 0.29	2.25 ± 0.55	1.79 ± 0.10
Evenness[3]	0.98 ± 0.00	0.98 ± 0.00	0.99 ± 0.00	0.99 ± 0.01	0.98 ± 0.00
Diversity[4]	3.34 ± 0.10	2.77 ± 0.17	2.83 ± 0.22	3.08 ± 0.24	2.84 ± 0.04

[1] N = total number of operational taxonomic units; [2] Richness = Margalef species richness: $d = (N\text{-}1)/ \log(n)$; [3] Evenness = Pielou's evenness: $J' = H'/ \log(N)$; [4] Diversity = Shannon's diversity index: $H' = -\Sigma (p_i (\ln p_i)$ where n = total number of individuals (total intensity units) and p_i = the proportion of the total number of individuals in the ith species.

[#] Values are expressed in terms of percentage relative abundance against the peak density at day 0.

n.d = not detected

disease resistance (Rawls et al. 2004; Bates et al. 2006; Round and Mazmanian 2009). These communities are sensitive to rearing environment, seasonal and diet changes, including probiotic supplementation (Merrifield et al. 2010a; Dimitroglou et al. 2011; Romero et al. 2014). The present literature available on the impact of probiotics on the intestinal microbiomes of fish has been investigated predominantly by culture-dependent means or semi-quantitative techniques such as PCR-DGGE.

The present study used a multidisciplinary approach consisting of culture-based techniques, PCR-DGGE and high-throughput sequencing. The culture-dependent methods employed demonstrated that probiotic feeding resulted in higher LAB, *Bacillus* spp. and enterococci counts in the GI tract of tilapia when compared to control-fed fish. Sequence analysis confirmed that these isolates from the AquaStar® Growout-fed fish were the specific probiotic species administered. Despite these significant increases in bacterial groups, there was no significant impact on the total viable counts at either time point.

However, since only a fraction of the total intestinal microbiota of fish is cultivable under laboratory conditions (Zhou et al. 2014), and early estimates suggest up to 50 % of the

bacterial community in the tilapia intestine is non-cultivable (Sugita et al. 1981), culture-independent methods were also utilised in the current investigation in order to provide a comprehensive overview of microbial communities. Here, PCR-DGGE revealed complex microbial communities in all replicates from both treatments. Sequencing of excised bands confirmed the presence of *B. subtilis*, *E. faecium*, *L. reuteri* and *P. acidilactici* in AquaStar® Growout-supplemented fish whilst these species were not detected in fish fed the control diet. At week four, SIMPER analyses revealed a significantly higher percentage similarity between replicates from the probiotic treatment when compared with the control replicates. Additionally at week eight, ANOSIM showed that the compositional dissimilarity between the groups was significantly greater than those within each group. This suggests that AquaStar® Growout can modulate the GI microbiome and may have a stabilising effect on the community.

The introduction of high-throughput sequencing technologies has increased our understanding of microbial diversity and function in complex environments, including the gastrointestinal tract of fish (van Kessel et al. 2011; Roeselers et al.

Fig. 5 Light (**a–d**), scanning electron (**e** and **g**) and transmission electron (**f** and **h**) micrographs of the mid-intestine of Nile tilapia fed either the control (**a**, **b** and **e**, **f**) or probiotic (**c**, **d** and **g**, **h**) diet at the end of the experimental period. Goblet cells (*arrowheads*) are filled with abundant acidic mucins (*blue*: **b** and **d**) in both treatments and abundant IELs (*arrows*) are present in the epithelia. Abbreviations used are *E* enterocytes, *LP* lamina propria, *L* lumen, *MV* microvilli. Light microscopy staining: **a**, **c** H & E; **b**, **d** Alcian Blue-PAS. *Scale bars*= 100 μm (**a–d**) 2 μm (**f** and **h**) or 1 μm (**e** and **g**)
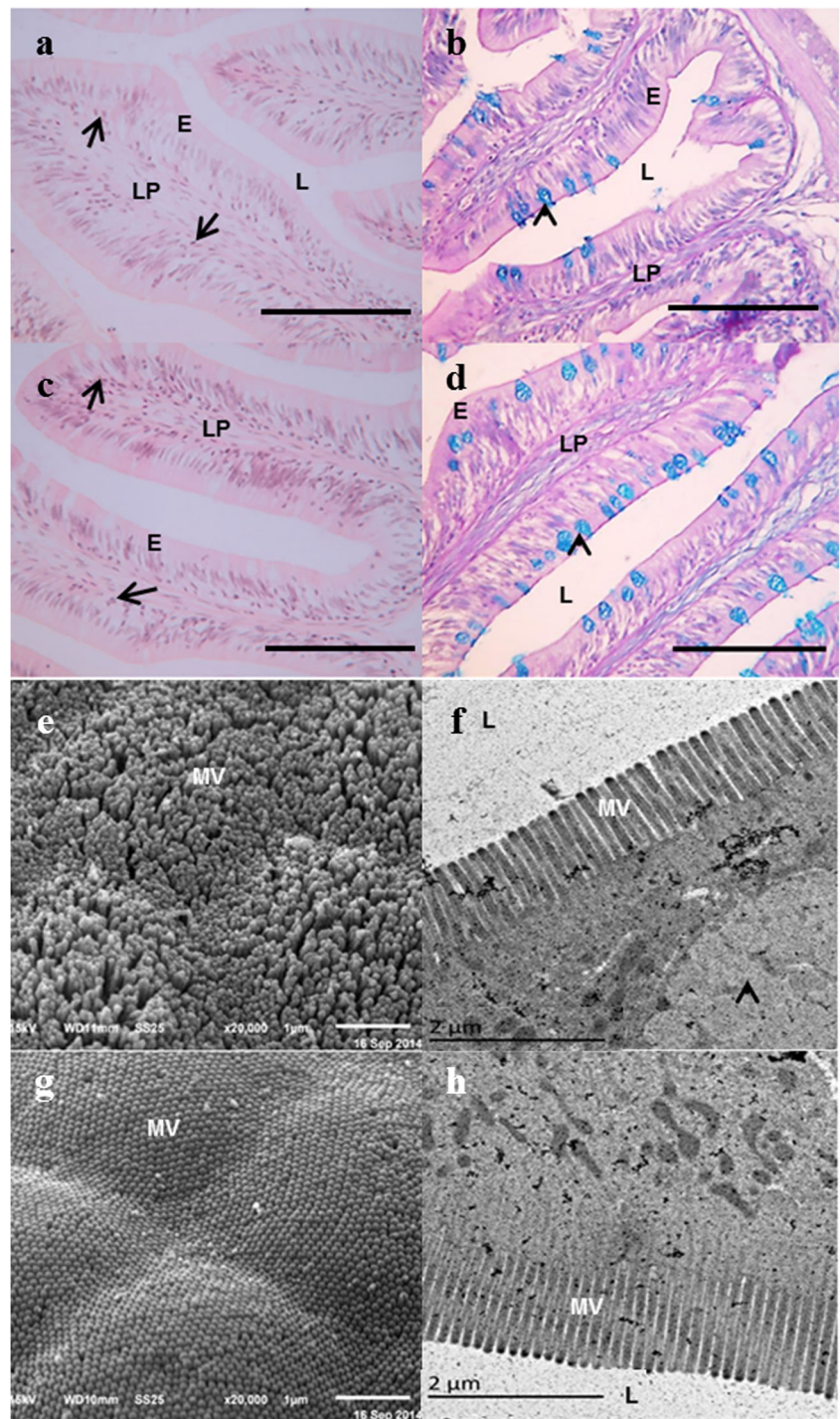
2011; Desai et al. 2012; Wu et al. 2012; Boutin et al. 2013; Carda-Diéguez et al. 2013; Wu et al. 2013; Ingerslev et al. 2014; Zarkasi et al. 2014; Falcinelli et al. 2015). At present, there is a paucity of information on the intestinal microbiome of tilapia using high-throughput sequencing; to the author's knowledge, this is the first study utilising high-throughput sequencing to assess the intestinal microbial communities in this fish species. In the present study, sequence libraries for both treatments displayed Good's coverage estimations of >0.99, indicating that the intestinal microbiota had been fully sampled. *Firmicutes* accounted for >99 % of 16S rRNA reads in the AquaStar® Growout-fed fish, and although they accounted for a significantly smaller proportion of the reads in the control-fed fish, they remained the most abundant phylum present. Concomitantly, *Proteobacteria* and *Cyanobacteria* were significantly more abundant in the

Table 5 Histological data from the GI tracts of tilapia fed control and AquaStar® Growout supplemented diets after four and eight weeks of experimental feeding

	Control	Probiotic	*P*- value
Week 4			
Mucosal fold length (µm)	265.53 ± 34.56	284.27 ± 28.06	0.34
Perimeter ratio (AU)	5.55 ± 0.46	5.97 ± 1.20	0.66
IEL levels (per 100 µm)	34.71 ± 3.39	35.28 ± 2.27	0.75
Goblet cells (per 100 µm)	5.65 ± 1.51	6.88 ± 0.83	0.08
Week 8			
Mucosal fold length (µm)	270.38 ± 51.29	282.04 ± 69.36	0.75
Perimeter ratio (AU)	5.36 ± 1.24	6.48 ± 0.74	0.09
IEL levels (per 100 µm)	32.68 ± 4.81[a]	40.01 ± 4.46[b]	0.02
Goblet cells (per 100 µm)	5.76 ± 0.41	6.23 ± 1.44	0.45
Microvilli length (µm)	1.19 ± 0.14	1.37 ± 0.19	0.08
Microvilli density (AU)	3.49 ± 0.75[a]	4.58 ± 0.69[b]	0.05
ASI (AU)	22.07 ± 3.85[a]	40.84 ± 5.17[b]	0.01

ASI Absorptive surface index

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

control fish along with other notable phyla present including *Fusobacteria*, *Actinobacteria* and *Bacteroidetes*. These phyla have all been detected in varying levels in herbivorous fish species (van Kessel et al. 2011; Roeselers et al. 2011; Wu et al. 2013) including tilapia (Zhou et al. 2009; Liu et al. 2013). BLAST searches using single representative sequences from *Cyanobacteria* revealed a high similarity to nucleotide sequences from soybean chloroplasts. It is possible that at least some of these reads may have been artefacts derived from the diets as opposed to the presence of *Cyanobacteria* populations.

At the genera level, the most abundant 16S rRNA reads belonged to *Enterococcus* and *Bacillus* followed by *Pediococcus*. At a lower level, *Lactobacillus* spp. were also detected. Confirming the cultivable and PCR-DGGE analyses, the administered probiotic species, *E. faecium*, *B. subtilis*, *P. acidilactici* and *L. reuteri*, were identified in the high-throughput sequence libraries from the probiotic-fed fish. In contrast, although these genera were present in the control-fed fish, with the exception of *E. faecium*, the probiotic species were not detected. *E. faecium* has routinely been detected as an indigenous constituent of the gut of a number of fish (Sun et al. 2009; Gopalakannan and Arul 2011; Desai et al. 2012; Sahnouni et al. 2012; Bourouni et al. 2012) and shellfish species (Cai et al. 1999) and its presence in the control tilapia in this experiment is indicative that this species is native to the tilapia intestine also. Members of the *Lactobacillus* and *Pediococcus* genera have also been reported as indigenous members of the intestinal microbiota of a number of fish species (Cai et al. 1999; Ferguson et al. 2010; Jatobá et al. 2011; Ringø et al. 2014). The relative abundance of a number of potential pathogens (*Legionella* spp., *Mycobacterium* spp. and *Streptococcus* spp.) was reduced, significantly in the case

of *Legionella*, by the application of dietary AquaStar® Growout. This topic warrants further investigation.

Despite the numerous significant differences in OTU abundances detected, 29 of the 69 (42 %) genera detected in this study were common to both treatment groups. This may be suggestive of a core microbiome, which, despite possible modulation in terms of abundance, persists within the intestine irrespective of probiotic treatment. This would infer that members of these genera are well adapted to the selective pressures present in the tilapia intestinal tract. Similarly, other studies have identified a core microbiome in fish species, which appear to be present when individuals are reared in different locations, different conditions or fed different diets (Roeselers et al. 2011; Wong et al. 2013).

Due to the absolute dominance (as a proportion of total number of reads) of the administered probiotics (i.e. *Enterococcus* and *Bacillus*), it is perhaps not surprising that the number of observed OTUs (those accounting for >0.01 % of the reads) and the Chao1 index were significantly lower in the probiotic group. Despite these changes, the diversity, as indicated by Shannon-Wiener Index, was not significantly different between the two treatments. This suggests that the apparent reduction of other OTUs may not necessarily be due to their absolute reduction in abundance, but possibly their relative decrease as a proportion of the total bacterial reads given the large number of probiotic 16S rRNA reads. Indeed, caution should be applied when interpreting high-throughput sequence libraries as 16S rRNA copy numbers can differ amongst bacterial species (Fogel et al. 1999); this can lead to incorrect conclusions when discussing true bacterial diversity and taxon abundance (Wintzingerode et al. 1997). For example, *Bacillus* and *Enterococcus* appear to be present at similar levels given the proportion of reads assigned to these genera in

the probiotic-fed fish; however, *Bacillus* strains have typically been reported to contain 10 copies of the 16S rRNA gene whereas *Enterococcus* spp. have frequently been reported to contain four copies (Fogel et al. 1999). Therefore, estimating the abundance (i.e. number of cells) of each bacterial species, relative to other species, is problematic.

The observed modulation of the intestinal microbiome in the present study influenced the host intestinal morphology. Histological analysis revealed an increased population of IELs in the mid-intestine of tilapia after eight weeks feeding on the AquaStar® Growout diet. Similar results have also been reported in tilapia fed monospecies probiotic applications of *P. acidilactici* (Standen et al. 2013) or *Lactobacillus rhamnosus* (Pirarat et al. 2011). Furthermore, after eight weeks, microvilli density was significantly higher in the mid-intestine of fish fed the probiotic when compared to control groups. Higher microvilli density may reduce the exposure of the tight junctions between enterocytes, and this may help to provide a more effective barrier against potential pathogens. Further, due to increased microvilli density, combined with numerical improvements of microvilli length and perimeter ratio, the absorptive surface area index was significantly improved in the probiotic-fed fish. Consequently, fish fed AquaStar® Growout may have a higher potential capacity for nutrient utilisation.

After the eight-week feeding trial, PCR-DGGE analysis was used to investigate the persistence of each of the probionts in the intestine after the cessation of probiotic feeding. All four probionts decreased in abundance after AquaStar® Growout supplementation had ceased but were still detected for a number of days post transition to the non-supplemented control diet. *E. faecium* was still detected for up to 6 days post reverting to the control diet. The remaining three probiotics were still present after 18 days of control feeding, demonstrating the temporal colonisation of the intestine of these species. Similarly, *P. acidilactici* could be detected in the tilapia intestine for at least 17 days after cessation of *P. acidilactici* provisions (Ferguson et al. 2010). The ability of other probiotics including *Carnobacterium* spp., *Lactobacillus* spp., *Lactococcus* spp., *Leuconostoc* spp. and *Bacillus* spp. to persist in the gastrointestinal tract of salmonids and catfish has been investigated, demonstrating temporal colonisation lasting from <3 days to >3 weeks (Nikoskelainen et al. 2003; Kim and Austin 2006; Balcázar et al. 2007; Ran et al. 2012). All these persistence assays followed shorter probiotic supplementation periods (between 7 and 32 days), compared to the current 56-day study. From such studies, it is evident that the length of time a probiont may remain in the intestine of fish, after probiotic feeding has ceased, is dependent on the probiotic species, host species, environmental factors, dosage and duration of probiotic supplementation.

In conclusion, all three microbiological methods used in the present study (culture based, PCR-DGGE and high-throughput sequencing) confirmed the presence of the probiotics in the intestine of the AquaStar® Growout-fed fish. Survival through the upper gastrointestinal tract is an essential requirement of any probiotic, since it must survive the gastric process in order to exert its beneficial effect in the intestine. Under these conditions, AquaStar® Growout can stimulate the localised immune response through the recruitment of IELs in the intestinal mucosa, which may result in better protection against localised pathogens. Intestinal translocation experiments and disease challenge studies are required to validate this hypothesis. Concomitantly with modulated microbiota and IEL levels, AquaStar® Growout treatment enhanced intestinal morphology by elevating the absorptive surface area.

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