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The effect of dietary probiotics on Nile tilapia, Oreochromis niloticus, health and growth performance

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The effect of dietary probiotics on Nile tilapia, *Oreochromis niloticus*, health and growth performance

By

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The effect of dietary probiotics on Nile tilapia, *Oreochromis niloticus*, health and growth performance

Benedict Standen

Three investigations were conducted in order to investigate the effect of dietary probiotics on tilapia (*Oreochromis niloticus*) growth performance, intestinal morphology, intestinal microbiology and immunity.

The first experiment demonstrated that *Bacillus subtilis*, *Lactobacillus reuteri* and *Pediococcus acidilactici* supplemented individually and as a mixed probiotic (in addition to *Enterococcus faecium*; AquaStar® Growout) were capable of modulating intestinal microbial populations as determined by culture dependent methods and DGGE. Furthermore, high-throughput sequencing reported that >99% of 16S rRNA reads in the mixed probiotic group belonged to the probiotic genera, predominantly assigned to *Enterococcus* (52.50%) and *Bacillus* (45.94%). Tilapia in the mixed probiotic group displayed significantly higher intraepithelial leucocyte (IEL) populations in the mid intestine when compared to the control and *L. reuteri* treatment. The mixed probiotic also improved microvilli density and had a higher absorptive surface area when compared to the control.

In the second trial, after six weeks of supplementing tilapia diets with AquaStar® Growout at 3g kg^{-1}, fish demonstrated significantly higher final weight, weight gain and SGR when compared to that of the control (void of probiotic) treatment or an initial probiotic feed (lasting two weeks) followed by control feeding. Probiotic supplementation at 3g kg^{-1} also caused an increase in the abundance of intestinal IELs and goblet cells and an up-regulation in the gene expression of intestinal caspase-3, PCNA and HSP70 and immunity genes TLR2, TNFα, IL-1β, TGFβ and IL-10 when compared with the expression of control replicates. These changes were not observed when supplementing tilapia diets with a lower dose (1.5g kg^{-1}), nor when supplementing the probiotic in either a pulsed manner or as an initial feed (two weeks) followed by control feeding.

Trial three revealed that the probiotic had a more discrete effect on the intestinal allochthonous microbiota as 16S rRNA reads assigned to probiotic genera only accounted for 5-10% of total reads. Nevertheless, the supplementation of dietary AquaStar® Growout at 3g kg^{-1} improved the localised immune response in tilapia, through the regulation of immunity genes TLR2, MYD88, NFκB, TNFα, IL-1β, TGFβ and IL-10, larger populations of goblet cells and a higher recruitment of IELs. Furthermore, the probiotic also improved the systemic immune response through the regulation of immunity genes (mentioned above) in the head kidney and significantly higher circulating leucocyte levels in whole blood. The extent of these changes were dependent on the probiotic treatment (i.e. continuously supplemented in feed or alternating weekly between probiotic at 3 g kg^{-1} and control feeding), the duration of feeding and the parameter investigated.

This research demonstrates that *B. subtilis*, *L. reuteri*, *P. acidilactici* and AquaStar® Growout can modulate the intestinal microbiota. In addition, AquaStar® Growout can improve intestinal morphology, growth performance and modulate both the localised and systemic immune responses of tilapia when supplemented through the feed at the appropriate dosage and feeding regime.

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List of abbreviations

ANOVA	Analysis of variance
AOAC	Association of Official Analytical Chemists
AU	Arbitrary units
BLAST	Basic local alignment search tool
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming units
DEPC	Diethylpyrocarbonate
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FCR	Feed conversion ratio
GALT	Gastro associated lymphoid tissue
HK	Head kidney
HSP	Heat shock protein
IEL	Intra epithelial leucocyte
IL-10	Interleukin 10
IL-1β	Interleukin 1 beta
IP	Intraperitoneal
LAB	Lactic acid bacteria
MAMPs	Microbial associated molecular patterns
MCH	Mean corpuscular haemoglobin
MCHC	Mean corpuscular haemoglobin concentration
MCV	Mean corpuscular volume
MRS	de Man, Rogosa and Sharpe
MS222	Tricaine methane sulphonate
MYD88	Myeloid differentiation factor 88
NFκB	Nuclear factor kappa B
NRC	Nation Research Council
OTU	Operational taxonomic unit
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PCV	Packed cell volume
PER	Protein efficiency ratio
PRR	Pattern recognition receptor
RNA	Ribonucleic acid
RT-PCR	Real time polymerase chain reaction
S & B	Slanetz and Bartley medium
SCFA	Short-chain fatty acid
SEM	Scanning electron microscopy
SGR	Specific growth rate

TAE	Tris-acetate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
TGFβ	Transforming growth factor beta
TLR	Toll like receptor
TNFα	Tumour necrosis factor alpha
TSA	Tryptone soy agar
TVC	Total viable count

Common genera abbreviations

A.	*Aeromonas*
B.	*Bacillus*
Ct.	*Cetobacterium*
E.	*Enterococcus*
Ed.	*Edwardsiella*
L.	*Lactobacillus*
Lc	*Lactococcus*
M.	*Micrococcus*
P.	*Pediococcus*
Ps.	*Pseudomonas*
S.	*Saccharomyces*
St.	*Streptococcus*

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Conferences attended and work presented

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Prebiotics and probiotics in medicine, veterinary sciences and aquaculture: the future (Keele University, UK September 2012)

BioMarine Convention (October 2012) – Participated as a note taker and formulated reports summarising discussions.

Aquaculture America Seattle (February 2014) – Presented a poster entitled "Dietary AquaStar® supplementation modulates intestinal health and promotes growth performance of Nile tilapia (*Oreochromis niloticus*)"

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Publications

Standen, B. T., Abid, A. (2011) Evaluation of probiotic bacteria in tilapia production. International Aquafeed 14, 16-18.

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Standen, B. T., Peggs, D. L., Rawling, M. D., Foey, A., Davies, S. J., Santos, G. A., Merrifield, D. L. (Under review). Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*. Fish Shellfish Immun

Peggs, D. L., **Standen, B. T.**, Waines, P. Rodiles, A., Harper, G *et al*. (Under review). Dietary induced changes to the intestinal morphology and microbiome of European seabass (*Dicentrarchus labrax*). J World Aquacult Soc

Chapter 1. Introduction

1.1 Aquaculture

The Food and Agriculture Organization of the United Nations (FAO) defines aquaculture as "the farming of aquatic organisms including fish, molluscs, crustaceans and aquatic plants where some sort of intervention is made to enhance production" (FAO 1995). In 2012 aquaculture production reached a record high of 90.4 million tonnes with an estimated value of US$144.4 billion (FAO 2014). Aquaculture is an important source of income and livelihood for millions of people worldwide as well as a crucial production sector for high-protein food and in 2013 global production of farmed fish for human consumption was 70.5 million tonnes (FAO 2014). In the last three decades alone (1980-2010) global food fish production has grown at an average annual rate of 8.8% (FAO 2012) and this increased by a further 5.8% in 2013 (FAO 2014). This consistent growth is attributed to an increasing demand for affordable protein sources, particularly in third world countries, coupled with declining stocks of capture fisheries. Indeed, farmed fish contributed to 42.2% of the total fish produced (including non-food uses) by both aquaculture and capture fisheries in 2012, an increase from previous years (Fig 1.1).

Global aquaculture can be categorised into inland aquaculture and mariculture. Inland aquaculture primarily uses freshwater whereas mariculture predominantly uses sea water. Since 1980 inland aquaculture has seen average annual growth rates of 9.2% (compared with 7.6% for mariculture) increasing its share in total aquaculture from 50% in 1980 to 63% in 2012. This contribution increases to 86% when taking into account farmed fish alone (FAO 2014). Inland aquaculture is expected to be the lead sector in achieving long-term food

security and meeting the added demands of population growth in many developing countries in the coming decades (FAO 2014).

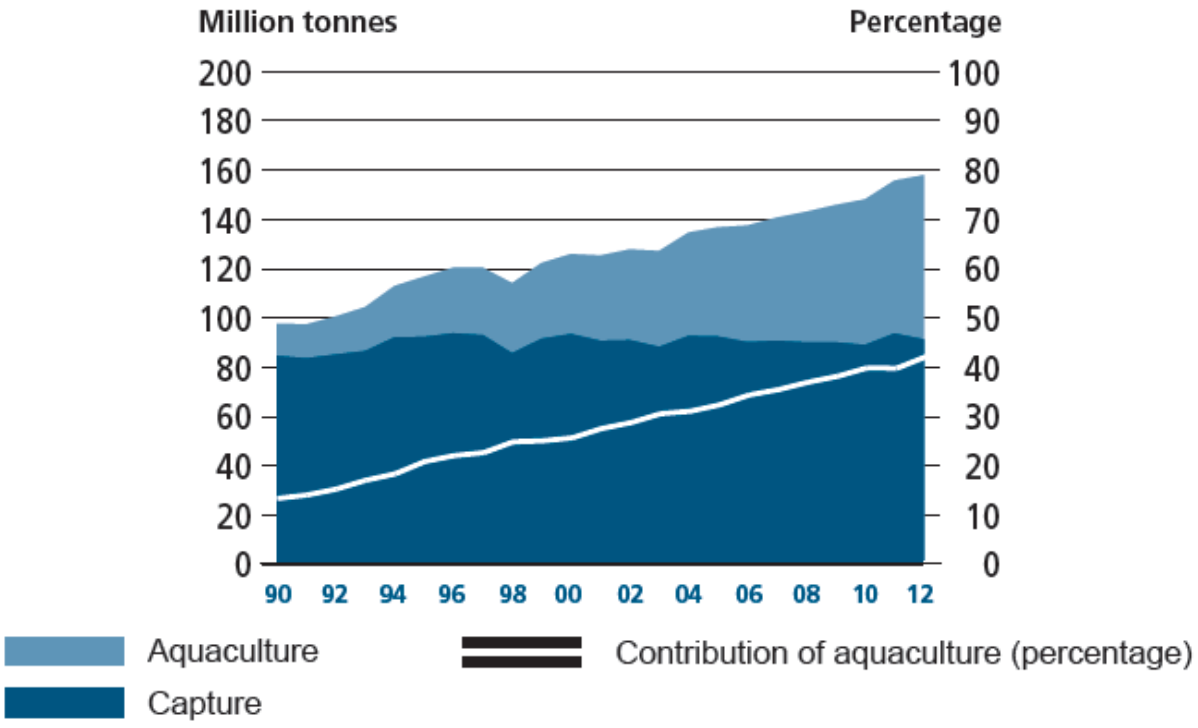


Figure 1.1: Share of aquaculture in total fish production. Source: FAO (2014).

1.1.1 Tilapia aquaculture

Tilapia are a freshwater species belonging to the Cichlidae family. They are native to Africa but today have a global presence due to aquaculture, recreational fishing, aquatic weed control and scientific research purposes (El-Sayed 2006). Tilapia have a number of attributes which make them a popular choice for aquaculture. These include fast growth, tolerance to a wide range of physical and environmental conditions, relative resistance to stress and disease, ability to reproduce in captivity, have a short generation time and are able to convert low cost feed into high quality protein (El-Sayed 2006). This has given tilapia a reputation of being the

'aquatic chicken'. The FAO has recorded tilapia production in 135 countries (FAO 2014), most notably China (~ 1.6 million tonnes), Indonesia (~ 947, 000 tonnes), Egypt (~ 636, 000 tonnes), the Philippines (~ 269, 000 tonnes) and Thailand (~ 213, 000 tonnes) (FIGIS 2013). During the 1950's to 1970's tilapia culture grew slowly, gaining momentum in the 1980's and in 1993 aquaculture production outpaced landings from capture fisheries. Today, technological innovations have seen the rapid growth in tilapia culture with total production over 4.8 million tonnes in 2013 and a value of over US$8.2 billion (FIGIS 2013) (Fig 1.2).

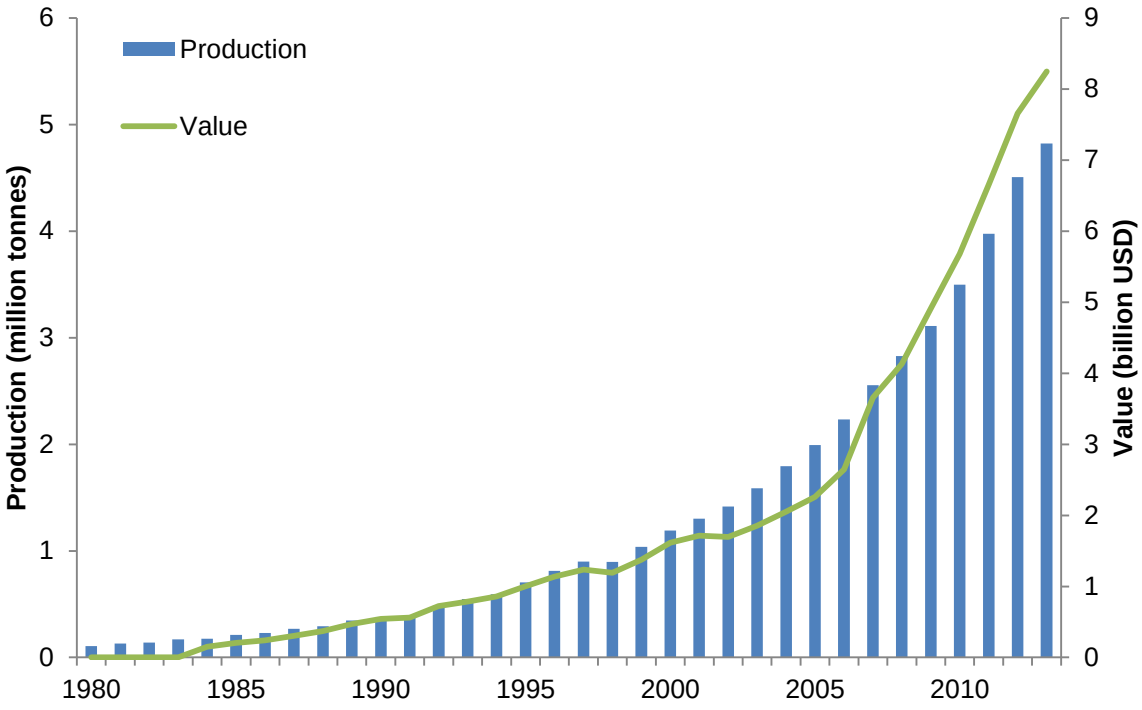


Figure 1.2: Global tilapia production and value between 1980 and 2013. Source: (FIGIS 2013).

The main species produced is Nile tilapia (*Oreochromis niloticus*; 3.4 million tonnes) but other popular species for culture include Mozambique tilapia (*Oreochromis mossambicus*;

34,206 tonnes) and various hybrids of which Nile tilapia and blue tilapia are most popular (*O. niloticus* x *Oreochromis aureus*; 414,475 tonnes) (Fig 1.3).

With an increasing pressure to farm species at lower trophic levels, the expansion of tilapia aquaculture reflects a positive future for the species. This is especially true with the increasing interest of many countries to farm tilapia such as the Americas, where there is a huge availability of freshwater resources. Technological innovation in genetics including GIFT (Genetically Improved Farmed Tilapia), GMT (Genetically Male Tilapia), GST (GenoMar Supreme Tilapia) and GET EXCEL strains have improved growth performance, temperature and salinity tolerance, colouration, stress and disease resistance. These improvements, along with others in genetics, nutrition, disease management, and farming systems have all contributed to an exciting and secure future for tilapia culture.

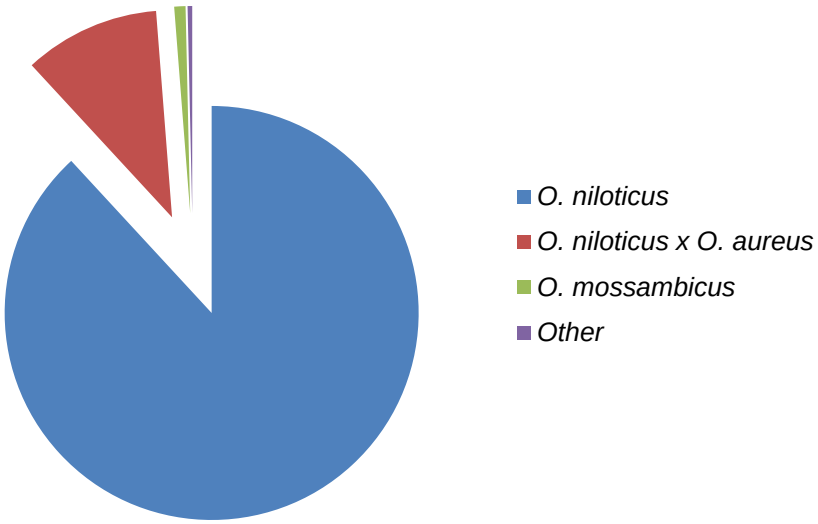


Figure 1.3: Aquaculture output by tilapia species groups in 2013. Source: (FIGIS 2013).

1.2 Stress and disease

The aquatic environment supports a number of opportunistic and secondary pathogens as well as beneficial microbes. However, the rearing technology for intensive production of fish creates a highly artificial environment which promotes bacterial growth and may affect the bacterial selection negatively. Thus, in intensive facilities, environmental conditions (oxygen levels, pH, water quality and temperature) and poor husbandry practises (inadequate nutrition, overfeeding, transportation and stocking density) can be stressful for the organisms involved, which can compromise the immune response and increase susceptibility to infection and disease. In addition to this, bacteria may be introduced via artificial or natural feeds, inlet water and less often via vertical transmission. These disease outbreaks can have large economic impacts in the leading fish producing countries (Bondad-Reantaso *et al*. 2005).

The main culprits that contribute to severe disease outbreaks in tilapia include *Aeromonas hydrophila*, *Edwardsiella tarda* and *Streptococcus iniae*. Certain opportunistic pathogens, including *A. hydrophila*, do not cause disease in a healthy fish; indeed, they are routinely found as minor components in the intestinal tract of healthy fish but emerge as pathogens under certain circumstances. One of the most commonly attributed causal factors for disease outbreaks in aquaculture scenarios is stress. As aquaculture intensifies, and fish densities increase, stress and disease are likely to play a more important role in years to come. Certainly, this is the case with streptococcosis, a septicaemic disease affecting a number of fish species including tilapia (Toranzo *et al*. 2005), caused by the etiological agents *St. iniae* and *St. agalactiae*. Streptococcosis has increased in prevalence over the last decade with annual losses estimated to be worth US$100 million in the 1990's (Shoemaker *et al*. 2001) and in the 2010's, over US$250 million (Amal and Zamri-Saad 2011).

The employment of membrane filters, ozone and/or UV contributes to the removal of pathogens but the total elimination of pathogens is difficult to achieve. Traditionally antibiotics and other antimicrobial chemicals have been used to combat disease. Abuse of these antimicrobials has resulted in the evolution of antimicrobial resistance amongst pathogenic bacteria (Baquero *et al*. 2008) as well as having residual effects in the host and environment. Furthermore the loss of a stable microbial balance via disinfection leaves an environment which is vulnerable to attack by opportunistic pathogenic bacteria. This has been reflected in the EU moratorium on the banning of antibiotic growth promoters in animal feeds, including fish (EU 2005).

Whilst being an effective strategy against certain diseases, vaccination can be expensive, labour intensive and can have variable results. As new pathogens constantly emerge and mutations arise within existing pathogens, vaccination is impractical and is not sufficient as a strategy to defend against all diseases. This has fuelled a growing trend to explore novel feed compounds to provide various functional attributes to the health of fish and shrimp, and has also helped facilitate consumer perceptions of bio-security and eco-friendly aquaculture. Therefore, the development of effective strategies for manipulating microbial communities to promote and sustain the health of the host have been explored.

1.3 Host-microbiota interactions within the gastrointestinal tract of fish

Aquatic animals are constantly in contact with the microbial composition and changes in their surrounding environment. Pathogens, many of which are opportunistic in nature, may be able to maintain themselves in the water column and proliferate independently of the host causing disease or rendering aquatic animals immunocompromised (Moriarty 1998). The GI tract is one of the key sites of interaction with the external world and is considered one of the

major portals and infection loci for a number of pathogens in fish (Ringø *et al*. 2007). The establishment of a normal microbiota is vital, as this can affect a wide range of biological processes including development and assembly of the gut associate lymphoid tissue (GALT), nutrient digestion and absorption, angiogenesis, epithelial integrity and renewal and activity of the enteric nervous system (Husebye *et al*. 1994; Stappenbeck *et al*. 2002; Rawls *et al*. 2004; Gómez and Balcázar 2008; Perez-Sanchez *et al*. 2010) to such an extent, some authors refer to it as an 'extra organ' (O' Hara and Shanahan 2006).

It is often difficult to fully assess host-microbe interactions, since microbial communities in fish reared in conventional systems are extremely dynamic which can cause experimental complications. The development of gnotobiotic models using germ-free (GF) animals has enabled researchers to have increased control of variables, enhanced reproducibility of results and more accurate experimental designs (Coates 1975). Successful gnotobiotic models have been developed in a number of fish including platyfish (*Platypoecilus maculatus*) (Baker *et al*. 1942), zebrafish (*Danio rerio*) (Pham *et al*. 2008), tilapia (Situmorang *et al*. 2014), salmonids (Trust 1974), European seabass (*Dicentrarchus labrax*) (Dierckens *et al*. 2009; Rekecki *et al*. 2009), turbot (*Scophthalmus maximus*) (Munro *et al*. 1995), Atlantic halibut (*Hippoglossus hippoglossus*) (Verner-Jeffreys *et al*. 2003), cod (*Gadus morhua*) (Forberg *et al*. 2011) and red drum (*Sciaenops ocellatus*) (Douillet and Holt 1994). Using GF zebrafish, Rawls *et al.* (2007) used a defective *Pseudomonas* containing deletions in genes relating to motility and pathogenesis, to observe host-microbe interactions when compared with non-mutant *Pseudomonas*. Mutant *Pseudomonas* were unable to interact with the host suggesting that flagella function, including swimming motility, is an important component in order for the host to detect and monitor such microbes.

In a novel investigation, Rawls *et al.* (2006) performed reciprocal transplants of the microbiotas of conventional zebrafish and mice into GF zebrafish and mice. Researchers

discovered that the microbial assemblages resembled that of the original community, however, differences in community structure between zebrafish and mice arise, at least in part, from selection pressures imposed within the intestinal habitat of each host. This suggests that each host species has a core microbiome, a concept which has been evidenced in fish (Roeselers *et al*. 2011; Wong *et al*. 2013).

1.3.1 Indigenous intestinal microbiota of tilapia

Our current understanding of the microbiota composition has predominantly been derived from farmed fish. To limit mortalities at an early life stage, intensive aquaculture practices often employ the disinfection of eggs and larvae. To some extent, this is perhaps counter-intuitive since fish microbiota are the first line of defence (Boutin *et al*. 2012). Bacteria initially colonise the chorion of the egg with any taxonomic differences likely to be caused through precursors of innate immunity (Llewellyn *et al*. 2014). Once the egg hatches, the initial colonization of the gut is derived from the intake of water by fry to maintain osmotic balance and from grazing on suspended particle's and egg debris (Reitan *et al*. 1998; Olafsen 2001). In adult fish the intestinal microbiome is more stable and can be separated into two distinct groups; autochthonous and allochthonous. Allochthonous microbiota, are associated with the digesta whereas autochthonous microbiota are potential residents and have more intimate associations with the epithelial mucosa in the stomach and/or intestine.

A number of authors have observed a large microbial diversity within the intestinal tract of tilapia. Cultivable bacterial levels were reported to be in the range of 10^6-10^8 CFU g^{-1} (Al-Harbi and Uddin 2003, 2005) although this may fluctuate depending on the season (Al-Harbi and Uddin 2004). Al-Harbi and Uddin (2003) enumerated the bacterial flora of pond water, pond sediment, tilapia gills and intestine and reported a comparable microbiota

between these environments, although there was a larger diversification in the tilapia intestine. In this study *Corynebacterium urealyticum*, *Shewanella putrefaciens* and *A. hydrophila* were the predominant bacterial species in tilapia intestines. Later studies by the same authors calculated between 77-87% of the total isolates were Gram-negative rods (Al-Harbi and Uddin 2004; Al-Harbi and Uddin 2005). These were identified predominantly as *A. hydrophila*, *Sh. putrefaciens*, *Escherichia coli*, *Vibrio cholerae*, *Pseudomonas* spp., *Photobacterium damselae* and *Pasteurella* spp. Al Harbi and Uddin, (2005) reported that *Vibrio* made up 58% of the total isolates; these were further identified as *Vibrio parahaemolyticus* , *Vibrio carchariae*, *Vibrio alginolyticus* and *Vibrio vulnificus*. Other constituents included *Bacillus* sp., *Cellulomonus* sp., *C. urealyticum* and *Streptococcus* sp. Using culture based techniques, Tsuchiya *et al*. (2008) identified *Cetobacterium somerae* as a major component in the tilapia intestine. This species was confirmed using 16S rRNA sequencing.

Culture dependent approaches are not sufficient on their own and it is more appropriate to use them in conjunction with culture independent approaches. He *et al*. (2010; 2013) used DGGE followed by 16S rRNA sequencing to identify multiple phyla present in the hybrid tilapia intestine including Proteobacteria, Actinobacteria, Cyanobacteria, Fusobacteria and Firmicutes. Specifically, *Lactobacillus plantarum* isolated from the tilapia intestine were able to inhibit the growth of several pathogens *in vitro* and improved the immune system and growth performance of tilapia *in vivo* (Jatobá *et al*. 2011). Fluorescent *in situ* hybridisation (FISH) has also been employed to identify and quantify specific taxa in the tilapia intestine. *Pseudomonas fluorescens* constituted ca. 15% of total bacterial abundance, *Lactobacillus brevis* and *Lactobacillus collinoides* constituted ca. 10% and *Lactobacillus coryniformis*, *Lactobacillus farciminis* and *Vibrio* spp. were present at approximately 5% of total bacterial abundance in the tilapia intestine (Del'Duca *et al*. 2015). *Lactobacillus* and

Weisella has been recovered in the intestine of Nile tilapia by other researchers (Merrifield *et al*. 2010b; Jatobá *et al*. 2011).

Besides bacteria, it is important to remember that there are other constituents in the intestinal tract, namely yeasts, Archaea and viruses. Despite this, very little work has been conducted investigating these in tilapia. In extreme environments, such as in deep sea fish, yeasts can account for a greater proportion of the cultivable microbiota than bacteria (Ohwada *et al*. 1980). Whilst this is likely to be an exception, yeasts have been routinely found in healthy fish, although the diversity and density is extremely variable (Gatesoupe 2007). It is important to remember however that yeast cells can be hundreds of times larger than bacterial cells (for example, 200-300 μm^3 for brewer's yeast vs 1 μm^3 for *Pseudomonas*) and therefore exert a greater effect than their lower CFU levels may suggest.

Using 16S rRNA clone libraries, van der Maarel *et al.* (1998) demonstrated that the digesta of grey mullet (*Mugil cephalus*) and flounder (*Platichthys flesus*) was dominated by group II marine Archaea. Furthermore, similar clones were also identified in water samples from two stations in the North Sea. A number of 16S rRNA sequences with 97.6-99.5% similarity to *Methanococcoides methylutens* (marine methanogenic Archaea) were also found in the intestine of flounder (van der Maarel *et al*. 1999). Conversely, Fidopiastis *et al.* (2006) and Smriga *et al*. (2010) reported negative PCR amplification when using Archaea specific primers in fish faeces.

It has been proposed that bacteriophages likely exert a strong selection pressure on the diversity and population structure of bacterial communities within the intestine (Breitbart *et al*. 2003) and these mechanisms could be exploited in order to reduce infectious diseases in aquaculture (Nakai and Park 2002). Whether bacteriophages are present in the intestines of healthy fish remain to be investigated fully but they have been identified from diseased ayu

(*Plecoglossus altivelis*) as well as fish commonly found in Mexico and Chile (Park *et al.* 2000; Bastías *et al*. 2010).

Future work should focus on identifying and quantifying populations of Archaea, bacteriophage and yeast in tilapia.

1.3.2 Role of GI microbiota in nutrition

The GI microbiota is involved in a number of nutritional functions including digestion, nutrient utilisation and the production of amino acids, enzymes, short-chain fatty acids (SCFA's), vitamins and minerals (Nayak 2010b). Smriga *et al.* (2010) suggested that in coral reef fish, members of Bacteroidetes, Firmicutes, Fusobacteria and Proteobacteria may contribute to digestion by producing a variety of enzymes. In a comprehensive review, Ray *et al*. (2012) described that the GI microbiota can produce an extensive range of enzymes which aid the digestive process including amylase, cellulase, lipase, protease, chitinases and phytase. Possible contributors to enzyme production include *Bacillus*, Enterobacteriaceae, *Acinetobacter*, *Aeromonas*, *Flavobacterium*, *Photobacterium*, *Pseudomonas*, *Vibrio*, *Microbacterium*, *Micrococcus*, *Staphylococcus*, other unidentified anaerobes and yeasts. However, as the authors highlight, in contrast to endothermic animals, it is difficult to conclude the exact contribution of the GI microbiota due to the complexity and variable ecology of the digestive tract of different fish species, the presence of a stomach or pyloric caeca and the relative length of the intestine. Mukherjee and colleagues (2014) isolated *B. subtilis*, *Bacillus methylotrophicus* and *Enterobacter hormaechei* from the intestinal tract of Indian major carp, *Catla catla*, demonstrating their enzymatic contribution by the production of amylase, protease, lipase, cellulase, phytase and xylanase.

Roeselers *et al*. (2011) used a pyrosequencing approach to identify Fusobacteria in the zebrafish intestine. The majority of these Fusobacteria sequences were closely aligned to *Ct. somerae* which has been isolated in many different fish species including zebrafish, rainbow trout, *Oncorhynchus mykiss*, common carp, *Cyprinus carpio*, goldfish, *Carassius auratus*, ayu, channel catfish, *Ictalurus punctatus*, largemouth bass, *Micropterus salmoides*, bluegill, *Lepomis macrochirus*, and tilapia (Kim *et al*. 2007; Tsuchiya *et al*. 2008; Larsen *et al*. 2014) and is known to produce large quantities of vitamin B_{12} (cobalamin). Consequently certain freshwater fish, including tilapia, where *Ct. somerae* is routinely found as an indigenous GI constituent, have no dietary requirement for this particular vitamin (Sugita *et al*. 1991; Tsuchiya *et al*. 2008; NRC 2011).

The microbiota of fish, particularly those of a herbivorous and omnivorous nature, use fermentation to convert carbohydrates into simpler compounds, such as SCFA's. The SCFA's produced are rapidly absorbed by the host and used for energy generation and biosynthesis (Stevens and Hume 1995). The importance of SCFA's to overall energy supply and metabolism has not yet been quantified but it is likely to be substantial in fish with a high fibre diet. Mountfort *et al.* (2002) estimated the rates of SCFA production in the hindgut of three species of temperate marine fish (*Odax pullus*, *Aplodactylus arctidens* and *Kyphosus sydneyanus*) concluding that acetate levels were highest followed by propionate and butyrate. The SCFA turnover rates were similar to those in the intestinal tracts of herbivorous reptiles and mammals, an interesting insight considering the ectothermic nature of fish. Clements *et al.* (2007) observed that the hindguts of these fish species were dominated by different taxonomic members of the bacterial group Clostridia. Clostridia are mostly polymer degraders, using polysaccharides and proteins as substrates producing SCFA's as fermentation products. This is consistent with the results of Mountfort *et al.* (2002). In addition to nutritional benefits, SCFA production by GI microbes has an added benefit of

creating an acidic, hostile environment preventing pathogenic invasion. Furthermore, in human studies SCFA's have been shown to inhibit cholesterol synthesis, reduce the risk of cardiovascular disease and certain cancers (Wong *et al*. 2006).

The contribution of the indigenous microbiota to nutrition is also evident at the transcriptional level. Using DNA microarrays, Rawls *et al*. (2004) demonstrated that the absence of a microbiota in GF zebrafish is associated with a compromised ability to utilise nutrients and the assumption of a metabolic state which shares features commonly associated with fasting.

1.3.3 Role of GI microbiota in immunity

To understand the function of the intestinal microbiota in the context of both barrier function and immunity, the fish immune system as a whole and how an immune response is provoked must be considered. Due to the antigenic nature of the aquatic environment, mucosal tissues (such as the intestine) are under constant threat and thus mucosal immunity is of utmost importance to the host. The GALT orchestrates a network of immunological and non-immunological defences which must provide protection against pathogens, whilst at the same time tolerating commensal organisms.

The immune system can be divided into two main branches, the innate and adaptive immune response. The innate immune system lacks the ability to acquire memory and specific recognition when confronted with foreign agents; this means however that the innate immune system has developed to be non-specific and is able to antagonise a wide range of pathogenic insults. Innate immunity in fish contributes to a larger proportion of overall immunity in fish when compared their mammalian counterparts (Ellis 2001; Whyte 2007).

This is due to the poikilothermic nature of fish; adaptive immune defences take considerable time to respond (antibody production in salmonids can take between 4-6 weeks, even at optimum temperatures, compared to just hours or days for the innate response) (Ellis 2001). The immediate lines of defence consist of a physical barrier of commensal bacteria and mucus. Indigenous microbes will compete with pathogens for adhesion sites on epithelial cells, energy and nutrients. Furthermore they will antagonise potential pathogens directly through the production of inhibitory compounds, for example bacteriocins. Mucus is vital to the defensive barrier and is produced at all mucosal surfaces including the intestinal tract where it is synthesised by goblet cells. Mucus functions to trap and remove pathogens, preventing their attachment to the epithelia and is consequently in a permanent state of translocation. In addition to mucin components (mucopolysaccharides) and glycoproteins, mucus also contains a number of secretory factors with a wide range of functions including pathogen antagonism (Ellis 2001; Whyte 2007). Commensal organisms can affect the mucus layer via alterations in mucin gene expression as well as mucus composition by modulating the local release of bioactive compounds (Deplancke and Gaskins 2001).

GI microbiota can also modulate the immune system to benefit the host, particularly through innate mechanisms. Indigenous bacteria in the lumen express microbe associated molecular patterns (MAMP's) which are recognised by pattern recognition receptors (PRR's). One of the most documented PRR's are toll-like receptors (TLR's). These receptors activate a signalling cascade which finely tunes the epithelial translation of a number of proteins including cytokines, chemokines and defensins. Such immune signals initiate, and regulate, intestinal inflammation and chemotaxis of immune cells. Further to this, the intestinal microbiota can affect the expression of several immunity related genes. Using DNA microarray comparisons of the gene expression in the intestinal tract of GF zebrafish larvae, Rawls *et al.* (2004) demonstrated that the microbiota regulated a number of genes involved in

innate immunity, including those coding for serum amyloid A1, C-reactive protein, complement component 3, glutathione peroxidase and myeloperoxidase. These genes were all up-regulated in conventional zebrafish, when compared to GF zebrafish. Furthermore, out of the 212 genes investigated, 59 were conserved in murine models. Given the great evolutionary distance and the differences in the microbiota composition, it could be speculated that commensal microbiota in other teleost species share a similar role.

1.3.4 Role of GI microbiota in intestinal development

Gnotobiotic studies in zebrafish highlight the role microbiota play on host epithelial development, maturation and function. Bates *et al*. (2006) observed that in the absence of microbiota the gut epithelium was arrested in aspects of its differentiation, revealed by the lack of brushborder alkaline phosphatase activity, the maintenance of immature patterns of glycan expression and a reduction in the numbers of goblet and endocrine cells. Interestingly, the introduction of a complex microbiota at later stages of development was enough to reverse the GF phenotypes. Furthermore, the introduction of heat-inactivated preparations of intestinal microbiota or bacterial lipopolysaccharides (LPS) could only restore alkaline phosphatase activity indicating that the host can respond to the microbiota via at least two distinct pathways. Rawls *et al.* (2004) linked the role of the zebrafish microbiota to epithelial renewal and enterocyte morphology. GF zebrafish had reduced rates of epithelial proliferation where the enterocytes were characterised by large nuclear vacuoles filled with clear electron-lucent material compared with electron-dense eosinophilic material in conventional fish. Similar results have been observed in European sea bass where, after nine days post hatching, the intestinal epithelium consisted of cuboidal to columnar enterocytes in conventional larvae, as opposed to cuboidal to squamous in GF larvae (Rekecki *et al*. 2009).

1.4 Probiotics: history and definitions

The word probiotic originates from the Latin words *pro* (for) and *bios* (life). In modern society, probiotics are fairly commonplace in health promoting "functional foods" for humans. Yet the probiotic concept was first theorised in the first half of the twentieth century by Metchnikoff who suggested that the longevity of Bulgarian peasants was linked to their heavy consumption of fermented milk (Metchnikoff 1907). Early research focused on how probiotics could be used as a remedy to intestinal disorders in infants. The probiotic concept reached the animal production industry in the 1960's initially in poultry feeds and in the last few decades aquaculture too (Gatesoupe 1999).

The etymology of the word 'probiotic' has evolved and differs greatly depending on the source. Fuller (1989) defined probiotics as "live microbial feed supplements which beneficially affects the host animal by improving its intestinal balance". Gatesoupe (1999) broadened this definition to encompass not just the improvement of the intestinal balance, since this is often not reported, but also to include the improvement of general health. Salminen (1999) argued that a probiotic need not be alive but in fact could be "any microbial preparation or the components of microbial cells" as long as it confers a benefit to the health of the host, thus introducing concepts of viable vs non-viable cells. Verschuere *et al*. (2000) identified that fish occupy a medium where pathogens are able to maintain themselves and proliferate (i.e. the water column) and argued that a probiotic may also act to improve the "ambient microbial community" or "the quality of the ambient environment". Microbial applications which improve water quality via the breakdown or organic matter have been termed bioaugmentation or bioremediation (Moriarty 1997; Moriarty 1998). Early definitions referred to probiotics as a feed supplement but it is now clear that they can also confer benefits to the host when administered via the water (Taoka *et al*. 2006; Zhou *et al*. 2010a).

Thus a probiotic can be considered "any microbial cell provided via the diet or rearing water that benefits the host fish, fish farmer or fish consumer, which is achieved, in part at least, by improving the microbial balance of the fish" (Merrifield *et al*. 2010a). Direct benefits to the host may manifest themselves as immunostimulation, increased disease resistance, reduced stress response, improved GI morphology and microbiota composition and benefits to the farmer or consumer as improved fish appetite, growth performance, feed and energy utilisation, carcass and flesh quality, and reduced malformations (Merrifield *et al*. 2010a). As well as having some favourable characteristics which can benefit the host (e.g. pathogen antagonism, production of digestive enzymes etc.), a suitable probiotic candidate must conform to a number of essential criteria as well as others which can be considered favourable as detailed in Merrifield *et al.* (2010a). Essential requirements are:

- must not be pathogenic to the host, other aquatic species or humans
- must be free of plasmid-encoded antibiotic resistance genes
- must be resistant to bile salts and low pH

Currently, any microbial application to be used in the EU must have undergone rigorous scrutiny by the European Food Safety Authority (EFSA). This is conducted through a Qualified Presumption of Safety (QPS) which is a generic risk assessment approach applied by EFSA (Leuschner et al., 2010). *Lactobacillus* is generally considered non-pathogenic; however its presence on the QPS list is under regular review since *L. rhamnosus* has been isolated from immunocompromised patients indicating that under certain conditions it can act as an opportunistic pathogen. *Pediococcus* species are generally accepted subject to antibiotic resistance tests. Another commonly used probiotic, *Bacillus*, is on the list subject to a proven safety record and the absence of toxigenic material (i.e. *B. anthracis* and *B. cereus* produce toxins and are not on the list). Also not on the list is *Enterococcus* which have a wide

prevalence of antibiotic resistance genes and can cause disease under certain scenarios. The inclusion of this genus of probiotic is therefore assessed on a case by case basis.

1.5 Probiotic use in aquaculture

Probiotics are now widely used in aquaculture. Rico and colleagues (2013) assessed the use of veterinary medicines, feed additives and probiotics in aquaculture facilities in Asia. Out of 252 facilities investigated, 74% of Thai and 8% of Chinese tilapia farms reported using probiotics. Interestingly, this compared with 9.7% and 16% of farms which routinely use antibiotics in Thailand and China, respectively. Consequently research investigating the supplementation of probiotics in fish and shellfish has increased in recent years. This has resulted in the publication of a number of recent reviews describing probiotic use in aquaculture in a generalised sense (Nayak 2010a; Dimitroglou *et al*. 2011; Martínez Cruz *et al*. 2012; C. De *et al*. 2014; Lazado and Caipang 2014b; Newaj-Fyzul *et al*. 2014; Pérez-Sánchez *et al*. 2014), in respect to specific fish groups (Merrifield *et al*. 2010a; Welker and Lim 2011; Hoseinifar *et al*. 2014; Lazado and Caipang 2014a; van Hai 2015) and shrimp (van Hai and Fotedar 2010).

Numerous probiotics have been investigated in tilapia studies. These have been isolated from a wide range of sources including the intestines of tilapia and their rearing systems (i.e. rearing water and sediment) (Aly *et al*. 2008a; Apún-Molina *et al*. 2009; Del'Duca *et al*. 2013; Eissa and Abou-El Gheit 2014) and even tilapia gonads (Aly *et al*. 2008a; Abd El-Rhman *et al*. 2009).

One of the main groups of bacteria which have been investigated as potential probiotics are lactic acid bacteria (LAB), so named because of their ability to produce lactic

406 acid as a product of fermentative metabolism (Ringø and Gatesoupe 1998). This group are
407 generally catalase negative and contain both rods (e.g. lactobacilli and carnobacteria) and
408 cocci (e.g. streptococci and enterococci). A number of LAB have proved effective probiotics
409 in tilapia including *Lactobacillus acidophilus*, *L. plantarum*, *Lactobacillus rhamnosus*,
410 *Lactococcus lactis*, *Enterococcus faecium* and *Pediococcus acidilactici* (Pirarat *et al*. 2006;
411 Ferguson *et al*. 2010; Zhou *et al*. 2010b; Jatobá *et al*. 2011; Pirarat *et al*. 2011; Ren *et al*.
412 2013; Standen *et al*. 2013; Villamil *et al*. 2014).

413 *Bacillus* spp. have also received considerable attention as suitable probiotic
414 candidates in tilapia, most notably *B. subtilis* but also *Bacillus amyloliquefaciens*, *Bacillus*
415 *coagulans*, *Bacillus firmus*, *Bacillus pumilus*, *Bacillus thermophylus*, *Bacillus licheniformis*
416 and *Bacillus toyoi* (Aly *et al*. 2008a; Zhou *et al*. 2010a; Mohamed and Ahmed Refat 2011;
417 Ridha and Azad 2012; Nakandakare *et al*. 2013; Mehrim *et al*. 2014; Ng *et al*. 2014; Telli *et*
418 *al*. 2014). Although a few yeasts have been investigated in other fish species, *Saccharomyces*
419 *cerevisae* is the only one to be assessed in tilapia (Abdel-Tawwab *et al*. 2008; Abdel-Tawwab
420 *et al*. 2010; Abdel-Tawwab 2012).

421 Controversially, genera which contain pathogens have been also been investigated as
422 potential probiotics in tilapia; these include *Citrobacter*, *Micrococcus*, *Pseudomonas* and
423 *Streptococcus* (Aly *et al*. 2008a; Abd El-Rhman *et al*. 2009; Ayyat *et al*. 2014; Eissa and
424 Abou-El Gheit 2014).

425 Probiotic investigations in tilapia have reported a number of benefits to the host
426 including modulation of the intestinal microbiota, improved growth performance, modulation
427 of the immune response and improved disease resistance and reproductive success. These
428 will be explored further in the subsequent sections.

429

1.6 Probiotic mechanisms of action

Due to the ethical and methodological limitations of animal studies, together with complicated relationships between possible modes of action, it is difficult to understand the exact mechanisms by which probiotics function. Theoretically, a probiotic bacterium need only possess a single mode of action. However, it has been suggested that multi-species probiotics can have advantages since they may provide complimentary modes of action, thus conferring multiple benefits to the host (Timmerman *et al*. 2004; Salinas *et al*. 2008).

1.6.1 Probiotic colonisation and microbiota modulation

The continual supplementation of probiotic cells can result in the temporal colonisation of the intestinal tract and modulation of the indigenous microbiota. Surprisingly, less than 25% of probiotic studies in tilapia assess the gut microbiota, and those that do are generally interested in probiotic recovery. It is important, but not essential depending on the definition, that a probiotic survives the gastric process in order to reach the intestine where it can exert its beneficial effects. Intestinal probiotic levels are typically much lower than the original supplementation dose. For example, Bucio Galindo (2009) aimed to quantify the kinetic passage of *L. plantarum* from feed to faeces in tilapia. After feeding, at time zero, there were no *L. plantarum* in the faeces, however levels peaked between 3.6 and 5.1 hours post probiotic feeding and following this the probiotic disappeared in an exponential decay pattern until it could not be detected after three days post ingestion. When supplemented at 10^8 CFU g^{-1} approximately 6.46% of probiotic cells could be recovered, proving this strain is capable of surviving the digestive process. When supplemented at a higher dose, 10^{11} CFU g^{-1}, daily ingestion of *L. plantarum* lead to a survival fraction equal to 10^9 CFU g^{-1} of dry matter faeces, similar numerical levels to the total cultivable anaerobic flora.

Other studies have reported that probiotic bacteria may persist in the tilapia intestine after reverting to control (non-supplemented) diets. Ferguson *et al*. (2010) used a DGGE approach to demonstrate that after reverting to a basal diet, *P. acidilactici* can persist in the gut for at least 17 days. Interestingly, this probiotic also provided antagonism towards unidentified bacterium EU697160 (previously isolated from the posterior intestine of Atlantic salmon, *Salmo salar*). 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). Thus it is evident 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. Persisting within the gut may enable any probiotic benefits to continue even when probiotic feeding has ceased. For example, the application of *B. amyloliquefaciens* significantly elevated intestinal total viable counts (TVC) when compared to the control of *Lactobacillus* sp. fed groups. These levels remained higher 61 days after the cessation of probiotic feeding (Ridha and Azad 2012).

Probiotic administration can alter the indigenous intestinal composition in fish, as well as total population levels. The effect of probiotics on TVC of aerobic bacteria within the intestine of tilapia is unclear since there are conflicting results. Some authors report that probiotics increase intestinal TVC (He *et al*. 2013; Ridha and Azad 2012), some report the reduction of TVC (Jatobá *et al*. 2011) whilst others report no differences (Ferguson *et al*. 2010; Standen *et al*. 2013). A culture dependent approach has also been employed to demonstrate that probiotics can increase the intestinal abundance of specific groups of bacteria including LAB, *Bacillus* sp. and yeast (Shelby *et al*. 2006; Ferguson *et al*. 2010; He

et al. 2013; Standen *et al*. 2013; Iwashita *et al*. 2015) and decrease *Pseudomonas aeruginosa* levels (Jatobá *et al*. 2011).

DGGE has also been utilised to demonstrate the effect of probiotics on the indigenous microbiota of tilapia (He *et al*. 2013; Liu *et al*. 2013). A clustering effect, indicating similarity, was observed between the microbiota of tilapia fed a control diet when compared to tilapia fed a *P. acidilactici* supplemented diet (Ferguson *et al*. 2010). This data suggests that a potential mechanism for the modulation of the microbiota, including the reduction of potential pathogens, may be via competitive exclusion. This is discussed in the next section.

1.6.2 Competitive exclusion

As discussed previously, probiotic bacteria may be able to colonize the intestine, at least temporarily, by adhering and growing within the intestinal mucus and mucosa, reducing the available receptor sites for the attachment of pathogens and stimulating their removal from infected regions (Verschuere *et al*. 2000). Irianto & Austin (2002) suggested that this application strategy may be beneficial when probiotics are administered to juvenile or first feeding fish, or to older animals immediately after antibiotic treatment. Considering this mode of action is widely accepted, there is a scarcity of research which focuses on this mechanism. Ren *et al*. (2013) used the intestinal sac model to demonstrate the *ex vivo* interactions of probiotic, *L. plantarum*, and pathogen, *A. hydrophila*, in the intestine of tilapia. Damage which was caused by the pathogen was alleviated by the pre-treatment of *L. plantarum*. The authors attributed this in part to competition for adhesion sites along with improved host immunity.

Probiotics can also compete for nutrients. Iron is essential to carry out vital physiological and metabolic processes for all microorganisms. Although abundant in nature it is not easily available in its preferred state since Fe^{2+} will undergo rapid oxidation to Fe^{3+} and finally forms insoluble ferric oxyhydroxide which cannot be taken up by microbes (Saha *et al*. 2012). Siderophores, iron-binding agents, are extracellular substances which allow the acquisition of this limited nutrient and are a mechanism of virulence for a number of pathogens (Saha *et al*. 2012). Brunt *et al*. (2007) recovered *Bacillus* sp. and *Aeromonas sobria* from the intestine of rainbow trout and carp, respectively, and demonstrated the effectiveness as probiotics for the control of infections caused by *Aeromonas salmonicida*, *Lactococcus garvieae*, *St. iniae*, *Vibrio anguillarum*, *Vibrio ordali* and *Yersinia ruckeri*. The authors hypothesised that the reductions in mortality were at least in part due to the probiotics' ability to produce siderophores, most notably *A. sobria*. Korkea-aho *et al*. (2011) investigated the ability of *Pseudomonas* M174 to inhibit the growth of *Flavobacterium psychrophilum* in iron sufficient and iron deficient media, as well as an *in vivo* challenge trial. Siderophore production was only detected in *Pseudomonas* cultures under iron limiting conditions. Growth inhibition of *Fl. psychrophilum* was observed using cell-free supernatants from *Pseudomonas* cultures and this antagonism was enhanced when cultured under iron deficient conditions. Furthermore, the application of *Pseudomonas* M174 significantly reduced mortality due to *Fl. psychrophilum* infection. These studies suggest siderophore production may be an important attribute for a potential probiotic, in order to provide antagonism against pathogens. It is also likely that probiotics compete with pathogens for other inorganic and organic nutrients too. To the author's knowledge, no work has been carried out investigating this in tilapia however.

1.6.3 Probiotic effects on nutrient utilisation, digestion and growth

Probiotic effects on growth performance are arguably the most widely investigated. This is also true for tilapia where multiple studies report mixed results regarding growth performance (Table 1.1). Improved growth performance has been reported in tilapia with the application of *B. coagulans, B. pumilus, B. subtilis, Bifidobacterium bifidum, E. faecium, L. acidophilus, L. plantarum, L. rhamonosus, Lc lactis, Micrococcus luteus, Ps. fluorescens, Rhodopseudomonas palustris, S. cerevisae* and *Streptococcus thermophilus* (Abdel-Tawwab *et al*. 2008; Aly *et al*. 2008b; Aly *et al*. 2008c; Wang *et al*. 2008; Abd El-Rhman *et al*. 2009; Zhou *et al*. 2010a; Zhou *et al*. 2010b; Gonçalves *et al*. 2011; Jatobá *et al*. 2011; Ayyat *et al*. 2014; Eissa and Abou-El Gheit 2014; Ridha and Azad 2015). These improvements can result in considerable economic gains (El-Haroun *et al*. 2006). The mechanisms which underpin these improvements have not been fully elucidated. Ghazalah *et al.* (2010) demonstrated that the supplementation of Biogen® (containing *B. subtilis* & *B. licheniformis*) significantly increased the final weight of tilapia even when diets contained a lower proportion of protein (27.5% in probiotic diets compared to 30% in control diets). Not only does this demonstrate that probiotics can improve protein digestibility but they can have further economic implications since diets could be produced with lower protein levels.

Probiotics can also elevate GI digestive enzyme activities in fish supplemented with probiotics. For example, Essa *et al.* (2010) reported improved growth performance in tilapia. Researchers observed elevated amylase, protease and lipase activities in tilapia supplemented with *B. subtilis* and/ or *L. rhamnosus* and elevated protease activity in fish supplemented with *S. cerevisae*.

Other potential mechanisms could include improved intestinal morphology. For example, after supplementing diets with *L. rhamnosus* for 30 days, Pirarat *et al*. (2011)

549 reported longer mucosal folds within the proximal intestine in tilapia fed the probiotic, when
550 compared to those receiving the control diet. Having said this, authors didn't observe any
551 differences with regards to growth performance. Further research is needed to optimise
552 dosage and feeding duration in order to achieve improved growth performance.

553 The developmental stage of the fish can also affect the efficacy of a probiotic with
554 regards to improving growth performance. Ridha and Azad (2015) supplemented diets for
555 juvenile (approx. 28g) and adult (approx. 94g) tilapia with *B. subtilis* and *L. acidophilus*
556 singularly and in combination for 15 weeks. When administered singularly *L. acidophilus*
557 failed to affect the SGR in juvenile tilapia; however, when administered to adult fish SGR
558 was significantly lower in probiotic fed fish when compared to the control treatment. The
559 administration of *B. subtilis* and the combination with *L. rhamnosus* improved final weight,
560 SGR and feed intake in juvenile tilapia. Furthermore, the probiotic mix also improved the
561 FCR in juveniles. However, these changes were not observed in adult fish, despite the dose
562 and feed duration being constant. This could be due to the fact that smaller fish have larger
563 scope for increased growth performance, relative to their original size within a given time
564 period. Furthermore, the intestinal microbiota and probiotic colonisation dynamics differs
565 depending on the developmental stage of the host (Llewellyn *et al*. 2014). Future work should
566 consider the developmental stage of the fish as this may affect probiotic efficacy.

567 Numerous studies have reported no difference in growth performance in tilapia after
568 probiotic treatment (Shelby *et al*. 2006; Abd El-Rhman *et al*. 2009; Ali *et al*. 2010; Zhou *et al*.
569 2010a; Ridha and Azad 2012; He *et al*. 2013; Liu *et al*. 2013; Standen *et al*. 2013; Ng *et al*.
570 2014; Telli *et al*. 2014; Ridha and Azad 2015) and some have reported impaired growth
571 performance (Shelby *et al*. 2006; Abd El-Rhman *et al*. 2009; Abumourad *et al*. 2013). It
572 should be noted that growth performance similar to control treatments (i.e. no difference in

573 growth performance) is not a negative result, if the probiotic can manifest other benefits such
574 as immune modulation and disease resistance which likely requires energy and resources.

575 **Table 1.1:** Tilapia studies reporting growth performance data, based on actual values, after probiotic administration.

Potential probiont	Dosage (Duration)	Observation	Reference
Nile tilapia			
ALL-LAC™ (containing *St. faecium** & *L. acidophilus*)	0.1% (63 days)	↑ SR, FW, SGR PER; ↓ FCR	(Lara-Flores *et al*. 2003)
B. amyloliquefaciens	10^8 CFU g^{-1} (99 days)	↔ FW, WG, SGR, FCR	(Ridha and Azad 2012)
B. coagulans	10^7 CFU ml^{-1} (40 days)	↑ FW, WG, SGR; ↔ SR	(Zhou *et al*. 2010a)
B. pumilus	10^6, 10^{12} CFU g^{-1} (2 months)	↑ WG (dose and duration dependent)	(Aly *et al*. 2008c)
B. subtilis	10^6 CFU g^{-1} (84 days)	↔ FW, FL, WG, FCR, FI, SR	(Telli *et al*. 2014)
B. subtilis	10^7 CFU ml^{-1} (40 days)	↔ FW, SR, WG, SGR	(Zhou *et al*. 2010a)
B. subtilis	10^7 CFU g^{-1} (2 months)	↑ WG; ↔ SR	(Aly *et al*. 2008b)
B. subtilis	10^8 CFU g^{-1} (105 days)	↑ FW, SGR, FI; ↔ FCR (juvenile fish): ↔ FW, SGR, FI, FCR (adult fish)	(Ridha and Azad 2015)
B. subtilis	10^8 CFU g^{-1} (84 days)	↔ FW, SGR, FI, FCR	(Ridha and Azad 2015)
B. subtilis + *Aspergillus oryzae* + *S. cerevisae*	0.5, 1% (28 days)	↓ FCR, ↔ FW, WG, FI (dose dependent)	(Iwashita *et al*. 2015)
B. subtilis + *L. acidophilus*	10^8 CFU g^{-1} (105 days)	↑ FW, SGR, FI; ↓ FCR (juvenile fish): ↔ FW, SGR, FI, FCR (adult fish)	(Ridha and Azad 2015)
B. subtilis + *L. acidophilus*	10^7 CFU g^{-1} (2 months)	↑ SR; ↔ WG	(Aly *et al*. 2008b)
Bacillus sp.	10^3 CFU ml^{-1} (134 days)	↑ FW, SGR; ↔ SR, WG	(Apún-Molina *et al*. 2009)
Bacillus sp. + presumptive LAB	Bacilli (10^3 CFU ml^{-1}), LAB (10^4 CFU g^{-1}) (134 days)	↑ FW, WG, SGR; ↔ SR	(Apún-Molina *et al*. 2009)
Bactocell PA10 MD (containing *P. acidilactici*)	10^8 CFU g^{-1} (63 days)	↓ WG; ↔ SR	(Shelby *et al*. 2006)
Bi. bifidum	10^5 CFU g^{-1} (98 days)	↑ FI; ↔ FW, WG, FCR, SR	(Ayyat *et al*. 2014)
Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.5, 1.5, 2.0, 2.5% (120 days)	↑ FW, WG, SGR, PER, PPV, ER; ↓ FCR; ↔ FI (dose dependent)	(El-Haroun *et al*. 2006)

Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.3% (98 days)	↑ FW, SGR, WG; ↓ FCR; ↔ SR (dependent on stocking density)	(Mehrim 2009)
Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.2% (4 months)	↑ FW, WG	(Ghazalah *et al*. 2010)
Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.1, 0.2, 0.3% (4 months)	↔ FW, WG, SGR (dose dependent)	(Ali *et al*. 2010)
Biomate SF-20 (containing *E. faecium*)	10^6 CFU g^{-1} (39 days)	↔ WG, SR	(Shelby *et al*. 2006)
Bio-Nutra 200 (containing *B. subtilis*, *As. oryzae* & yeast)	0.1, 0.2, 0.3% (150 days)	↑ FW, WG, SGR, PPV; ↓ FCR; ↔ PER (dose dependent)	(Salem 2010)
Bioplus 2B (containing *B. Subtilis* & *B. licheniformis*)	Exp. I: 10^6 CFU g^{-1} (39 days) Exp. II: 10^8 CFU g^{-1} (63 days)	↔ WG, SR (Experiment I) ↓ WG; ↑ SR (Experiment II)	(Shelby *et al*. 2006)
BioSaf™ (containing *S. cerevisae*)	0.1% (63 days)	↑ SR, FW, SGR, PER; ↓ FCR	(Lara-Flores *et al*. 2003)
Biostim (containing *Lactobacillus sporogenes*, L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*)	10^8 CFU g^{-1} (84 days)	↑ FW, SGR; ↓ FCR; ↔ FI	(Ridha and Azad 2015)
Biostim (containing *L. sporogenes*, L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*) + *B. subtilis*	10^8 CFU g^{-1} (84 days)	↑ SGR; ↔ FW, FI, FCR	(Ridha and Azad 2015)
Biotics (containing *B. subtilis, L. acidophilus, S. cerevisae* & *As. oryzae*)	0.1, 0.2% (120 days)	↑ FW, WG, SGR, SR; ↓ FCR (dose dependent)	(Ahmed *et al*. 2014)
E. faecium	10^7 CFU ml^{-1}(40 days)	↑ FW, WG; ↔ SR	(Wang *et al*. 2008)
HydroYeast Aquaculture® (yeast ,*L. acidophilus, Bifidobacterium longum, B.*	0.5, 0.1, 0.15% (56 days)	↑ SR (males), *K*-factor (females) (dose dependent)	(Mehrim *et al*. 2014)

*thermophylus & St. faecium**)			
L. acidophilus	10^8 CFU g^{-1} (105 days)	↔ FW, SGR, FI, FCR (juvenile fish): ↔ FW, FI, FCR; ↓ SGR (adult fish)	(Ridha and Azad 2015)
L. acidophilus	10^7 CFU g^{-1} (2 months)	↑ WG; ↔ SR	(Aly *et al*. 2008b)
L. acidophilus	10^4 CFU g^{-1} (98 days)	↑ FW, WG, FI; ↔ FCR, SR	(Ayyat *et al*. 2014)
L. acidophilus + *Bi. Bifidum* + *St. thermophilus* + *S. cerevisae*	10^5 CFU g^{-1} (98 days)	↑ FW, WG, FI; ↔ FCR, SR	(Ayyat *et al*. 2014)
L. acidophilus + *Bi. bifidum* + *St. thermophilus*	10^5 CFU g^{-1} (98 days)	↑ FW, WG, FI; ↔ FCR, SR	(Ayyat *et al*. 2014)
Lactobacillus sp.	10^8 CFU g^{-1} (99 days)	↔ FW, WG, SGR, FCR	(Ridha and Azad 2012)
L. plantarum	10^6 CFU $^{-1}$ (30 days)	↑ WG; ↑ FCR; ↔ FW, FI, PER, SR	(Abumourad *et al*. 2013)
L. plantarum	10^8 CFU g^{-1} (84 days)	↑ FW, FER; ↔ SR	(Jatobá *et al*. 2011)
L. rhamnosus	10^{10} CFU g^{-1} (30 days)	↔ FW, WG, SGR, FCR	(Pirarat *et al*. 2011)
L. rhamnosus	10^{10} CFU g^{-1} (3 weeks)	↑ FW; ↔ WG, SGR, FER	(Gonçalves *et al*. 2011)
Lc. lactis	10^7 CFU ml^{-1} (40 days)	↑ FW, WG; ↔ SR	(Zhou *et al*. 2010b)
Levucell SB 20 (containing *S. cerevisae*)	10^8 CFU g^{-1} (63 days)	↓ WG; ↔ SR	(Shelby *et al*. 2006)
M. luteus	10^7 CFU g^{-1} (90 days)	↑ FW, WG; ↓ FCR; ↔ SGR, FI, PER, SR	(Abd El-Rhman *et al*. 2009)
M. luteus + *Pseudomonas* sp.	10^7 CFU g^{-1} (90 days)	↔ FW, WG, SGR, FI, FCR, PER, SR	(Abd El-Rhman *et al*. 2009)
Organic Green™ (containing *L. acidophilus, B. subtilis, Saccharomyces* & *As. oryzae*)	0.1, 0.2% (2 months)	↑ WG (dose and duration dependent)	(Aly *et al*. 2008c)
P. acidilactici	10^7 CFU g^{-1} (32 days)	↑ SR; ↔ FW, WG, SGR, FCR, PER	(Ferguson *et al*. 2010)
P. acidilactici	10^6 CFU g^{-1} (42 days)	↔ FW, WG, SGR, FCR, FI, PER, *K*-factor	(Standen *et al*. 2013)
PAS TR® (containing *B. subtilis* and *B. toyoi*)	0.4% (63 days)	↑ FW; ↓ FCR; ↔ FL, FI, WG, SR	(Nakandakare *et al*. 2013)

Presumptive LAB	10^4 CFU g^{-1} (134 days)	↑ FW, WG, SGR; ↔ SR	(Apún-Molina *et al*. 2009)
Ps. fluorescens	10^8 CFU g^{-1} (45 days)	↑ FW, WG, SGR	(Eissa and Abou-El Gheit 2014)
Pseudomonas sp.	10^7 CFU g^{-1} (90 days)	↓ FW, WG, SGR, PER, SR; ↔ FI, FCR	(Abd El-Rhman *et al*. 2009)
R. palustris	10^7 CFU ml^{-1} (40 days)	↑ FW, WG, SGR; ↔ SR	(Zhou *et al*. 2010a)
S. cerevisae	10^6 CFU g^{-1} (98 days)	↑ FW, WG, FI; ↔ FCR, SR	(Ayyat *et al*. 2014)
S. cerevisiae	0.025, 0.05, 0.1, 0.2, 0.5% (84 days)	↑ FW, WG, SGR, FI, PER; ↓ FCR; ↔ SR	(Abdel-Tawwab *et al*. 2008)
S. cerevisiae	0.05, 0.1, 0.2, 0.5% (84 days)	↑ FW, FI; ↔ SGR, FCR, SR	(Abdel-Tawwab 2012)
St. thermophilus	10^5 CFU g^{-1} (98 days)	↑ FI; ↔ FW, WG, FCR, SR	(Ayyat *et al*. 2014)
Hybrid tilapia			
B. licheniformis	0.1, 0.3% (56 days)	↔ FW, WG, SGR, FCR, FL, SR	(Ng *et al*. 2014)
B. subtilis	10^5 CFU g^{-1} (56 days)	↔ FW, WG, FCR, SR	(He *et al*. 2013)
B. subtilis	0.1, 0.3% (56 days)	↔ FW, WG, SGR, FCR, FL, SR	(Ng *et al*. 2014)
Bacillus spp. + *Pediococcus* spp.	0.1% (56 days)	↔ FW, WG, SGR, FCR, FL, SR	(Ng *et al*. 2014)
L. acidophilus	10^5, 10^7, 10^9 CFU g^{-1} (35 days)	↔ WG, FCR, SR	(Liu *et al*. 2013)
L. brevis	10^5, 10^7, 10^9 CFU g^{-1} (35 days)	↔ WG, FCR, SR	(Liu *et al*. 2013)
Galilee tilapia (*Sarotherodon galilaeus* L.)			
S. cerevisiae	1% (6 weeks)	↑ FW, WG, SGR, FI, PER; ↓ FCR; ↔ SR	(Abdel-Tawwab *et al*. 2010)

Upward facing arrows indicate increasing values whilst downward facing arrows indicate decreasing values. Horizontal arrows indicate no change. Green arrows represent improvements whilst red indicates a negative effect on growth performance.

* *St. faecium* and *L. sporogenes* have now been reclassified to *E. faecium* and *B. coagulans* respectively.

FCR = Feed conversion ratio
FER = Feed efficiency ratio
FI = Feed intake
FL = Final length
FW = Final weight
K-factor = Condition factor
PER = Protein efficiency ratio
SGR = Standard growth rate
SR = Survival rate
WG = Weight gain

1.6.4 Probiotic production of inhibitory substances

Probiotics can produce a wide range of chemical substances that have bactericidal or bacteriostatic effects on other microbial populations. These compounds include antibiotics, bacteriocins, siderophores, lysozymes, proteases, hydrogen peroxide, organic acids, ammonia and diacetyl (Verschuere *et al*. 2000). The presence of these substances is thought to aid the barrier function against opportunistic pathogens.

Agar diffusion methods, or variations of, are often used to assess the production of inhibitory substances *in vitro*. Cell free supernatants from *Bacillus* sp., *L. acidophilus* and *L. plantarum* have shown antagonism against *Vibrio* sp., *A. hydrophila* and *Ps. fluorescens* (Apún-Molina *et al*. 2009; Abumourad *et al*. 2013; Villamil *et al*. 2014). Other authors have used whole cell preparations to demonstrate the *in vitro* antagonism of *Bacillus* sp., *B. subtilis*, *B. pumilus*, *Citrobacter freundii*, *Enterococcus* sp., *L. acidophilus*, *L. brevis*, *Lc. lactis*, *M. luteus* and *Pseudomonas* sp. to pathogens *A. hydrophila*, *Ps. fluoresecns*, *Pseudomonas putida* and *St. iniae* (Aly *et al*. 2008a; Aly *et al*. 2008b; Abd El-Rhman *et al*. 2009; Zhou *et al*. 2010b; Del'Duca *et al*. 2013; Liu *et al*. 2013).

Mukherjee and colleagues (2014) screened a number of intestinal bacterial strains to investigate their ability to produce inhibitory intracellular, extracellular, whole cell and heat-killed cellular components. Out of 208 strains investigated, four showed antagonism against four or more pathogens. Intracellular, extracellular, whole cell and heat-killed cellular components of *B. subtilis* showed antagonism against *A. hydrophila*, *A. salmonicida*, *Pseudomonas* sp., *Ps. fluorescens* and *Ps. putida* but not *Aeromonas veronii*. Equally all cellular components of *B. methylotrophicus* and *En. hormaechei* provided antagonism against *A. hydrophila*, *A. salmonicida* and *Ps. fluorescens*.

1.6.5 Probiotic effects on immunity

Many studies have investigated the effect of probiotics on the immune system in tilapia, focusing on both the innate and adaptive immune response as well as the localised (i.e. intestine) and systemic (whole organism) response (Table 1.2). At the systemic level, probiotics have commonly been reported to improve serum lysozyme, alternative complement and bactericidal activities, total immunoglobulin (Ig) levels, elevate circulating leucocyte levels, modulate the proportion of leucocyte subpopulations, enhance head kidney chemiluminescence activity, respiratory burst activity and modulate cytokine gene expression in immunologically important organs (Pirarat *et al*. 2006; Taoka *et al*. 2006; Aly *et al*. 2008b; Aly *et al*. 2008c; Wang *et al*. 2008; Ali *et al*. 2010; Ferguson *et al*. 2010; Zhou *et al*. 2010a; Zhou *et al*. 2010b; Jatobá *et al*. 2011; Pirarat *et al*. 2011; Ridha and Azad 2012; Liu *et al*. 2013; Villamil *et al*. 2014; Iwashita *et al*. 2015; Ridha and Azad 2015). Other studies have demonstrated that haematological profiles can also be modified by probiotics (Taoka *et al*. 2006; Abdel-Tawwab *et al*. 2008; Aly *et al*. 2008b; Abd El-Rhman *et al*. 2009; Mehrim 2009; Ferguson *et al*. 2010; Abdel-Tawwab 2012; Eissa and Abou-El Gheit 2014; Mehrim *et al*. 2014; Ng *et al*. 2014; Telli *et al*. 2014; Iwashita *et al*. 2015).

At the localised level, *L. rhamnosus* significantly elevated the abundance of intraepithelial leucocytes (IEL's) and acidophilic granulocytes in the proximal intestine of tilapia, as well as significantly increasing populations of goblet cells in the distal intestine, when fed at 10^{10} CFU g^{-1} for 30 days (Pirarat *et al*. 2011). Larger populations of IEL's and goblet cells were also been reported in the mid intestine of tilapia supplemented with *P. acidilactici* (Standen *et al*. 2013). The improvements to the epithelial barrier function, reported by Pirarat *et al*. (2011), may be the mechanism behind the reduced intestinal damage following an *Aeromonas* challenge after tilapia were supplemented with the same probiotic, *L.*

rhamnosus (Ngamkala *et al*. 2010). Protective effects of *L. plantarum* have also been demonstrated using an *ex vivo* model (Ren *et al*. 2013). Using the intestinal sac method researchers observed that damage caused by *A. hydrophila* could be alleviated by *L. plantarum*, demonstrating its probiotic potential.

Within the intestine, probiotics can affect the gene expression of both pro- and anti-inflammatory cytokines (He *et al*. 2013; Liu *et al*. 2013; Ren *et al*. 2013; Standen *et al*. 2013). The probiotic modulation of cytokine expression is not limited to the intestine, indeed authors have reported similar results in the spleen and head kidney (Pirarat *et al*. 2011; Liu *et al*. 2013; Villamil *et al*. 2014). An up-regulation of both head kidney and spleen IL-1β and transferrin could explain reduction in mortality when tilapia were exposed to *A. hydrophila* (Villamil *et al*. 2014). The observations by Liu *et al*. (2013) reveal that these effects can be complex and can be affected by probiotic species, dose, duration of supplementation and the organ and gene of interest. These results demonstrate that probiotics can provide elevated resistance to pathogens and improve the immune-readiness of the host, were it to come into contact with a potential pathogen. Certainly, Pirarat *et al*. (2006) suggested that protection against *Ed. tarda* infection after supplementation with *L. rhamnosus* was accomplished by enhancing the alternative complement activity. Complement components facilitate chemotaxis, opsonisation and pathogen destruction (Holland and Lambris 2002) and provides an important link between the innate and adaptive immune system (Morgan *et al*. 2005). To the authors' knowledge, only two studies have investigated the adaptive immune response in tilapia through the measurement of total immunoglobulins (Ig's) (Shelby *et al*. 2006; Ridha and Azad 2012). The abundance of serum Ig's was increased after supplementing tilapia diets with *B. amyloliquefaciens* and *Lactobacillus* sp. (Ridha and Azad 2012) but no difference was observed after supplementing tilapia diets with commercial probiotics Bactocell PA10 MD, Biomate SF-20, Bioplus 2B or Levucell SB20 (Shelby *et al*. 2006).

639 **Table 1.2:** Tilapia studies reporting immunological data, based on actual values, after probiotic administration.

Probiotic	Dose (duration)	Parameters investigated	Reference
Nile tilapia			
B. amyloliquefaciens	10^8 CFU g^{-1} (99 days)	↑ SLys, SOD, TIg, HAT; ↔ SPro, WBC, RBC, HB, HT, Neut, Lymph, Mono	(Ridha and Azad 2012)
B. coagulans	10^7 CFU ml^{-1} (40 days)	↑ SPro, SGlo, SOD, CAT, RBA, MPO; ↔ SAlb, A/G, T-AOC, SLys	(Zhou *et al*. 2010a)
B. pumilus	10^6, 10^{12} CFU g^{-1} (2 months)	↑ RBA, WBC, Lymph, Mono; ↓ Neut, Eosin; ↔ HT, Baso (dose and duration dependent)	(Aly *et al*. 2008c)
B. subtilis	10^7 CFU g^{-1} (56 days)	↑ RBA, SLys, SBA; ↔ HT	(Aly *et al*. 2008b)
B. subtilis	10^6 (84 days)	↓ HT; ↑ SLys, MCHC, Throm, IP; ↔ RBC, Hg, MCV, MCH, WBC, Neut, Mono, SGlu, SCort, PA	(Telli *et al*. 2014)
B. subtilis	10^7 CFU ml^{-1} (40 days)	↑ SOD, CAT, RBA; ↔ SPro, SAlb, SGlo, A/G, T-AOC, SLys, MPO	(Zhou *et al*. 2010a)
B. subtilis	10^8 CFU g^{-1} (105 days)	↑ SOD, PA; ↔ SLys, ACA, HAT (dependent on developmental stage)	(Ridha and Azad 2015)
B. subtilis	10^8 CFU g^{-1} (84 days)	↑ SLys, ACA, PA; ↔ SOD, HAT	(Ridha and Azad 2015)
B. subtilis + *As. oryzae* + *S. cerevisae*	0.5, 1% (28 days)	↑ RBA, HT, Lymph, Mono, SGlu, SCort, SAlb; ↓ RBC; ↑↓ Throm; ↔ HB, MCV, MCHC, WBC, Neut, SPro, SGlo (dose and duration dependent)	(Iwashita *et al*. 2015)
B. subtilis + *L. acidophilus*	10^7 CFU g^{-1} (56 days)	↑ HT (dependent on duration), RBA, SLys, SBA	(Aly *et al*. 2008b)
B. subtilis + *L. acidophilus*	10^8 CFU g^{-1} (105 days)	↑ PA; ↓ SOD; ↔ SLys, ACA, HAT (dependent on developmental stage)	(Ridha and Azad 2015)

B. subtilis + *L. acidophilus, Clostridium butyricum* + *S. cerevisiae*	1% (30 days)	↑ SPro. SLys, LLys, HB, SBA; ↓ RBA; ↔ SMLys (dependent on probiotic viability and route of administration)	(Taoka *et al*. 2006)
Bactocell PA10 MD (containing *P. acidilactici*)	10^8 CFU g^{-1} (63 days)	↔ SLys, TIg, ACA	(Shelby *et al*. 2006)
Bi. bifidum	10^5 CFU g^{-1} (98 days)	↓ SAlb, AST; ↔ SPro, SGlo, ALT	(Ayyat *et al*. 2014)
Bi. bifidum + *L. acidophilus* + *St. thermophiles* + *S. cervisae*	10^5 CFU g^{-1} (98 days)	↔ SPro, SGlo, ALT, SAlb, AST	(Ayyat *et al*. 2014)
Bi. bifidum + *L. acidophilus* + *St. thermophilus*	10^5 CFU g^{-1} (98 days)	↔ SPro, SGlo, ALT, SAlb, AST	(Ayyat *et al*. 2014)
Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.1, 0.2, 0.3% (4 months)	↑ WBC, Lymph; ↓ Mono, Eosin	(Ali *et al*. 2010)
Biogen® (containing *B. subtilis* & *B. licheniformis*)	0.3% (98 days)	↑ HB, RBC, HT, WBC, SPro, SAlb, SGlo, A/G; ↔ MCV, MCH, MCHC, Throm, Lymph, Mono, Neut, Eosin (dependent on stocking density)	(Mehrim 2009)
Biomate SF-20 (containing *E. faecium*)	10^6 CFU g^{-1} (39 days)	↔ SLys, TIg	(Shelby *et al*. 2006)
Bio-Nutra 200 (containing *B. subtilis*, *As. oryzae* & yeast)	0.1, 0.2, 0.3% (150 days)	↑ SPro, SGlo; ↓ SAlb, ALT; ↔ A/G, AST (dose dependent)	(Salem 2010)
Bioplus 2B (containing *B. Subtilis* & *B. licheniformis*)	Exp. I: 10^6 CFU g^{-1} (39 days) Exp. II: 10^8 CFU g^{-1} (63 days)	↔ SLys, TIg (Experiment I): ↔ SLys, TIg, ACA (Experiment II)	(Shelby *et al*. 2006)
Biostim (containing *L. sporogenes*, L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*)	10^8 CFU g^{-1} (84 days)	↑ SLys, ACA, PA; ↔ SOD, HAT	(Ridha and Azad 2015)

Biostim (containing *L. sporogenes**, *L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*) + *B. subtilis*	10^8 CFU g^{-1} (84 days)	↑ PA; ↓ ACA; ↔ SLys, SOD, HAT	(Ridha and Azad 2015)
E. faecium	10^7 CFU ml^{-1}(40 days)	↑ ACA, SLys, MPO, RBA; ↔ SPro, SAlb, SGlo, A/G	(Wang *et al*. 2008)
HydroYeast Aquaculture® (yeast ,*L. acidophilus, Bi. longum, B. thermophylus & Streptococcus faecium**)	0.5, 0.1, 0.15% (56 days)	↑ RBC, HT, WBC, HB, Spr, SAl, SGl; ↓ A/G; ↔ MCV, MCH, MCHC, Throm (dose dependent)	(Mehrim *et al*. 2014)
L. acidophilus	10^7 CFU g^{-1} (56 days)	↑ RBA, SLys, SBA; ↔ HT	(Aly *et al*. 2008b)
L. acidophilus	10^8 CFU g^{-1} (105 days)	↑ SOD, PA; ↔ SLys, ACA, HAT (dependent on developmental stage)	(Ridha and Azad 2015)
L. acidophilus	10^4 CFU g^{-1} (98 days)	↓ SAlb; ↔ SPro, SGlo, ALT, AST	(Ayyat *et al*. 2014)
Lactobacillus sp.	10^8 CFU g^{-1} (99 days)	↑ SLys, SOD, TIg, HAT; ↔ SPro, WBC, RBC, HB, HT, Neut, Lymph, Mono	(Ridha and Azad 2012)
L. acidophilus	10^6 CFU g^{-1} (15 days)	↑ Cyt, TFE	(Villamil *et al*. 2014)
L. plantarum	10^6 CFU $^{-1}$ (30 days)	↑ SPro; ↓ LDH; ↔ SGlu, AST, ALT, Cyt	(Abumourad *et al*. 2013)
L. plantarum	10^8 CFU g^{-1} (12 weeks)	↑ Throm, WBC, Lymph; ↔ SGlu, HT, RBC, Neut, Mono	(Jatobá *et al*. 2011)
L. plantarum	10^9 CFU ml^{-1} (ex vivo)	↑ Cyt, HSP70	(Ren *et al*. 2013)
L. rhamnosus	10^{10} CFU g^{-1} (30 days)	↑ IEL, AG, GC, ACA, Cyt, SBA; ↓ IP, SLys; ↔ PA, HK-CA	(Pirarat *et al*. 2011)
L. rhamnosus	10^8, 10^{10} CFU g^{-1} (28 days)	↑ ACA; ↔ CA, SLys	(Pirarat *et al*. 2006)
Lc. lactis	10^7 CFU ml^{-1} (40 days)	↑ SPro, SGlo, RBA, SLys; ↔ SAlb, A/G	(Zhou *et al*. 2010b)
Levucell SB 20 (containing *S. cerevisae*)	10^8 CFU g^{-1} (63 days)	↔ SLys, TIg, ACA	(Shelby *et al*. 2006)

M. luteus	10^7 CFU ml^{-1} (90 days)	↔ RBC, HB, HT, SGlu, SPro, AST, ALT, LDH	(Abd El-Rhman *et al*. 2009)
M. luteus + *Pseudomonas sp.*	10^7 CFU ml^{-1} (90 days)	↓ RBC, HT, SPro, AST, ALT, LDH; ↑ SGlu; ↔ HB;	(Abd El-Rhman *et al*. 2009)
Organic Green™ (containing *L. acidophilus, B. subtilis, Saccharomyces* & *As. oryzae*)	0.1, 0.2% (2 months)	↑ RBA,; ↓ Neut, Eosin; ↔ HT, WBC, Lymph, Baso, Mono (dose and duration dependent)	(Aly *et al*. 2008c)
P. acidilactici	10^7 CFU g^{-1} (32 days)	↓ HT; ↑ WBC, SLys; ↔ Hg, RBC, MCV, MCH, MCHC, SAlb, SGlo, A/G, SPro, ACA, IEL	(Ferguson *et al*. 2010)
P. acidilactici	10^6 CFU g^{-1} (6 weeks)	↑ Cyt, IEL, Neut, Mono; ↔ HT, Hg, RBC, WBC, MCV, MCH, MCHC, SLys, GC, Lymph, Throm	(Standen *et al*. 2013)
Ps. fluorescens	10^8 CFU g^{-1} (45 days)	↑ RBC, HB, WBC, HT, Lymph, Mono, MCHC, SPro, SGlo, SGlu, AST, ALT, LDH; ↓ MCV, MCH, SAlb	(Eissa and Abou-El Gheit 2014)
Pseudomonas sp.	10^7 CFU ml^{-1} (90 days)	↓ RBC, HB, HT, SPro, AST, ALT, LDH; ↑ SGlu,	(Abd El-Rhman *et al*. 2009)
R. palustris	10^7 CFU ml^{-1} (40 days)	↑ SOD, CAT, RBA, MPO; ↔ SPro, SAlb, SGlo, A/G, T-AOC, SLys	(Zhou *et al*. 2010a)
S. cerevisae	10^6 CFU g^{-1} (98 days)	↑ AST; ↓ SAlb; ↔ SPro, SGlo, ALT	(Ayyat *et al*. 2014)
S. cerevisiae	0.025, 0.05, 0.1, 0.2, 0.5% (84 days)	↑ RBC, HB, Ht, SGlu, SLip, SPr, SAl, SGl (dose dependent)	(Abdel-Tawwab *et al*. 2008)
S. cerevisiae	0.05, 0.1, 0.2, 0.5% (84 days)	↑ RBC, HB, HT, SGlu, SLip, SPr, BA (dose dependent)	(Abdel-Tawwab 2012)
St. thermophilus	10^5 CFU g^{-1} (98 days)	↔ SPro, SGlo, ALT, SAlb, AST	(Ayyat *et al*. 2014)
Hybrid tilapia			
B. licheniformis	0.1, 0.3% (56 days)	↔ HT	(Ng *et al*. 2014)

B. subtilis	10^5 CFU g^{-1} (56 days)	↑ Cyt; ↓ HSP70	(He *et al*. 2013)
B. subtilis	0.1, 0.3% (56 days)	↑ HT (dose dependent)	(Ng *et al*. 2014)
Bacillus spp. + *Pediococcus* spp.	0.1% (56 days)	↔ HT	(Ng *et al*. 2014)
L. acidophilus	10^5, 10^7, 10^9 CFU g^{-1} (35 days)	↑↓ Cyt, HSP70 (dose and time dependent)	(Liu *et al*. 2013)
L. brevis	10^5, 10^7, 10^9 CFU g^{-1} (35 days)	↑↓ Cyt, HSP70 (dose and time dependent)	(Liu *et al*. 2013)
Galilee tilapia			
S. cerevisiae	1% (6 weeks)	↑ SGlu, SPr, SLip; ↔ Creat, AST, ALT	(Abdel-Tawwab *et al*. 2010)

Upward facing arrows indicate increasing values whilst downward facing arrows indicate decreasing values. Horizontal arrows indicate no change.

* *St. faecium* and *L. sporogenes* have now been reclassified to *E. faecium* and *B. coagulans* respectively.

A/G = serum albumin: globulin ratio
ACA = alternative complement activity
ALT = alanine aminotransferase
AST = aspartate aminotransferase
Baso = basophils
CAT = catalase
Creat = creatinine
Cyt = cytokine expression
Eosin = eosinophils
GC = goblet cell
HAT = haemagglutination titer
HB = Haemoglobin
HK-CA = head kidney chemiluminescence activity
HSP70 = heat shcok protein 70
HT = Haematocrit
IEL = intraepithelial leucocyte
IP = index of phagocytosis
LDH = lactate dehydrogenase
LLys = liver lysozyme
Lymph = lymphocytes
MCH = mean corpuscular haemoglobin
MCHC = mean corpuscular haemoglobin concentration
MCV = mean corpuscular volume
Mono = monocytes
MPO = myeloperoxidase
Neut = Neutrophils
PA = phagocytic activity
RBA = respiratory burst activity
RBC = red blood cells
SAlb = serum albumin
SBA = serum bactericidal activity
SCort = serum cortisol
SGlo = Serum globulin
SGlo = serum globulin
SGlu = serum glucose
SLip = serum lipid
SLys = Serum lysozyme
SMLys = skin mucus lysozyme
SOD = superoxide dismutase
SPro = serum protein
T-AOC = total antioxidant competency
TFE = transferrin expression
Throm = thrombocytes
TIg = Total immunoglobulins
WBC = white blood cells

1.6.6 Probiotic effects on disease resistance

The use of probiotics as biological control agents for disease is fairly well established in aquaculture (Newaj-Fyzul *et al*. 2014). Probiotics can provide antagonism towards pathogens, either directly through competitive mechanisms and the production of inhibitory substances or indirectly by modulating the hosts own immune system. Numerous researchers have investigated disease resistance after administering probiotics in tilapia, with many reporting positive data (Table 1.3). The majority of disease challenge investigations use an intraperitoneal (IP) injection to administer the pathogen. As Merrifield *et al.* (2010a) discussed, this route of infection by-passes one of the possible methods, probiotic competitive exclusion within the intestine, which could reduce or even prevent the infection in the first place. In effect, IP challenges demonstrate the effect of probiotics on infected fish, thus it is likely that probiotics have been undervalued when it comes to assessing disease resistance.

In respect to probiotic investigations in tilapia, most challenge trials have focused on improving disease resistance against *A. hydrophila* with some studies reporting lower cumulative mortality after the application of *B. firmus*, *B. subtilis*, *Ci. freundii*, *L. acidophilus*, *L. brevis*, *M. luteus*, *Pseudomonas* sp. and *S. cerevisae* (Villamil *et al*. 2014; Abd El-Rhman *et al*. 2009; Abdel-Tawwab 2012; Aly *et al*. 2008b). In hybrid tilapia, *L. acidophilus* failed to reduce mortalities whereas *L. brevis* was capable of reducing mortalities when fish were exposed to *A. hydrophila* (Liu *et al*. 2013). Aly *et al.* (2008a) demonstrated that whilst *B. firmus* conferred limited protection against *A. hydrophila*, *B. pumilus* and *Ci. freundii* conferred better protection as did a mixture of the three probiotics, as indicated by significantly higher survival. Other probiotic mixes have been effectively utilised to reduce mortalities in tilapia upon exposure to *A. hydrophila* (Iwashita *et al*. 2015) whilst others appear to be ineffective (Aly *et al*. 2008c; Ayyat *et al*. 2014).

Aly *et al*. (2008b) investigated the protective effects of *B. subtilis* and *L. acidophilus*, alone and in combination, against three bacterial pathogens, *A. hydrophila*, *Ps. fluorescens* and *St. iniae*. Both probiotics, when supplemented singularly, increased the relative level of protection against all three pathogens. Furthermore, when supplemented together, the probiotics provided the highest level of protection against each pathogen. *B. subtilis*, *B. licheniformis*, both administered singularly and in combination, have also been used to provide protection against *Streptococcus agalactiae* when using an immersion challenge in hybrid tilapia (Ng *et al*. 2014).

To the authors' knowledge, only two studies have investigated the effect of probiotics on resistance to edwardsiellosis, cause by the aetiological agent *Ed. tarda*. Protective effects by *L. rhamnosus* and a multi-species application consisting of *B. subtilis*, *L. acidophilus*, *Clostridium butyricum* and *S. cerevisae* have been demonstrated (Pirarat *et al*. 2006; Taoka *et al*. 2006). Additionally, Taoka *et al*. compared the administration of probiotics through the diet, or added to the water supply. Although the cumulative mortality was doubled in the water supply group when compared to the dietary route, these differences were not significant.

Disease resistance studies in tilapia have primarily focused on the antibacterial effect of probiotics. There is evidence to suggest that probiotics can be used to protect fish against non-bacterial pathogens but such information is not yet available in tilapia. In shrimp (*Litopenaeus vannamei*), it has been demonstrated that *Bacillus* sp. can confer protection against white spot syndrome virus (WSSV) (Li *et al*. 2009). The protection of rainbow trout from *Ichthyophthirius multifiliis* (Ich), an epidermal protozoan parasite, has also been successful (Pieters *et al*. 2008). However, *B. subtilis* and *Bacillus cereus* were unable to control parasitic infections by *Gyrodactylus* spp., *Apiosoma* spp., *Epistylis* sp., *Ich. multifiliis* or *Ambiphyra* spp in tilapia (Marengoni *et al*. 2015). Very little work has been conducted on

how probiotics could be used to counter fungal insults, nonetheless early evidence suggest that they may also have antifungal characteristics (Lategan and Gibson 2003; Lategan *et al*. 2004). Clearly probiotics have the capability of providing protection from bacterial pathogens. It is not unreasonable to assume therefore that by stimulating the host's immune system they can also have a protective effect against other types of pathogen such as viruses, protozoa or fungi. More research is needed to explore this in all commercially important fish species, including tilapia.

695 **Table 1.3:** Tilapia studies reporting disease resistance data using challenge trials, based on actual values, after probiotic administration.

Pathogen	Probiotic	Challenge	Observation	Reference
Nile tilapia (*O. niloticus*)				
A. hydrophila	*B. subtilis*, *As. oryzae* & *S. cerevisae*	IP injection	↓ Mortality; ↑ RLP	(Iwashita *et al*. 2015)
A. hydrophila	*L. acidophilus*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*Bi. bifidum*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*St. thermophilus*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*S. cerevisae*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*L. acidophilus*, *Bi. bifidum* & *St. thermophilus*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*L. acidophilus*, *Bi. bifidum*, *St. thermophilus* & *S. cerevisae*	IP injection	↔ Mortality, RLP	(Ayyat *et al*. 2014)
A. hydrophila	*L. acidophilus*	IP injection	↓ Mortality	(Villamil *et al*. 2014)
A. hydrophila	*L. rhamnosus*	Oral EI	↓ Intestinal damage ↔ Mortality	(Ngamkala *et al*. 2010)
A. hydrophila	*M. luteus*	IP injection	↓ Mortality; ↔ Morbidity	(Abd El-Rhman *et al*. 2009)
A. hydrophila	*Pseudomonas* sp.	IP injection	↓ Mortality; ↔ Morbidity	(Abd El-Rhman *et al*. 2009)
A. hydrophila	*Ps. fluorescens*	IP injection	↓ Mortality	(Eissa and Abou-El Gheit 2014)
A. hydrophila	*S. cerevisae*	IP injection	↓ Mortality	(Abdel-Tawwab *et al*. 2008)
A. hydrophila	*S. cerevisae*	IP injection	↓ Mortality	(Abdel-Tawwab 2012)
A. hydrophila	*B. subtilis*	IP injection	↑ RLP	(Aly *et al*. 2008b)
A. hydrophila	*L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
A. hydrophila	*B. subtilis* & *L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
A. hydrophila	*B. pumilus*	IP injection	↔ RLP	(Aly *et al*. 2008c)
A. hydrophila	Organic Green (containing *L. acidophilus, B. subtilis, Saccharomyces* & *As. oryzae*)	IP injection	↔ RLP	(Aly *et al*. 2008c)
A. hydrophila	*B. pumilus*	IP injection	↓ Mortality	(Aly *et al*. 2008a)
A. hydrophila	*B. firmus*	IP injection	↓ Mortality	(Aly *et al*. 2008a)

A. hydrophila	*Ci. freundii*	IP injection	↓ Mortality	(Aly *et al*. 2008a)
A. hydrophila	*B. pumilus, B. firmus* & *Ci. freundii*	IP injection	↓ Mortality	(Aly *et al*. 2008a)
Ed. tarda	*B. subtilis*, *L. acidophilus*, *Ch. butyricum* & *S. cerevisae*	IP injection	↓ Mortality	(Taoka *et al*. 2006)
Ed. tarda	*L. rhamnosus*	IP injection	↓ Mortality	(Pirarat *et al*. 2006)
Flavobacterium columnare	*B. subtilis*	IM injection	↓ Mortality	(Mohamed and Ahmed Refat 2011)
Proteus vulgaris	*B. subtilis*	IP injection	↓ Mortality	(Ridha and Azad 2015)
P. vulgaris	*L. acidophilus*	IP injection	↔ Mortality	(Ridha and Azad 2015)
P. vulgaris	*B. subtilis* + *L. acidophilus*	IP injection	↓ Mortality	(Ridha and Azad 2015)
P. vulgaris	Biostim (containing *L. sporogenes*, L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*)	IP injection	↓ Mortality	(Ridha and Azad 2015)
P. vulgaris	Biostim (containing *L. sporogenes*, L. acidophilus*, *B. subtilis*, *B. licheniformis* & *S. cerevisae*) + *B. subtilis*	IP injection	↓ Mortality	(Ridha and Azad 2015)
Ps. fluorescens	*L. plantarum*	IP injection	↔ Mortality	(Abumourad *et al*. 2013)
Ps. fluorescens	*B. subtilis*	IP injection	↑ RLP	(Aly *et al*. 2008b)
Ps. fluorescens	*L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
Ps. fluorescens	*B. subtilis* & *L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
St. iniae	*B. subtilis, As. oryzae* & *S. cerevisae*	IP injection	↓ Mortality; ↑ RLP	(Iwashita *et al*. 2015)
St. iniae	Biomate SF-20 (containing *E. faecium*)	IP injection	↔ Mortality	(Shelby *et al*. 2006)
St. iniae	Bioplus 2B (containing *B. Subtilis* & *B. licheniformis*)	IP injection	↔ Mortality	(Shelby *et al*. 2006)
St. iniae	Levucell SB 20 (containing *S. cerevisae*)	IP injection	↔ Mortality	(Shelby *et al*. 2006)
St. iniae	Bactocell PA10 MD (containing *P. acidilactici*)	IP injection	↔ Mortality	(Shelby *et al*. 2006)
St. iniae	*B. subtilis*	IP injection	↑ RLP	(Aly *et al*. 2008b)

St. iniae	*L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
St. iniae	*B. subtilis* & *L. acidophilus*	IP injection	↑ RLP	(Aly *et al*. 2008b)
Hybrid tilapia				
A. hydrophila	*L. acidophilus*	Immersion	↔ Mortality	(Liu *et al*. 2013)
A. hydrophila	*L. brevis*	Immersion	↓ Mortality	(Liu *et al*. 2013)
St. agalactiae	*B. subtilis*	Immersion	↓ Mortality	(Ng *et al*. 2014)
St. agalactiae	*B. licheniformis*	Immersion	↓ Mortality	(Ng *et al*. 2014)
St. agalactiae	*Bacillus* sp. & *Pediococcus* sp.	Immersion	↓ Mortality	(Ng *et al*. 2014)

Upward facing arrows indicate increasing values whilst downward facing arrows indicate decreasing values. Horizontal arrows indicate no change. Green arrows represent improvements whilst red indicates a negative effect on disease resistance.

* Now reclassified as *B. coagulans*

IP = Intraperitoneal injection

EI = Endotracheal intubation

IM = Intramuscular injection

RLP = Relative level of protection

1.6.7 Probiotic effects on reproduction

The numbers of fish which have been domesticated are increasing continuously, due to the development of aquaculture for food fish and ornamental aquaculture. One of the biggest limiting factors in the expansion of aquaculture is broodstock management and ultimately obtaining sufficient fry which satisfy both quantity and quality assurances. Only a single study has investigated how probiotics may affect the reproductive success in tilapia (Mehrim *et al*. 2014). The authors fed male and female tilapia with diets containing increasing levels (0, 5, 10 and 15 g kg^{-1}) of Hydroyeast Aquaculture®, a commercial probiotic containing *L. acidophilus*, *Bi. longhun*, *B. thermophylus*, *St. faecium* (now reclassified as *E. faecium*) and yeast together with oligosaccharides and enzymes. After eight weeks, there were increasing concentrations of testosterone and progesterone in male and female serum, respectively, with increasing dose of probiotic. The highest dose resulted in significantly higher testes weight and gonad somatic index (GSI) in male fish. Further to this, the sperm count was elevated in the highest dose when compared to the control treatment (179.33 ± 7.96 vs 91.00 ± 2.51 x 10^6 ml^{-1}, respectively). These sperm also showed significantly higher viability, lower abnormalities and lower mortalities when compared with the control treatment. Although GSI and egg number remained unchanged in female fish, the ova weight was significantly higher in the treatments receiving 5 and 10 g kg^{-1} of the probiotic, with the highest being observed in 10 g kg^{-1}. Consequently, absolute and relative fecundity were both improved in these probiotic groups, when compared to the control treatment, with the highest in tilapia receiving 10 g kg^{-1}.

Probiotics, including *B. subtilis* and *L. rhamnosus*, have also been reported to improve reproductive success in poecilid fish, zebrafish and killifish (Ghosh *et al*. 2007; Gioacchini *et al*. 2010; Gioacchini *et al*. 2011; Lombardo *et al*. 2011; Chitra and Krishnaveni 2013).

1.7 Conclusions

The literature available provides clear evidence that a wide range of probiotic applications can improve growth performance, modulate intestinal microbiota and intestinal morphology, stimulate the host's immune system and improve reproductive success. Furthermore they can improve disease resistance, rendering them an important asset in the battle to reduce the usage of antibiotics and other chemical therapies. Their efficacy depends on a number of factors including probiotic and host species, dosage, duration of feeding, mode of supplementation and environmental conditions and these may provide a basis for varied results. Future work must focus on applications of probiotics, their industrial scale up as well as determining the mechanisms involved which will enable aqua culturists to use probiotics to their maximum efficiency.

1.8 Thesis aims and objectives

The overall aim of this project was to investigate the effects of probiotics (specifically those in AquaStar® Growout) on tilapia health and growth performance, define the mechanisms behind their action and investigate how its efficacy is affected by its application regime (i.e. monospecies vs multispecies), dosage and feeding regime.

As discussed in section 1.3, the establishment of the 'normal' microbiota and the intimate relationships with the host epithelial cells effectively primes regulatory mechanisms and stimulates gastric development. However, these populations in fish are unstable and are often subject to microbial imbalance, termed 'dysbiosis'. Probiotics offer an attractive approach to fortify these intestinal microbial communities. Despite this, many studies fail to

assess the impact of probiotics on resident communities within the intestine. This is true for tilapia and consequently it will be one of the objectives of the current study.

It is also essential that the host is capable of mounting an immune response were it to come into contact with a pathogen. Therefore, determining the effects of probiotics on the localised mucosal immune response as well as the systemic immune response is key. In addition, the probiotic effect on growth performance will also be assessed.

Chapter 2. General materials and methods

The following analytical protocols were fundamental to the experimental analyses and were carried out as described unless otherwise stated. Other methods relating to specific trials (including specific dietary formulations) are described in the relevant chapters. Unless otherwise specified, all materials, chemicals and reagents were supplied by Sigma-Aldrich and Fisher Scientific Ltd. Where the work was carried out in Plymouth, all experimental work involving fish was carried out under the Home Office project license # 30/2644 and personal license # 30/9994. Approval was also given by Plymouth University ethical committee.

2.1 Aquarium facilities and water quality management

Trial's I and II were conducted in system 'A' (Fig 2.1) at the Aquatic Animal Nutrition and Health Research Aquarium, Plymouth University. The system is a closed freshwater recirculation system (RS) with a total holding capacity of ~6,000 l. The experimental set-up comprised of 18 x 150 l square fibreglass tanks, each provided with recirculated aerated water at a rate of ~450 l h^{-1}. An automated 12 h light: 12 h dark photoperiod was maintained throughout both trials. The system received a constant 30 l h^{-1} flow of groundwater to maintain losses from evaporation and reduce the potential build-up of nitrogenous wastes. All system and water parameters were monitored and maintained to meet the requirements of tilapia. Biological filtration was provided by a submerged filter bed. All nitrogenous wastes were monitored weekly using an automated discrete analyser (DR 2800, HACH) and cuvettes for ammonia, nitrite and nitrate (LCK 304, 341 and 340, respectively). The build-up of these compounds (as well as background probiotic levels) was prevented by

weekly water changes (~20% of system volume). Acceptable levels were considered to be < 0.1mg l^{-1}, < 1.0 mg l^{-1} and <50 mg l^{-1} for ammonia, nitrite and nitrate, respectively. Regular water changes also aided to minimise the build-up of background probiotic levels. Prior to water entering the biological filter, plastic brushes trapped and removed any solid waste; these were rinsed weekly to prevent the build-up of solid waste.

System pH, dissolved oxygen (DO) and temperature were monitored daily (HQ40d, HACH). Temperature was kept at 28 ± 1 °C by an inline thermostatically controlled inline heater (Elecro) and pH was maintained at 6.8 ± 1. To maintain the appropriate pH, sodium bicarbonate ($NaHCO_3$) was added to buffer the system water. DO was maintained > 6 mg l^{-1} saturation by a supply of compressed air delivered to each individual tank via air stones and perforated pipes to the sump water.

Figure 2.1: System A located at Plymouth University. Water circulates between experimental tanks (A), through mechanical (B) and biological (C) filtration and is held in the sump (D) before returning to experimental tanks.

2.2 Experimental fish

Three separate batches of tilapia (*Oreochromis niloticus*) were obtained to conduct three trials. For trial's I and II, fish were sourced from Fishgen Ltd. and for Trial III, fish were sourced from Manit Farm, Thailand. Upon arrival, water quality parameters (DO, pH, temperature and ammonia) were taken and a gradual water change took place using water from the holding system. After an hour, fish were transferred to fibreglass tanks and allowed to recover in darkness for 12 hr before light levels were slowly increased to full light intensity. Fish were fed a commercial feed with a pellet size suitable for the size of fry *ad libitum* for a minimum of six weeks of acclimation until random distribution into tanks for experimental trials. Grading (± 1.5% biomass) and provisional distribution into experimental tanks occurred 3-4 days prior to the experimental start date in order to reduce stress. Prior to fish sampling (apart from weighing) all tilapia were euthanized by overdose (300 mg l^{-1}) of tricaine methane sulfonate (MS222; Pharmaq, Fordingbridge, UK). Once fish had lost equilibrium they were concussed, followed by destruction of the brain.

2.3 Feed and weighing

For trial's I and II, iso-nitrogenous and iso-lipidic diets were formulated using Feedsoft Professional® to meet the known requirements of tilapia (NRC 2011). 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). The lyophilised probiotic powder was added, at the levels described in the respective chapters, at the expense of corn starch. Diets were dried for 24 hours in an air convection oven set to 44°C, broken up by hand to form suitably sized pellets and kept in air tight refrigerated

containers prior to use. Probiotic viability was checked using selective media (de Man, Rogosa and Sharpe (MRS) media, *Bacillus* selective agar and Slanetz and Bartley (S & B) media for *Lactobacillus*/*Pediococcus*, *Bacillus* and *Enterococcus* spp., respectively) by spread plating 10-fold serial dilutions and calculating the colony forming units (CFU) by counting statistically viable plates (i.e. 20-200 colonies). Control diets were also plated out on each medium to ensure they were void of probiotic contamination. Fresh diets were produced at the trial midpoint to ensure high probiotic viability. Specific dietary information can be found in each experimental chapter.

All fish were weighed at day zero and fed relative to percentage biomass per day in four equal rations at ~0830, 1130, 1430 and 1730. Daily feed was adjusted on a weekly basis by batch weighing following a 24 hr starvation period. Feed input was adjusted accordingly in the event of any mortality.

2.4 AOAC protocols for proximate analysis

In each trial, diets and, where indicated, fish carcasses were analysed for proximate analysis for the determination of moisture, ash, protein, lipid and gross energy according to AOAC (1995) protocols. Unless specified samples were analysed in triplicate.

2.4.1 Moisture

For the determination of moisture content, samples were weighed into metal dishes (for diet samples) or aluminium foil trays (fish carcasses) and left uncovered in a drying oven set to 105°C for 72 hours, or until a constant weight was achieved. Samples were transferred to a desiccator to cool and re-weighed. The difference in weight accounted for the loss of

moisture, calculated by ((WW – DW)/ WW)*100, where WW is wet weight (g) and DW is dry weight (g).

2.4.2 Ash

For the determination of ash (total inorganic or mineral) content, samples (~ 500mg) were weighed into pre-weighed porcelain crucibles and placed in a muffle furnace at 550°C for 8 hours until a light grey ash results, or until a constant weight was achieved. Samples were cooled in a desiccator and re-weighed. The difference in weight accounted for the loss of organic material, calculated by ((SR – CW)/ SW)*100, where SR is sample residue (including crucible weight, g), CW is crucible weight (g) and SW is the original sample weight (g)

2.4.3 Protein

For the determination of protein content, the Kjeldahl method was used. Samples (~ 200mg) were weighed in triplicate and transferred to Kjeldahl tubes. Two blank (empty) tubes were processed alongside to account for any influence the reagents may have on the procedure. Two samples of acetanilide standard were used (theoretical nitrogen content = 10.36%) which corrected for the efficiency of nitrogen extraction. Additionally, two samples of casein were used to validate nitrogen and protein content. Catalyst tablets were added to each tube and 10ml of 0.1M sulphuric acid was added. Tubes were transferred to the Kjehldahl digestion block where they were heated to 105°C for 15 min, 225°C for 60 min and 380°C for 45 min. Samples were then distilled and titrated using the Vadopest 40 automatic distillation unit (Gerhadt Laboratory Instruments, Bonn, Germany). Total nitrogen was

determined by ((ST – BT)* 0.2* 1.4007)/ SW where ST is the sample titre (ml), BT is the blank titre (ml), SW is sample weight (mg), 0.2 is the acid molarity and 1.4007 is the molecular weight of nitrogen. The efficiency was corrected for using results from acetanilide samples and crude protein was calculated by TN*6.25 where TN is total nitrogen and 6.25 is the conversion factor.

2.4.4 Lipid

For the determination of lipid content, samples (~ 3g) were weighed into extraction thimbles, plugged with cotton wool and placed into a beaker, along with anti-bumping granules. One hundred and forty millilitres of petroleum ether was added and the beakers placed on the Soxtherm unit (Gerhadt Laboratory Instruments, Bonn, Germany), heated to 150°C for 30 minutes, rinsed for 45 minutes and left to evaporate. Beakers were left in a fume hood for at least an hour until all traces of solvent had dissolved and the beaker was re-weighed. The increase in beaker weight accounted for the extracted lipid, calculated by (LW/ SW)*100 where LW is lipid weight (determined from weight increase of beaker, g) and SW is the original sample weight (g).

2.4.5 Gross energy

For the determination of gross energy, samples (~ 1g) were analysed in duplicate using a bomb calorimeter (Parr 1356, Parr Instruments Co, USA). Samples were crafted into pellets and placed inside a stainless steel container and filled with 30 bar (435 PSI) of oxygen. The sample was electronically ignited through a wired connection inside the decomposition vessel and burned. The heat created by the combustion process was transferred to the

surrounding water jacket where it was detected. This information was then converted into the energy value of the sample.

2.5 Growth performance and nutrient utilisation

Growth performance and feed utilisation were assessed by final weight (FW), net weight gain (NWG), percentage increase (%I), feed intake (FI), specific growth rate (SGR), feed conversion ratio (FCR), protein efficiency ratio (PER) and survival. Calculations were carried out using the formulae: NWG = FW - IW; %I = ((100/ IW)*FW) - 100; SGR = 100 ((ln FW - ln IW)/T); FCR = FI/WG and PER = WG/PI, where FW = final weight (g), IW = initial weight (g), T = duration of feeding (days), FI = feed intake, WG = wet weight gain (g) and PI = protein ingested.

2.6 Fish dissection

Fish were dissected under aseptic conditions. After ensuring fish were healthy by means of an external examination, the entire outside of the fish was wiped down with 70% ethanol. A single cut was made with a sterile scalpel along the underside of the fish to open the peritoneal cavity. The entire gastrointestinal tract was carefully removed, trimmed of any lipid and the mid-intestine was identified by dividing the intestine into three equal portions. Digesta was isolated from the entire mid- intestine. Where mucosa samples were needed (as appropriate) the samples were taken from the most anterior part of the mid-intestine.

2.7 Histological appraisal

2.7.1 Sample preparation and paraffin wax embedding

Fish were sampled for histological appraisal of the mid-intestine. Tissue samples (approximately 5mm) were fixed in 10% buffered formalin and transferred to 70% ethanol after 48 hours. Samples were then dehydrated in a series of graded ethanol concentrations (Leica TP1020) prior to embedding. Samples were embedded in paraffin wax (Leica EG1150 H) with two or three samples per wax block.

2.7.2 Sectioning and staining

Multiple sections (5µm thick) were cut from each wax block with a microtome (Leica), mounted onto glass slides and left to dry overnight. Sections were then cleared with histolene and rehydrated in a series of graded ethanol concentrations. Multiple sets of sections were stained with haematoxylin and eosin (H & E) or Alcian Blue-PAS (AB- PAS) and dehydrated once again. Sections were cleared in histolene and mounted with a cover slip and DPX mountant. Sections were examined and photographed using a Leica digital microimaging device (DMD108, Leica Microsystems) and analysed using Image J v1.46r (National Institute of Health, USA). Intraepithelial leucocytes (IEL's) and goblet cells were quantified over a standardised distance of 100 µm from sections stained with H & E and AB-PAS, respectively. In both cases, typically 10 folds were measured per section and averaged. To measure perimeter ratio (arbitrary units, AU), the external and inside (lumen) perimeter of each section was measured and was calculated by LP/ EP where LP = lumen perimeter (µm) and EP = external perimeter (µm) (Fig 2.2).

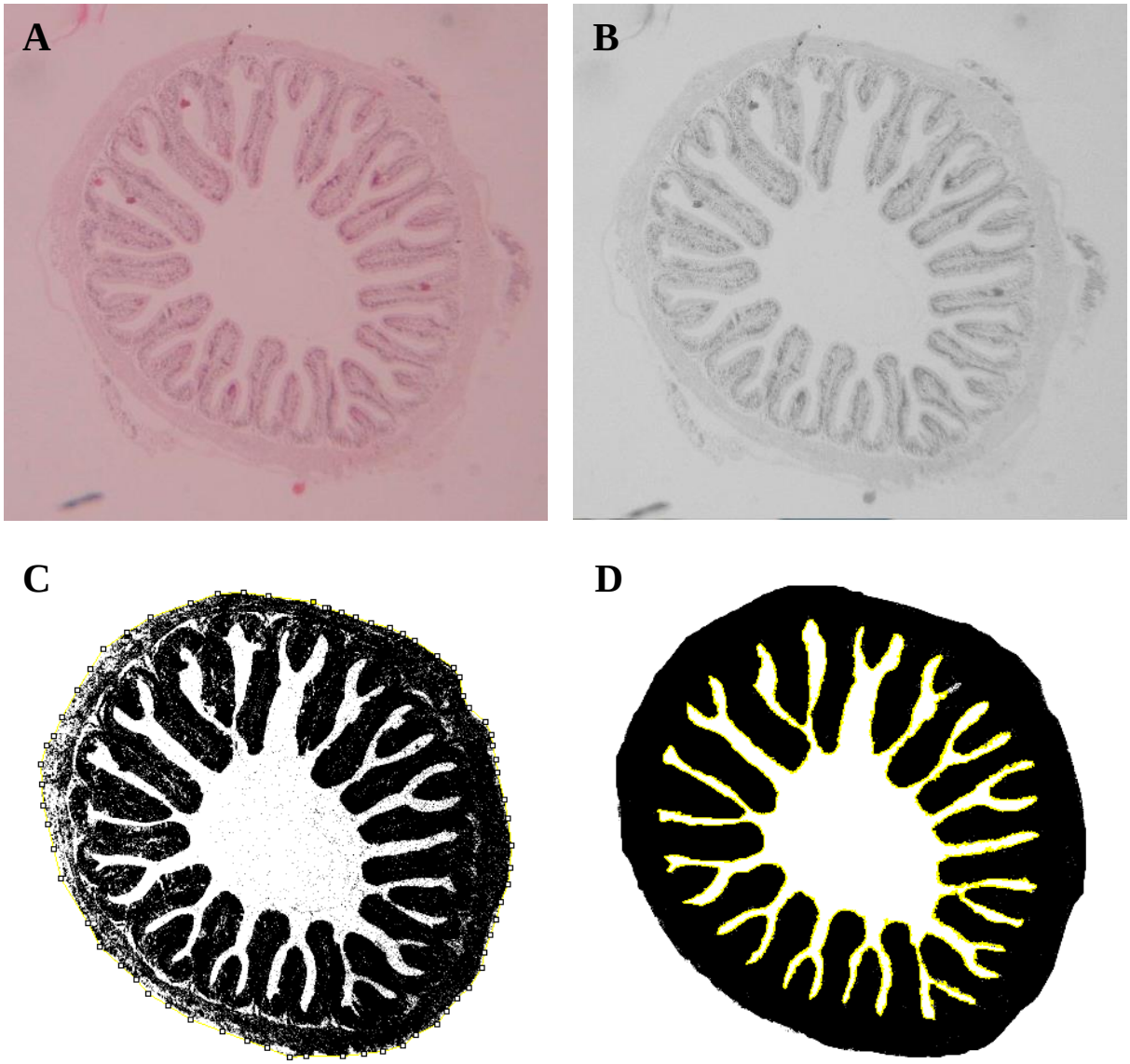


Figure 2.2: Procedure for measuring intestinal perimeter ratio. Images of transverse cross sections are loaded into Image J (A), transformed to 8-bit (B) and the threshold function applied to obtain a black and white image (C). Image was adjusted to account for sectioning artefacts (D) and both the lumen and external perimeter measured (yellow).

2.8 Microbiological analyses

2.8.1 Culture dependent analyses

Samples were serially diluted with phosphate buffered saline (PBS) and 20µl was spotted onto duplicate MRS, S&B and *Bacillus* selective agar using the Miles and Misra method (Miles *et al*. 1938) to assess bacterial populations. Tryptone soya agar (TSA) was used to determine the total aerobic heterotrophic bacterial populations. Plates were incubated for 72 hours at 28°C and CFU were calculated by counting colonies from statistically viable plates (between 3-30 colonies). Representative subsets of probiotics were identified by using 16S rRNA gene sequence analysis by using primers 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- GGT TAC CTT GTT ACG ACT T -3'). DNA was obtained from the colonies as described in section 2.8.2. The following reagents were included in each PCR tube: 1 µl primer 27F and 1491R (50 pmol μl^{-1}; Eurofins MWG, Germany), 25 µl MyTaq^{TM} Red Mix (Bioline), 20 µl DEPC treated water (Ambion) and 3 µl DNA template. Thermal cycling was conducted using a Techne TC-312 thermal cycler (Techne, Staffordshire, UK) under the following conditions: 94°C for 10 min, then 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 60 s. PCR products were checked on an agarose gel (section 2.9), cleaned (QIAquick PCR Purification Kit, Qiagen) and then sequenced by GATC-Biotech (Konstanz, Germany). The nucleotide sequence was submitted to a BLAST search to retrieve the closest known alignment identities for the partial 16S rRNA sequences.

2.8.2 DNA extraction from individual colonies

In order to obtain DNA from colonies, microLYSIS® Plus (Microzone$^{\mu}$, Sussex, UK) was used as a DNA release buffer according to manufacturer's instructions. One colony was

mixed with 20µl of microLYSIS® and placed in a thermal cycler TC-512 (Techne, Staffordshire, UK) under the following conditions: 65°C for 15 min, 96°C for 2 min, 65°C for 4 min, 96°C for 1 min, 65°C for 1 min, 96°C for 30 s and a final hold at 10°C. Extracted DNA was used as a template for immediate PCR or stored at -20°C for later use.

2.8.3 DNA extraction from intestinal samples

DNA was extracted from digesta and mucosa samples using the QIAmp® Stool Mini Kit (Qiagen) with some modifications to the manufacturer's instructions. All centrifugation steps were performed at maximum speed (17,000 *g*) in a benchtop microcentrifuge and all reagents were molecular grade. To 100-200mg of sample, 500µl lysozyme (50 mg ml^{-1} in TE buffer) was added for 30 min at 37°C to enhance the lysis of Gram positive bacteria. Samples were homogenised and 700 µl of buffer ASL was added, vortexed for 1 min and heated for 5 min at 90°C. Samples were vortexed again and centrifuged for 1 min. Eight hundred µl of the supernatant was transferred to a new microcentrifuge tube and half an Inhibitex tablet was added and immediately vortexed to remove PCR inhibitors. After leaving to stand for 1 min at room temperature samples were centrifuged for 3 min. The supernatant was transferred to a new microcentrifuge tube and then centrifuged again for a further 3 min. Three hundred and fifty µl of the supernatant was added to 15 µl of proteinase K (Qiagen) in a fresh microcentrifuge tube followed by 350 µl of buffer AL. Samples were mixed by inversion and incubated for 60 min at 56°C. Samples were cooled to room temperature before clean-up using the phenol- chloroform method. An equal amount (350 µl) of chloroform and phenol were added, mixed by inversion and centrifuged for 10 min. The supernatant (~650 µl) was mixed with an equal volume of chloroform in a new microcentrifuge tube and mixed by inversion before another 10 min centrifugation. The supernatant (~600 µl) was transferred to

a new microcentrifuge tube and 0.8 volumes (~480 µl) of ice cold isopropanol added and mixed by inversion. After 15 min standing at room temperature samples were centrifuged for 10 min. The supernatant was discarded and the pellet washed twice with 500 µl of cold 70% ethanol. The pellet was left to air dry for 5 min and suspended overnight in 30 µl of water. Samples were checked for quality and quantity of DNA with a Nanodrop 2000 (Thermo Scientific, Wilmington, USA) and the integrity was checked using a 1.5% agarose gel (described in section 2.9). Samples were either used immediately for downstream procedures or stored at -20°C until further use.

2.8.4 DGGE

PCR amplification of the 16S rRNA V3 region was conducted using the reverse primer P2 (5'- ATT ACC GCG GCT GCT GG -3') and the forward primer P3 (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAGGCAGCAG-3') (Muyzer *et al*. 1993). The following reagents were included in each PCR tube: 1 µl primer P2 and P3 (50 pmol μl^{-1}; Eurofins MWG, Germany), 15 µl MyTaq^{TM} Red Mix (Bioline), 11 µl DEPC treated water (Ambion) and 2 µl DNA template. Thermal cycling was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: 94°C for 10 min, then 35 cycles at 94°C for 30 s, 53°C for 30 s and 72°C for 60 s. DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad laboratories, Italy). Nineteen µl of PCR product from each sample was run on a 10% polyacrylamide gel (16 cm × 16 cm × 1 mm) with a denaturing gradient of 40–60% (where 100% denaturant is 7 M urea and 40% formamide). The gel was run at 65 V for 16.5 h at 60 °C in 1 × TAE buffer (66 mM Tris, 5 mM Na acetate, 1 mM EDTA). Visualization of the DGGE bands was achieved with SYBR Gold staining (Molecular Probes, Eugene, OR, USA).

The gel was scanned in a Bio-Rad universal hood II (Bio-Rad laboratories, Italy) and optimized for analyses by enhancing contrast and greyscale. Bands of interest were identified based on high peak intensities and/ or the co-migration of known probiotic bands. DNA was eluted from excised bands into 20µl of molecular grade water and stored at 4°C. This served as a template for PCR using the same primers and thermal cycling conditions minus the –GC clamp for sequence analysis. PCR products were cleaned (QIAquick PCR Purification Kit; Qiagen) and sequenced by GATC-Biotech (Konstanz, Germany). The nucleotide sequences were submitted to a BLAST search to retrieve the closest known alignment identities for the partial 16S rRNA sequences.

2.8.5 High-throughput sequencing

PCR amplification of the 16S rRNA V1-V2 region was conducted using primers 338R (5'- GCW GCC WCC CGT AGG WGT -3') and 27F (5'- AGA GTT TGA TCM TGG CTC AG -3'). The following reagents were included in each PCR tube: 0.5 µl primer 338R and 27F (50 pmol μl^{-1}; Eurofins MWG, Germany), 15 µl MyTaq™ Red Mix (Bioline), 19.5 µl DEPC treated water (Ambion) and 4 µl DNA template (diluted 1/10 in molecular grade water). Thermal cycling was conducted using a TC-512 thermal cycler (Techne, Staffordshire, UK) under the following conditions: initial denaturation at 94°C for 7 min, then 10 cycles at 94°C for 30 s, touchdown of 1°C per cycle from 62 -53°C for 30 s and 72°C for 30 s. A further 20 cycles were performed at 94°C for 30 s, 53°C for 30 s and 72°C for 30 s before a final extension for 7 min at 72°C. 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™, 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 and a 318™ chip (Life Technologies™, USA) on an Ion Torrent Personal Genome Machine (Life Technologies™, USA). Sequences were binned by sample and filtered within the PGM software to remove low quality reads. Data were then exported as FastQ files.

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 unity (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 OTU's were identified with a minimum pairwise identity of 95% and representative sequences from the OTU's were aligned using PyNAST (Caporaso *et al*. 2010a). Single 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).

2.9 Agarose gel electrophoresis

Prior to downstream procedures, for quality control, all DNA extractions and PCR products were run on a 1.5% agarose gel. RNA samples were run on a 1% agarose gel. Agarose was mixed with 1 x tris acetate- EDTA (TAE) buffer and dissolved using a microwave. After cooling the TAE/ agarose mix to 50°C, SYBR Safe DNA stain was added (1 µl per 10 ml of molten agarose gel). Unless otherwise specified gels were poured and ran in an electrophoresis tank for 40 min at 90 V. Typically 5 µl of sample and 5 µl of Hyper ladder IV (Bioline, London, UK) were used and ran alongside positive and negative controls to check the amplicon length, as well as any possible contamination.

2.10 Haemato-immunology

Blood was taken from the caudal vein using a 25 gauge needle and a 1 ml syringe. Whole blood was collected in a 1.5 ml microcentrifuge tube where it was used for downstream procedures. Whole blood was left at 4°C overnight and then centrifuged at 3, 600 *g* for 10 minutes. Serum was removed and stored at -80°C until use.

2.10.1 Haematocrit

Whole blood haematocrit levels were determined by the microhaematocrit method. Whole blood was collected in heparinised capillary tubes and subsequently centrifuged at 10, 500 *g* for 5 minutes (Centurion haematocrit centrifuge). Haematocrit was measured as percentage packed cell volume (% PCV) using a Hawksley haematocrit reader.

2.10.2 Haemoglobin

The colorimetric determination of blood haemoglobin was determined using Drabkin's cyanide- ferricyanide solution. Five µl of whole blood was mixed with 1 ml of Drabkin's reagent (1: 200). Each sample was transferred to a cuvette and measured using a spectrophotometer at 540 nm. Drabkin's solution (with no blood) was used to blank the spectrophotometer. The sample haemoglobin levels were determined from a standard curve of lyophilised haemoglobin porcine powder and calculated using the formula HC = ((OD_{540} – 0.0002)/ 6.6137) x 200 where HC = haemoglobin concentration (g dl^{-1}), OD_{540} = absorbance of sample at 540 nm, 0.0002 = y- intercept of standard curve, 6.6137 = slope of standard curve and 200 = dilution factor.

In addition to calculating the haematocrit and haemoglobin, the mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and mean corpuscular volume (MCV) was calculated according to the following formulae: MCH (pg)= (HB*10)/RBC, MCHC (g dl^{-1}) = (HB*100)/HT and MCV (fL) = (HT*10)/RBC where HB = haemoglobin concentration (g dl^{-1}), RBC = red blood cells (10^6) and HT = haematocrit (%PCV).

2.10.3 Blood cell counts

Twenty µl of whole blood was added to 980 µl of Dacie's solution (1/50 dilution; 1 ml formaldehyde, 3.13g trisodium citrate, 0.1 g brilliant cresyl blue and made up to 100 ml with distilled water) and gently mixed to disperse the blood cells. Ten µl of sample was placed on a Neubauer haemocytometer in order to enumerate erythrocytes and leucocytes.

2.10.4 Serum lysozyme activity

Serum lysozyme activity was analysed using the turbidometric method, based on the lysis on *Micrococcus lysodeikticus*. Briefly, 10 µl of serum was added to 190 µl of 0.04 M sodium phosphate buffer (pH 6.2) containing lyophilised *M. lysodeikticus* at 0.2 mg ml^{-1}. Reactions were carried out on a 96- well plate in triplicate. Columns 11 and 12 contained 200µl of sodium phosphate buffer/ *M. lysodeikticus* mix (no serum) as a background control. The reduction in absorbance at 540 nm was measured at one and six minutes in a microplate reader (VersaMax^{TM}, Molecular Devices) at 25°C. A unit of lysozyme activity was defined as a decrease in absorbance of 0.001 per minute.

2.11 Gene expression

Gene expression samples were immediately immersed in RNALater at a ratio of 1:4 and stored at -80°C until use. Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. Briefly, tissue was placed in 1 ml Trizol reagent, vortexed for 30 s and left to stand at room temperature to ensure the complete dissociation of the nucleotide complexes. Two hundred µl of chloroform was added, vortexed for 15 s and left to stand at room temperature for 10 min. Samples were then centrifuged at 12,000 *g* for 15 min.

The aqueous supernatant was removed and 500 µl of isopropanol was added, mixed by pipetting and left to stand for 10 min. Samples were then centrifuged at 14,000 *g* for 15 min. This isopropanol step was repeated twice to ensure the maximum amount of RNA was precipitated. The supernatant was removed and the pellet washed by adding 1 ml of 70% ethanol. Samples were gently mixed by pipetting and then centrifuged at 10,000 *g* for 10 minutes. This 70% ethanol step was repeated twice to ensure all impurities were removed. The ethanol was removed and pellets left to stand for 5 min before the addition of 30 µl of nuclease free water. All centrifugation steps were conducted at 4°C. RNA concentration and purity was measured spectophotometrically (NanoDrop Technologies) and RNA integrity was checked by running each sample on a 1% agarose gel (Section 2.9). Any samples with DNA contamination (as indicated by a smear) 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 $E = 10(^{-1/slope})$. Duplicate PCR reactions (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 (Bustin *et al*. 2009). 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 in order to standardise the

results by eliminating variation in mRNA and cDNA quantity and quality. Reference genes were imported into GeNorm to assess the optimal number, and choices of reference genes.

2.12 Statistical analyses

All statistical analyses were carried out using Statgraphics Centurion XVI (Warrenton, VA, USA). All data are presented as mean ± standard deviation. All data were checked for normality and equality of variance using Kolmogorov- Smirnov and Bartlett's test, respectively. Typically a one way analysis of variance (ANOVA) and *post hoc* Tukey's honest significant difference (HSD) were used for the analysis where normal assumptions were met. 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. All percentage data were transformed using arcsine function prior to statistical analysis. In all cases significance was accepted at $P < 0.05$. 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 ecological parameters (number of OTU's, species richness, evenness and diversity) were analysed using Primer V6 software (PRIMER-E Ltd, Ivybridge, UK). Margalef species richness (*d*), Pielous evenness (*J'*) and Shannon's diversity index (*H'*) were used to assess species richness, evenness and diversity respectively as determined by the following formulae: $d = (N\text{-}1)/\log(n)$, $J' = H'/\log(N)$ and $H' = -\Sigma (p_i (\ln p_i)$ where N = the total number of OTU's, n = total number of individuals (total intensity units) and p_i = the proportion of the total number of individuals in the $_i$th species. High-throughput sequencing data were uploaded to Stamp

v2.0.8 and t-test/ ANOVA with *post hoc* Tukey's HSD were used to distinguish differences at each taxonomic level.

Chapter 3. Effect of monospecies and multispecies probiotic supplementation on growth performance, intestinal microbiota and health in tilapia, *Oreochromis niloticus*

Abstract

The intestinal microbiota, intestinal morphology, health status and growth performance of tilapia (*O. niloticus*) were investigated. Tilapia (55.03 ± 0.44g) were fed either a control diet (CON) or a probiotic diet containing *B. subtilis* (BS-5), *Lactobacillus reuteri* (LR-5), *P. acidilactici* (PA-5) singularly or as a multispecies probiotic (with the addition of *E. faecium*; AQ-5) for eight weeks. Culture dependent analyses revealed higher levels of allochthonous and autochthonous lactic acid bacteria, enterococci and *Bacillus* spp. in the corresponding probiotic treatment in all cases except for LR-5. After eight weeks, the compositional dissimilarity of the microbial profiles in treatment CON was significantly different to all probiotic treatments. Equally microbial communities in AQ-5 replicates were significantly dissimilar to those of LR-5 and PA-5. High-throughput sequencing revealed that the AQ-5 treatment significantly reduced the number of operational taxonomic units and species richness when compared to CON. Significantly higher proportions of reads belonging to Proteobacteria and Cyanobacteria were detected in the CON group whereas AQ-5 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 AQ-5 fish. After four weeks significantly higher goblet cells were observed in the mid-intestine of tilapia in AQ-5 when compared with

groups BS-5 and LR-5. AQ-5 acted to recruit a larger abundance of intra-epithelial leucocytes when compared with treatments CON and LR-5. The supplementation of AQ-5 in tilapia diets also led to higher microvilli density and a higher absorptive surface area index when compared with CON. These data suggest that both mono- and multispecies probiotics can modulate the intestinal microbiota, but the multispecies probiotic showed higher efficacy when modulating the intestinal morphology and mucosal barrier function in tilapia.

3.1 Introduction

Considering that a probiotic can exert 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 (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).

It has been shown in higher vertebrates that multispecies probiotic mixes may have an advantage over monospecies probiotics (Timmerman *et al*. 2004). This is particularly true if the probiotic species have complimentary modes of action, thus providing synergism and conferring multiple benefits to the host. Evidence suggests that this may also be true for fish. After individual challenge trials with *A. hydrophila* and *Ps. fluorescens*, Aly *et al*. (2008) reported higher survival after one month in tilapia fed a dietary formulation containing *B. subtilis* and *L. acidophilus*, compared to a control diet, or diets containing the probiotic strains individually. The authors also observed that the viability of *L. acidophilus* in feed was significantly enhanced when mixed with *B. subtilis*. This suggests that diets containing more

than one probiotic may have the potential for prolonged shelf life, an important consideration for industrial scale up.

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. These studies primarily used culture based approaches to enumerate probiont levels, and to a lesser extent total cultivable communities. More recently 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 focus of this study was to assess the effects of monospecies and multispecies probiotic supplementation on the gastrointestinal (GI) microbiota of tilapia using a multidisciplinary approach, including high-throughput sequencing. In addition, the impact of the probiotic on the host intestinal morphology, growth performance and haemato-immunology were assessed.

3.2 Materials and methods

3.2.1 Experimental design and diet preparation

Six-hundred and eighty tilapia were randomly distributed to 17 150L fibreglass tanks (40 fish per tank; average weight = 55.03 ± 0.44g). Five diets were formulated and pelleted as

described in Section 2.3 (Tables 3.1 and 3.2). Experimental treatments were as follows: control (basal diet; *n* = 4), *B. subtilis* (basal diet + *B. subtilis*; *n* = 3), *L. reuteri* (basal diet + *L. reuteri*; *n* = 3), *P. acidilactici* (basal diet + *P. acidilactici*; *n* = 3) and Aquastar® Growout (basal diet + Aquastar® Growout; *n* = 4). All probiotics were supplied as a lyophilised product and added to the dry ingredients at 5 g kg^{-1} at the expense of corn starch. All diets were assessed for proximate composition as described in section 2.4 (Table 3.2). The probiotic trial was conducted for eight weeks followed by an 18 day post cessation of probiotic feed to assess probiont persistence in the intestine.

3.2.2 Growth performance and carcass composition

Prior to the start of the trial, twelve fish were pooled into four samples to assess initial proximate carcass composition as described in section 2.4. At the end of the experimental period two fish per tank were pooled into a single sample (thus *n* = 4 for CON and AQ-5 and *n* = 3 for BS-5, LR-5 and PA-5) to assess final proximate carcass composition. Growth performance and feed utilisation were assessed by means of final weight, weight gain, feed intake, protein efficiency ratio (PER), feed conversion ratio (FCR), specific growth rate (SGR), percentage increase and survival as described in section 2.5. Additionally, condition factor was assessed after four and eight weeks of experimental feeding by the formula $(100*FW)/FL^3$ where FW = final weight (g) and FL = final length (cm).

3.2.3 Intestinal microbiological analyses

At weeks four and eight, two fish per tank were euthanized, dissected and digesta and intestinal mucosa isolated as described in section 2.6.

3.2.4 Culture dependent analyses

Digesta from the mid-intestine was isolated and pooled by tank (thus $n = 4$ for CON and AQ-5 and $n = 3$ for BS-5, LR-5 and PA-5) to assess allochthonous populations. Mucosa samples were removed aseptically, washed with sterile PBS, homogenised and processed on an individual fish basis (thus $n = 8$ for CON and AQ-5 and $n = 6$ for BS-5, LR-5 and PA-5) to assess autochthonous bacterial populations. Culture dependent analyses were carried out according to section 2.8.1 to assess allochthonous and autochthonous bacterial populations. In addition to being spotted onto TSA, MRS, S+B and *Bacillus* selective agar, samples were also spotted onto *Aeromonas* selective agar to enumerate aeromonads.

Table 3.1: Codes assigned to dietary treatments.

Code	Dietary treatment
CON	Control (basal diet)
BS-5	Basal diet supplemented with *B. subtilis* at 5g kg^{-1}
LR-5	Basal diet supplemented with *L. reuteri* at 5g kg^{-1}
PA-5	Basal diet supplemented with *P. acidilactici* at 5g kg^{-1}
AQ-5	Basal diet supplemented with AquaStar® Growout at 5g kg^{-1}

Table 3.2: Dietary formulation and proximate composition (%).

	CON	BS-5	LR-5	PA-5	AQ-5
Fishmeal[a]	5.00	5.00	5.00	5.00	5.00
Soyabean meal[b]	37.26	37.26	37.26	37.26	37.26
Corn Starch[c]	24.28	23.78	23.78	23.78	23.78
Lysamine pea protein[d]	5.00	5.00	5.00	5.00	5.00
Glutalys[d]	10.00	10.00	10.00	10.00	10.00
Wheat bran[e]	10.00	10.00	10.00	10.00	10.00
Fish oil	4.50	4.50	4.50	4.50	4.50
Corn oil	2.95	2.95	2.95	2.95	2.95
Vitamin & mineral premix[f]	0.50	0.50	0.50	0.50	0.50
CMC-binder[c]	0.50	0.50	0.50	0.50	0.50
B. subtilis[g]	0.00	0.50	0.00	0.00	0.00
L. reuteri[g]	0.00	0.00	0.50	0.00	0.00
P. acidilactici[g]	0.00	0.00	0.00	0.50	0.00
AquaStar® Growout[g]	0.00	0.00	0.00	0.00	0.50
Proximate composition (% as fed basis)					
Dry matter	92.89	93.48	91.98	92.78	92.10
Protein	35.74	36.61	35.59	35.82	35.88
Lipid	10.06	9.63	9.74	9.63	9.82
Ash	4.19	4.36	4.19	4.18	4.22
Energy (MJ kg^{-1})	20.06	19.81	19.63	19.95	20.00

[a] Herring meal LT92 – United Fish Products Ltd., Aberdeen, UK.
[b] Biomar (48% protein), 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.

3.2.5 Culture independent analyses

DNA was extracted from digesta samples using the QIAamp Stool Mini Kit (Qiagen) as described in section 2.8.3. DGGE was carried out on all digesta samples (thus n = 4 for CON and AQ-5 and n = 3 for BS-5, LR-5 and PA-5) to assess allochthonous bacterial communities as described in section 2.8.4. Additionally, high-throughput sequencing was conducted on replicates from CON and AQ-5 treatments (n = 4) as described in section 2.8.5 to assess allochthonous microbial populations.

3.2.6 Persistence of the probiotics after reverting to non-supplemented diet

After reverting the AQ-5 treated fish to the control diet at the end of the trial (eight weeks), two fish per tank from treatment AQ-5 (n = 4) were sampled on days 3, 6, 9 and 18 post cessation of AquaStar® Growout feeding to assess probiotic persistence within the intestine by DGGE analysis as described in section 2.8.4. Presumptive probiotic bands were excised for sequence analysis as described in section 2.8.4.

3.2.7 Intestinal histology

At weeks four and eight, two fish per tank (thus n = 8 for CON and AQ-5 and n = 6 for BS-5, LR-5 and PA-5) were sampled for histological appraisal of the mid-intestine as described in section 2.7. In addition to the perimeter ratio, the abundances of IEL's and goblet cells, the mucosal fold length was calculated by measuring each mucosal fold within a section and calculating the average.

After eight weeks, the mid-intestines from two fish per tank from CON and AQ-5 treatments (n = 8) were sampled for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). Intestinal samples (ca. $2mm^2$) were washed in 1% S-carboxymethyl-L-cysteine for 30 s (SEM only) to remove any mucus before fixation in 2.5% glutaraldehyde in sodium cacodylate buffer (0.1M pH 7.2). Fixative was removed from SEM samples and rinsed twice with 0.1M sodium cacodylate buffer each for 15 min. Samples were then dehydrated in increasing ethanol concentrations (30%, 50%, 70%, 90%) for 15 min each followed by 100% ethanol twice for a further 15 min each. Samples were critically point dried with ethanol as the intermediate fluid and CO_2 as the transition fluid (Emitech K850, Kent, UK). Dried samples were mounted onto aluminium stubs and gold sputter coated (Emitech K550, Kent, UK). Samples were screened with a JSM 6610 LV (Jeol, Tokyo, Japan) SEM.

TEM samples were rinsed twice with sodium cacodylate buffer (0.1M pH 7.2) for 15 min each in order to remove the fixative. Samples were secondary fixed for 1 hr with osmium tetroxide (OsO_4) and then rinsed again with sodium cacodylate buffer (0.1M pH 7.2) to remove residual OsO_4. Samples were dehydrated in increasing ethanol concentrations (30%, 50%, 70%, 90%) each for 15 min and then twice in 100% ethanol for 15 min each. Over the course of 72 hr the ethanol was replaced with resin by means of decreasing the ethanol: resin ratio by submersion in 30% resin (70% ethanol) for 24 hr, 50% resin (50% ethanol) for a minimum of 5 hr, 70% resin (30% ethanol) for a minimum of 5 hr and finally 100% resin for 24 hr. Samples were placed in beem capsules and polymerisation of the resin occurred overnight at 70°C. Blocks were trimmed and semi-thin sections (0.5 μm) were cut with a glass knife and stained with methylene blue for initial examination under a light microscope. Ultrathin sections (~90 nm) were cut with a diamond knife and placed on copper grids. Sections were stained with saturated uranyl acetate solution for 30 min, rinsed with distilled

water and post-stained with Reynold lead citrate for 30 min. Stained sections were screened with a 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. SEM micrographs were taken at x 20,000 magnification. Micrographs were converted to 8-bit and then the foreground was differentiated from the background (space between microvilli) by the threshold function. The ratio of white/ black (i.e. foreground/ background) was calculated to give a microvilli density measure (arbitrary units; AU). Typically, 10 representative micrographs were analysed per sample. In order to measure microvilli length, 10 random well orientated microvilli (magnification x 20,000) were measured per micrograph, with typically 10 images analysed per sample.

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

3.2.8 Haemato-immunological analyses

Blood sampling was conducted after four and eight weeks of experimental feeding. Haematocrit, haemoglobin, MCV, MCH, MCHC, blood cell counts and lysozyme (n = 8 for CON and AQ-5 and n = 6 for BS-5, LR-5 and PA-5) was investigated as described in section 2.10.

3.2.9 Statistical analyses

Statistical analyses were carried out as described in section 2.12.

3.3 Results

3.3.1 Growth performance and carcass composition

Growth performance was assessed after eight weeks of feeding experimental diets by means of final weight, weight gain, feed intake, PER, FCR, SGR, percentage increase, condition factor and survival (Table 3.3). Tilapia in all groups showed excellent survival (> 99%) and good appetites resulting in positive growth performance. There were no significant differences in final weight, weight gain, percentage increase or feed intake between any of the treatments ($P > 0.05$). SGR's also remained unaffected ($P > 0.05$) by dietary treatment, ranging from 1.27 in BS-5 and AQ-5 to 1.35 in CON and LR-5. Furthermore there were no significant differences in feed utilisation parameters, FCR and PER ($P > 0.05$). The condition factor remained unaffected by treatments at both four and eight weeks of experimental feeding ($P > 0.05$). There were no significant differences observed in moisture, ash, lipid or protein content between dietary treatments (Table 3.4).

Table 3.3: Growth performance of tilapia after eight weeks of feeding experimental diets.

	CON	BS-5	LR-5	PA-5	AQ-5
Initial weight (g fish^{-1})	55.04 ± 1.29	54.63 ± 0.94	55.02 ± 0.78	55.83 ± 0.52	54.61 ± 0.93
Average weight (g fish^{-1})	103.93 ± 3.96	99.42 ± 0.94	104.07 ± 5.48	102.09 ± 6.76	99.43 ± 8.41
Weight gain (g fish^{-1})	48.89 ± 2.93	44.79 ± 1.88	49.05 ± 4.76	46.26 ± 6.46	44.82 ± 7.97
Feed intake (g fish^{-1})	70.13 ± 2.38	68.70 ± 0.92	70.44 ± 1.96	69.76 ± 2.16	68.10 ± 2.52
PER	0.91 ± 0.05	0.78 ± 0.06	0.90 ± 0.11	0.84 ± 0.15	0.82 ± 0.18
FCR (g g^{-1})	1.44 ± 0.04	1.58 ± 0.08	1.47 ± 0.12	1.51 ± 0.15	1.57 ± 0.25
SGR (% day^{-1})	1.35 ± 0.05	1.27 ± 0.06	1.35 ± 0.09	1.28 ± 0.13	1.27 ± 0.17
Percentage increase (%)	188.79 ± 4.17	182.06 ± 4.87	189.06 ± 7.59	182.80 ± 11.10	181.99 ± 14.04
Condition factor (week 4)	1.91 ± 0.07	1.91 ± 0.11	1.90 ± 0.13	1.93 ± 0.03	1.90 ± 0.16
Condition factor (week 8)	2.02 ± 0.10	1.99 ± 0.09	2.13 ± 0.29	1.94 ± 0.06	1.98 ± 0.20
Survival (%)	100 ± 0.00	99.17 ± 1.18	99.17 ± 1.18	100.00 ± 0.00	100.00 ± 0.00

Table 3.4: Proximate carcass composition (%) of tilapia of tilapia prior to ‘Day 0’ and after eight weeks of feeding experimental diets.

	Initial fish	CON	BS-5	LR-5	PA-5	AQ-5
Moisture	70.74 ± 1.32	66.66 ± 0.91	67.05 ± 1.34	66.24 ± 0.60	68.43 ± 1.74	65.72 ± 0.45
Ash*	14.54 ± 1.07	11.46 ± 0.84	11.88 ± 0.87	11.61 ± 0.82	12.14 ± 0.97	11.75 ± 0.43
Lipid*	28.67 ± 1.92	34.15 ± 2.59	30.42 ± 3.38	32.43 ± 2.64	29.48 ± 2.83	32.88 ± 1.52
Protein*	53.63 ± 1.81	53.09 ± 1.77	54.03 ± 3.00	52.10 ± 1.25	53.90 ± 2.49	53.38 ± 2.03

* Parameters reported as percentage of dry weight matter.

3.3.2 Culture dependent analyses

The effect of dietary probiotics on the allochthonous and autochthonous intestinal bacteria was determined using culture based methods at weeks four and eight. TVC, LAB, *Bacillus* spp., enterococci and *Aeromonas* spp. counts were enumerated on TSA, MRS, *Bacillus* spp., S & B and *Aeromonas* spp. selective media, respectively (Table 3.5). No significant differences were observed in TVC levels between the treatments at either time points, with allochthonous levels ranging between log 6-8 CFU g^{-1} and autochthonous levels slightly lower, between log 5-7 CFU g^{-1}. There were no differences between allochthonous or autochthonous *Aeromonas* levels at either time point.

After feeding experimental diets, there was significantly higher allochthonous LAB counts in treatments PA-5 and AQ-5 when compared with CON and LR-5 (week four) and also BS-5 after eight weeks. After four weeks the lowest autochthonous LAB levels were observed in the CON fed fish, these were significantly lower than all probiotic treatments. At both time points the administration of *B. subtilis* in the diets caused a significantly higher abundance of autochthonous LAB in the intestine of tilapia when compared to CON and LR-5 groups. At week four significantly higher autochthonous LAB levels were found in PA-5, but in both cases there was no significant differences between this treatment and AQ-5.

At week eight, significantly higher allochthonous *Bacillus* levels were present in AQ-5 and BS-5 fed fish when compared to CON, LR-5 and PA-5. Autochthonous *Bacillus* were not detected in CON, LR-5 or PA-5. Significantly higher levels were present in treatments BS-5 and AQ-5 when compared to the remaining three treatments, but there were no differences between these two groups.

After four weeks of feeding experimental diets, both allochthonous and autochthonous enterococci were not detected in BS-5, LR-5 or PA-5. Despite detectable levels being present in the digesta of the CON fed fish this was not significantly different between the monospecies probiotic groups due to their presence in only one out of four replicates. In both cases, there was a significantly higher abundance of enterococci in AQ-5 when compared to all other treatments. After eight weeks, enterococci were not detected in CON, PA-5 (allochthonous) and LR-5 (autochthonous). Both allochthonous and autochthonous levels were significantly higher in the AQ-5 treatment when compared to all other treatments.

Subsets of these isolates were confirmed as the probiotics administered by 16S rRNA sequence analysis and by migration to the same position as known *B. subtilis, E. faecium, L. reuteri* and *P. acidilactici* samples in a DGGE.

227 **Table 3.5:** TVC (log CFU g^{-1}) of allochthonous (D) and autochthonous (M) heterotrophic aerobic bacteria, LAB, *Bacillus* spp., enterococci, and

228 *Aeromonas* spp. in the GI tract of tilapia fed experimental diets after four and eight weeks of feeding experimental diets.

	Week	Region	CON	BS-5	LR-5	PA-5	AQ-5
TVC	4	D	5.81 ± 2.22	7.72 ± 0.24	7.50 ± 0.15	7.71 ± 0.36	8.17 ± 0.67
		M	5.85 ± 1.02	6.64 ± 0.80	7.10 ± 1.23	6.37 ± 1.40	6.30 ± 0.48
	8	D	7.36 ± 0.61	6.31 ± 1.47	5.55 ± 1.89	6.64 ± 0.49	6.93 ± 0.53
		M	4.93 ± 0.65	4.58 ± 0.66	4.40 ± 0.77	4.93 ± 0.76	4.78 ± 0.43
LAB	4	D	3.34 ± 0.37^{a}	4.20 ± 1.10^{ab}	3.01 ± 0.44^{a}	6.58 ± 1.37^{b}	5.91 ± 0.98^{b}
		M	2.80 ± 0.20^{a}	4.39 ± 0.86^{b}	3.09 ± 0.36^{c}	4.84 ± 0.89^{d}	4.64 ± 1.45^{bcd}
	8	D	3.10 ± 0.69^{a}	3.07 ± 0.52^{a}	3.22 ± 0.73^{a}	6.62 ± 0.84^{b}	6.41 ± 0.73^{b}
		M	n.d^{a}	2.78 ± 0.18^{b}	n.d^{a}	4.66 ± 0.41^{c}	4.43 ± 0.99^{c}
Bacillus spp.	4	D	*	*	*	*	*
		M	*	*	*	*	*
	8	D	2.66 ± 0.77^{a}	4.03 ± 1.55^{ab}	3.47 ± 0.32^{a}	2.51 ± 0.71^{a}	6.39 ± 0.45^{b}
		M	n.d^{a}	2.60 ± 1.33^{b}	n.d^{a}	n.d^{a}	4.79 ± 1.15^{b}
Enterococci	4	D	2.78 ± 0.13^{a}	n.d^{a}	n.d^{a}	n.d^{a}	5.09 ± 1.51^{b}
		M	n.d^{a}	n.d^{a}	n.d^{a}	n.d^{a}	5.09 ± 1.51^{b}
	8	D	n.d^{a}	2.89 ± 0.16^{b}	3.11 ± 0.59^{b}	n.d^{a}	6.28 ± 0.16^{c}
		M	n.d^{a}	2.77 ± 0.15^{b}	n.d^{a}	n.d^{a}	4.20 ± 0.92^{c}
Aeromonas spp.	4	D	4.34 ± 1.32	4.94 ± 1.16	4.45 ± 0.34	5.22 ± 1.09	4.27 ± 1.57
		M	3.03 ± 0.53	3.56 ± 0.99	3.88 ± 1.61	4.05 ± 0.87	3.67 ± 0.93
	8	D	6.89 ± 0.37	6.95 ± 0.26	6.38 ± 0.17	6.51 ± 0.48	6.07 ± 0.31
		M	4.63 ± 0.92	4.51 ± 0.67	4.22 ± 0.53	4.55 ± 0.72	3.79 ± 0.75

229 n.d. = not detected

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

231 * no data available due to overgrowth on the plates.

3.3.3 DGGE

The influence of dietary probiotics on the intestinal microbial diversity in tilapia was investigated using DGGE at weeks four and eight. At both time points, DGGE analysis revealed complex microbial communities in both treatments with samples containing 21-32 OTU's (Figs 3.1 and 3.2). Bands of interest were isolated from DGGE fingerprints at both time points and sequencing results confirmed the presence of each four probiotic species in the respective treatments (Tables 3.6 and 3.7). Table 3.8 displays the microbial ecological parameters derived from the DGGE fingerprints from fish fed experimental diets for four weeks. There were no differences in number of OTU's present, species richness and diversity or SIMPER analyses ($P > 0.05$). Furthermore, ANOSIM indicated no significant pairwise dissimilarity's between dietary treatments. Similarly, the number of OTU's, species richness and diversity and SIMPER analyses remained unaffected after eight weeks of probiotic supplementation ($P > 0.05$; Table 3.9). However, after eight weeks, ANOSIM revealed a significant dissimilarity between the intestinal microbiota from fish in CON when compared with BS-5, LR-5, PA-5 and AQ-5 (dissimilarity = 39.37, 40.64, 39.65 and 52.09 respectively; $P = 0.03$). The intestinal microbiota from AQ-5 was also significantly dissimilar to that of LR-5 and PA5 (dissimilarity = 45.83 and 52.59 respectively; $P = 0.03$) and approaching significance when compared to that of BS-5 (dissimilarity = 46.37; $P = 0.06$). There were no further differences between the other pairwise comparisons (Table 3.9).

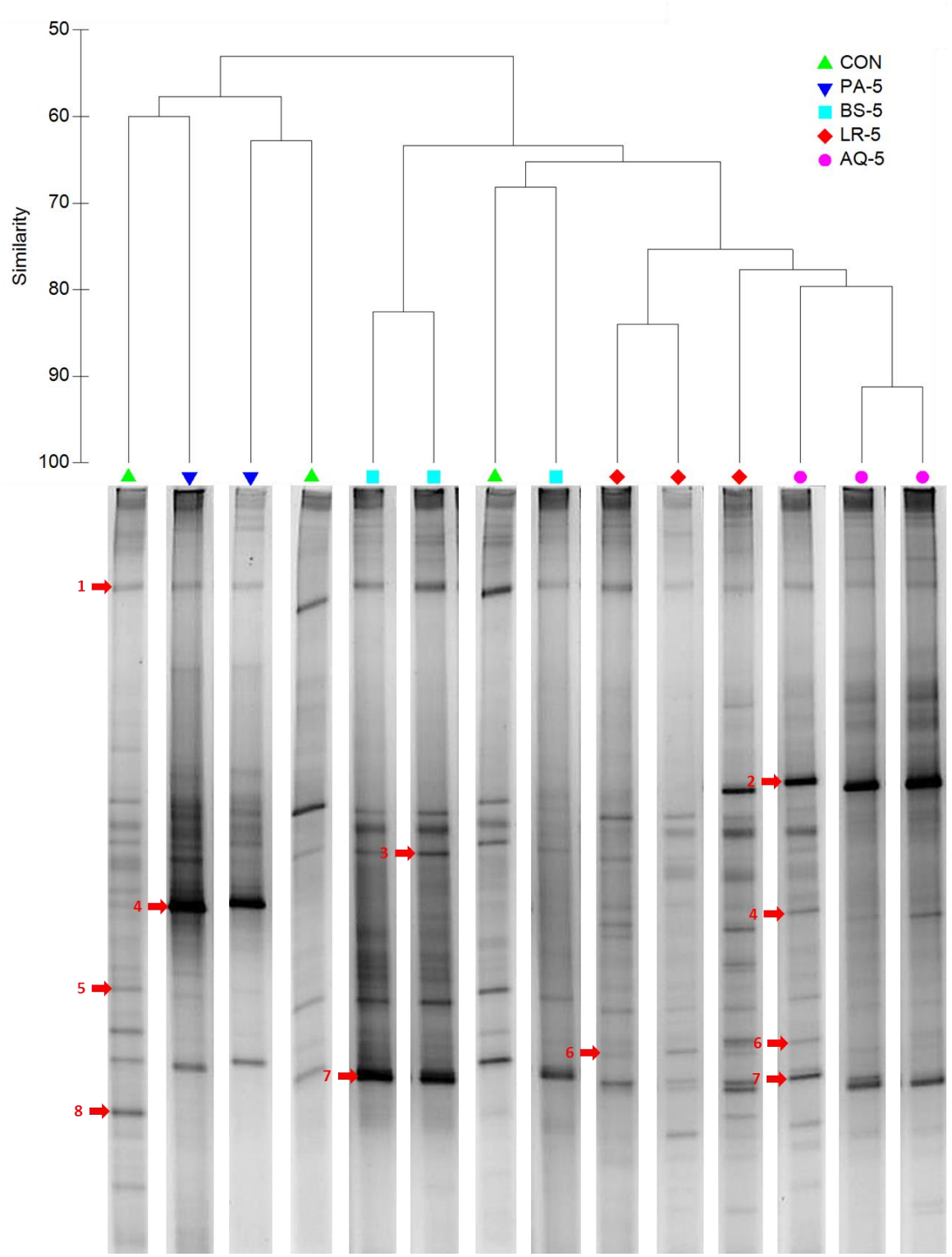


Figure 3.1: Dendrogram representing the relatedness of the microbial communities in the digesta of tilapia after four weeks of feeding with experimental diets. DGGE fingerprints below represent amplified products from the V3 region of the samples which correspond to those used in the dendrogram. Band numbers correspond to those in Table 3.6.

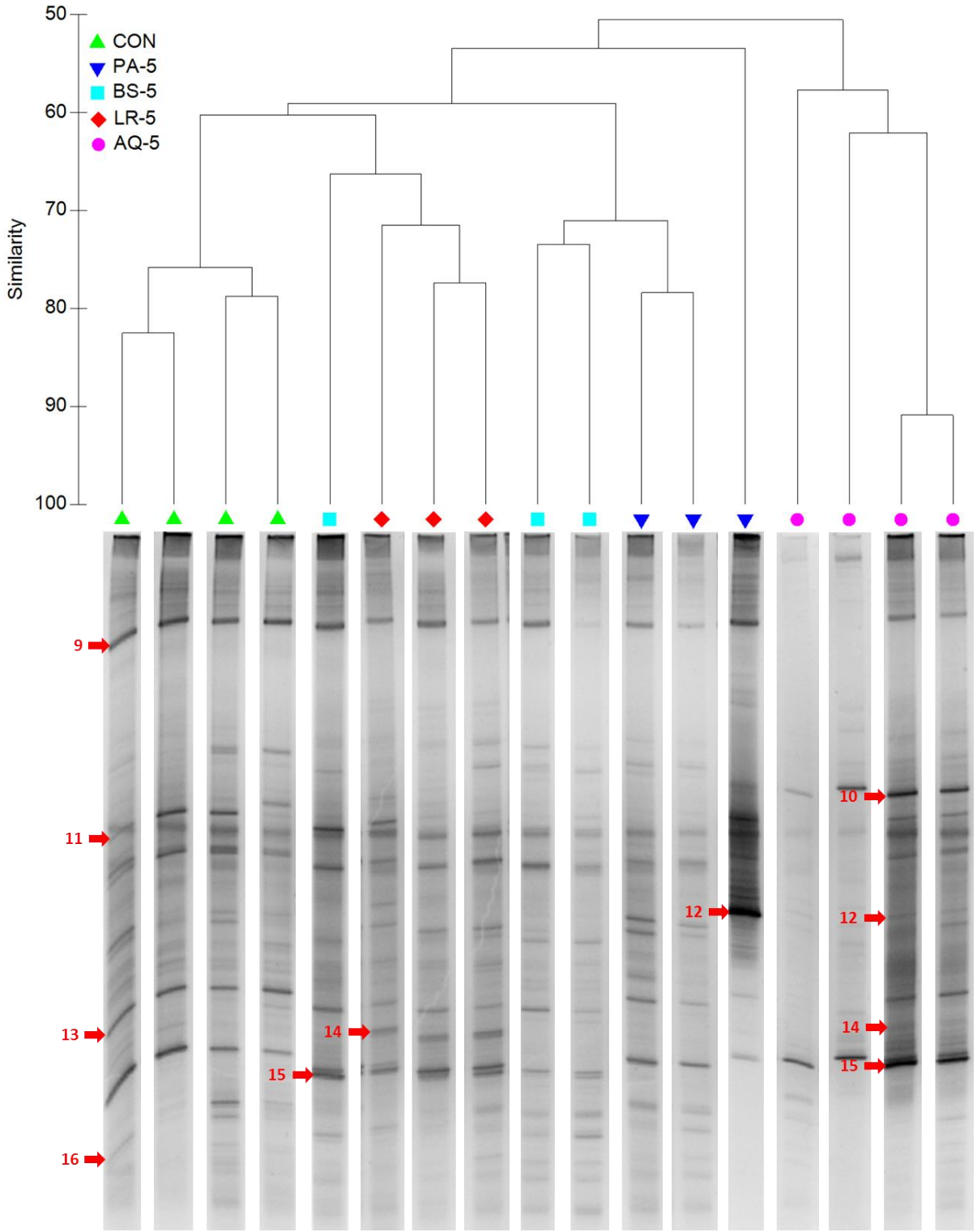


Figure 3.2: Dendrogram representing the relatedness of the microbial communities in the digesta of tilapia after eight weeks of feeding with experimental diets. DGGE fingerprints below represent amplified products from the V3 region of the samples which correspond to those used in the dendrogram. Band numbers correspond to those in Table 3.7.

264 **Table 3.6:** Excised bands from DGGE after four weeks (Fig 3.1) of feeding experimental
265 diets with their closest known alignment identities retrieved from NCBI-BLAST searches.

Band	Closest relative	Identity (%)	Treatments/ replicates present
1	*Lactobacillus aviarius*	99	CON(3/3) PA-5 (2/2) BS-5 (3/3) LR-5 (3/3) AQ-5 (3/3)
2	*Enterococcus faecium*	100	CON(2/3) PA-5 (0/2) BS-5 (1/3) LR-5 (3/3) AQ-5 (3/3)
3	*Lactobacillus sakei*	98	CON(3/3) PA-5 (2/2) BS-5 (3/3) LR-5 (3/3) AQ-5 (3/3)
4	*Pediococcus acidilactici*	92	CON(3/3) PA-5 (2/2) BS-5 (1/3) LR-5 (3/3) AQ-5 (3/3)
5	*Lactobacillus frumenti*	100	CON(3/3) PA-5 (2/2) BS-5 (3/3) LR-5 (3/3) AQ-5 (3/3)
6	*Lactobacillus reuteri*	95	CON(1/3) PA-5 (0/2) BS-5 (2/3) LR-5 (3/3) AQ-5 (1/3)
7	*Bacillus subtilis*	100	CON(3/3) PA-5 (2/2) BS-5 (3/3) LR-5 (2/3) AQ-5 (3/3)
8	*Mycobacterium* sp.	100	CON(3/3) PA-5 (0/2) BS-5 (3/3) LR-5 (3/3) AQ-5 (3/3)

266

267 **Table 3.7:** Excised bands from DGGE after eight weeks (Fig 3.2) of feeding experimental
268 diets with their closest known alignment identities retrieved from NCBI-BLAST searches.

Band	Closest relative	Identity (%)	Treatments/ replicates present
9	*Lactobacillus aviarius*	100	CON(4/4)
			PA-5 (3/3)
			BS-5 (3/3)
			LR-5 (3/3)
			AQ-5 (4/4)
10	*Enterococcus faecium*	100	CON(3/4)
			PA-5 (1/3)
			BS-5 (2/3)
			LR-5 (3/3)
			AQ-5 (4/4)
11	*Lactobacillus crispatus*	98	CON(4/4)
			PA-5 (2/3)
			BS-5 (2/3)
			LR-5 (2/3)
			AQ-5 (0/4)
12	*Pediococcus acidilactici*	99	CON(0/4)
			PA-5 (3/3)
			BS-5 (0/3)
			LR-5 (0/3)
			AQ-5 (2/4)
13	*Pseudomonas stutzeri*	93	CON(4/4)
			PA-5 (3/3)
			BS-5 (3/3)
			LR-5 (3/3)
			AQ-5 (3/4)
14	*Lactobacillus reuteri*	97	CON(3/4)
			PA-5 (1/3)
			BS-5 (1/3)
			LR-5 (3/3)
			AQ-5 (3/4)
15	*Bacillus subtilis*	100	CON(0/4)
			PA-5 (0/3)
			BS-5 (2/3)
			LR-5 (3/3)
			AQ-5 (4/4)
16	*Lactobacillus rhamnosus*	94	CON(4/4)
			PA-5 (2/3)
			BS-5 (3/3)
			LR-5 (3/3)
			AQ-5 (0/4)

Table 3.8: Microbial community analysis of the intestinal allochthonous bacterial populations of tilapia from DGGE fingerprints after four weeks of feeding experimental diets.

	Microbial ecological parameters				Similarity (ANOSIM)		
	N	Richness	Diversity	SIMPER (%)	*R*- value	*P*- value	Dissimilarity (%)
CON	24.67 ± 4.19	7.37 ± 0.92	3.19 ± 0.18	63.39 ± 1.46			
BS-5	21.33 ± 2.49	6.64 ± 0.56	3.05 ± 0.12	69.92 ± 10.40			
LR-5	26.67 ± 2.49	7.81 ± 0.54	3.28 ± 0.09	76.50 ± 5.41			
PA-5	23.50 ± 7.50	7.07 ± 1.66	3.10 ± 0.33	59.57 ± 0.00			
AQ-5	28.33 ± 0.47	8.17 ± 0.10	3.34 ± 0.02	83.52 ± 5.81			
Pairwise comparisons							
CON vs BS-5					0.65	0.10	43.42
CON vs LR-5					0.82	0.10	43.59
CON vs PA-5					0.42	0.10	41.12
CON vs AQ-5					0.69	0.10	42.08
BS-5 vs LR-5					0.63	0.10	36.54
BS-5 vs PA-5					0.83	0.10	50.21
BS-5 vs AQ-5					0.67	0.10	35.47
LR-5 vs PA-5					0.92	0.10	48.00
LR-5 vs AQ-5					0.22	0.30	23.25
PA-5 vs AQ-5					1.00	0.10	48.21

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 ($P < 0.05$).

Table 3.9: Microbial community analysis of the intestinal allochthonous bacterial populations of tilapia from DGGE fingerprints after eight weeks of feeding experimental diets.

	Microbial ecological parameters				Similarity (ANOSIM)		
	N	Richness	Diversity	SIMPER (%)	*R*- value	*P*- value	Dissimilarity (%)
CON	32.25 ± 2.38	8.99 ± 0.50	3.47 ± 0.07	77.44 ± 3.87			
BS-5	27.00 ± 3.74	7.88 ± 0.80	3.29 ± 0.14	65.79 ± 5.43			
LR-5	29.00 ± 3.27	8.31 ± 0.69	3.36 ± 0.11	73.49 ± 3.48			
PA-5	27.33 ± 3.30	7.95 ± 0.71	3.30 ± 0.12	60.27 ± 13.08			
AQ-5	29.00 ± 4.24	8.30 ± 0.90	3.36 ± 0.14	64.72 ± 12.00			
Pairwise comparisons							
CON vs BS-5					0.82	0.03	39.37
CON vs LR-5					0.95	0.03	40.64
CON vs PA-5					0.56	0.03	39.65
CON vs AQ-5					0.98	0.03	52.09
BS-5 vs LR-5					0.72	0.10	41.13
BS-5 vs PA-5					0.09	0.30	38.93
BS-5 vs AQ-5					0.65	0.06	46.37
LR-5 vs PA-5					0.48	0.10	43.26
LR-5 vs AQ-5					0.75	0.03	45.83
PA-5 vs AQ-5					0.89	0.03	52.59

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 ($P < 0.05$).

3.3.4 High-throughput sequencing

Microbial communities from the digesta of tilapia in treatments CON and AQ-5 were further investigated using high-throughput sequencing analyses. A total of 1,609,610 sequence reads were obtained from the Ion Torrent® PGM; after removing low quality reads 68,161 ± 2,701 and 38,444 ± 4,135 sequences were obtained for AQ-5 and CON groups, 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 OTU's detected in the samples are representative of the sampled population (Table 3.10).

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 3.3a). Consequently there was a significantly higher number of OTUs and species richness (Chao1) in the control group when compared to probiotic fed fish (Table 3.10). The PCoA plot demonstrates a clear separation between each treatment (Fig 3.3b) suggesting that there is clear dissimilarity between the intestine microbiota of fish fed control diets compared with fish fed the AQ-5 diet. This is supported by the UPGMA which shows clear differentiation between CON and AQ-5 replicates, with replicates clustering by treatment (Fig 3.3c). Fig 3.3d illustrates that 40 genera were present (i.e. accounting for >0.01% of the reads) in control samples which were not present in AQ-5 samples. Twenty-nine genera were common to samples in both CON and AQ-5 treatments.

299 **Table 3.10:** Number of raw reads, reads assigned to OTU's, Goods coverage and diversity/ richness indices of allochthonous intestinal
300 microbiota composition between CON and AQ-5 treatments after eight weeks of experimental feeding.

Treatment	Reads (pre trimming)	Reads assigned (post trimming)	Good's Coverage	Number of OTU's	Shannon's diversity index	Chao1 Index
CON	244,815 ± 46,578	38,444 ± 4,135	1.00 ± 0.00	129.49 ± 10.44[a]	4.04 ± 0.71	136.08 ± 10.74[a]
AQ-5	157,588 ± 8518	68,161 ± 2,701	0.99 ± 0.00	90.16 ± 10.66[b]	3.87 ± 0.07	114.29 ± 9.87[b]

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

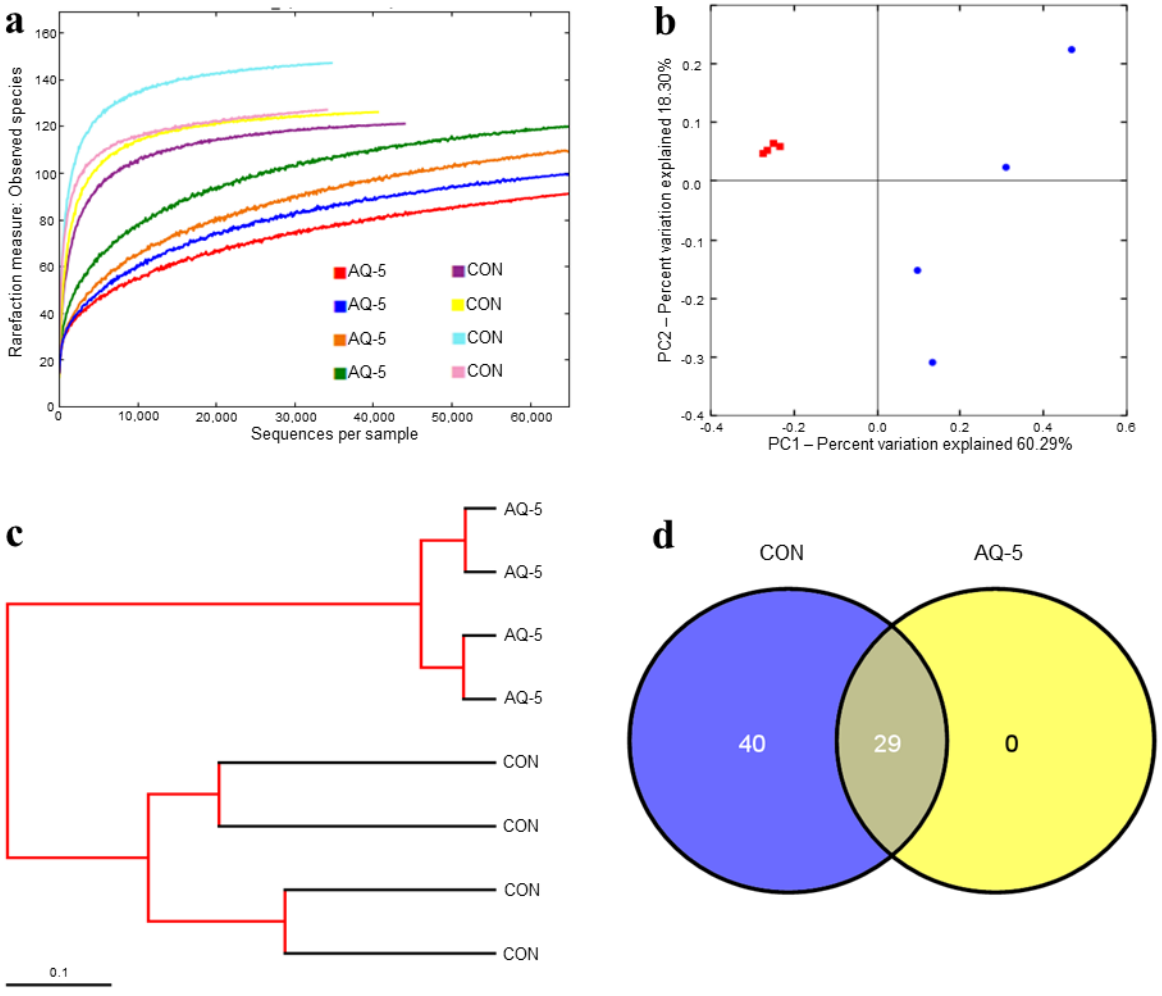


Figure 3.3: Bacterial community composition and relatedness in the digesta of tilapia fed either a CON or AQ-5 diet for eight weeks. (a) Comparison of rarefaction curves between allochthonous intestinal microbiota composition between fish fed a control diet or probiotic diet after eight weeks of experimental feeding. (b) PCoA plots where data points represent samples from fish fed a control diet (blue circles) and probiotic diet (red squares). (c) UPMGA showing hierarchical clustering of intestinal microbiota from fish 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) assigned to control replicates, probiotic replicates and genera which are common in both treatments.

Fig 3.4 shows the major bacterial constituents in the digesta of fish fed either a CON or AQ-5 based diet at the phylum and genus levels. Firmicutes accounted for > 99% of 16S reads in probiotic fed fish. Firmicutes were also the dominant phyla in the digesta of CON 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 AQ-5 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 abundance was lower and not significantly different between treatments.

The relative abundance of reads assigned to *Enterococcus* was significantly (P = < 0.001) higher in AQ-5 fed fish when compared to CON 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 CON samples when compared to the AQ-5 fed fish (P < 0.05). In the AQ-5 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. *Bacillus* (34%) also comprised a large component of the allochthonous microbiota in CON fish and low levels of *Enterococcus* (1.35%), *Lactobacillus* (1.04%) and *Pediococcus* (0.15%) sequence reads were also present. BLAST searches using single representative sequences belonging to each of these genera confirmed the presence of *P. acidilactici*, *B. subtilis* and *L. reuteri* in AQ-5 samples; however, these species were not present in the CON samples. In the CON 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 CON group; however they were found at lower levels in the AQ-5 treatment (0.02% and 0.31% respectively; $P > 0.05$). *Streptococcus* accounted for 0.48% of the reads in CON samples but was not detected in 3 out of 4 replicates of the AQ-5 treatment (present at 0.01% in the fourth replicate).

3.3.5 Persistence of probiotics after reverting to the control diet

The persistence of each probiotic in the intestine of the AQ-5 fed fish was assessed by DGGE analysis on 3, 6, 9 and 18 days post reverting to the control diet (Fig 3.5). *E. faecium* was present six 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 3.5). Sequence analysis confirmed that these OTUs were the respective probiotic species. The number of OTU's, species richness, species evenness and diversity of microbial communities were altered after reverting to the control diet; these parameters all 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 3.5).

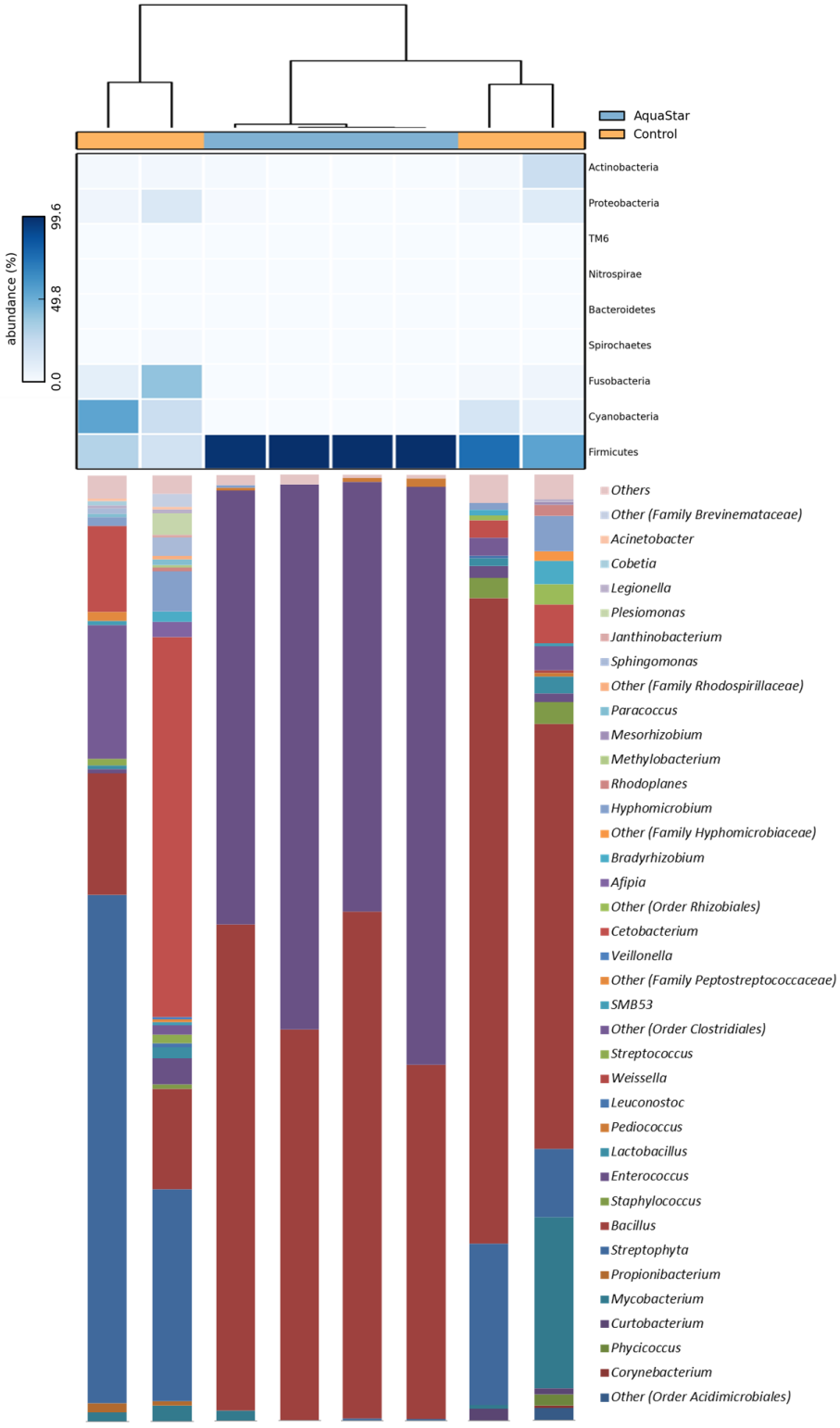
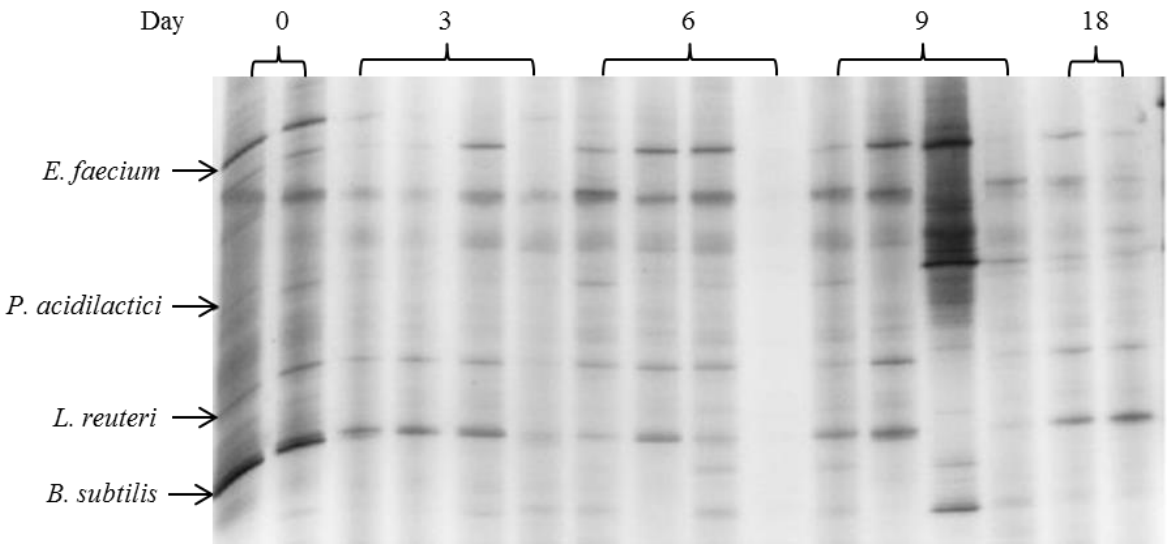


Figure 3.4: Comparison of allochthonous intestinal microbiota composition between fish fed a CON or AQ-5 diet after eight weeks of experimental feeding. Heatmap shows bacterial OTU's assigned at the phylum level and bars show OTU's assigned at the genus level (> 0.25 % of reads). Each sample represents pooled microbial communities of two fish.



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

[#] Values are expressed in terms of percentage relative abundance against the peak density at day 0.
n.d = not detected

Figure 3.5: DGGE fingerprints showing the persistence of the probiotic bacteria administered within the intestinal tract of tilapia, previously from the AQ-5 treatment, after reverting to the CON diet. Numbers above lanes indicate a pooled sample from two fish on the day post reverting to the basal diet. The table shows microbial diversity and percentage band intensity of *E. faecium*, *P. acidilactici*, *L. reuteri* and *B. subtilis* (relative to day 0) from DGGE fingerprints of the probiotic fed fish after reverting to the control diet.

3.3.6 Intestinal histology

Light microscopy was used to examine the mid-intestine of tilapia after four and eight weeks of feeding experimental diets. Fish from all dietary treatments displayed an intact epithelial barrier with extensive mucosal folds extending into the lumen, with each fold consisting of simple lamina propria housing abundant IEL's and numerous goblet cells (Fig 3.6). At week four there were no differences in mucosal fold length, perimeter ratio or IEL abundance (Table 3.11). There were significantly higher numbers of goblet cells present in the epithelia of fish in treatment AQ-5 when compared with those in treatments BS-5 and LR-5 (P = <0.01) but were not different to treatments CON or PA-5. These differences in goblet cell abundance were not apparent after eight weeks. Equally there were no differences in mucosal fold length or perimeter ratio after eight weeks. IEL abundance was significantly elevated in the AQ-5 treatment (40.01 ± 4.46) when compared to treatments CON (32.68 ± 4.81) and LR-5 (32.00 ± 3.03; P = 0.02). Treatments BS-5 and PA-5 were not significantly different from CON, AQ-5 or indeed each other (Table 3.11).

After eight weeks, treatments CON and AQ-5 were analysed by SEM and TEM to assess microvilli density and length, respectively. 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 (Fig 3.7). The microvilli density in the intestine of the AQ-5 fed fish (4.58 ± 0.69) was significantly higher than the CON fed fish (3.49 ± 0.75; P < 0.05) (Table 3.11). Numerical increases (P = 0.08) in microvilli length (AQ-5 = 1.37 ± 0.19μm vs. CON = 1.19 ± 0.14μm) and the perimeter ratio (P = 0.09), combined with a significant increase in microvilli density, resulted in a significantly (P = 0.01) higher absorptive surface area index (ASI) of the AQ-5 fed fish (40.84 ± 5.17) compared to those receiving the CON diet (22.07 ± 3.85) (Table 3.11).

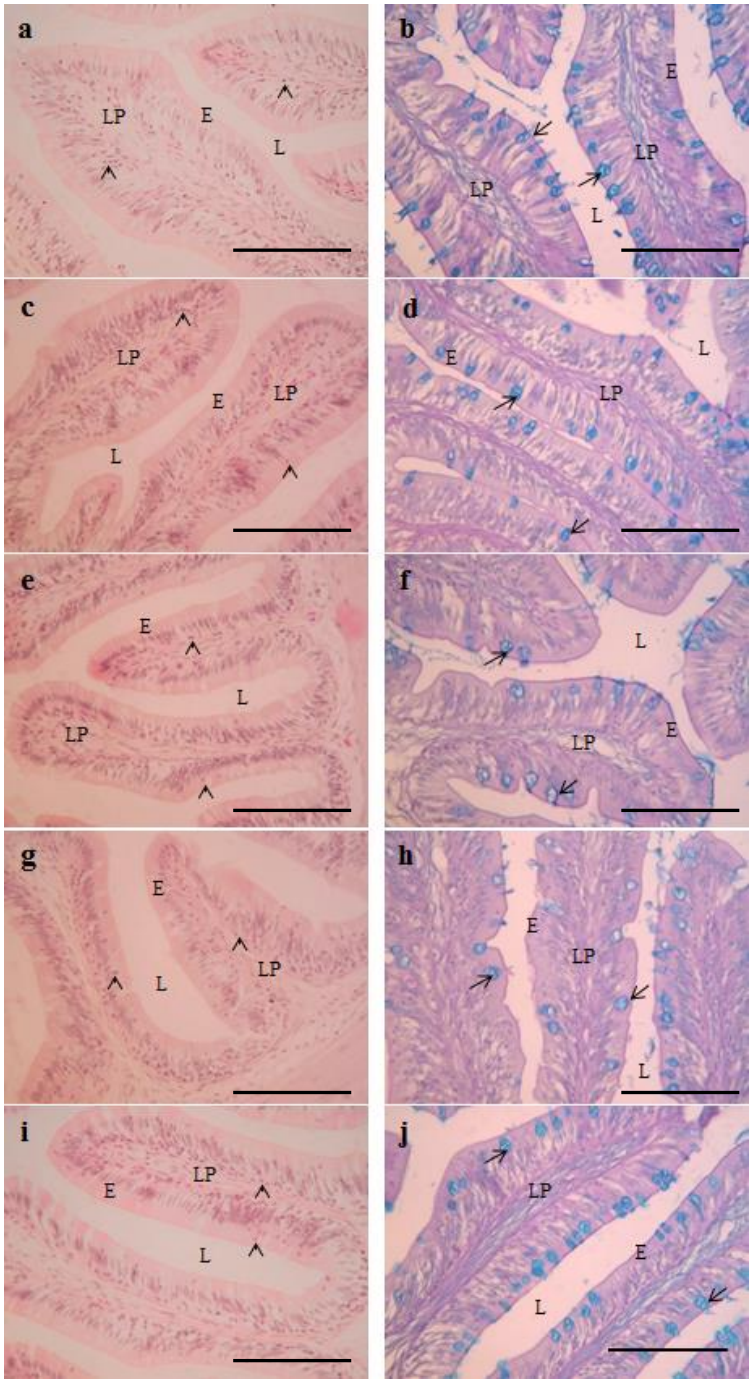


Figure 3.6: Light micrographs of the mid intestine of tilapia fed CON (a & b), BS-5 (c & d), LR-5 (e & f), PA-5 (g & h) or AQ-5 (i & j) diet after eight weeks. Goblet cells (arrows) are filled with abundant acidic mucins in all treatments and abundant IELs (arrowheads) are present in the epithelia. Abbreviations used are E: enterocytes; LP: lamina propria, L: lumen. Light microscopy staining: a, c, e, g, i: H & E; b, d, f, h, j: Alcian-Blue-PAS. Scale bars = 100µm.

Table 3.11: Histological data from the mid-intestine of tilapia after four and eight weeks of experimental feeding.

	CON	BS-5	LR-5	PA-5	AQ-5
Week 4					
Mucosal fold length (µm)	265.53 ± 34.56	300.10 ± 56.67	264.96 ± 34.27	301.71 ± 55.15	284.27 ± 28.06
Perimeter ratio (AU)	5.55 ± 0.46	6.42 ± 1.13	6.52 ± 1.02	6.47 ± 1.35	5.98 ± 1.20
IEL's (per 100 µm)	34.71 ± 3.39	31.92 ± 2.62	33.30 ± 2.78	34.81 ± 5.16	35.28 ± 2.27
Goblet cells (per 100 µm)	5.65 ± 1.51^{ab}	4.90 ± 0.61^{a}	4.93 ± 0.65^{a}	5.17 ± 0.46^{ab}	6.88 ± 0.83^{b}
Week 8					
Mucosal fold length (µm)	270.38 ± 51.29	295.12 ± 28.22	262.70 ± 53.10	300.48 ± 41.96	282.04 ± 69.36
Perimeter ratio (AU)	5.36 ± 1.24	5.87 ± 0.97	5.82 ± 1.04	6.70 ± 1.14	6.48 ± 0.74
IEL's (per 100 µm)	32.68 ± 4.81^{a}	38.55 ± 5.87^{ab}	32.00 ± 3.03^{a}	35.25 ± 2.57^{ab}	40.01 ± 4.46^{b}
Goblet cells (per 100 µm)	5.76 ± 0.41	5.65 ± 0.99	5.14 ± 0.68	5.19 ± 0.59	6.23 ± 1.44
Microvilli length (µm)	1.19 ± 0.14	-	-	-	1.37 ± 0.19
Microvilli density (AU)	3.49 ± 0.75^{a}	-	-	-	4.58 ± 0.69^{b}
ASI*	22.07 ± 3.85^{a}	-	-	-	40.84 ± 5.17^{b}

$^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

* absorptive surface index

\- no data collected

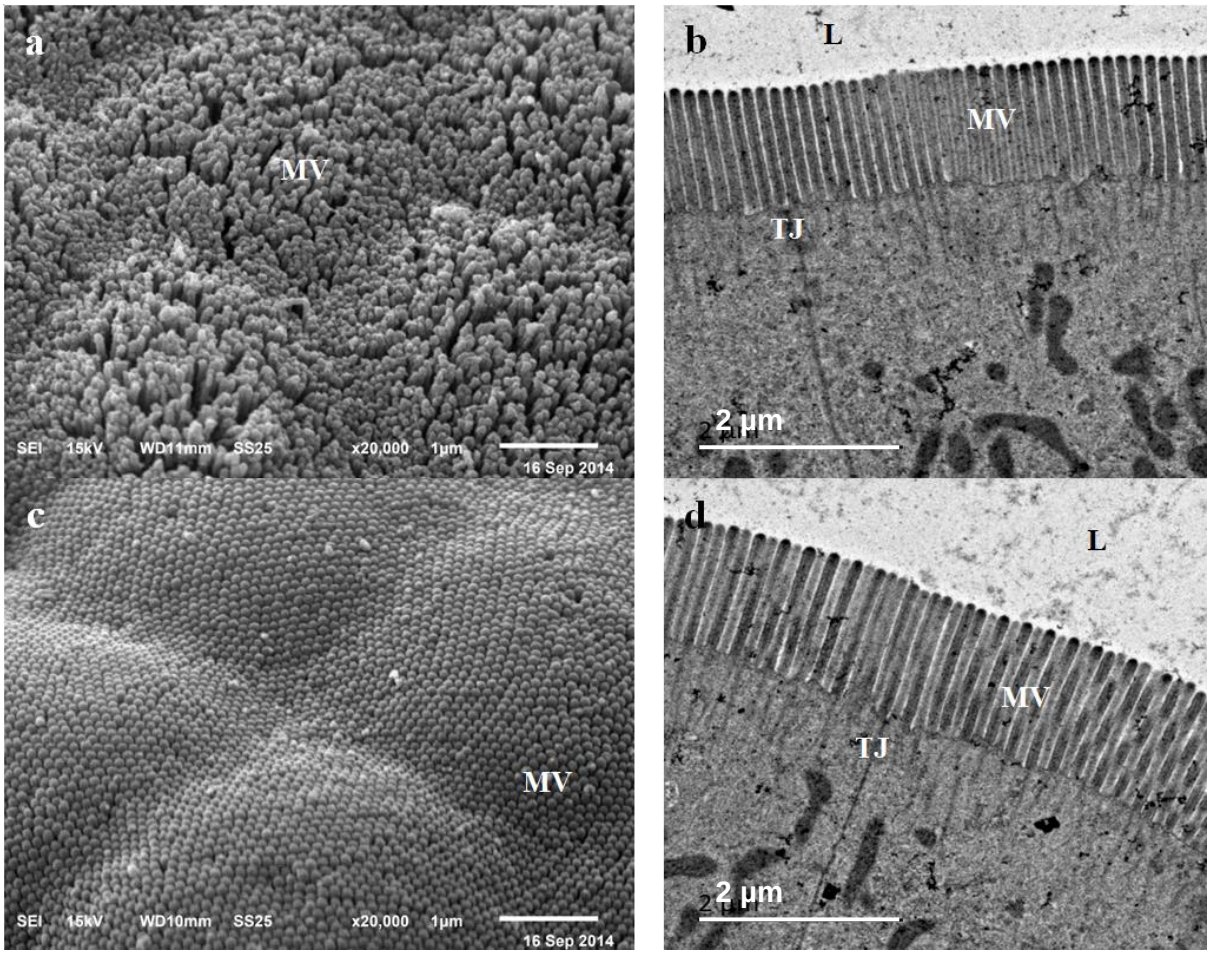


Figure 3.7: Scanning electron (a and c) and transmission electron (b and d) micrographs of the mid-intestine of Nile tilapia fed CON (a and b) or AQ-5 (c and d) diets at the end of the experimental period. Abbreviations used are L: lumen; TJ: tight junction; MV: microvilli. Scale bars = 1 µm (a and c), 2 µm (b and d).

3.3.7 Haemato-immunological analyses

Blood was taken from tilapia at weeks four and eight in order to assess haemato-immunological parameters. There were no significant differences in haematocrit, haemoglobin, erythrocytes, leucocytes, MCV, MCH, MCHC or serum lysozyme activity after four weeks of feeding on the experimental diets (Table 3.12). After eight weeks MCV was

422 significantly higher in the CON fed fish when compared to LR-5, PA-5 and AQ-5 ($P < 0.05$).
423 The MCV of fish in treatment BS-5 was not significantly different to that of any other
424 treatment. Haematocrit, haemoglobin, RBC, WBC, MCH, MCHC and serum lysozyme
425 activity remained unchanged by dietary treatment after eight weeks (Table 3.12).

Table 3.12: Haemato-immunological parameters in tilapia after four and eight weeks of feeding experimental diets.

	CON	BS-5	LR-5	PA-5	AQ-5
Week 4					
Haematocrit (%PCV)	41.80 ± 3.19	40.67 ± 2.21	38.83 ± 2.73	38.33 ± 1.25	37.00 ± 2.10
Haemoglobin (g dl^{-1})	9.66 ± 1.89	10.30 ± 0.90	9.45 ± 1.73	9.32 ± 1.07	8.86 ± 1.20
RBC (10^6 μl^{-1})	1.81 ± 0.72	1.20 ± 0.92	1.85 ± 0.88	1.75 ± 0.94	1.65 ± 0.83
WBC (10^3 μl^{-1})	28.78 ± 10.79	23.52 ± 16.52	25.67 ± 9.38	27.54 ± 9.79	29.63 ± 12.70
MCV (fL)	379.16 ± 252.03	536.71 ± 272.91	327.06 ± 246.82	333.79 ± 249.60	364.64 ± 252.48
MCH (pg)	76.66 ± 62.07	135.71 ± 76.75	85.52 ± 64.42	107.68 ± 114.13	84.83 ± 63.96
MCHC (g dl^{-1})	25.94 ± 1.79	23.67 ± 4.22	26.12 ± 1.80	24.22 ± 3.66	24.24 ± 2.46
Serum lysozyme activity (AU)	312.20 ± 52.25	301.18 ± 66.12	353.82 ± 32.61	316.19 ± 62.09	312.96 ± 72.93
Week 8					
Haematocrit (%PCV)	37.50 ± 2.29	35.17 ± 4.10	36.33 ± 2.21	35.67 ± 1.70	37.20 ± 3.37
Haemoglobin (g dl^{-1})	8.96 ± 1.17	8.88 ± 0.53	9.15 ± 1.27	8.14 ± 1.65	8.47 ± 1.66
RBC (10^6 μl^{-1})	1.55 ± 0.61	2.02 ± 0.68	2.06 ± 0.18	2.09 ± 0.39	2.21 ± 0.24
WBC (10^3 μl^{-1})	20.11 ± 7.80	23.05 ± 8.72	23.85 ± 7.85	19.81 ± 5.37	29.80 ± 6.21
MCV (fL)	323.24 ± 178.34^{a}	231.39 ± 141.72^{ab}	165.13 ± 10.45^{b}	191.84 ± 50.07^{b}	174.14 ± 16.97^{b}
MCH (pg)	74.50 ± 48.00	54.66 ± 30.97	43.54 ± 3.19	39.74 ± 7.20	39.00 ± 9.05
MCHC (g dl^{-1})	23.72 ± 3.51	24.15 ± 1.96	26.56 ± 3.41	20.91 ± 4.46	22.52 ± 3.88
Serum lysozyme activity (AU)	207.26 ± 90.51	277.58 ± 58.96	286.82 ± 120.19	257.58 ± 96.67	239.12 ± 97.06

$^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

3.4 Discussion

The intestinal microbiomes of fish are complex communities which have been demonstrated to impact host health, mucosal development and differentiation, metabolism, nutrition and disease resistance (Rawls *et al*. 2004; Bates *et al*. 2006; Round and Mazmanian 2009). These communities are sensitive to rearing environment, seasonal and dietary changes, including probiotic supplementation (Merrifield *et al*. 2010; 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 DGGE.

The present study used a multidisciplinary approach consisting of culture based techniques, DGGE and high-throughput sequencing. Culture dependent approaches demonstrated that each probiotic treatment was able to modulate the allochthonous and autocthonous microbial populations within the intestine. Each probiotic was recovered in the intestine using culture methods with the exception of *L. reuteri*. However, this species was detected when using more sensitive culture independent techniques suggesting that this species may have low survivability during diet preparation or through the upper intestinal tract. Despite differences on selective agar, dietary treatment did not affect intestinal total viable counts.

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 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, DGGE revealed complex microbial communities in all treatments. Sequencing of excised bands confirmed the presence of *B. subtilis*, *E.*

452 *faecium*, *L. reuteri* and *P. acidilactici* in the corresponding treatments. At week eight,
453 ANOSIM indicated that the intestinal microbiota of fish in the CON treatment was
454 significantly dissimilar to all probiotic treatments illustrating that both the mono- and
455 multispecies probiotics tested can modulate the intestinal microbiota of tilapia.

456 The introduction of high-throughput sequencing technologies has increased our
457 understanding of microbial diversity and function in complex environments, including the GI
458 tract of fish (van Kessel *et al*. 2011; Roeselers *et al*. 2011; Desai *et al*. 2012; Wu *et al*. 2012;
459 Boutin *et al*. 2013; Carda-Diéguez *et al*. 2013; Wu *et al*. 2013; Ingerslev *et al*. 2014; Zarkasi
460 *et al*. 2014; Falcinelli *et al*. 2015; Lyons *et al*. 2015). At present, there is a paucity of
461 information on the intestinal microbiome of tilapia using high-throughput sequencing; to the
462 authors knowledge this is the first study utilising high-throughput sequencing to assess the
463 intestinal microbial communities in tilapia. In the present study, sequence libraries for both
464 treatments assessed (CON and AQ-5) displayed Good's coverage estimations of >0.99,
465 indicating that the intestinal microbiota had been fully sampled. Firmicutes accounted for >
466 99% of 16S rRNA reads in the AQ-5 fish and although they accounted for a significantly
467 smaller proportion of the reads in the control fed fish they remained the most abundant
468 phylum present. Concomitantly, Proteobacteria and Cyanobacteria were significantly more
469 abundant in the control fish along with other notable phyla present including Fusobacteria,
470 Actinobacteria and Bacteroidetes. These phyla have all been detected in varying levels in
471 omnivorous fish species (van Kessel *et al*. 2011; Roeselers *et al*. 2011; Wu *et al*. 2013)
472 including tilapia (Zhou *et al*. 2009; Liu *et al*. 2013). BLAST searches using single
473 representative sequences from Cyanobacteria revealed high similarity to nucleotide
474 sequences from soybean chloroplasts. It is possible that at least some of these reads may have
475 been artefacts derived from the diets as opposed to the presence of Cyanobacteria populations.

At the genera level, in AQ-5, the most abundant 16S rRNA reads belonged to *Enterococcus* and *Bacillus* followed by *Pediococcus*. At a lower level, *Lactobacillus* spp. was also detected. Confirming the 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 AQ-5 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. 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 the 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 OTU's 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 OTU's 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. 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 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 IEL's in the mid-intestine of tilapia after eight weeks feeding in treatment diet AQ-5 when compared with CON and LR-5 groups. Similar results were obtained by Salinas *et al*. (2008) who discovered higher populations of acidophilic granulocytes and Ig^+ populations in intestinal mucosa of gilthead sea bream, *Sparus aurata*, when supplementing diets with *Lactobacillus delbrüeckii* ssp. *lactis* and *B. subtilis* when compared to the control treatment, or each treatment containing the probiotics singularly. Other studies have also reported a larger abundance of intestinal IEL's in tilapia fed monospecies probiotic applications of *P. acidilactici* (Standen *et al*. 2013) and *L. rhamnosus* (Pirarat *et al*. 2011).

Furthermore, after eight weeks microvilli density was significantly higher in the mid-intestine of treatment AQ-5 when compared to treatment CON. 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 to this, increased microvilli density, combined with numerical improvements of microvilli length and perimeter ratio, significantly improved the absorptive surface area index in the AQ-5 fed fish. Here, no differences were observed in growth performance. As long as growth is not impaired, this result (i.e. no difference in growth performance) should be considered a positive outcome since the probiotics can manifest other benefits such as immune modulation or improvements in gut morphology which likely uses energy and resources. Further work is needed to optimise of dose and feeding regime to investigate whether the changes observed here could result in improved growth performance.

After the eight week feeding trial, 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 showed decreasing trends 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 six 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, after the dietary supplementation of *P. acidilactici* for 32 days, *P. acidilactici* could be detected in the tilapia intestine for at least 17 days after cessation of probiotic feeding (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 tilapia, salmonids and catfish has been investigated, demonstrating temporal colonisation lasting from < 3 days to > 3 weeks (Nikoskelainen *et al*. 2003; Kim

548 and Austin 2006; Balcázar *et al*. 2007; Ran *et al*. 2012; Ridha and Azad 2012). Thus it is
549 evident the length of time a probiont may remain in the intestine of fish, after probiotic
550 feeding has ceased, is dependent on the probiotic species, host species, environmental factors,
551 dosage and duration of probiotic supplementation.

552 In conclusion, all three microbiological methods used in the present study (culture
553 based, DGGE and high-throughput sequencing) confirmed the probiotic presence in the
554 intestine of the corresponding treatment. Survival through the upper GI tract is an essential
555 requirement of any probiotic, since probiotic cells must survive the gastric process in order to
556 exert their beneficial effect in the intestine. Furthermore, each probiotic, whether applied
557 singularly or as a mix, was able to modulate the intestinal microbiota in tilapia. Under these
558 conditions AquaStar® Growout can stimulate the localised immune response through the
559 recruitment of IEL's (when compared to CON) and the higher abundance of goblet cells
560 (when compared to BS-5 and LR-5) in the intestinal mucosa, which may result in better
561 protection against localised pathogens. Intestinal translocation experiments and disease
562 challenge studies are required to validate this hypothesis. Future work should focus on
563 elucidating the mechanisms which underpin these localised immunological changes using a
564 transcriptomic approach. Concomitantly with modulated microbiota and IEL levels,
565 AquaStar® Growout treatment enhanced intestinal morphology by elevating the absorptive
566 surface area when compared to CON. In addition, the probiotics contained in the AquaStar®
567 Growout product are able to remain in the gut after reverting to the basal diet suggesting
568 these benefits may continue to persist after probiotic feeding has ceased. The benefits
569 observed were not at the expense of growth performance which was not affected by dietary
570 treatment.

Chapter 4. Dietary administration of a commercial mixed-species probiotic improves growth performance and modulates the intestinal immunity of tilapia, *Oreochromis niloticus*

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. After six weeks of supplementing the probiotic at 3g kg^{-1}, an up-regulation of intestinal caspase-3, PCNA and HSP70 mRNA levels fed fish was observed when compared to the CON. 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 after six weeks. 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} for six weeks can improve tilapia growth and elevate the intestinal immunological status of the host.

4.1 Introduction

Using high-throughput sequencing, it has previously been reported that AquaStar® Growout, supplemented through tilapia diets at 5 g kg^{-1}, can result in the relative dominance of probiotic 16S rRNA reads (>99% of total reads; Chapter 3) in the tilapia intestine, but it is unclear what implications this has on localised host immunity. It is of paramount importance that aquaculture stocks are healthy and capable of mounting an effective immune response were the host to come into contact with a potential pathogen. The intestinal 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 (Foey and Picchietti 2014). Probiotics can have beneficial implications 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 as described in Chapter 3, and also by inducing the expression of pro-inflammatory cytokines (e.g. TNFα and IL-1β), thus maintaining the capacity of recognising and responding to pathogens, and regulatory cytokines (e.g. TGFβ and IL-10) for the maintenance of mucosal tolerance (Pirarat *et al*. 2011; He *et al*. 2013; Liu *et al*. 2013; Ren *et al*. 2013; Standen *et al*. 2013; Villamil *et al*. 2014). These cytokines are the end products to complex molecular pathways which is initiated by TLR's recognising their corresponding pathogen associated molecular pattern (PAMP) (Cerf-Bensussan and Gaboriau-Routhiau 2010). 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 IL-1β, TNFα, IL-8 and TGFβ expression (Abid *et al*. 2013; Sun *et al*. 2014).

The current investigation aimed to evaluate multiple doses and feeding regimes of AquaStar® Growout, on intestinal immunity, growth performance, intestinal integrity and intestinal microbiology.

4.2 Materials and methods

4.2.1 Experimental design and dietary preparation

Five hundred tilapia were randomly distributed to 10 150L fibreglass tanks (50 fish per tank; average weight = 29.02 ± 0.33g). Three diets were formulated and pelleted as described in section 2.3 (Table 4.1). Treatments were as follows; control (basal diet void of AquaStar® Growout), low probiotic dose (basal diet supplemented with AquaStar® Growout at 1.5g kg^{-1}), high probiotic dose (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 the basal diet) and initial probiotic feeding (first two weeks AquaStar® Growout feeding at 1.5g kg^{-1} followed by remainder of the trial on the basal diet). Fish were fed experimental diets for six weeks at a rate of 1- 5% biomass per day in four equal rations. All diets were assessed for proximate composition as described in section 2.4 (Table 4.1). Water quality parameters were monitored and maintained as described in section 2.1. Diet codes were assigned to each treatment for ease of analysis (Table 4.2). After four weeks, treatments were reduced from triplicate to duplicate (i.e. n = 2) in order to increase stocking densities and reduce tilapia aggression.

Table 4.1: Dietary formulation and proximate 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.

4.2.2 Growth performance and carcass composition

Prior to the start of the trial, nine fish were pooled into three samples to assess initial proximate carcass composition as described in section 2.4. At the end of the experimental period four fish per tank were pooled into two samples (n = 4) to assess final proximate carcass composition. Growth performance and feed utilisation were assessed by final weight, weight gain, feed intake, SGR, FCR and PER as described in section 2.5.

Table 4.2: Codes assigned to dietary treatments.

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

4.2.3 RT-PCR

The mid-intestine was sampled from two fish per tank after three weeks (n = 6) and four fish per tank after six weeks (n = 8) to assess gene expression of caspase-3, PCNA, HSP70, TLR2, TGFβ, IL-10, TNFα and IL-1β as described in section 2.11. Primer sequences and efficiencies are reported in Table 4.3. Cycle threshold (Ct) values were imported into the relative expression software tool (REST©) (Pfaffl *et al*. 2002) where experimental treatments were each compared to the control. Data were reported as fold change.

4.2.4 Intestinal histology

Two fish per tank were sampled at weeks three (n = 6) and six (n = 8) to asses perimeter ratio, IEL and goblet cell abundance as described in section 2.7.

4.2.5 Culture dependent analyses

Digesta was isolated and pooled from two fish per tank after three weeks (n = 3). After six weeks, digesta was removed from four fish per tank and pooled into two samples

(thus n = 4) to assess allochthonous level of LAB, enterococci, *Bacillus* and TVC. Samples were processed as described in section 2.8.1.

4.2.6 DGGE

DNA was extracted using the QIAamp Stool Mini Kit (Qiagen) as described in section 2.8.3. DGGE was carried out on all digesta samples from week three (n = 3) and week six (n = 4) to assess allochthonous bacterial communities as described in section 2.8.4.

4.2.7 *Haemato-immunological analyses*

Blood samples were taken to assess haematocrit and haemoglobin after six weeks (n = 10). Furthermore serum was collected to assess serum lysozyme activity (n = 20). All sampling and analyses was carried out according to section 2.10.

4.2.8 Statistical analyses

Statistical analyses were carried out as described in section 2.12.

120 **Table 4.3:** Primer sequences 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

4.3 Results

4.3.1 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 4.4). 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. There were no significant differences in proximal composition between carcass moisture, ash, lipid, protein or energy (Table 4.5).

Table 4.4: Growth performance of tilapia after six weeks of feeding 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}

* Parameters reported as percentage of dry weight matter.

$^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

Table 4.5: Proximate carcass composition (%) of tilapia of tilapia prior to 'Day 0' and after six weeks of feeding experimental diets.

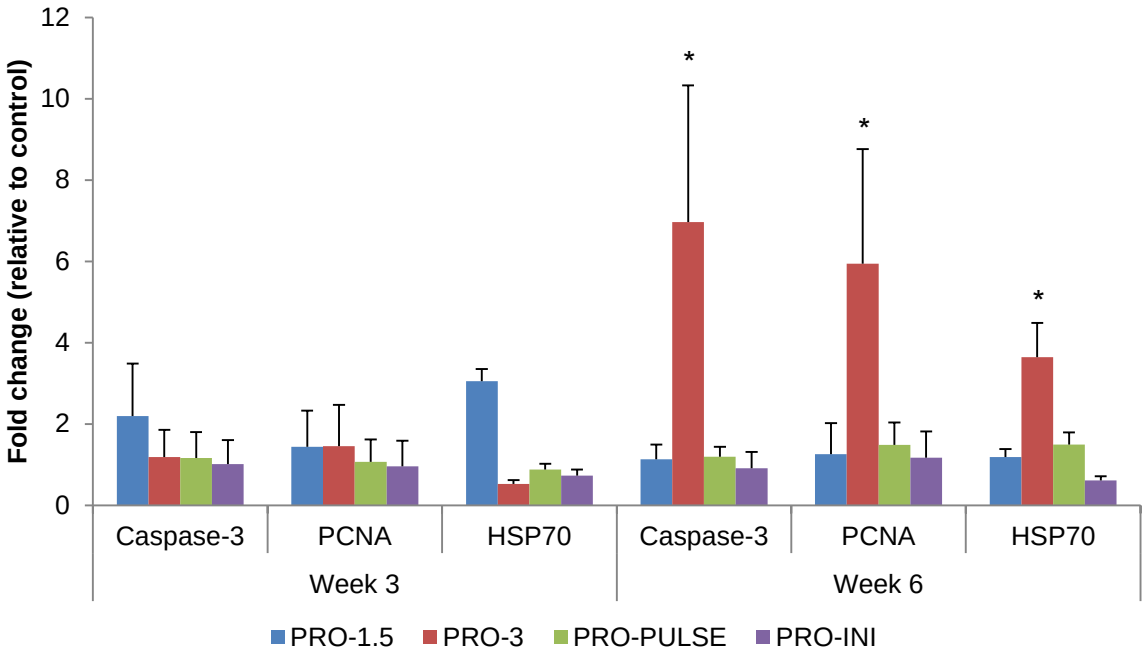
	Initial fish	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Moisture	77.41 ± 0.32	68.75 ± 0.44	68.97 ± 0.78	69.41 ± 0.89	69.81 ± 1.14	68.72 ± 0.59
Ash*	16.64 ± 0.43	9.88 ± 0.37	10.17 ± 0.49	9.67 ± 0.31	10.52 ± 0.74	10.20 ± 0.08
Lipid*	18.95 ± 0.87	34.68 ± 0.53	32.42 ± 0.78	34.94 ± 1.79	32.67 ± 1.68	33.78 ± 0.73
Protein*	58.19 ± 0.20	52.03 ± 0.42	53.41 ± 0.52	52.48 ± 1.50	54.43 ± 1.32	52.90 ± 1.38
Energy*	-	24.67 ± 0.15	24.39 ± 0.45	24.72 ± 0.46	24.56 ± 0.36	25.05 ± 0.16

* Parameters reported as percentage of dry weight matter.

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 after six weeks of experimental feeding which were up-regulated approximately seven fold in PRO-3 when compared to the control group ($P = 0.001$; Fig 4.1). 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 after six weeks of supplementation ($P = < 0.001$ and 0.028 respectively). Caspase-3, PCNA and HSP70 gene expression levels did not differ between any experimental treatments when compared to the control treatment at week three (Fig 4.1).

TLR2, pro- and anti-inflammatory gene expression analyses after three weeks did not reveal any significant differences between the experimental treatments when compared to the control treatment (Fig 4.2). After six weeks, changes in immunity gene expression were revealed (Fig 4.2). TLR2 was significantly up-regulated more than four fold in PRO-3 when compared to the control treatment ($P = 0.004$). The pro-inflammatory cytokines TNFα and IL-1β were up-regulated approximately 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 cytokines IL-10 and TGFβ 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 PRO-1.5, PR0-PULSE and PRO-INI when compared to the control treatment with any of the investigated genes of interest ($P > 0.05$).



158

159 **Figure 4.1:** Relative mid-intestinal gene expression of caspase-3 and PCNA and HSP70 after
160 three and six weeks of feeding experimental diets. Values are reported in fold change when
161 compared against the expression in the control treatment (set to 1.0). Asterisks highlight
162 significant differences ($P < 0.05$) when compared to the control treatment within the same
163 sampling period.

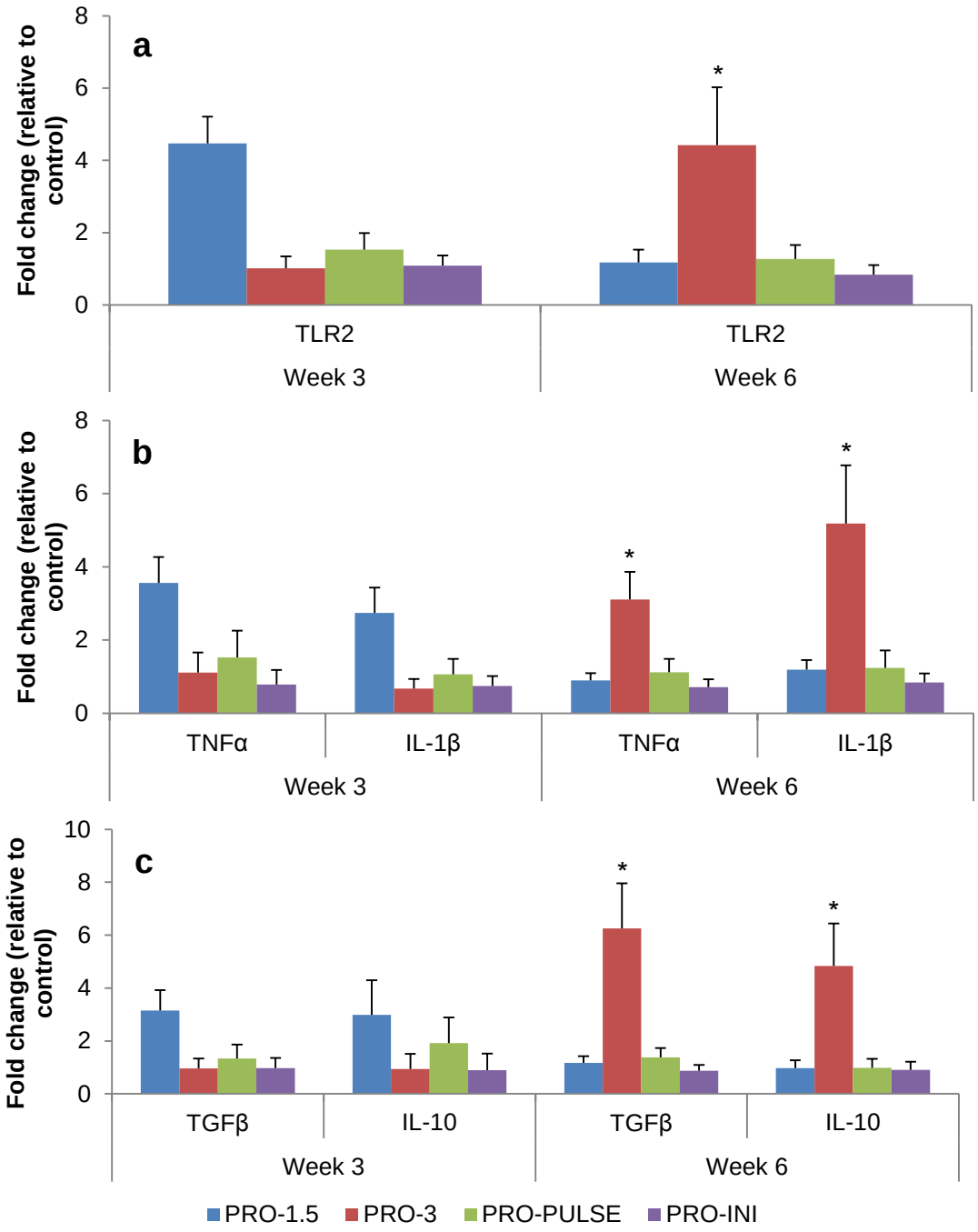


Figure 4.2: Relative gene expression of intestinal TLR2 (a) pro-inflammatory cytokines TNFα and IL-1β (b) and anti-inflammatory cytokines IL-10 and TGFβ (c) after three and six weeks of feeding experimental diets. Values are reported in fold change when compared against the expression in the control treatment (set to 1.0). Asterisks highlight significant differences ($P < 0.05$) when compared to the control treatment within the same sampling period.

4.2.3 Intestinal histology

At week three and six, light microscopy was used to examine the perimeter ratio, IEL and goblet cell levels from the mid-intestine (Table 4.6). At both time points fish from all dietary treatments had an intact epithelial barrier with extensive mucosal folds, abundant IEL's and numerous goblet cells. After three weeks of feeding experimental diets, perimeter ratio, IEL and goblet cell abundance remained unaffected by dietary regime (P = 0.771, 0.246 and 0.477 respectively). After six weeks, tilapia in different treatments showed altered perimeter ratios (P = 0.007). The highest perimeter ratio was recorded in PRO-INI which was significantly higher than PRO-1.5 but not CON, PRO-3 or PRO-PULSE. Perimeter ratio in PRO-3 was also significantly higher when compared to the lower probiotic dose, PRO-1.5. However, perimeter ratio remained unchanged between treatments PRO-1.5, CON, and PRO-PULSE. After six weeks, IEL and goblet cell abundance remained unchanged by dietary treatment in groups CON, PRO-1.5, PRO-PULSE and PRO-INI. However, IEL levels were significantly elevated in PRO-3 when compared to treatments CON, PRO-PULSE and PRO-INI (P = 0.003) but not PRO1.5. PRO-3 also contained significantly larger populations of goblet cell when compared to all other treatments after six weeks (P < 0.001). No differences were observed in IEL or goblet cell abundance after three weeks (Table 4.6).

Table 4.6: Histological data from the mid-intestine of tilapia after three and six weeks of experimental feeding.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Week 3					
Perimeter ratio (AU)	2.94 ± 0.92	3.34 ± 0.60	3.10 ± 0.70	3.40 ± 0.88	3.58 ± 0.93
IEL's (per 100 µm)	31.06 ± 3.54	34.63 ± 4.42	36.55 ± 2.99	32.55 ± 2.76	32.91 ± 5.25
Goblet cells (per 100 µm)	4.22 ± 0.93	4.43 ± 0.88	4.37 ± 0.72	3.83 ± 0.54	3.66 ± 0.89
Week 6					
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 ($P < 0.05$).

4.2.4 Culture dependent analyses

The effect of AquaStar® Growout treatments on the allochthonous aerobic heterotrophic bacteria was determined using culture based methods at week three and six (Table 4.7). No significant differences were observed in TVC levels between the treatments at either time point with allochthonous levels approximately log 6-7 CFU g^{-1} for each treatment (week three $P = 0.349$ and week six $P = 0.993$).

After three weeks of feeding experimental diets, there were significantly higher intestinal LAB in PRO-3 when compared to CON, PRO-PULSE and PRO-INI ($P = 0.001$) but no different to PRO-1.5. LAB were only detected in two out of three replicates in PRO-1.5 and not detected at all in CON, PRO-PULSE and PRO-INI replicates. Thus there were no significant differences between these four treatments. Levels of enterococci showed a similar pattern. The abundance of allochthonous enterococci was greatest in PRO-3 and PRO-1.5. Enterococci were not detected in treatments CON or PRO-INI and in just one out of three replicates in PRO-PULSE. Consequently, enterococci levels were significantly higher in PRO-3 and PRO-1.5 when compared to those detected in other treatments ($P = <0.001$), but not different from each other. *Bacillus* levels remained unchanged between dietary regimes ($P = 0.727$; Table 4.7).

After six weeks, highest LAB levels were observed in the digesta of PRO-3, these were significantly higher than CON and PRO-INI ($P = 0.006$) but not PRO-1.5 or PRO-PULSE. Similarly, PRO-3 resulted in the highest *Bacillus* levels which were significantly higher than those found in PRO-PULSE but not in other treatments ($P = 0.026$). LAB and *Bacillus* populations were no different in treatments CON, PRO-1.5, PRO-PULSE and PRO-INI. Furthermore, enterococci levels were significantly higher in PRO-3 when compared to CON, PRO-PULSE and PRO-INI. Despite being numerically higher, they were no different

214 to enterococci levels recovered in PRO-1.5 digesta (Table 4.7). Representative subsets of
215 probiotics were confirmed by using 16S rRNA gene sequence analysis.

216

217 **Table 4.7:** Allochthonous TVC, LAB, enterococci and *Bacillus* spp. (log CFU g^{-1}) in the
218 intestinal tract of tilapia after three and six weeks of experimental feeding.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Week 3					
TVC	6.29 ± 0.19	6.01 ± 0.53	7.07 ± 0.53	6.15 ± 0.18	6.77 ± 0.53
LAB	n.d^{a}	2.93 ± 1.92^{ab}	6.18 ± 0.99^{b}	n.d^{a}	n.d^{a}
Bacillus spp.	4.77 ± 0.22	5.00 ± 0.21	5.89 ± 1.42	4.84 ± 0.19	4.95 ± 0.18
Enterococci	n.d^{a}	3.98 ± 0.59^{b}	5.83 ± 0.98^{b}	1.16 ± 1.21^{a}	n.d^{a}
Week 6					
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}

219 n.d = not detected
220 $^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

221

222 *4.2.5 DGGE*

223 The influence of probiotic treatment on the allochthonous intestinal microbial
224 diversity in tilapia was investigated using DGGE after three and six weeks. Suspected
225 probiotic bands were identified by migration to the same position as known *B. subtilis*, *E.*
226 *faecium*, *L. reuteri* and *P. acidilactici* samples. Presumed probiotic bands were isolated from
227 DGGE gels and sequencing confirmed the presence of all four probiotic species from
228 probiotic fingerprints. Tables 4.8 and 4.9 display the microbial ecological parameters derived
229 from the DGGE fingerprints after three and six weeks, respectively.

Table 4.8: Microbial community analysis of the intestinal allochthonous bacterial populations of tilapia from DGGE fingerprints after three weeks of feeding experimental diets.

	Microbial ecological parameters				Similarity (ANOSIM)		
	N	Richness	Diversity	SIMPER (%)	*R*- value	*P*- value	Dissimilarity (%)
CON	15.67 ± 1.89	5.32 ± 0.46	2.74 ± 0.13	78.82 ± 5.69			
PRO-1.5	16.00 ± 6.53	5.34 ± 1.58	2.68 ± 0.45	65.56 ± 12.27			
PRO-3	16.00 ± 2.16	5.40 ± 0.52	2.76 ± 0.14	74.84 ± 7.30			
PRO-PULSE	24.00 ± 2.45	7.23 ± 0.54	3.17 ± 0.10	91.65 ± 2.95			
PRO-INI	16.67 ± 1.25	5.57 ± 0.30	2.81 ± 0.08	79.83 ± 4.44			
Pairwise comparisons							
CON vs PRO-1.5					0.57	0.10	38.86
CON vs PRO-3					0.65	0.10	33.18
CON vs PRO-PULSE					1.00	0.10	33.94
CON vs PRO-INI					0.78	0.10	30.52
PRO-1.5 vs PRO-3					-0.11	0.70	25.12
PRO-1.5 vs PRO-PULSE					0.35	0.20	30.46
PRO-1.5 vs PRO-INI					0.22	0.10	32.33
PRO-3 vs PRO-PULSE					0.56	0.10	26.79
PRO-3 vs PRO-INI					0.19	0.10	24.92
PRO-PULSE vs PRO-INI					0.78	0.10	25.07

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 ($P < 0.05$).

235 **Table 4.9:** Microbial community analysis of the intestinal allochthonous bacterial populations of tilapia from DGGE fingerprints after six weeks
236 of feeding 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

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

At week three, numerically PRO-PULSE displayed highest values with regards to number of OTU's, species richness and diversity indices observed in allochthonous intestinal microbial communities (Table 4.8). However, these differences were not significant (P = 0.147, 0.169 and 0.278 respectively). Replicates within all treatments showed a high similarity percentage (SIMPER) and no differences were detected between treatments (P = 0.055). Similarly, no differences were observed when pair wise comparisons were assessed by means of ANOSIM. Replicates from CON, PRO-PULSE and PRO-INI showed tight clustering indicating a high similarity (Fig 4.3). Apart from one replicate from PRO-3, both the continuously supplemented probiotic groups (PRO-1.5 and PRO-3) showed a looser clustering effect, indicating their similarity to each other along with their dissimilarity to treatments CON, PRO-PULSE and PRO-INI.

After six weeks, PRO-PULSE again demonstrated the highest values with respect to number of OTU's, species richness or diversity indices. However, there were no significant differences between treatments (P = 0.083, 0.086 and 0.102 respectively; Table 4.9). 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 revealed no differences with regards to SIMPER analyses. ANOSIM revealed that the microbial communities within PRO-3 was 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; Table 4.9). This can be visualised in Fig 4.4 where there is a loose clustering effect of the communities from replicates assigned to each treatment. Replicates from treatments CON, PRO-PULSE and PRO-INI showed loose clustering although there was a certain amount of overlap

between these three treatments. Two out of four replicates from PRO-1.5 showed high similarity to those from PRO-3, whereas the remaining two replicates are more similar to the other treatments (Fig 4.4).

4.2.6 Haemato-immunological analyses

Blood samples were assessed for haematocrit, haemoglobin and serum lysozyme activity after six weeks of feeding experimental diets (Table 4.10). Both haematocrit or haemoglobin values were within the expected range for tilapia but they were not affected by dietary regime. However, differences were observed in serum lysozyme. Serum lysozyme activity was significantly lower in PRO-1.5 when compared with activity in CON and PRO-PULSE but not when compared with PRO-3 or PRO-INI ($P = 0.034$). Activity did not differ between treatments CON, PRO-3, PRO-PULSE or PRO-INI.

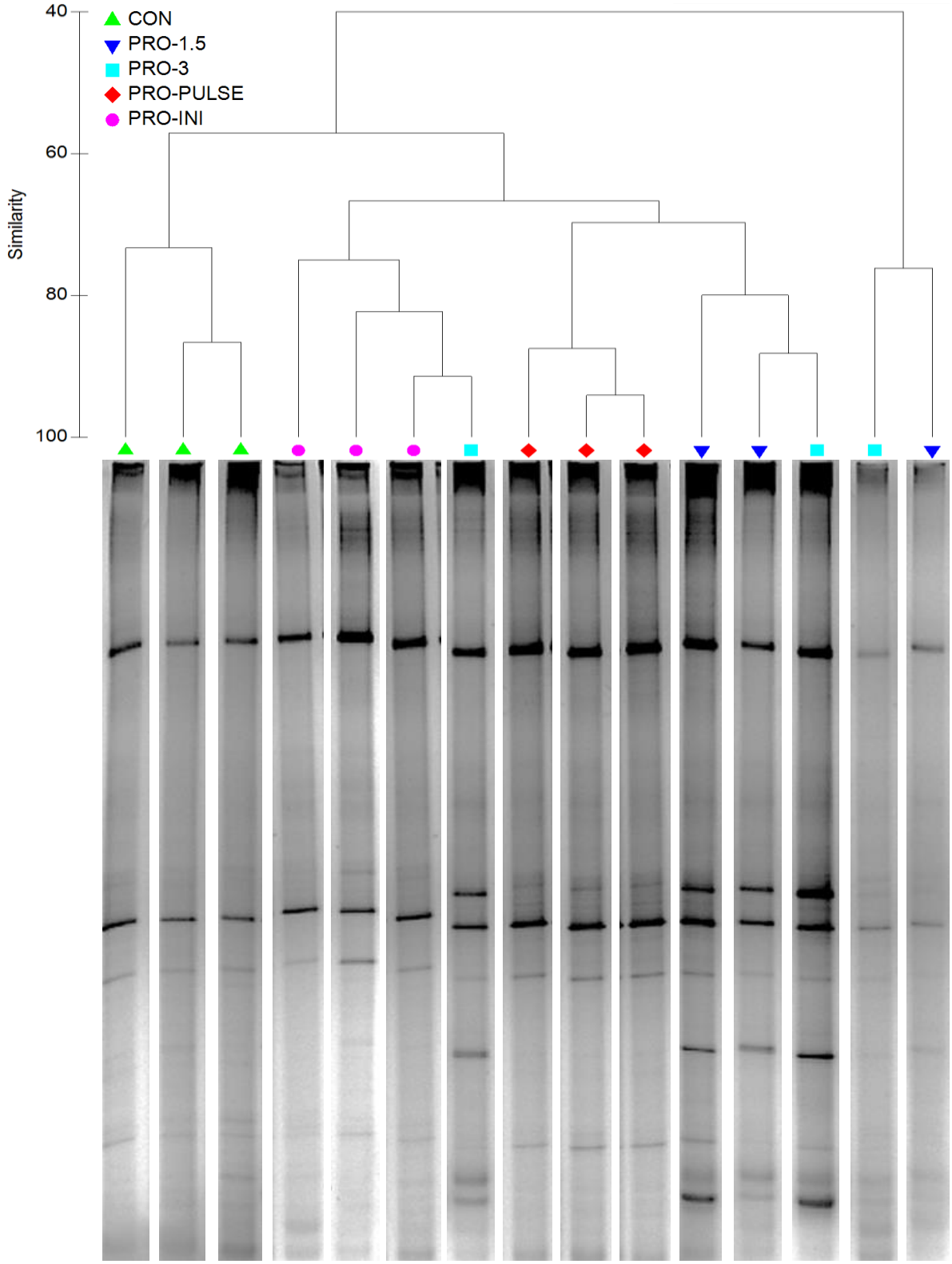


Figure 4.3: Dendrogram representing the relatedness of the microbial communities in the digesta of tilapia after three weeks of feeding with experimental diets. DGGE fingerprints below represent amplified products from the V3 region of the samples which correspond to those used in the dendrogram.

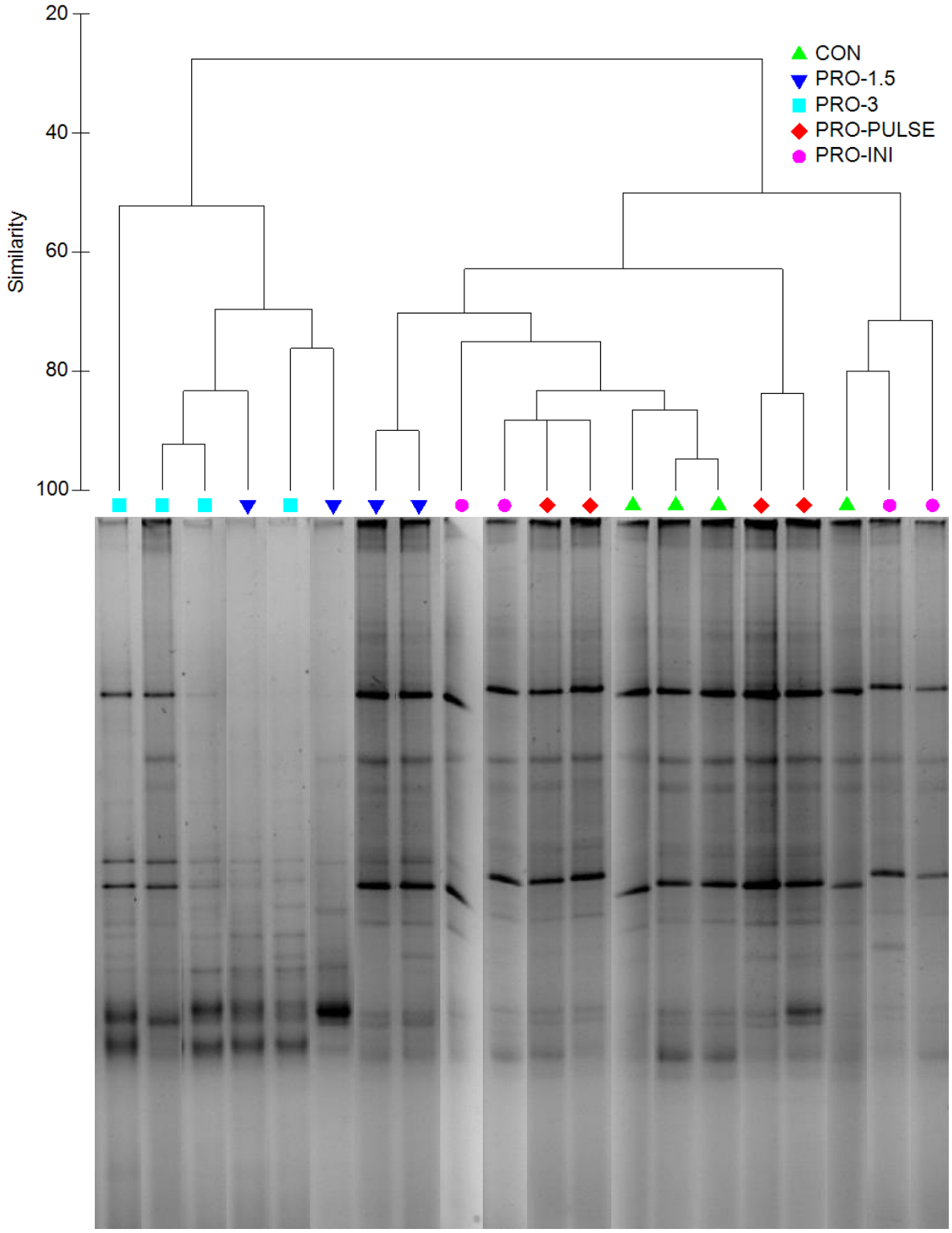


Figure 4.4: Dendrogram representing the relatedness of the microbial communities in the digesta of tilapia after six weeks of feeding with experimental diets. DGGE fingerprints below represent amplified products from the V3 region of the samples which correspond to those used in the dendrogram.

Table 4.10: Haemato-immunological data from tilapia after six weeks of experimental feeding.

	CON	PRO-1.5	PRO-3	PRO-PULSE	PRO-INI
Haematocrit (%PCV)	37.30 ± 2.45	39.60 ± 3.29	37.80 ± 2.86	38.00 ± 1.58	38.11 ± 3.18
Haemoglobin (g dl^{-1})	7.24 ± 1.70	8.32 ± 1.09	8.12 ± 0.83	7.62 ± 1.87	8.42 ± 0.83
Serum lysozyme activity (AU)	335.50 ± 40.30[a]	295.43 ± 55.75[b]	344.04 ± 100.71[ab]	345.85 ± 43.80[a]	308.80 ± 60.57[ab]

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

4.4 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 has previously been reported to improve growth performance of rainbow trout (Giannenas *et al*. 2015). Although there is no data regarding the growth promoting effects of AquaStar® Growout in tilapia, *Bacillus* spp., *Enterococcus* spp. and *Lactobacillus* spp., either singularly or in combination with other species have been reported to improve tilapia growth (Lara-Flores *et al*. 2003; El-Haroun *et al*. 2006; Wang *et al*. 2008; Apún-Molina *et al*. 2009; Mehrim 2009; Essa *et al*. 2010; Zhou *et al*. 2010; Jatobá *et al*. 2011; Abumourad *et al*. 2013; Ayyat *et al*. 2014). The mechanisms which underpin these improvements are only partly described. Previous work on tilapia suggests that Aquastar® Growout may improve the potential for growth by increasing absorptive surface area (Chapter 3). Probiotics may also be important in the production of digestive enzymes. Essa *et al*. 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* (Essa *et al*. 2010). Probiotics have also shown antagonism to a number of pathogens (Aly *et al*. 2008). It could be hypothesised that probiotics are capable of exerting a similar growth promoting mechanism as antibiotic growth promoters, through the inhibition of sub-clinical infections.

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 (Norouzitallab *et al*. 2015). 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 (Basu *et al*. 2002). 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. Many authors have reported lower expression of HSP70 after probiotic administration in fish (Avella *et al*. 2010a; Avella *et al*. 2010b; Avella *et al*. 2011) including tilapia (He *et al*. 2013). 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. After six weeks, intestinal HSP70 gene expression was significantly higher in PRO-3 when compared to the control. Using an *ex vivo* approach, Ren *et al*. (2013) 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 *L. plantarum*, as well as a mix of *A. hydrophila* and *L. plantarum* to the intestinal sac caused an upregulation of HSP70 (Ren *et al*. 2013). Similar results were reported by Liu *et al*. (2013) after the supplementation of hybrid tilapia diets 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 intestinal HSP70 was significantly up-regulated, down-regulated after 20 days and not different after 35 days when compared to the control treatment.

After six weeks, 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 (Voll *et al*. 1997). Since the GI tract is one of the key sites of interaction with the external environment (Ringø *et al*. 2007) 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 an issue. 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 (Foey and Picchietti 2014). 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 (Foey and Picchietti 2014).

TLR2 gene expression was up-regulated after six weeks 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 (Takeuchi *et al*. 1999), 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 *St. iniae* and *St. agalactiae*. Indeed, TLR2 was up-regulated in Mrigal carp (*Cirrhinus mrigala*) following *Streptococcus uberis* infection as well as *A. hydrophila* infection (Basu *et al*. 2012), 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 (Abid *et al*. 2013; Sun *et al*. 2014). Sun *et al*. (2014) reported an upregulation in both TLR2 and TLR5 in grouper after *Psychrobacter* sp. supplementation. Furthermore, authors demonstrated a higher expression of pro-inflammatory genes IL-1β and IL-8, and 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} for six weeks when compared to the control treatment. Importantly, 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 (Pirarat *et al*. 2011; He *et al*. 2013; Liu *et al*. 2013; Ren *et al*. 2013; Standen *et al*. 2013; Villamil *et al*. 2014). 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 which may have contributed to significantly lower mortality when exposed to *A. hydrophila* (Liu *et al*. 2013; Villamil *et al*. 2014).

This 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 studies where TGFβ was up-regulated after probiotic administration (He *et al*. 2013; Liu *et al*. 2013). 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 (Perez-Sanchez *et al*. 2011).

Histological analyses revealed a significantly larger population of IEL's in the mid intestine of tilapia after six weeks 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* (Standen *et al*. 2013), *L. rhamnosus* (Pirarat *et al*.

2011) or AquaStar® Growout (Chapter 3). Probiotic administration has led to increased IEL abundance in other commercially important fish species including European sea bass and gilthead sea bream (Salinas *et al*. 2008; Picchietti *et al*. 2009). Whilst the type of IEL cannot be eluded to in this study, Picchietti *et al*. (2009) characterised elevated T-cells and acidophilic granulocytes in the posterior intestine of European sea bass. Likewise, Salinas *et al*. (2008) reported higher levels of acidophilic granulocytes and Ig^+ cells in the posterior intestine of gilthead sea bream when fed a mixed probiotic (*Lactobacillus delbrüeckii* ssp *lactis* + *B. subtilis*). 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 in PRO-3 when compared to all other treatments after six weeks of probiotic supplementation. 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. In addition to mucin components (mucopolysaccharides) and glycoproteins, mucus also contains a number of secretory factors with a wide range of functions including pathogen antagonism (Ellis 2001; Whyte 2007). Applications of *L. rhamnosus* and *P. acidilactici* have also been reported to increase the number of goblet cells in tilapia (Pirarat *et al*. 2006; Standen *et al*. 2013). It remains to be seen however whether probiotics can modulate the compositional components within intestinal mucus.

This study was successful in recovering each probiotic species from tilapia digesta, a requirement which is important for potential probiotic candidates. Furthermore, probiotic supplementation was capable of modulating the composition of intestinal microbiota. This confirms results obtained in Chapter 3 where the effect of dietary AquaStar Growout® on the

intestinal microbiota in tilapia was demonstrated using high-throughput sequencing. Probiotic modulation of intestinal microbiota has also been achieved after dietary supplementation by *B. subtilis, P. acidilactici, B. amyloliquefaciens, E. faecium, Lactobacillus* sp. *L. plantarum, L. brevis, L. acidophilus* and *S. cerevisae* as well as multi-species applications (Shelby *et al*. 2006; Ferguson *et al*. 2010; Ridha and Azad 2012; He *et al*. 2013; Liu *et al*. 2013; Standen *et al*. 2013; Iwashita *et al*. 2015).

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 increased number of goblet cells and IEL's in the intestine, which may ultimately retard pathogen infection and translocation. Future work should focus on investigating the temporal effect of AquaStar® Growout on the localised immune response, as well as investigating the systemic immune response. Furthermore, it is important to study the molecular pathways which link TLR's with cytokines, as this could provide important information regarding probiotic mechanisms of action.

Chapter 5. Dietary supplementation of AquaStar® Growout elicits both a localised and systemic immune response in tilapia, *Oreochromis niloticus*

Abstract

The immunological status, intestinal morphology, intestinal microbiology and growth performance of tilapia, *O. niloticus*, were investigated after dietary administration of the commercial probiotic AquaStar® Growout. Tilapia (41.43 ± 0.18g) were split into three treatments; control (CON), continuous probiotic feeding (PRO-3) and pulsed probiotic feeding (PRO-PULSE) and fed for five weeks. At weekly intervals samples of intestine and head-kidney (HK) were taken to assess the gene expression of TLR2, MYD88, NFκB and inflammatory cytokines. TLR2, TNFα, IL-1β, TGFβ and IL-10 were all up-regulated by probiotic treatments. The gene expression of MYD88 was not affected by dietary treatment in the intestine, but was up-regulated in probiotic treatments in the HK. Furthermore, the gene expression of intestinal NFκB was significantly down-regulated in both probiotic treatments when compared to the control. However, no differences were observed with NFκB mRNA levels in the HK indicating that cytokines are activated by different molecular pathways depending on the organ. The extent of the up/down-regulation in the genes of interest was dependent on the probiotic treatment (i.e. continuous or pulsed), the duration of feeding (i.e. two, three, four or five weeks) and the tissue investigated (i.e. intestine or HK). Histological appraisal revealed that after five weeks, tilapia in PRO-3 demonstrated a significantly larger abundance of intraepithelial leucocytes (IELs) and goblet cells when compared to CON. Within whole blood of fish, there were significantly higher circulating leucocytes in PRO-3 when compared with CON. Together these results demonstrate that AquaStar® Growout can improve the barrier function as well as the localised and systemic immune response. These

improvements may provide the host with greater protection when challenged with a potential pathogen. High-throughput sequencing revealed that dietary treatment had little effect on the microbial communities within the intestine. This highlights that microbial modulation is not always necessary when bringing benefits to immune responses. Importantly, all the improvements described were at no detriment to growth performance which remained unaffected by probiotic supplementation.

5.1 Introduction

Probiotics have been successful in protecting tilapia from a number of pathogens including *A. hydrophila, Ed. tarda, Fl. columnare, Pr. vulgaris, Ps. fluorescens, St. iniae* and *St. agalactiae* (Pirarat *et al*. 2006; Aly *et al*. 2008a; Mohamed and Ahmed Refat 2011; Abdel-Tawwab 2012; Ng *et al*. 2014; Villamil *et al*. 2014; Iwashita *et al*. 2015; Ridha and Azad 2015). The mechanisms behind elevated protection are unclear and likely differ depending on probiotic application. Probiotics may limit pathogenic invasion via the production of inhibitory substances (Apún-Molina *et al*. 2009; Abumourad *et al*. 2013; Villamil *et al*. 2014), or by reducing the available receptor sites or nutrients which are needed by pathogens (Verschuere *et al*. 2000; Ren *et al*. 2013). It has also been shown that the administration of inactivated bacteria can also bring immune benefits to the host (Taoka *et al*. 2006). Considering inactivated bacteria could not compete for adhesion sites or nutrients, nor could they produce inhibitory substances, it is clear that probiotics can activate and modulate the mucosal immune system. The dietary supplementation of *L. rhamnosus, P. acidilactici* and AquaStar® Growout have previously been reported to reinforce the intestinal barrier defence in tilapia by means of increasing IELs and goblet cells (Pirarat *et al*. 2011; Standen *et al*. 2013; Chapters 3 and 4).

There is a paucity of information detailing the molecular interactions between probiotics and mucosal immunity in fish. The recognition of microbes in the intestine is mediated by PRRs, including TLRs. TLRs recognise a broad range of microbe/pathogen associated molecular patterns (MAMPs/PAMPs) and are expressed according to location of MAMP/PAMP exposure. Teleosts express multiple TLRs including 1, 2, 3, 4, 5, 5S, 7, 8, 9, 13, 14, 18, 19, 20, 21, 22 and 23 which recognise and respond to both bacterial and viral ligands (Rebl *et al*. 2010). Once bound, adaptor proteins, such as myeloid differentiation factor 88 (MYD88) are recruited. This induces the activation of transcription factors such as nuclear factor-kappa B (NFκB) which results in the production of cytokines and antimicrobial proteins (Zhu *et al*. 2013). In a recent study, Sun *et al.* (2014) demonstrated that grouper fed viable *Psychrobacter* sp. resulted in an up-regulation of intestinal TLR2, TLR5, MYD88 and cytokines IL-1β, TGFβ and IL-8. Furthermore, Chapter 4 reported that supplementing tilapia diets with AquaStar® Growout at 3 g kg^{-1} resulted in an up-regulation of intestinal gene expression of TLR2 and a corresponding up-regulation in cytokines TNFα, IL-1β, TGFβ an IL-10.

As well as improvements to localised immunity as highlighted in Chapters 3 and 4, probiotic supplementation can improve the systemic immunity in tilapia. These benefits can manifest themselves via modifications to blood constituents (such as serum lysozyme activity, circulating leucocytes etc.) or through the gene expression of cytokines in other immuno-important organs such as the HK and spleen (Taoka *et al*. 2006; Aly *et al*. 2008b; Mehrim 2009; Ali *et al*. 2010; Pirarat *et al*. 2011; Ridha and Azad 2012; Liu *et al*. 2013; Villamil *et al*. 2014; Ridha and Azad 2015).

The aim of this trial was to assess the temporal effects of probiotics on both the localised and systemic immune system through the analysis of intestinal and HK gene

expression, haemato-immunology and histology. Intestinal microbiology and growth performance were also investigated.

5.2 Materials and methods

5.2.1 Experimental design and dietary preparation

Four hundred and fifty tilapia were randomly distributed to nine 500L concrete tanks (50 fish per tank; average weight = 41.43 ± 0.18g) which were supplied by freshwater sourced from the local river system, on a flow-through basis (Fig 5.1). Commercial diets (No. 461; 32% protein, 5% lipid) were obtained from INTEQC Feed Co. Ltd., Thailand. The commercial diet was ground in a blender to form a fine powder, sieved to remove large particles and weighed into 1kg batches. AquaStar® Growout was added to the ground diet at 3g kg^{-1} (Table 5.1). The diet was mixed thoroughly to ensure a homogenous mix and warm water was added to form a consistency suitable for cold press extrusion. Once extruded, diets were placed in aluminium trays and dried in an air convection oven for 24 hours. The basal diet served as the control diet and was prepared in the same manner, without the addition of probiotic. Diets were analysed for proximate composition as described in section 2.4 (Table 5.1). Treatments were as follows; control (basal diet void of AquaStar® Growout), continuous probiotic feeding (continuous feeding of the basal diet supplemented with AquaStar® Growout at 3g kg^{-1}) and probiotic pulse feeding (alternating weekly between AquaStar® Growout feeding at 3g kg^{-1} and the basal diet). Diet codes were assigned for ease of analysis (Table 5.2). Fish were fed experimental diets for five weeks at a rate of 2.5- 5% biomass per day in four equal rations and sampling took place at weekly intervals (two, three, four and five weeks). Water quality parameters were monitored and maintained as described in section 2.1 with the exception of water temperature which was approximately 31 ± 1°C.

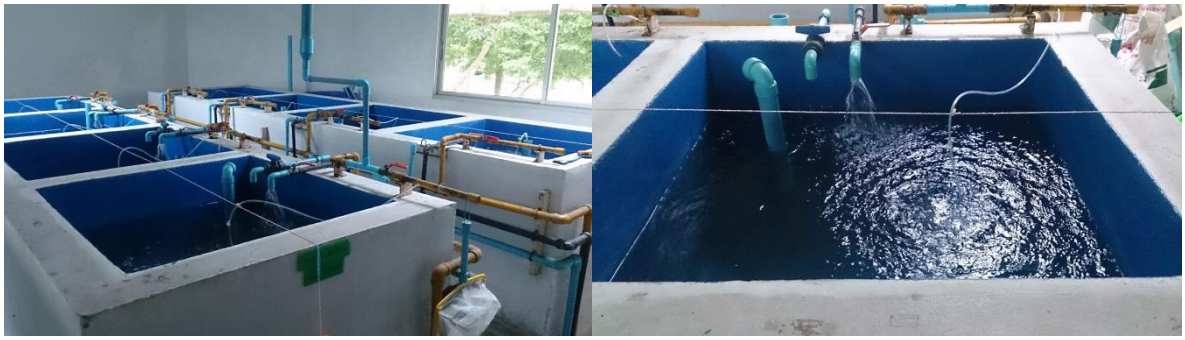


Figure 5.1: Flow through system where tilapia were held during trial III. The experimental system consisted of concrete tanks with a capacity of 500L. Water originated from the local river system and was monitored daily (pH, DO and temperature) and weekly (nitrogen waste) to ensure conditions were appropriate for tilapia.

5.2.2 Growth performance

Growth performance and feed utilisation were assessed by final weight, weight gain, feed intake, SGR, FCR and PER as described in section 2.5.

Table 5.1: Dietary formulation and proximate composition (%).

	Basal	3g kg^{-1}
Commercial feed[a]	100.00	99.70
AquaStar® Growout[b]	0.00	0.30
Proximate composition (% as fed basis)		
Moisture	6.80 ± 0.11	6.84 ± 0.02
Crude protein	33.97 ± 0.51	34.16 ± 0.20
Lipid	6.06 ± 0.60	6.87 ± 0.22
Ash	13.32 ± 0.04	13.26 ± 0.01
Fibre	3.36 ± 0.12	3.58 ± 0.14
Energy (MJ kg^{-1})	18.08 ± 0.31	17.77 ± 0.06

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

[b] Biomin Holding GmbH, Industriestrasse 21, 3130 Herzogenburg, Austria.

Table 5.2: Codes assigned to dietary treatments.

Dietary code	Diet
CON	Continuous feeding of basal diet (without probiotic)
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 3g kg^{-1} and the basal diet

5.2.3 RT-PCR

At each sampling point, the mid-intestine and HK were isolated from three fish per tank (*n* = 9). Samples were stored separately in RNALater and kept at -20°C until transportation. Upon arrival in Plymouth, samples were immediately transferred to -80°C. Both organs were assessed for the gene expression of TLR2, MYD88, NFκB, TNFα, IL-1β TGFβ and IL-10. HK samples were processed as described in section 2.11. RNA was extracted from intestinal samples in the same manner as described in section 2.11. RT-PCR of intestinal samples were performed using the SYBR Green method in an iQ5 iCycler thermal cycler (Bio-Rad) and duplicate PCR reactions were set on a 96 well plate. Each reaction contained 1 µl of diluted (1/20 in molecular grade water) cDNA, 5 µl of 2X concentrated iQ™ SYBR Green Supermix (Bio-Rad), 0.3 µM of forward and 0.3 µM of reverse primer. The thermal profile for all reactions was 3 min at 95°C, and then 45 cycles of 20 s at 95°C, 20 s at 60°C and 20 s at 72°C. All quality control steps occurred as described in section 2.11. Primer sequences and efficiencies for intestinal and HK samples are displayed in Tables 5.3 and 5.4, respectively. The data obtained for both tissues (HK and intestine) were analysed using the iQ5 optical system software version 2.0 (Bio-Rad).

Table 5.3: Primer sequences for RT-PCR of intestinal samples.

Gene	Forward 5’ - 3’	Reverse 5’ – 3’	Amplicon size	Tm (°C)	E-value	GenBank number
β-actin	GGGTCAGAAAGACAGCTACGTT	CTCAGCTCGTTGTAGAAGGTGT	144	56.7	1.94	XM_003443127.2
TLR2	GCAGTGCCTTGAGTCTTGATC	ACCGTGGAGATCGAGAACCT	101	59.6	1.92	XM_005460165
MYD88	AGCTCGAAGTAAACGCCTGAT	ACAAATGGTGAGGAAGCGTAAA	85	57.2	1.91	KJ130039
NFκB	CACTCGTCCGACTGCTCTAG	TCTCCTCCAGCTCCCGATAC	82	61.4	1.98	XM_005462791.1
TNFα	TTCAGGGTGATCTGCGG	CCCAGGTAAATGGCGTTGTA	197	54.9	1.91	NM_001279533.1
IL-1β	TGGTGACTCTCCTGGTCTGA	GCACAACTTTATCGGCTTCCA	86	58.7	1.97	XM_005457887.1
TGFβ	TGCCAAGGTGCTTAACAGGT	ATCCCCGACGTTACTCCGTA	118	57.3	1.94	XM_003459454.2
IL-10	CTTCTCAGACCGTCCTCCTG	AGGAGTCTTCGACGGACTGA	216	57.0	1.94	XM_003441366.2

Table 5.4: Primer sequences for RT-PCR of HK samples.

Gene	Forward 5’ - 3’	Reverse 5’ – 3’	Amplicon size	Tm (°C)	E-value	GenBank number
β-actin	TGACCTCACAGACTACCTCATG	TGATGTCACGCACGATTTCC	89	58.8	2.03	KJ126772.1
GAPDH	CCGATGTGTCAGTGGTGGAT	GCCTTCTTGACGGCTTCCTT	82	59.4	1.98	JN381952.1
EF1α	TGATCTACAAGTGCGGAGGAA	GGAGCCCTTTCCCATCTCA	80	58.4	2.00	AB075952.1
TLR2	GCAGTGCCTTGAGTCTTGATC	ACCGTGGAGATCGAGAACCT	101	59.6	1.99	XM_005460165
MYD88	AGCTCGAAGTAAACGCCTGAT	ACAAATGGTGAGGAAGCGTAAA	85	57.2	2.05	KJ130039
NFκB	CACTCGTCCGACTGCTCTAG	TCTCCTCCAGCTCCCGATAC	82	61.4	1.67	XM_005462791.1
TNFα	CCAGAAGCACTAAAGGCGAAGA	CCTTGGCTTTGCTGCTGATC	82	59.9	1.99	AY428948.1
IL-1β	TGGTGACTCTCCTGGTCTGA	GCACAACTTTATCGGCTTCCA	86	58.7	1.92	XM_005457887.1
TGFβ	GTTTGAACTTCGGCGGTACTG	TCCTGCTCATAGTCCCAGAGA	80	59.8	1.89	XM_003459454.2
IL-10	CTGCTAGATCAGTCCGTCGAA	GCAGAACCGTGTCCAGGTAA	94	59.6	1.99	XM_003441366.2

5.2.4 Intestinal histology

At each sampling point, three fish per tank (n = 9) were sampled to asses perimeter ratio, IEL and goblet cell abundance as described in section 2.7.

5.2.5 Haemato-immunological analyses

At each sampling point, blood samples were taken to assess haematocrit, haemoglobin, blood cell counts, MCV, MCH and MCHC (n = 9). Serum was collected to assess serum lysozyme activity (n = 15). All sampling and analyses was carried out according to section 2.10. Furthermore leucocyte differential counts were conducted to determine the proportions of circulating white blood cells (n = 9). For this, a blood smear was prepared by the addition of 5 µl of whole blood onto a clean slide. A second slide was used to smear the blood sample down the length of the slide in order to achieve a monofilm layer of cells. Blood smears were left to dry, fixed in methanol for 15 min and stained using May Grünwald stain (diluted 1:1 with Sorensen's buffer, pH 6.8). Slides were then rinsed in Sorensen's buffer and counter stained with Giemsa satin (diluted 1:9 with Sorensen's buffer, pH 6.8). After a final rinse in buffer, slides were left to dry and cover slips were added using DPX mountant. All blood smears were examined under 400 x magnification. One hundred white blood cells were counted to quantify the proportions of lymphocytes, monocytes and granulocytes (Fig 5.2).

5.2.6 High-throughput sequencing

After four and five weeks, digesta was isolated from the mid-intestine of tilapia from two fish per tank (n = 6). All microbiology samples were stored and transported to Plymouth University in 100% molecular grade ethanol. DNA was extracted using the QIAamp Stool

Mini Kit (Qiagen) as described in section 2.8.3 with an extra half hour for the lysozyme pre-treatment. High-throughput sequencing was used to assess allochthonous bacterial communities as described in section 2.8.5.

5.2.7 Statistical analyses

Statistical analyses were carried out as described in section 2.12.

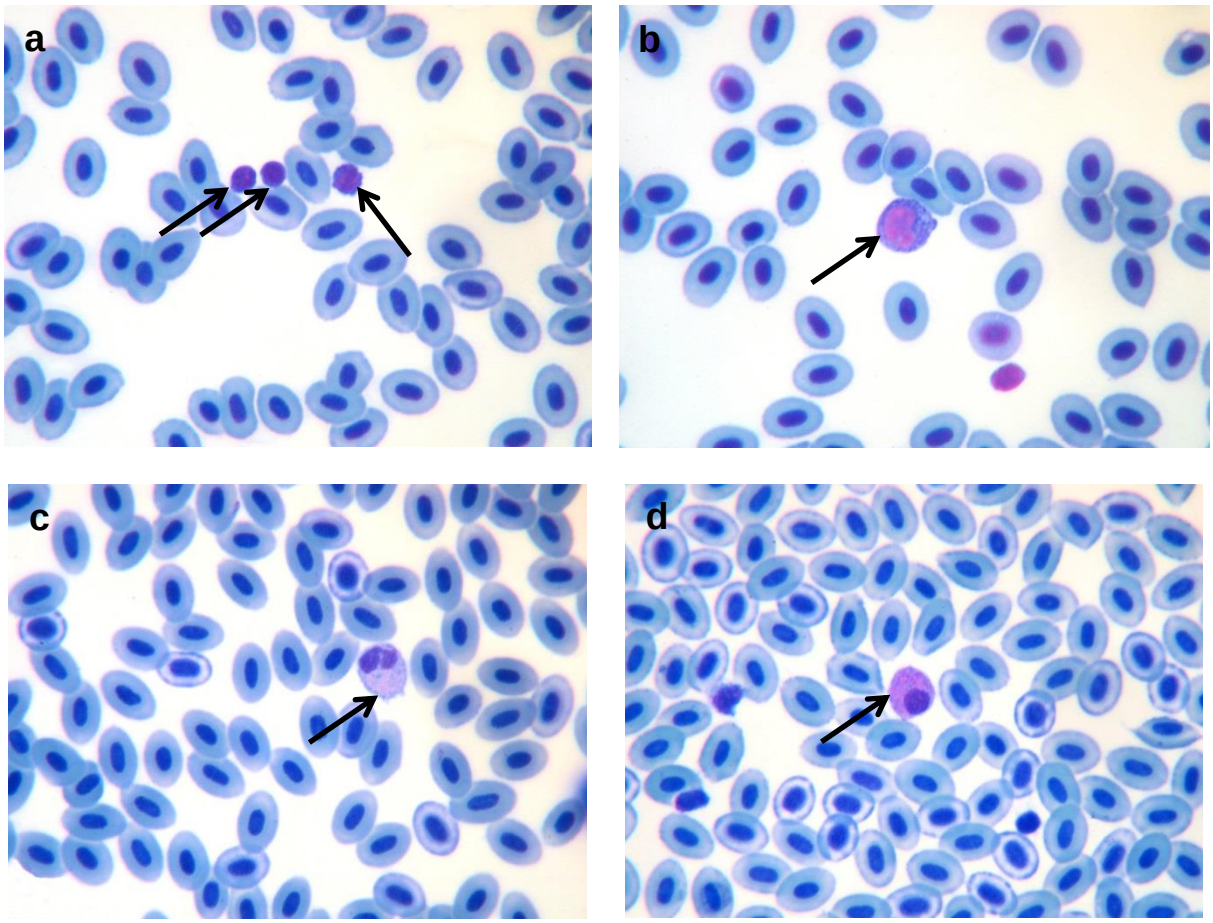


Figure 5.2: Characterisation of tilapia leucocyte sub-populations stained with May Grünwald and Giemsa: (a) lymphocyte, (b) monocyte, (c) granulocyte (neutrophil) and (d) granulocyte (eosinophil).

5.3 Results

5.3.1 Growth performance

Growth performance was assessed by means of final weight, weight gain, feed intake, PER, FCR and SGR (Table 5.5). Fish in all treatments showed excellent growth characteristics with 100% survival in each treatment. No differences were observed between treatments in any parameter measured.

Table 5.5: Growth performance of tilapia after five weeks of feeding experimental diets.

	CON	PRO-3	PRO-PULSE
Initial weight (g fish^{-1})	41.43 ± 0.09	41.21 ± 0.12	41.65 ± 0.23
Average weight (g fish^{-1})	103.86 ± 2.48	106.72 ± 1.51	107.92 ± 2.69
Weight gain (g fish^{-1})	62.42 ± 2.49	65.51 ± 1.44	66.27 ± 2.60
Feed intake (g fish^{-1})	65.08 ± 0.31	66.37 ± 1.35	66.56 ± 1.13
PER	3.19 ± 0.25	3.21 ± 0.13	3.27 ± 0.03
FCR (g g^{-1})	0.99 ± 0.04	0.99 ± 0.02	0.98 ± 0.00
SGR (% day^{-1})	3.06 ± 0.02	3.17 ± 0.04	3.17 ± 0.08
Survival	100 ± 0.00	100 ± 0.00	100 ± 0.00

5.3.2 RT-PCR

Relative intestinal gene expression of TLR2, MYD88 and NFκB were analysed at each time point. After two and five weeks of experimental feeding, the intestinal gene expression of TLR2 was significantly higher in PRO-3 when compared with both CON and PRO-PULSE. At these time points, there was no difference in gene expression levels between CON and PRO-PULSE treatments. After three and four weeks of experimental feeding, TLR2 expression was significantly higher in PRO-PULSE when compared with CON, but significantly lower than PRO-3, where gene expression was highest (Fig 5.3a). The intestinal gene expression of MYD88 was not affected by dietary treatment (Fig 5.3b). Initially, after

two weeks of experimental feeding, intestinal gene expression of NFκB was similar in all treatments. By week three the abundance of NFκB mRNA had significantly reduced in both probiotic treatments, PRO-3 and PRO-PULSE, when compared with CON. At week three there was no difference between PRO-3 and PRO-PULSE. After four and five weeks, similar results were observed with regards to the gene expression of intestinal NFκB. At both of these time points the expression was significantly higher in the CON fed fish when compared to both PRO-3 and PRO-PULSE, but PRO-PULSE samples demonstrated significantly higher expression than PRO-3 samples (Fig 5.3c).

The relative intestinal expression of cytokines TNFα, IL-1β, IL-10 and TGFβ were also analysed at weeks two, three, four and five. At each time point the gene expression of TNFα was significantly higher in PRO-3 fed fish when compared with PRO-PULSE. In each case, expression levels observed in PRO-PULSE samples were significantly higher than CON samples which had the lowest mRNA levels (Fig 5.4a). With the exception of week two, similar results were observed with regards to intestinal gene expression of IL-1β (Fig 5.4b). The highest expression levels were observed in PRO-3 samples, these were significantly higher than those of the PRO-PULSE which in turn had significantly higher expression levels when compared to CON. At week two there were no differences in IL-1β expression between dietary treatments.

After two weeks of experimental feeding, the abundance of TGFβ mRNA was significantly higher in treatment PRO-3 when compared to both PRO-PULSE and CON. There were no differences in expression levels between PRO-PULSE and PRO-3. After three and four weeks, TGFβ expression levels remained unaffected by dietary treatment. After five weeks, both probiotic treatments, PRO-3 and PRO-PULSE, resulted in significantly lower levels of TGFβ mRNA levels when compared with CON but expression was not different between PRO-3 and PRO-PULSE (Fig 5.4c). At each time point the intestinal expression of

IL-10 was highest in PRO-3. At week two, gene expression was significantly higher in PRO-3 when compared to both CON and PRO-PULSE; no differences were observed between these treatments however. After weeks three, four and five, IL-10 gene expression was significantly higher in PRO-3 samples when compared to PRO-PULSE, which in turn displayed significantly higher expression levels than those in CON (Fig 5.4d).

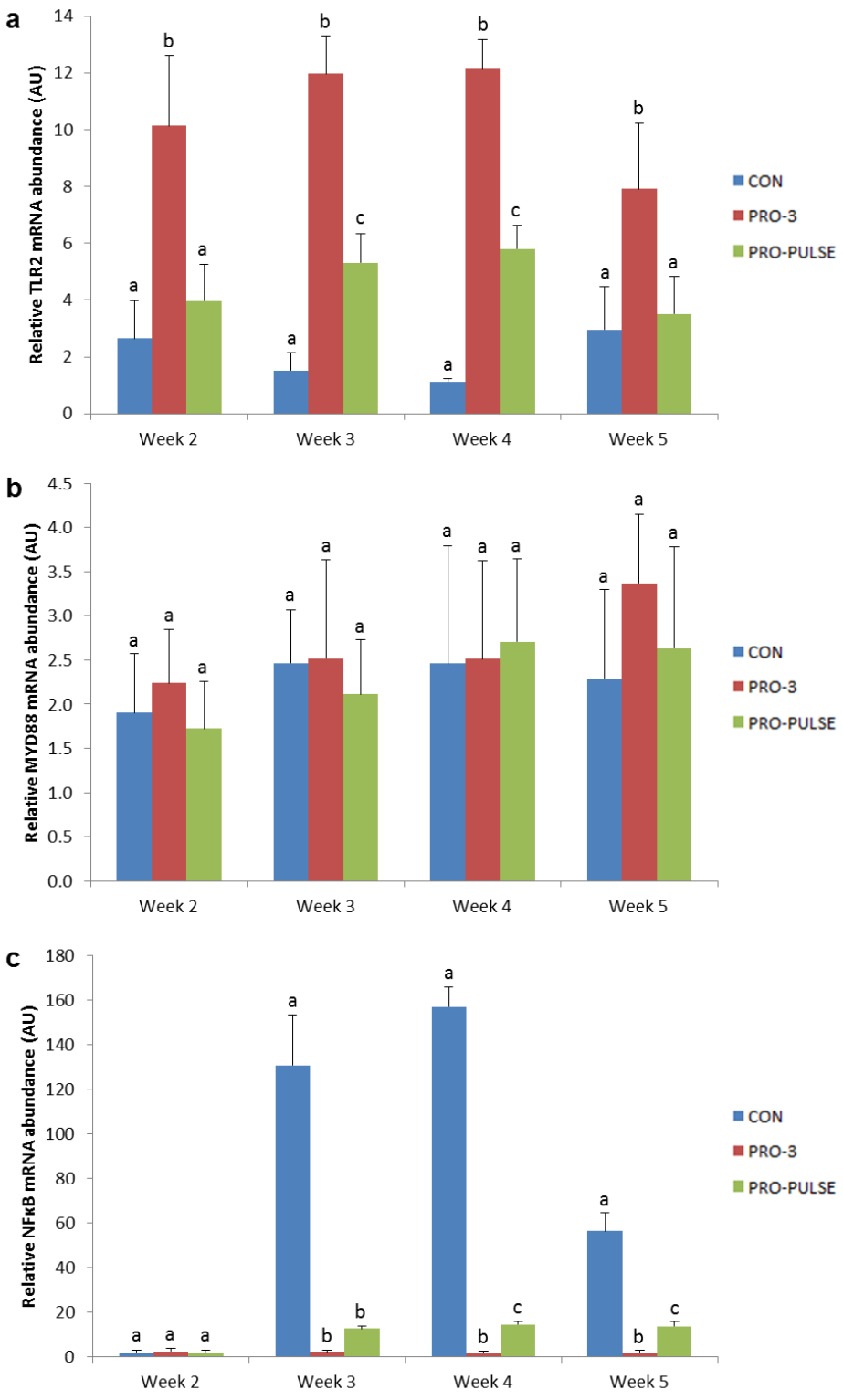


Figure 5.3: Effects of dietary treatment on the relative gene expression of intestinal TLR2 (a), MYD88 (b) and NFκB (c) after different feeding periods. Values are presented as means ± standard deviation. Different superscripts within each time point indicate a significant difference ($P < 0.05$).

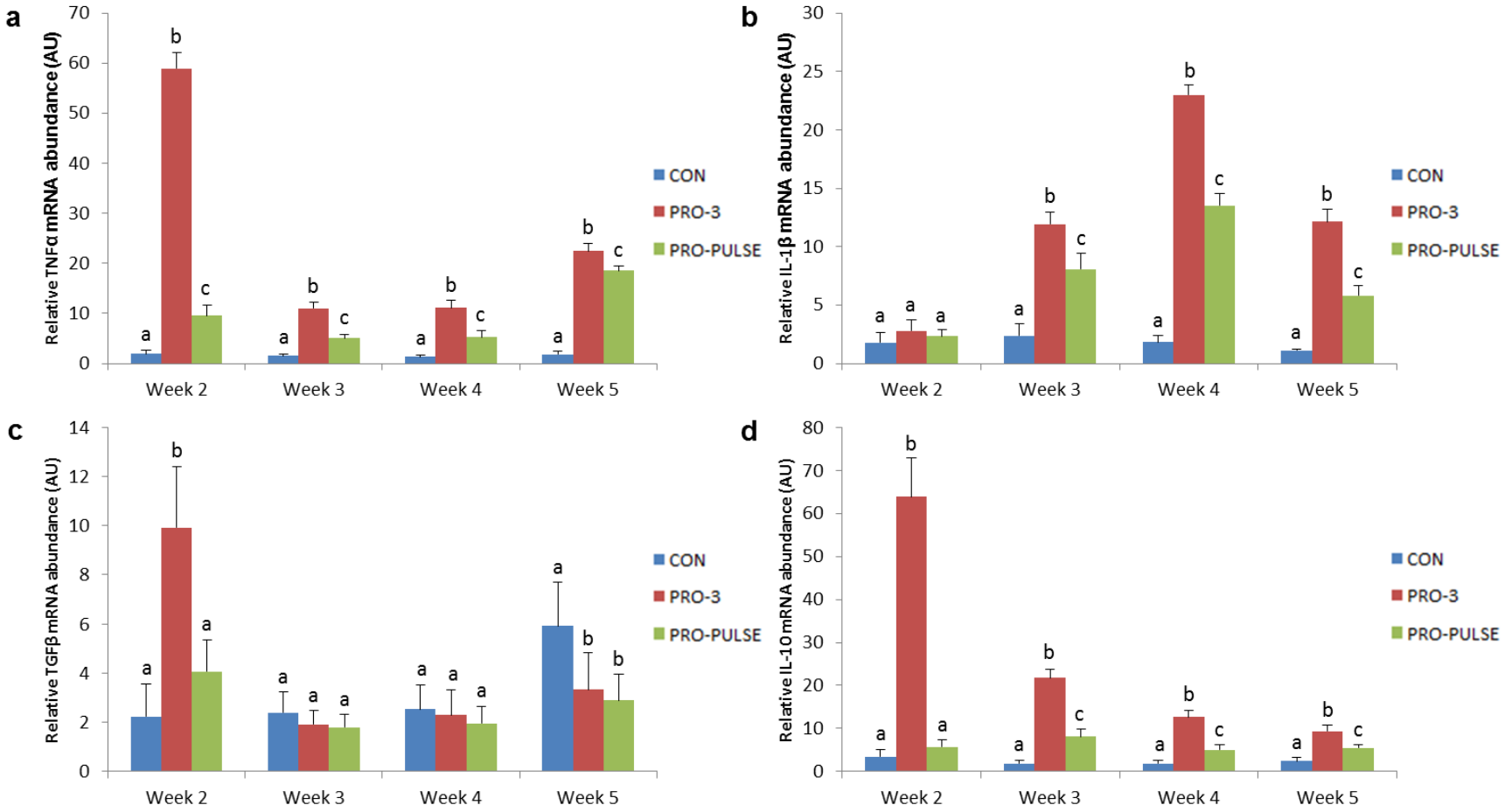


Figure 5.4: Effects of dietary treatment on the relative gene expression of intestinal TNFα (a), IL-1β (b), TGFβ (c) and IL-10 (d) after different feeding periods. Values are presented as means ± standard deviation. Different superscripts within each time point indicate a significant difference ($P < 0.05$).

To evaluate whether dietary probiotics can affect systemic immunity, the gene expression of TLR2, MYD88, NFκB, TNFα, IL-1β, TGFβ and IL-10 were assessed in HK tissues. The dietary supplementation of probiotics did not result in any changes to the HK TLR2, MYD88, NFκB or cytokine gene expression until week five. Furthermore no obvious patterns emerged. After five weeks, the continuous supplementation of AquaStar Growout® (PRO-3) significantly increased the HK gene expression of both TLR2 and MYD88 when compared to CON. The relative HK gene expression of TLR2 in PRO-PULSE treated fish did not differ from that of either PRO-3 or CON treatments (Fig 5.5a). In the case of MYD88, the elevated expression in PRO-3 was not statistically different to that of PRO-PULSE, but expression levels were significantly higher in PRO-PULSE when compared to that of the CON samples (Fig 5.5b). The relative abundance of NFκB in the HK was not affected by dietary treatment at any time point assessed (Fig 5.5c).

The relative expression of TNFα was significantly up-regulated in treatments PRO-3 and PRO-PULSE when compared to CON. Despite the highest expression being observed in PRO-3, there were no significant differences between the two probiotic treatments with regards to TNFα gene expression (Fig 5.6a). The HK expression of pro-inflammatory gene IL-1β was also significantly higher in PRO-3 when compared with CON but not PRO-PULSE. PRO-PULSE revealed numerically higher expression of IL-1β than that of CON but there were no significant differences between the two treatments (Fig 5.6b). The HK of fish belonging to PRO-3 revealed a significant up-regulation of TGFβ and IL-10 when compared to CON. In both cases the expression of TGFβ and IL-10 in PRO-PULSE did not differ from either PRO-3 or CON treatments (Figs 5.6c and 5.6d, respectively).

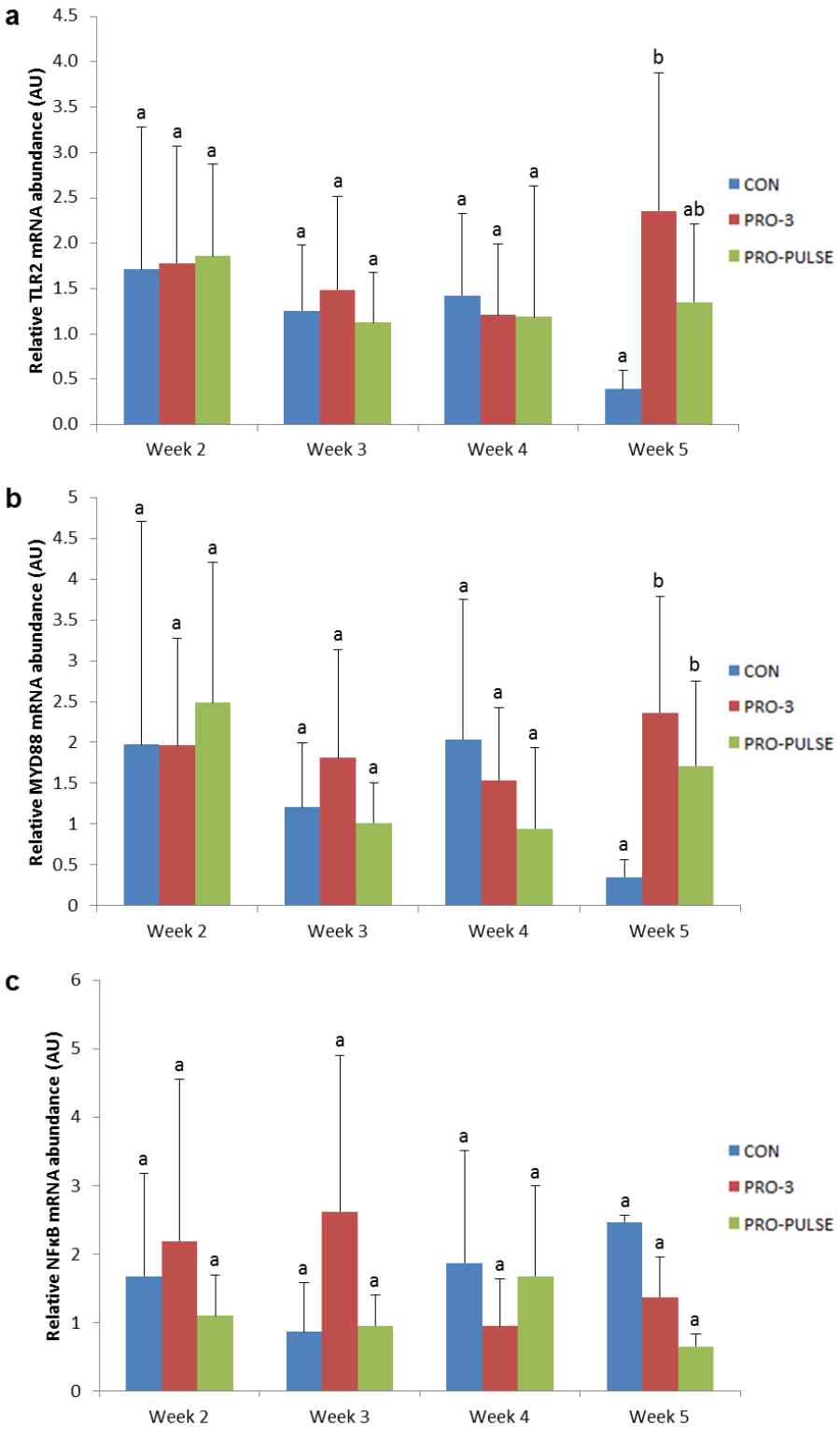


Figure 5.5: Effects of dietary treatment on the relative gene expression of HK TLR2 (a), MYD88 (b) and NFκB (c) after different feeding periods. Values are presented as means ± standard deviation. Different superscripts, within each time point, indicate a significant difference ($P < 0.05$).

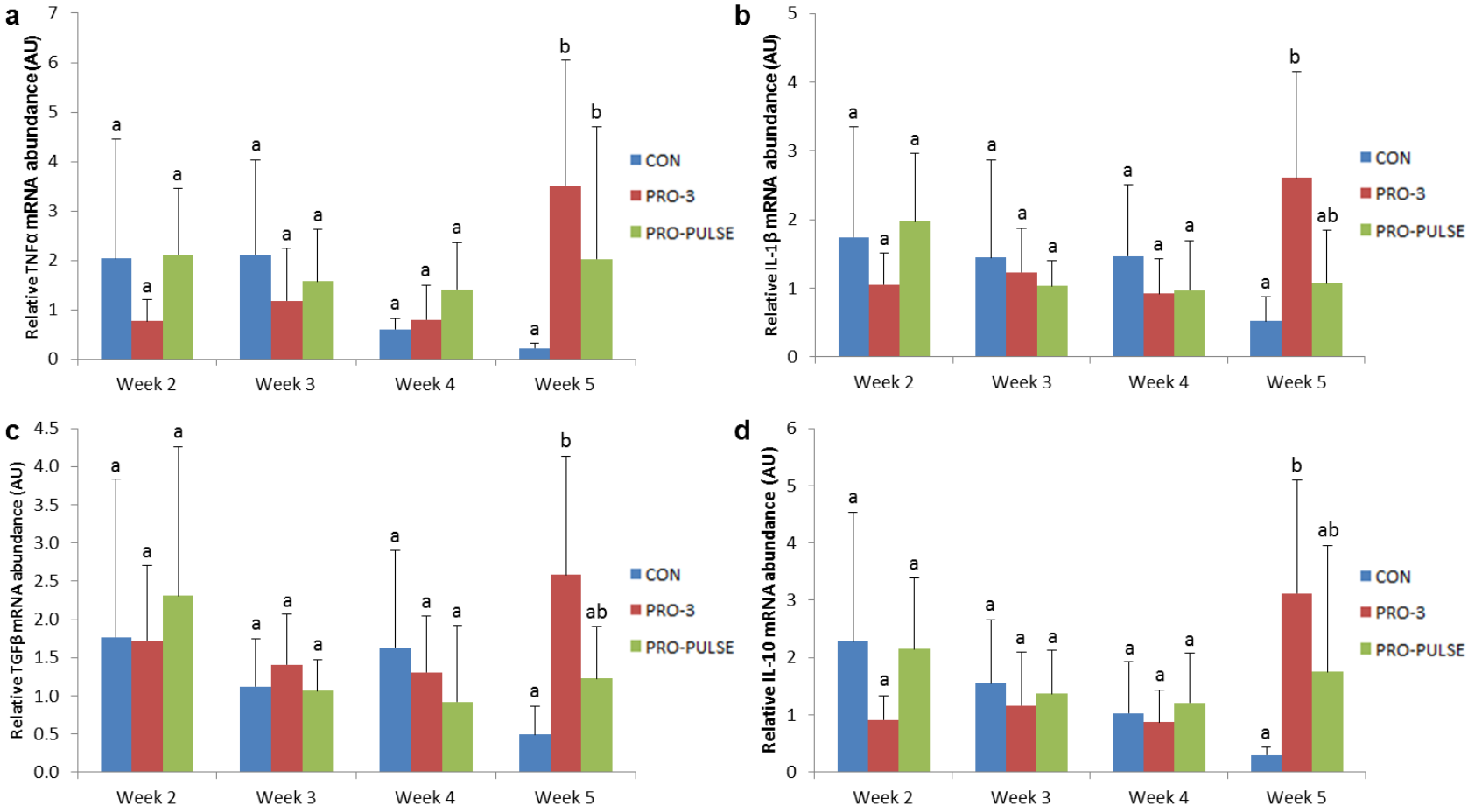


Figure 5.6: Effects of dietary treatment on the relative gene expression of HK TNFα (a), IL-1β (b), TGFβ (c) and IL-10 (d) after different feeding periods. Values are presented as means ± standard deviation. Different superscripts, within each time point, indicate a significant difference ($P < 0.05$).

5.3.3 Intestinal histology

At each sampling time point, a histological appraisal of the mid-intestine of tilapia was conducted by the measurement of the perimeter ratio, IEL and goblet cell numbers (Table 5.6). With the exception of week four, the perimeter ratio was highest in the probiotic treatments when compared with the control, however, no statistical difference was observed at any time point. The abundance of IELs and goblet cells were not affected by dietary treatment from weeks two to four. In probiotic treatments, IELs showed an increasing abundance through the investigation and after five weeks there were significantly higher populations of IELs in treatment PRO-3 (40.95 ± 7.04 per 100 µm) when compared to CON (29.50 ± 4.59 per 100 µm), but were not different to PRO-PULSE (33.43 ± 6.98 per 100 µm). CON and PRO-PULSE treatments did not differ from each other with regards to IEL abundance. The same patterns were observed with respect to the abundance of goblet cells residing in the mid-intestine of tilapia. In probiotic treatments, the number of goblet cells showed a gradual increase as the trial progressed until week five where they were significantly more abundant in PRO-3 (7.55 ± 2.49 per 100 µm) when compared to CON (4.77 ± 1.46 per 100 µm) but not PRO-PULSE (5.70 ± 2.05 per 100 µm). CON and PRO-PULSE treatments did not differ from each other with regards to goblet cell abundance.

Table 5.6: Histological data from the mid-intestine of tilapia after two, three, four and five weeks of experimental feeding.

	CON	PRO-3	PRO-PULSE
Week 2			
Perimeter ratio (AU)	2.68 ± 0.50	3.25 ± 1.08	2.90 ± 0.54
IEL's (per 100 µm)	30.22 ± 2.52	31.17 ± 3.74	29.93 ± 3.58
Goblet cells (per 100 µm)	3.79 ± 0.53	4.06 ± 0.67	4.38 ± 0.63
Week 3			
Perimeter ratio (AU)	2.70 ± 0.71	2.97 ± 0.88	3.33 ± 0.96
IEL's (per 100 µm)	31.43 ± 2.95	29.84 ± 1.76	31.23 ± 3.56
Goblet cells (per 100 µm)	4.59 ± 0.43	5.07 ± 0.82	5.16 ± 0.54
Week 4			
Perimeter ratio (AU)	3.70 ± 1.02	3.52 ± 0.73	3.38 ± 0.46
IEL's (per 100 µm)	30.60 ± 3.00	34.82 ± 2.66	33.37 ± 4.93
Goblet cells (per 100 µm)	3.71 ± 0.98	5.14 ± 1.17	4.52 ± 1.32
Week 5			
Perimeter ratio (AU)	3.28 ± 0.53	3.49 ± 1.27	3.52 ± 0.65
IEL's (per 100 µm)	29.50 ± 4.59^{a}	40.95 ± 7.04^{b}	33.43 ± 6.98^{ab}
Goblet cells (per 100 µm)	4.77 ± 1.46^{a}	7.55 ± 2.49^{b}	5.70 ± 2.05^{ab}

$^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

5.3.4 Haemato-immunological analyses

At each sampling time point, the haemato-immunological status of tilapia was assessed by the measurement of haematocrit, haemoglobin, erythrocyte and leucocyte counts, MCV, MCH, MCHC and serum lysozyme activity (Table 5.7). After two weeks of experimental feeding, the haemoglobin concentration was significantly higher in PRO-PULSE fed fish (11.67 ± 1.38 g dl^{-1}) when compared with CON fed fish (9.30 ± 1.32 g dl^{-1}), but it was not different to PRO-3 (10.70 ± 0.96 g dl^{-1}). As a result of this, MCHC was also significantly higher in the PRO-PULSE treatment (30.37 ± 3.57 g dl^{-1}) when compared to both CON and PRO-3 treatments (22.60 ± 2.57 and 25.78 ± 1.65 g dl^{-1}). Haematocrit, erythrocyte levels, leucocyte levels, MCV, MCH and serum lysozyme activity remained unaffected by dietary treatment after two weeks.

295 **Table 5.7:** Haemato-immunological data from tilapia after experimental feeding at each
296 sampling point.

	CON	PRO-3	PRO-PULSE
Week 2			
Haematocrit (%PCV)	41.11 ± 3.03	41.56 ± 3.17	38.78 ± 4.98
Haemoglobin (g dl^{-1})	9.30 ± 1.32[a]	10.70 ± 0.96[ab]	11.67 ± 1.38[b]
RBC (10^6 μl^{-1})	1.26 ± 0.42	1.40 ± 0.43	1.55 ± 0.32
WBC (10^3 μl^{-1})	10.71 ± 0.66	10.38 ± 0.95	10.65 ± 1.16
MCV (fL)	379.48 ± 174.70	324.29 ± 103.01	259.18 ± 53.39
MCH (pg)	86.69 ± 45.30	83.06 ± 24.93	78.42 ± 18.38
MCHC (g dl^{-1})	22.60 ± 2.57[a]	25.78 ± 1.65[a]	30.37 ± 3.57[b]
Serum lysozyme activity (AU)	356.08 ± 79.25	365.82 ± 100.27	385.38 ± 84.27
Week 3			
Haematocrit (%PCV)	40.67 ± 3.46[ab]	43.78 ± 2.25[b]	40.00 ± 2.11[a]
Haemoglobin (g dl^{-1})	11.69 ± 1.55	13.27 ± 1.55	12.62 ± 1.29
RBC (10^6 μl^{-1})	1.47 ± 0.52	1.41 ± 0.32	1.27 ± 0.25
WBC (10^3 μl^{-1})	12.67 ± 1.92	12.17 ± 0.64	12.00 ± 1.93
MCV (fL)	311.72 ± 103.55	326.91 ± 81.60	329.70 ± 75.98
MCH (pg)	93.53 ± 43.42	98.86 ± 26.08	103.77 ± 24.29
MCHC (g dl^{-1})	28.91 ± 4.36	30.23 ± 2.47	31.53 ± 2.52
Serum lysozyme activity (AU)	389.95 ± 95.82	420.51 ± 100.08	409.92 ± 77.36
Week 4			
Haematocrit (%PCV)	41.67 ± 2.67	39.67 ± 3.68	39.00 ± 3.06
Haemoglobin (g dl^{-1})	12.48 ± 2.69	11.70 ± 1.57	11.06 ± 1.53
RBC (10^6 μl^{-1})	1.44 ± 0.38[a]	1.24 ± 0.22[ab]	0.95 ± 0.18[b]
WBC (10^3 μl^{-1})	14.06 ± 5.37	15.46 ± 2.33	10.90 ± 1.05
MCV (fL)	314.46 ± 93.36[a]	329.66 ± 61.31[ab]	426.64 ± 89.97[b]
MCH (pg)	95.14 ± 36.90	97.03 ± 19.87	122.37 ± 33.79
MCHC (g dl^{-1})	29.87 ± 5.41	29.63 ± 3.88	28.58 ± 4.87
Serum lysozyme activity (AU)	406.62 ± 102.15	438.07 ± 105.57	474.57 ± 80.59
Week 5			
Haematocrit (%PCV)	42.25 ± 2.05	43.43 ± 3.70	45.00 ± 3.43
Haemoglobin (g dl^{-1})	11.43 ± 2.10	10.14 ± 2.39	10.59 ± 1.05
RBC (10^6 μl^{-1})	1.09 ± 0.39	1.17 ± 0.21	0.92 ± 0.18
WBC (10^3 μl^{-1})	10.29 ± 3.43[a]	16.62 ± 3.96[b]	12.79 ± 3.66[ab]
MCV (fL)	432.01 ± 148.66	379.24 ± 82.32	530.37 ± 121.31
MCH (pg)	113.63 ± 28.17	91.51 ± 32.42	120.25 ± 27.88
MCHC (g dl^{-1})	26.78 ± 5.62	21.50 ± 1.75	23.86 ± 2.87
Serum lysozyme activity (AU)	290.68 ± 85.74	345.54 ± 65.73	282.43 ± 91.53

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

After three weeks of experimental feeding, only haematocrit presented a statistical difference. Haematocrit was significantly higher in PRO-3 treatment (43.78 ± 2.25 %PCV) when compared with PRO-PULSE (40.00 ± 2.11 %PCV), but was not different to the CON fed fish (40.67 ± 3.46 %PCV). Haemoglobin, erythrocyte and leucocyte levels, MCV, MCH, MCHC and serum lysozyme activity remained unaffected by dietary treatment after three weeks.

After four weeks erythrocytes were significantly more abundant in CON fed fish ($1.44 \pm 0.38 \times 10^6$ μl^{-1}) when compared with PRO-PULSE ($0.95 \pm 0.18 \times 10^6$ μl^{-1}) but was not different to PRO-3 ($1.24 \pm 0.22 \times 10^6$ μl^{-1}). Consequently, MCV was significantly lower in CON (314.46 ± 93.36 pg) when compared with PRO-PULSE (426.64 ± 89.97 pg) but was not different to PRO-3 (329.66 ± 61.31 pg). Haematocrit, haemoglobin, leucocyte levels, MCH, MCHC and serum lysozyme activity remained unaffected by dietary treatment after four weeks.

After five weeks, leucocyte levels were significantly higher in PRO-3 ($16.62 \pm 3.96 \times 10^3$ μl^{-1}) when compared with CON ($10.29 \pm 3.43 \times 10^3$ μl^{-1}), but were not different to PRO-PULSE ($12.79 \pm 3.66 \times 10^3$ μl^{-1}). Haematocrit, haemoglobin, erythrocyte levels, MCV, MCH, MCHC and serum lysozyme activity remained unaffected by dietary treatment after five weeks of experimental feeding.

With the exception of week three, where samples were damaged during transportation, the abundance of leucocyte sub-populations (lymphocytes, monocytes and granulocytes) were also investigated at each time point (Table 5.8). No significant differences were observed in the proportions of circulating lymphocytes, monocytes or granulocytes after two and four weeks of experimental feeding. After five weeks, blood from PRO-3 treated fish had significantly higher proportions of circulating monocytes (14.17 ± 4.54%) when compared to

fish in PRO-PULSE (7.83 ± 3.44%). The proportion of granulocytes was significantly higher in blood from CON (2.17 ± 1.17%) samples when compared to PRO-PULSE (0.67 ± 0.75%).

Table 5.8: Circulatory leucocyte proportions of tilapia fed experimental diets at each sampling point.

	CON	PRO-3	PRO-PULSE
Week 2			
Lymphocytes	84.00 ± 4.90	82.50 ± 5.25	86.67 ± 4.68
Monocytes	11.17 ± 5.05	14.83 ± 4.37	11.17 ± 3.89
Granulocytes	4.83 ± 1.57	2.67 ± 2.56	2.17 ± 1.21
Week 3			
Lymphocytes	-*	-*	-*
Monocytes	-*	-*	-*
Granulocytes	-*	-*	-*
Week 4			
Lymphocytes	87.83 ± 5.76	87.83 ± 2.41	93.00 ± 2.52
Monocytes	10.00 ± 4.08	10.50 ± 2.57	6.17 ± 2.34
Granulocytes	2.17 ± 1.95	1.67 ± 1.70	0.83 ± 0.90
Week 5			
Lymphocytes	88.67 ± 4.97	84.67 ± 3.68	91.50 ± 3.95
Monocytes	9.17 ± 3.67^{ab}	14.17 ± 4.54^{b}	7.83 ± 3.44^{a}
Granulocytes	2.17 ± 1.17^{a}	1.17 ± 0.75^{ab}	0.67 ± 0.75^{b}

* No data available due to sample damage during transportation
$^{a, b}$ Different superscripts indicate a significant difference ($P < 0.05$).

5.3.5 High-throughput sequencing

Microbial communities from the digesta of tilapia were investigated after four and five weeks using high-throughput sequencing analyses. A total of 1,288,759 and 1,715,037 sequence reads were obtained from the Ion Torrent® PGM after four and five weeks, respectively (Table 5.9). After removing low quality reads, 19,821 ± 2,997, 18,351 ± 6,145 and 23,470 ± 3,528 sequences were obtained after four weeks, and after five weeks, 11,969 ± 3,513, 15,016 ± 13,429 and 14,696 ± 1,671 sequences were obtained for CON, PRO-3 and PRO-PULSE groups, respectively. These sequences were used for downstream analyses.

Good's coverage estimators for all treatments were 1.00 at both time points indicating that sufficient sequencing coverage was achieved and that the OTU's detected in the samples were representative of the microbial community.

After four weeks, the numbers of OTU's detected were significantly lower in PRO-PULSE when compared to CON and PRO-3. There were no differences between CON and PRO-3. Both diversity and species richness (as indicated by Shannon and Chao1 indices, respectively) were lowest in the PRO-PULSE treatment. Diversity was significantly lower in this treatment when compared with PRO-3 but not CON. However, the diversity of microbial communities remained the same in PRO-3 and CON. Species richness was significantly lower in PRO-PULSE when compared to CON but was not different to PRO-3. No differences were observed in species richness between the two probiotic treatment, PRO-3 and PRO-PULSE (Table 5.9).

After five weeks, allochthonous microbial communities from the PRO-PULSE fed fish revealed numerically the highest number of observed species, diversity and species richness indices. However, there were no statistical differences in each parameter between experimental treatments (Table 5.9).

Table 5.9: Number of raw reads, reads assigned to OTU's, Goods coverage and diversity/ richness indices of allochthonous intestinal microbiota after four and five weeks of experimental feeding.

	CON	PRO-3	PRO-PULSE
Week 4			
Reads (pre trimming)	69,908 ± 7,646	65,784 ± 24,087	79,102 ± 13,440
Reads assigned (post trimming)	19,821 ± 2,997	18,351 ± 6,145	23,470 ± 3,528
Good's Coverage	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Number of OTU's	59.50 ± 1.84[a]	62.42 ± 3.88[a]	54.31 ± 2.91[b]
Shannon's diversity index	3.48 ± 0.30[ab]	3.62 ± 0.16[a]	3.23 ± 0.07[b]
Chao1 Index	67.04 ± 2.84[a]	70.24 ± 5.02[ab]	60.56 ± 4.39[b]
Week 5			
Reads (pre trimming)	69,685 ± 17,362	61,986 ± 15,527	72,387 ± 7,829
Reads assigned (post trimming)	11,969 ± 3,513	15,016 ± 13,429	14,696 ± 1,671
Good's Coverage	1.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00
Number of OTU's	42.57 ± 5.12	43.45 ± 4.15	45.88 ± 3.08
Shannon's diversity index	2.98 ± 0.22	2.99 ± 0.39	3.19 ± 0.25
Chao1 Index	47.90 ± 5.19	48.91 ± 3.14	51.47 ± 2.29

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

After four weeks the PRO-PULSE group had just completed a week of basal feeding. High-throughput analyses after four weeks demonstrated that rarefaction curves approached the saturation phase in all treatments at approximately 5,000- 10,000 sequence reads (Fig 5.7a). The PCoA plot and dendrogram (Fig 5.7b and 5.7c, respectively) demonstrated little clustering effect between each the replicates from each treatment. Fig 5.7d illustrates that 32 genera were identified (i.e. accounting for >0.01% of the reads). Twenty three of these genera were found in all treatments. One of these genera (an unknown genera from the Family Rhodobacteraceae) was unique to CON replicates, four (*Enterococcus*, *Lactobacillus*, *Pediococcus* and an unknown genera belonging to the Family Enterococcaceae) were unique to PRO-3 and no genera were unique to PRO-PULSE replicates. Three genera were present in both CON and PRO-3 treatments (*Nocardia*, *Rhodoplanes* and *Deefgea*) and a further one genera was found in both CON and PRO-PULSE treatments (*Aeromonas*).

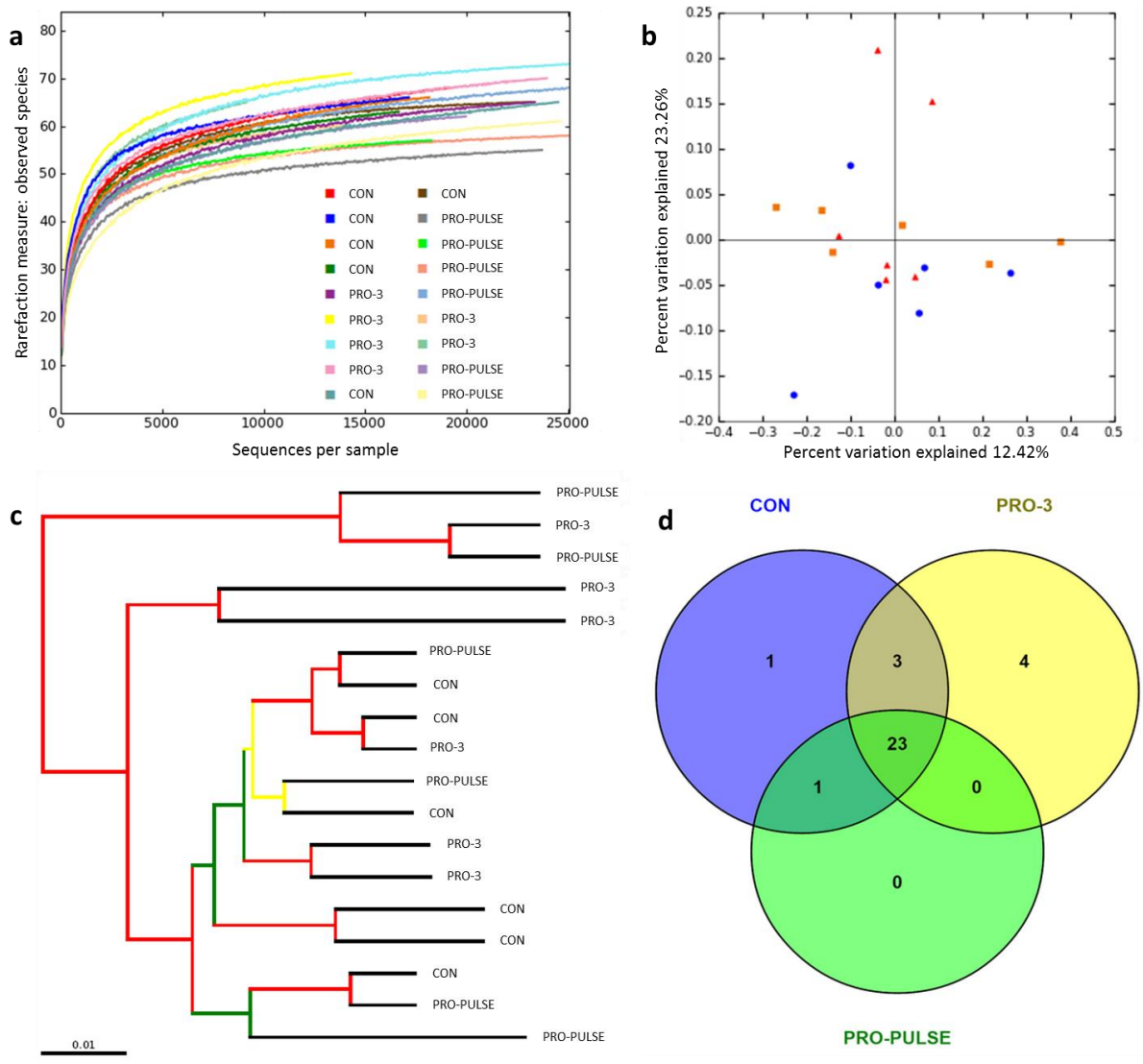


Figure 5.7: Bacterial community composition and relatedness in the digesta of tilapia fed experimental diets after four weeks. (a) Comparison of rarefaction curves between allochthonous intestinal microbiota composition between fish fed experimental treatments. (b) PCoA plots where data points represent samples from fish in treatment CON (red triangles), PRO-3 (blue circles) and PRO-PULSE (orange squares). (c) Dendrogram showing the relatedness of intestinal microbiota from treatment replicates. Bootstrap values are indicated by green (25-50%), yellow (50-75%) or red (75- 100%) branches. (d) Venn diagram showing the number of genera (accounting for >0.01% reads averaged from six replicates) assigned to each treatment. The PRO-PULSE treatment had just completed a week of basal feeding, without probiotic.

After the fifth week of experimental feeding, the PRO-PULSE treatment had just completed a week of probiotic feeding. After five weeks, high-throughput analyses demonstrated that rarefaction curves approached the saturation phase in all treatments at approximately 4,000- 6,000 sequence reads (Fig 5.8a). Similarly to week four, after five weeks the PCoA plot and dendrogram demonstrated little clustering effect between each the replicates from each treatment (Fig 5.8b and 5.8c). Fig 5.8d illustrates that 24 genera were identified (i.e. accounting for >0.01% of the reads). Twenty two of these genera were found in all treatments. No genera were unique to individual treatments, one genera (*Aquicella*) was common to both CON and PRO-3 treatments and one genera (*Pediococcus*) was common to both PRO-3 and PRO-PULSE treatments.

Fig 5.9 shows the major bacterial constituents in the digesta of fish fed experimental diets at the phylum and genus levels after four weeks. At this time point the PRO-PULSE treatment had just completed a week of basal feeding, without probiotic. In all treatments the most abundant 16S reads belonged to the phylum Fusobacteria (CON = 70.38 ± 3.7%, PRO-3 = 66.84 ± 10.46% and PRO-PULSE = 72.93 ± 11.26%), followed by Proteobacteria (CON = 23.72 ± 3.85%, PRO-3 = 17.12 ± 5.08% and PRO-PULSE = 21.91 ± 10.93%). The abundance of reads assigned to these phyla did not differ significantly between treatments. Reads assigned to Firmicutes were the next most abundant. PRO-3 replicates contained a significantly higher proportion of reads assigned to Firmicutes (15.62 ± 7.11%) when compared with those in CON (5.33 ± 2.94%) and PRO-PULSE (4.75 ± 3.43%). Reads assigned to Firmicutes in CON and PRO-PULSE treatments did not differ from each other. Reads belonging to Actinobacteria and Cyanobacteria were present in lower proportions and did not differ significantly between treatments.

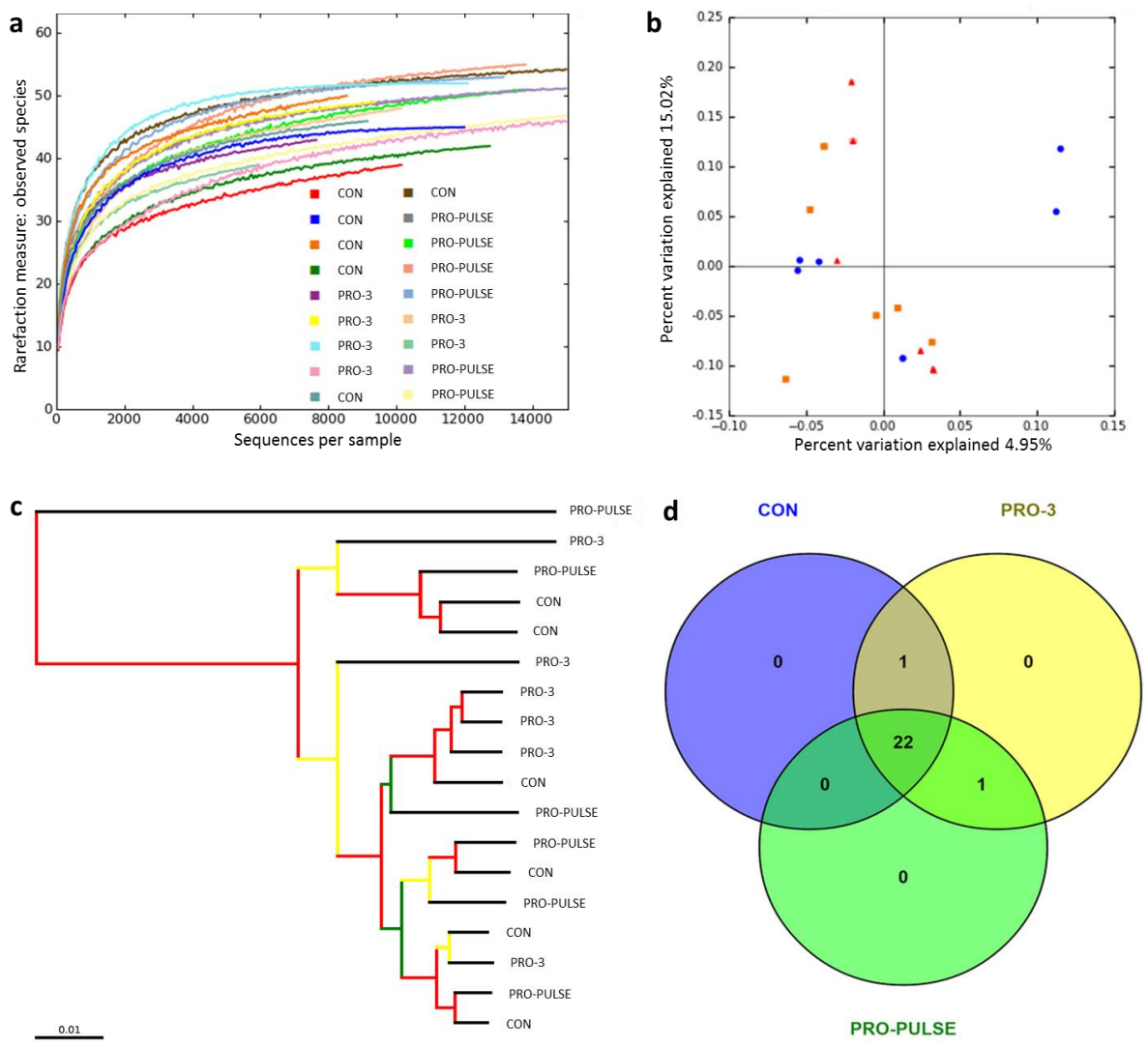


Figure 5.8: Bacterial community composition and relatedness in the digesta of tilapia fed experimental diets after five weeks. (a) Comparison of rarefaction curves between allochthonous intestinal microbiota composition between fish fed experimental treatments. (b) PCoA plots where data points represent samples from fish in treatment CON (red triangles), PRO-3 (blue circles) and PRO-PULSE (orange squares). (c) Dendrogram showing the relatedness of intestinal microbiota from treatment replicates. Bootstrap values are indicated by green (25-50%), yellow (50-75%) or red (75- 100%) branches. (d) Venn diagram showing the number of genera (accounting for >0.01% reads averaged from six replicates) assigned to each treatment. The PRO-PULSE treatment had just completed a week of probiotic feeding.

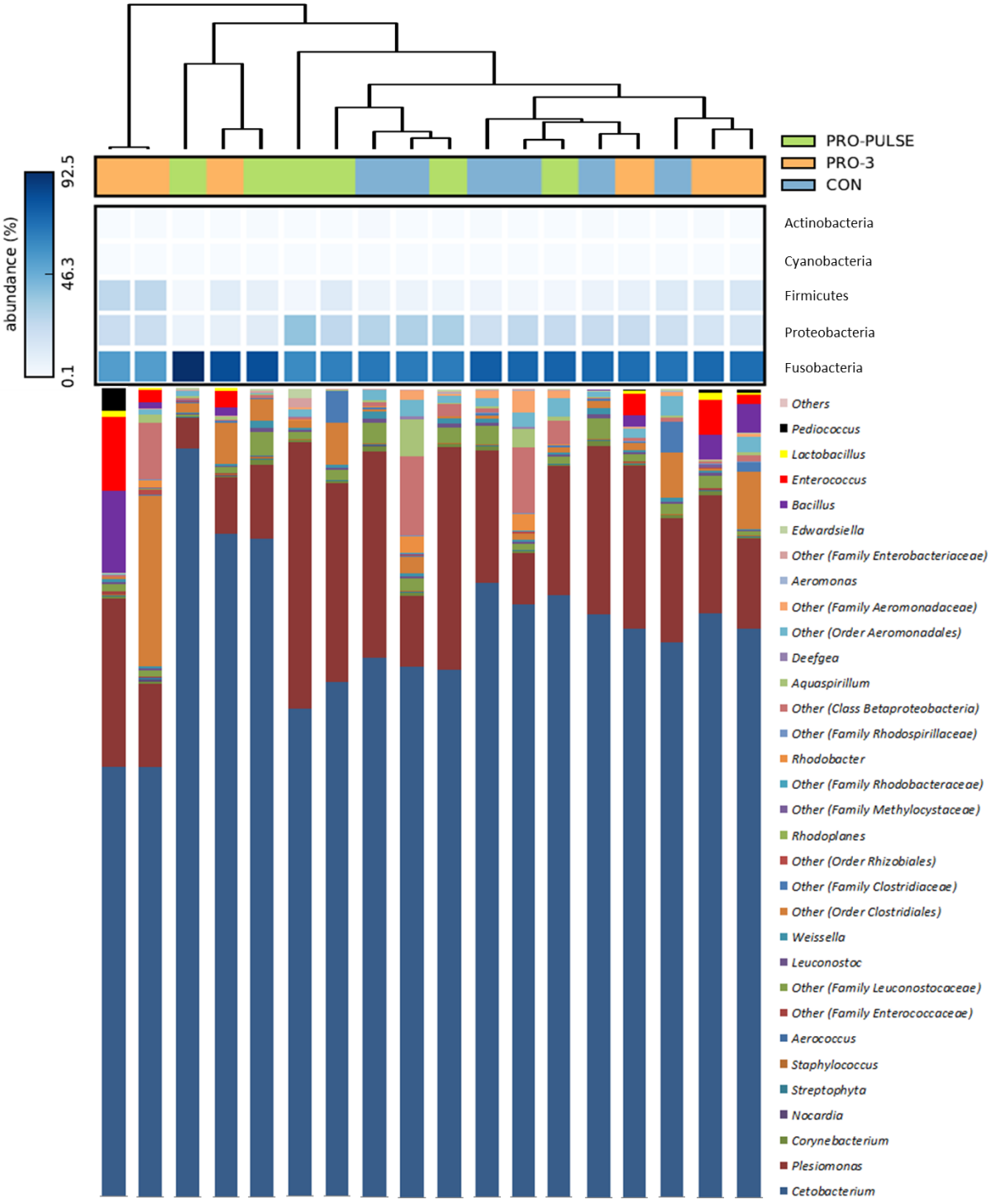


Figure 5.9: Comparison of allochthonous intestinal microbiota composition between fish fed dietary treatments after four weeks of experimental feeding. Heatmap shows bacterial OTU's assigned at the phylum level and bars show OTU's assigned at the genus level (showing genera accounting for >0.01%). The PRO-PULSE treatment had just completed a week of basal feeding, without probiotic.

After four weeks, at the genera level, proportionally, the most abundant reads in each treatment were assigned to *Cetobacterium* (CON = 70.38 ± 3.70%, PRO-3 = 66.84 ± 10.46% and PRO-PULSE = 72.93 ± 11.26%) followed by *Plesiomonas* (CON = 15.56 ± 6.53%, PRO-3 = 14.00 ± 5.10% and PRO-PULSE = 18.99 ± 10.31%; Fig 5.9). Reads belonging to both *Cetobacterium* and *Plesiomonas* were not significantly different between treatments. Proportions of reads assigned to *Bacillus*, *Enterococcus* and *Lactobacillus* were significantly higher in PRO-3 (3.33 ± 3.23, 3.47 ± 2.73 and 0.51 ± 0.24, respectively) when compared to CON and PRO-PULSE. Reads assigned to *Lactobacillus* were not detected in either CON or PRO-PULSE treatments whereas reads assigned to *Enterococcus* were only detected in 4/6 replicates (averaging 0.01%) and 2/6 replicates (averaging <0.01%) in CON and PRO-PULSE treatments, respectively. Reads assigned to *Bacillus* were detected in low proportions in all replicates from CON (0.06 ± 0.06%) and PRO-PULSE treatments (0.03 ± 0.01%). Reads belonging to *Pediococcus* were recovered in all PRO-3 replicates (0.63 ± 0.97%) but were only detected in 1/6 replicates from both CON and PRO-PULSE treatments at ca. 0.01%. However, *Pediococcus* abundance was not statistically different between treatments. Reads assigned to *Aeromonas* were significantly lower in PRO-3 (0.01 ± 0.01%) when compared to CON (0.04 ± 0.03%) but not PRO-PULSE (0.01 ± 0.01%). Reads assigned to other genera were not significantly different between dietary treatments after four weeks.

Fig 5.10 shows the major bacterial constituents in the digesta of fish fed experimental diets at the phylum and genus levels after five weeks. At this time point, the PRO-PULSE treatment had just completed a week of probiotic feeding. Reads belong to Fusobacteria were the most abundant in digesta samples after five weeks of experimental feeding (CON = 81.26 ± 6.39%, PRO-3 = 80.69 ± 8.78% and PRO-PULSE = 72.71 ± 14.28%). Reads assigned to Firmicutes (CON = 9.86 ± 7.08%, PRO-3 = 11.74 ± 6.16% and PRO-PULSE = 15.38 ± 8.27%) and Proteobacteria (CON = 8.38 ± 2.47%, PRO-3 = 7.27 ± 3.34% and PRO-PULSE = 11.46 ± 7.55%) were the second and third most abundant, respectively. Reads assigned to Cyanobacteria and Actinobacteria were also present in smaller quantities. The abundance of reads assigned to each phylum was not statistically different between the dietary treatments.

After five weeks, at the genera level, proportionally, the most abundant reads in each treatment were assigned to *Cetobacterium* (CON = 81.26 ± 6.91%, PRO-3 = 80.69 ± 9.48% and PRO-PULSE = 72.71 ± 15.43%) followed by *Plesiomonas* (CON = 6.62 ± 3.32%, PRO-3 = 5.62 ± 3.67% and PRO-PULSE = 10.86 ± 8.47%; Fig 5.10). Reads belonging to both *Cetobacterium* and *Plesiomonas* were not significantly different between treatments. The proportions of reads assigned to *Enterococcus* were significantly higher in PRO-PULSE (6.95 ± 5.95%) when compared to CON (0.81 ± 1.11%) but was no different to PRO-3 (2.28 ± 1.08%). Reads assigned to *Bacillus* and *Pediococcus* genera were numerically higher in PRO-PULSE (1.89 ± 1.15% and 0.26 ± 0.47%, respectively) when compared with PRO-3 (1.00 ± 0.94% and 0.06 ± 0.12%, respectively). In CON replicates, *Bacillus* accounted for 0.55 ± 0.28% of the total reads and *Pediococcus* was only found in a single replicate at <0.01%. Reads assigned to *Lactobacillus* were highest in PRO-3 treatment (1.21 ± 1.34%) when compared with both PRO-PULSE (0.60 ± 0.36%) and CON (0.10 ± 0.15%). Besides *Enterococcus*, there were no significant differences in the number of reads assigned to each genus between treatments after five weeks of experimental feeding.

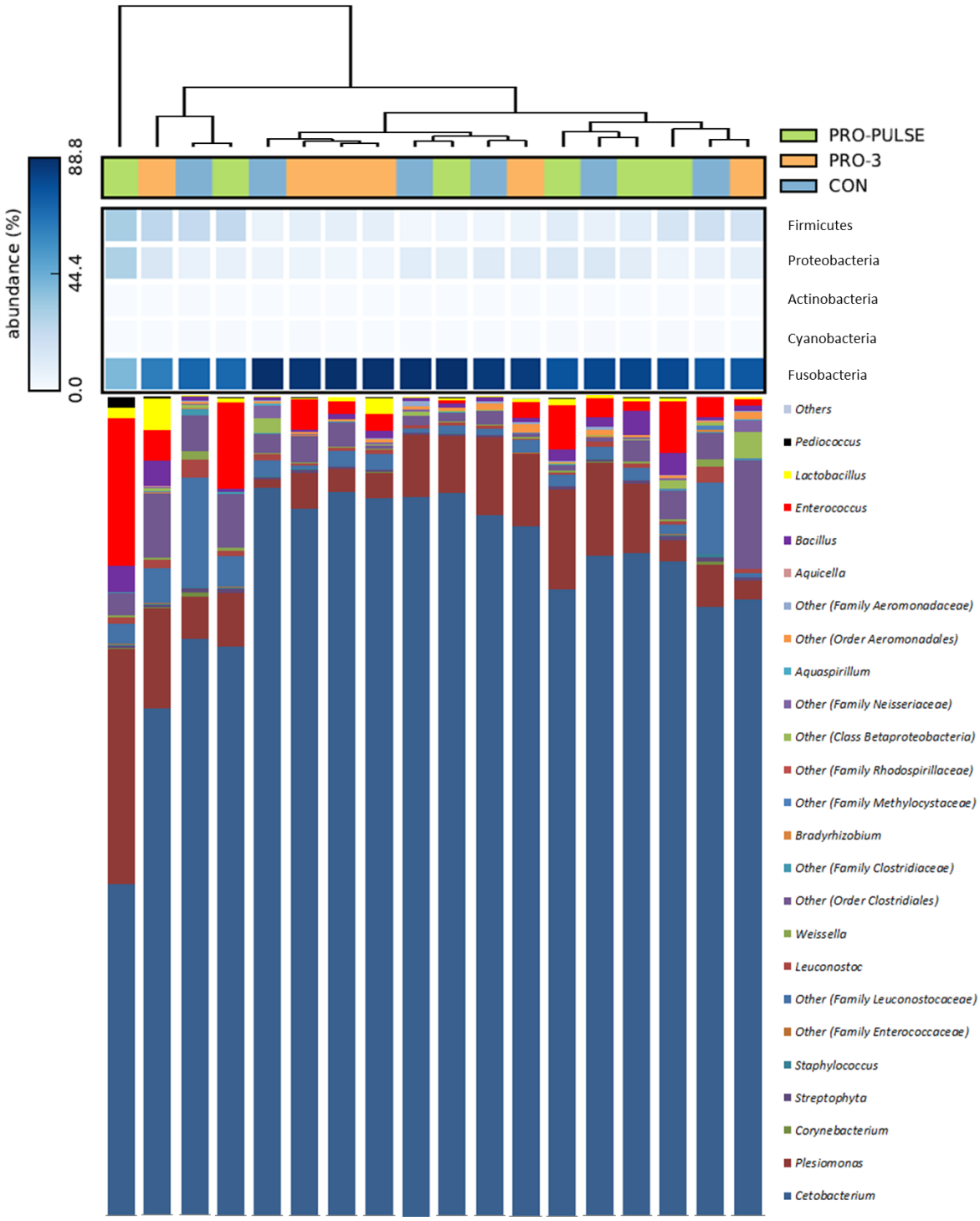


Figure 5.10: Comparison of allochthonous intestinal microbiota composition between fish fed dietary treatments after five weeks of experimental feeding. Heatmap shows bacterial OTU's assigned at the phylum level and bars show OTU's assigned at the genus level (showing genera accounting for >0.01%). The PRO-PULSE treatment had just completed a week of probiotic feeding.

5.4 Discussion

The intestine provides a key site of interaction with the external world and is a major portal for pathogenic invasion in fish (Ringø *et al*. 2007). It is essential therefore that the GALT is robust and provides an effective immunological barrier against pathogenic invasion. One of the ways that probiotics may improve barrier function is by regulating molecular mechanisms within the intestine through the activation of mucosal immunity. TLRs help direct the immune response by activating signalling cascades that increase the expression of soluble mediators, which recruit and regulate the immune and inflammatory cells eventually initiating or enhancing the immune responses (Perez-Sanchez *et al*. 2010). Studies in grouper have demonstrated that after 60 days, probiotics *Psychrobacter* spp. and *Bacillus pumilus* can regulate the gene expression of TLR1, TLR2 and TLR5 (Sun *et al*. 2014; Yang *et al*. 2014). Furthermore, in Chapter 3, supplementing tilapia diets at 3 g kg^{-1} resulted in the up-regulation of intestinal TLR2 gene expression. In the current study, after just two weeks, intestinal TLR2 was significantly up-regulated in treatment PRO-3 when compared to CON. This pattern persisted throughout all sampling points. Furthermore after three and four weeks TLR2 was significantly up-regulated in PRO-PULSE when compared with CON, although the up-regulation was greatest in PRO-3. This up-regulation likely comes from the interaction between TLR2 and its agonist, lipoteichoic acid which is present on the extracellular membranes of the probiotic species used.

Collectively, TLR signalling is largely divided into two pathways: MYD88 dependent and TRIF dependent pathways (Kawai and Akira 2007a). Here, the gene expression of intestinal MYD88 was not affected by dietary treatment. Similarly, after 60 days, groupers fed diets supplemented with *B. pumilus* showed no differences with regards to intestinal gene expression of MYD88 (Yang *et al*. 2014). These studies suggest that at the intestinal level probiotics may activate a MYD88 independent pathway in order to regulate cytokine

508 expression. Within the intestine, NF-κB was significantly down-regulated in probiotic groups
509 when compared to the control after three, four and five weeks, with the greatest down-
510 regulation occurring in PRO-3. Work conducted by Chang and Nie (2009) suggest that
511 another group of the PRR family, peptidoglycan recognition proteins (PGRPs), could mediate
512 several intracellular signalling pathways and also significantly inhibit NF-κB activity. TLR2
513 may work with PGRPs in the recognition of bacterial components, including peptidoglycan
514 (Zhu *et al*. 2013). Since TLR2 and PGRPs share a common function, an increased expression
515 of TLR2, as observed in probiotic treatments here, may also result in the inhibition of NF-κB.
516 Future work should be conducted to further elucidate this mechanism. As well as NF-κB,
517 signalling pathways initiated by TLRs can use mitogen-activated protein kinase (MAPK)
518 followed by activation protein 1 (AP-1) which can regulate inflammatory cytokine genes
519 (Kawai and Akira 2007b).

520 Further changes were observed in the transcription of immune genes in the HK, but
521 only after five weeks. This suggests that there is a lag phase of at least three weeks between
522 the probiotic modulating the localised immune response in the intestine and the systemic
523 response in the HK. Similar to the intestine, HK levels of TLR2 mRNA were significantly
524 higher in PRO-3 when compared with CON. However, unlike the intestine, HK gene
525 expression of MYD88 was also significantly higher in PRO-3 and PRO-PULSE when
526 compared with CON suggesting that the production of inflammatory cytokines is through a
527 MYD88 dependent pathway in the HK. The HK gene expression of NFκB was not
528 significantly different between treatments; similar to the intestine, signalling pathways
529 initiated by TLRs within the HK may rely on MAPK and AP-1 transcription factors.

530 In the present study, the pro-inflammatory cytokines TNFα and IL-1β were
531 significantly up-regulated in both probiotic treatments and both tissues, when compared to
532 the control. The extent of this up-regulation was dependent on the probiotic treatment (i.e.

continuous vs pulsed supplementation), duration of feeding as well as the organ being investigated (i.e. either intestine or HK). Higher levels of pro-inflammatory cytokines may be indicative of a host which has superior immune readiness. It has been reported that other probiotics can up-regulate the gene expression of both intestinal and HK TNFα and IL-1β in tilapia (Pirarat *et al*. 2011; He *et al*. 2013; Standen *et al*. 2013). This could have been a contributory factor in providing tilapia with protection after being exposed to *A. hydrophila* and *Ps. fluorescens* (Abumourad *et al*. 2013; Liu *et al*. 2013; Ren *et al*. 2013; Villamil *et al*. 2014).

Initially, intestinal gene expression of TGFβ and IL-10 were significantly up-regulated in probiotic groups. This pattern of gene expression was also observed in the HK, but only after five weeks. This suggests that the host does not recognise the probiotics as a threat; thus an increase in anti-inflammatory cytokines such as TGFβ and IL-10 may be part of mucosal tolerance. Although the intestinal expression of TGFβ was highest in PRO-3 after two weeks, after three and four weeks no differences were observed. Furthermore, after five weeks TGFβ was significantly down-regulated in probiotic treatments. Liu *et al*. (2013) also investigated the gene expression of TGFβ in the intestine, HK and spleen at three time points after supplementing tilapia diets with *L. brevis* and *L. acidophilus*. Liu and colleagues observed a complex relationship between the probiotic and the host which resulted in both the up and down-regulation of TGFβ depending on probiotic species, feeding duration and tissue of interest. Together, these studies highlight the complex temporal effect of probiotic administration on intestinal, HK and splenic immune gene expression.

In addition, significantly higher IEL's were observed in the mid-intestine of fish in PRO-3 when compared with CON after five weeks. It would be tempting to suppose that these leucocytes were primarily macrophages since these are potent producers of inflammatory cytokines (Mills and Ley 2014). Furthermore, the serum of tilapia in PRO-3

contained the highest proportions of circulating monocytes at all sampling points; it is these monocytes which migrate from the bloodstream into tissues where they differentiate into macrophages. Along with an increased abundance of IEL's, PRO-3 treated fish also exhibited a significantly higher number of goblet cells when compared to CON after five weeks. Together, these data suggest an improved barrier function, lending increased protection to the host against invading pathogens. Other studies have reported increased IEL and goblet cell abundance after probiotic supplementation after time periods ranging from 30 days to eight weeks (Pirarat *et al*. 2011; Standen *et al*. 2013; Chapters 3 and 4).

Further evidence of probiotic modulation of the systemic immune system was observed from haemato-immunology samples. After five weeks, PRO-3 blood samples contained a significantly higher number of white blood cells when compared to CON. White blood cells are essential components to both the innate and adaptive immune response and consequently a higher abundance implies a stronger immune system. After supplementing tilapia diets with *Ps. fluorescens,* Eissa and Abou-El Gheit (2014) observed a higher abundance of circulating leucocytes when compared to fish being fed non-supplemented diets. An increase in leucocytes could have been at least partially responsible for the reduced mortality observed when the tilapia were challenged via IP injection with *A. hydrophila* in the same study.

Importantly, the improvements to the tilapia immune response were not detrimental to growth performance which remained unaffected by dietary treatment. This is in agreement with other studies which report similar results after probiotic feeding of tilapia (Shelby *et al*. 2006; Abd El-Rhman *et al*. 2009; Zhou *et al*. 2010; Pirarat *et al*. 2011; He *et al*. 2013; Liu *et al*. 2013; Standen *et al*. 2013; Ng *et al*. 2014) Chapter 3). However, this was in contrast to Chapter 4 where AquaStar® Growout was reported to improve growth in tilapia after six weeks of supplementation at 3g kg^{-1}.

It is important that a probiotic survives the gastric process and reaches the intestine where it can exert its beneficial properties. Chapter 3 demonstrated that supplementing tilapia diets with AquaStar® Growout at 5g kg^{-1} resulted in the relative dominance (~99%) of probiotic 16S rRNA reads. In the current study, between 5-10% of reads in probiotic fed fish were assigned to probiotic genera. This discrepancy could be due to the different doses administered in the feed (5 g kg^{-1} in Chapter 3 and 3 g kg^{-1} here). In addition, it is well known that multiple biotic and abiotic factors may affect the gut microbiota and the colonisation dynamics of probiotics, including diet and environment (Ghanbari *et al*. 2015). Tilapia were fed an experimental diet in Chapter 3 and kept at 28°C, but fed a commercial diet and maintained at >30°C here. Although considerably less abundant (in relative terms) than in Chapter 3, it is clear that probiotics do not need to be dominant members of the intestinal microbiota in order to exert beneficial effects upon the host. This is supported by other studies where, after probiotic supplementation, researchers have reported low probiotic recovery, whilst reporting benefits to intestinal immunity and growth performance (Sáenz de Rodrigáñez *et al*. 2009; Yang *et al*. 2012; He *et al*. 2013; Liu *et al*. 2013). This said, the relatively small number of reads assigned to the probiotic species were enough to result in a significantly higher abundance of Firmicutes in PRO-3 when compared to both PRO-PULSE and CON after four weeks. High-throughput sequencing also revealed that regardless of treatment, reads belonging to *Cetobacterium* dominated the intestinal microbiota. This was further identified as *Ct. somerae*. This species is an important contributor to the production of vitamins, particularly vitamin B_{12}, and is routinely found in the intestines of many fish species, including tilapia (Tsuchiya *et al*. 2008; He *et al*. 2013; Liu *et al*. 2013; Larsen *et al*. 2014; Chapter 3).

This is the first study which has investigated the TLR signalling pathway after probiotic supplementation in tilapia. Under the current experimental conditions, AquaStar®

608 Growout is capable of localised immune modulation after two weeks (or less) and after
609 longer feeding periods (between four and five weeks) can modulate systemic immunity in the
610 HK. Furthermore, the probiotic application was capable of improving parameters that likely
611 lead to improved intestinal barrier function. Since innate immunity is non-specific, the
612 improvements described here suggest that the host could be better equipped to retard a wide
613 range of pathogens, increasing their resistance to multiple infections and diseases. Future
614 studies should test this hypothesis by assessing intestinal resistance to enteropathogens
615 through challenge trials. These improvements were greatest when supplementing the
616 probiotic continuously as opposed to via a pulsed regime.

Chapter 6: General discussion

The intestinal microbiota is important for a number of host functions, including nutrition and digestion, localised immunity and gut development (Rawls *et al*. 2004; Bates *et al*. 2006; Rawls *et al*. 2007). One of the most important goals in studying the intestinal microbiota of fish is to develop effective strategies for manipulating microbial communities to promote and sustain the health of the host. The continual supplementation of probiotic cells can result in the temporal colonisation of the intestinal tract and modulation of the indigenous microbiota. In Chapters 3 and 4, the intestinal microbiota was investigated using culture based methods. The results from both trials revealed that the probiotics tested, whether supplemented as monospecies or a multispecies application and regardless of feeding regime tested, had no effect on the total viable counts. Changes were detected in allochthonous (Chapters 3 and 4) and autochthonous (Chapter 3 only) populations when microbial communities were enumerated on selective media, namely MRS, S & B and *Bacillus* selective agar. The limitations of culture based methods, mainly cultivability issues, are well known (Zhou *et al*. 2014). Several methods for culture-independent microbial analyses have been utilised to assess the intestinal microbiota of fish, but the most widely used technique is DGGE, a semi-quantitative approach separating OTU amplicons based on nucleotide denaturing properties (Ferguson *et al*. 2010; He *et al*. 2013; Liu *et al*. 2013). Chapters 3 and 4 used a DGGE approach to assess the effect of dietary probiotics on the allochthonous microbial communities in tilapia. However, it can often be difficult to accurately identify OTU's present in environmental samples using this technique.

The introduction and development of high-throughput sequencing technologies has increased our understanding of microbial diversity in complex environments, including the intestinal tract of fish (van Kessel *et al*. 2011; Roeselers *et al*. 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; Lyons *et al*. 2015). These techniques offer researchers the ability to obtain large numbers of sequence reads in a relatively short period of time, yielding datasets which are orders of magnitude larger than those produced by other culture-independent approaches. Chapters 3 and 5 investigated the effect of dietary AquaStar® Growout on the allochthonous intestinal communities of tilapia using an Ion Torrent PGM. In Chapter 3, 16S reads belonging to probiotic genera accounted for >99% of the total reads in the AQ-5 treatment. In Chapter 5 reads belonging to probiotic genera only accounted for 5- 10% of the total reads, depending on the duration of feeding and probiotic feeding regime. An obvious reason for this could be that tilapia diets were supplemented with a higher dose in Chapter 3 (5 g kg^{-1}) when compared to tilapia diets in Chapter 5 (3 g kg^{-1}). It is well known that multiple biotic and abiotic factors may affect the gut microbiota and the colonisation dynamics of probiotics, including diet and environmental factors (Ghanbari *et al*. 2015). Tilapia were fed an experimental diet in Chapter 3 and kept at 28°C, but fed a commercial diet and maintained at >30°C in Chapter 5.

Analyses of intestinal bacterial communities have almost exclusively relied on 16S rRNA sequencing due to its universal phylogenetic distribution. Caution must be applied when interpreting this type of data since each bacterial strain can contain multiple copies of the 16S subunit (Wintzingerode *et al*. 1997). This can lead researchers to underestimate the abundance of taxa with lower 16S rRNA copy numbers such as Acidobacteria and overestimate taxa with higher 16S rRNA copy numbers such as Gammaproteobacteria and Firmicutes (Větrovský and Baldrian 2013). Větrovský and Baldrian also highlighted that whilst it is assumed copies of rRNA genes within an organism are the same, it has been demonstrated that 16S sequences from the same species or even the same genome can differ.

This makes it possible to compare the abundance of the same OTU between treatments, but care should be taken when comparing multiple OTU's within the same treatment.

The presence of several similar taxa (e.g. Proteobacteria, Firmicutes and Bacteroidetes) across such a large range of fish species and geographic locations suggest these bacterial groups may be important to host gut functions and contribute towards a core gut microbiome. Roeselers *et al*. (2011) hypothesised that the shared microbiota is shaped by evolutionary conserved aspects of the intestinal tract anatomy, physiology and immunity. Upon feeding rainbow trout fishmeal or plant based diets for 10 months, Wong *et al*. (2013) observed that despite large differences in growth performance, fillet quality and fish welfare, the intestinal microbiota composition remained fairly stable. Microbiological analyses from the current studies, specifically from high-throughput sequencing datasets, suggest tilapia may also have a core microbiome. This work demonstrates that regardless of diet, treatment, environment and rearing conditions certain OTU's such as *Bacillus*, *Enterococcus*, *Cetobacterium*, *Plesiomonas*, *Staphylococcus*, *Leuconostoc*, *Weisella* and *Bradyrhizobium* may populate the intestinal tract of tilapia.

It has been demonstrated *in vitro* that certain probiotics can be antagonistic towards pathogens (Aly *et al*. 2008a; Aly *et al*. 2008b; Abd El-Rhman *et al*. 2009; Apún-Molina *et al*. 2009; Zhou *et al*. 2010b; Del'Duca *et al*. 2013; Liu *et al*. 2013; Villamil *et al*. 2014). Few studies have assessed the *in vivo* ability of probiotics to antagonise pathogens. High-throughput sequencing data from the current study demonstrated that probiotic supplementation can significantly lower the abundance of 16S rRNA reads assigned to genera which contain pathogens including *Legionella* (Chapter 3) and *Aeromonas* (Chapter 5). Reads assigned to *Mycobacterium* and *Streptococcus* were also detected at lower abundances in AQ-5 when compared to the control in Chapter 3. This microbial shift could be through direct pathogen antagonism via the production of inhibitory compounds, competition for adhesion

sites and nutrients or indirectly via the host's immune system. Since the gut is a major portal of entry and infection loci for a number of pathogens, including aeromonads, the relative reduction in abundance through probiotic supplementation could have important connotations when fighting disease. This topic warrants further investigation.

High-throughput sequencing techniques have revolutionised our understanding of the fish intestinal microbiome and has provided a general overview of the taxonomic profile of the microbiota; however, metagenomic analyses will provide researchers with information regarding the entire genetic potential of the microbiota (Ghanbari *et al*. 2015). It is more difficult to investigate the microbiome's contribution towards functionality and metabolic processes. An appreciation of this functionality is essential in generating a complete view of the ecology and functional capacity of the gut microbiome. Transcriptomic and proteomic approaches have been used on microbial samples for several years to assess their metabolic potential and activity, respectively. These approaches have been applied and termed 'metatranscriptomics' and 'metaproteomics'. Metatranscriptomics is the study of all of the mRNA sequences from complex microbial ecosystems to determine the active bacterial processes expressed whilst metaproteomics identifies all of the total proteins present in environmental samples. Metabolomics approaches identify and quantify the metabolites and other small molecules in a complex microbial system. It is only through a multi-disciplined and holistic approach, termed "meta'omics" (Fig 6.1) which will lead to improved mechanistic models of microbial community structure and function (Sorek and Cossart 2010; Franzosa *et al*. 2015). The link between community structure and functional analyses has only been made in a small number of fish intestinal microbiota studies (Xing *et al*. 2013; Xia *et al*. 2014). Xing *et al*. (2013) investigated the taxonomic and functional metagenomic profile of the turbot intestinal tract microbiome, reporting the dominance of Firmicutes and Proteobacteria. When predicting the metabolic potential the microbiome, researchers

observed that the genes present coded for core metabolic functions including carbohydrate and protein metabolism, amino acid and vitamin production and RNA metabolism. Xia *et al*. (2014) also reported the dominance of Proteobacteria and Firmicutes, as well as Bacteroidetes in the intestine of Asian seabass, *Lates calcarifer*. Further to this, authors determined that in response to starvation, genes relating to transcription and cell division were depleted and genes associated with organelle biogenesis and immunity were enriched. These studies provide the first insights into the functional contribution of the microbiome. Probiotic studies must utilise these methodologies to elucidate the interactions between probiotics, the hosts microbiome and the host.

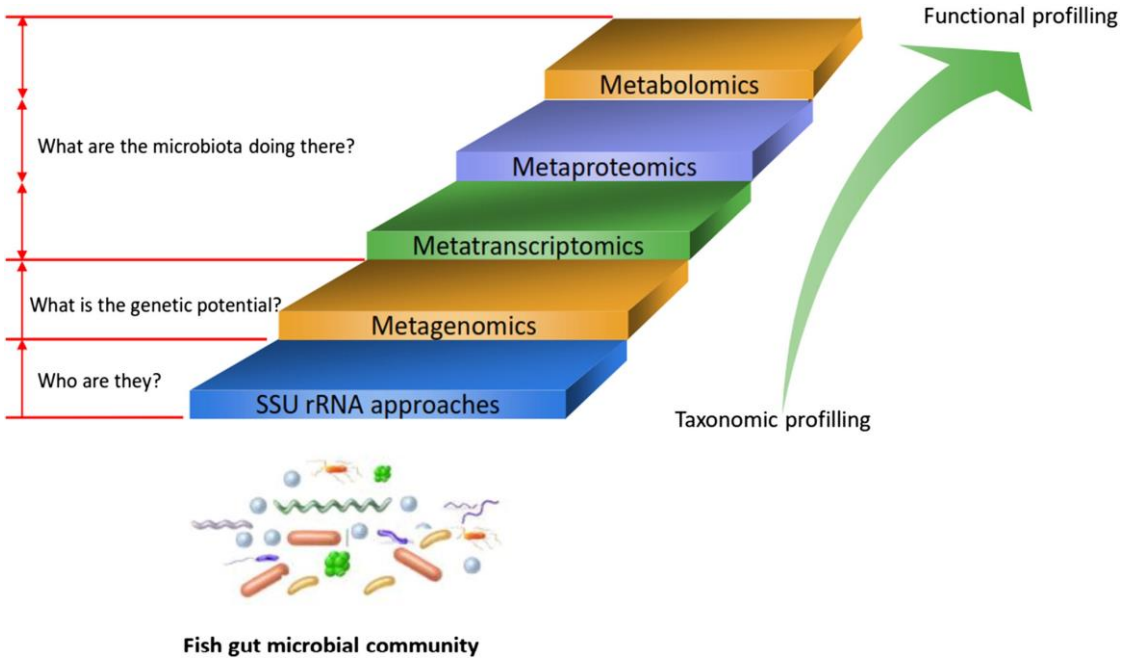


Figure 6.1: Simplified model demonstrating the meta'omic approach which will enable researchers to elucidate microbiota structure and function of environmental samples, including intestinal microbiota. Metagenomic approaches should be complemented by the parallel detection of mRNA transcripts expressed (metatranscriptomics), translated proteins (metaproteomics) and the metabolites produced (metabolomics). Source: Ghanbari *et al*. (2015).

Multiple studies in tilapia have reported an improvement in growth performance after probiotic supplementation (Abdel-Tawwab *et al*. 2008; Aly *et al*. 2008b; Aly *et al*. 2008c; Wang *et al*. 2008; Abd El-Rhman *et al*. 2009; Zhou *et al*. 2010a; Zhou *et al*. 2010b; Gonçalves *et al*. 2011; Jatobá *et al*. 2011; Ayyat *et al*. 2014; Eissa and Abou-El Gheit 2014; Ridha and Azad 2015). In Chapter 4, a significant improvement in final weight, weight gain and SGR was observed in tilapia which were fed diets supplemented with AquaStar® Growout at 3g kg^{-1} for six weeks when compared with the control treatment and the initial probiotic feeding group. Work conducted in Chapter 3 suggests that this improved growth performance could be a result of an increased absorptive surface area, through improved perimeter ratio and longer and more numerous microvilli. The same dosage (3g kg^{-1}) did not result in significantly improved growth performance in Chapter 5, possibly due to a shorter feeding period (5 weeks as opposed to 6 weeks) or a different diet. Alternatively, this could be a consequence of introducing 'noise' into the data by the weekly removal of biomass for sampling purposes. Importantly, the focus of the third trial (Chapter 5) was not growth performance, but immunological benefits brought about by probiotic supplementation. It should be noted that growth performance similar to control treatments (i.e. no difference in growth performance) can be considered a positive outcome if the probiotic can manifest other benefits such as immune modulation or improvements in gut morphology, as observed in these chapters, which would likely use energy and resources. Only detrimental effects in growth performance can be considered negative as observed in Shelby *et al*. (2006), Ridha & Azad (2015), Abumourad *et al*. (2013) and Abd El-Rhman *et al*. (2009).

The present study indicated that the assessed probiotics may stimulate, or enhance, a localised immune response through an enhanced infiltration of IELs in the intestine. This was observed after eight weeks in Chapter 3, six weeks in Chapter 4 and five weeks in Chapter 5. Considering no changes were detected after four weeks in Chapters 3 and 5 it is likely that

this effect occurs between four and five weeks after the onset of probiotic feeding. Whilst these results are in line with other probiotic studies in tilapia (Pirarat *et al*. 2011; Standen *et al*. 2013), the type of IELs were not characterised here. Using *Artemia* as a vector for probiotic administration, Picchietti *et al*. (2008) used polyclonal antibodies ORa and mAb G7 to determine specific IEL populations in the intestinal mucosa of gilthead seabream. Picchietti and colleagues observed higher abundances of Ig^+ leucocytes and acidophilic granulocytes after both *L. fructivorans* and *L. plantarum* supplementation. Further to this, PRO-3 treatments in both Chapters 4 and 5 contained significantly higher goblet cell levels in the mid-intestine when compared to the control group after six and five weeks, respectively. Considering there was no difference after four weeks in Chapter 5, it is likely this effect also manifests between four and five weeks after the initiation of probiotic feeding. Together, larger populations of IELs and goblet cells residing in the tilapia intestine likely improve the barrier function, ultimately retarding pathogen attachment and their subsequent infection. Future work should investigate this further using translocation experiments such as the *ex vivo* intestinal sac method. After supplementing tilapia diets with *L. plantarum* and exposing the intestinal sacs to *A. hydrophila*, Ren *et al*. (2013) assessed a number of parameters within the intestinal sac (apical membrane). Unfortunately, no effort was made to quantify *A. hydrophila* levels on the basolateral side of the intestinal sac, which would enable researchers to fully assess pathogen translocation. This should be a focus of future research.

The present studies investigated the probiotic effect on intestinal and head kidney gene expression. Chapters 4 and 5 both reported an up-regulation in pro-inflammatory cytokines IL-1β and TNFα after feeding tilapia diets supplemented at 3g kg^{-1}. This up-regulation was also observed in the pulsed regime (3 g kg^{-1}) in Chapter 5 but not in the pulsed treatment (at 1.5 g kg^{-1}) in Chapter 4. Importantly, the up-regulation in pro-inflammatory genes was not excessive, as inferred by the corresponding up-regulation in anti-inflammatory

genes TGFβ and IL-10. Furthermore, histological analyses from intestinal sections from both Chapter 4 and 5 revealed no signs of inflammation or damage, suggesting that this extra mRNA is not translated in proteins associated with inflammation. When taking this into consideration, an increased abundance of pro-inflammatory mRNA levels is indicative of a host which is in a higher state of immune readiness. In addition, anti-inflammatory cytokines TGFβ and IL-10 were also up-regulated in Chapters 4 and 5, although the extent of this was also dependent on dose and feeding regime. These changes are likely indicative of tolerance mechanisms where the host does not interpret the presence of the probiotics as a threat. Changes in the intestinal gene expression were observed after two weeks of experimental feeding in Chapter 5 suggesting that these effects are fairly rapid. Conversely, changes in head kidney (HK) gene expression were only evident after five weeks (Chapter 5) of supplementation where an up-regulation of pro and anti-inflammatory cytokines was observed. After supplementing hybrid tilapia diets with two *Lactobacillus* species, Liu *et al*. (2013) observed changes in intestinal and HK gene expression levels of TNFα, IL-1β and TGFβ after 10 days. It could be speculated that the differences in the onset of gene expression by probiotics is dependent on the duration of feeding, feeding regime, probiotic species, host species and the tissue of interest. Considering these innate mechanisms have the potential of responding within a short period of time, future work should focus on the first few hours-days of probiotic feeding, as well as longer term probiotic feeding. As well as the intestine and HK, it is important to consider other immune-relevant tissues with regards to gene expression such as the spleen. To the author's knowledge, only three studies have investigated splenic gene expression after probiotic supplementation in tilapia (Pirarat *et al*. 2011; Liu *et al*. 2013; Villamil *et al*. 2014). Future research should utilise high-throughput methodologies such as RNA-Seq and microarrays which would enable researchers to investigate multiple target genes from a large number of samples.

The majority of gene expression conducted in fish probiotic studies focuses on the transcription of cytokines; however, these are only the end products to complex molecular pathways involving receptors (including TLR2), adaptor proteins (including MYD88) and transcription factors (including NFκB). Therefore efforts should be made to investigate the pathway rather than just the end products. Supplementing tilapia diets with AquaStar® Growout at 3g kg^{-1} significantly up-regulated intestinal TLR2 gene expression. TLR2 is activated by lipoteichoic acid, a major constituent in Gram-positive bacteria. This up-regulation is likely a result of the increased abundance of Gram-positive probiotic bacteria and is particularly important since tilapia are susceptible to Gram-positive pathogens such as *St. iniae* and *St. agalactiae.* Interestingly, Chapter 5 demonstrated that cytokine production is driven by different molecular pathways in the intestine and the HK. In both cases, cytokine production was initiated by TLR2. In the intestine, the gene expression of MYD88 was not affected, suggesting that cytokine production is driven by a MYD88 independent pathway, perhaps relying on the adaptor protein TRIF instead (Kawai and Akira 2007a). In the HK the MYD88 dependent pathway was activated suggesting that cytokine production is driven by this adaptor protein. Furthermore, in both tissues NFκB was down-regulated in both probiotic treatments suggesting that MAPK and AP-1 are the transcription factors which link the adaptor proteins with the cytokines (Kawai and Akira 2007b). Further research should be conducted to confirm this. Importantly, all future research in this area should consider the entire molecular pathway as opposed to investigating cytokines as lone molecules since this provides more information on host-probiotic interactions.

Despite providing a unique perspective regarding the transcriptomics in tissues of interest, an important aspect is to determine how gene expression is correlated to protein translation. Early work conducted in yeast by Gygi and colleagues (1999) determined that the correlation between mRNA and protein levels was insufficient to predict protein expression

levels from quantitative mRNA data. Since an up-regulation at the transcription level, may not represent an increase in the final protein, proteomics will contribute greatly to our understanding of gene function (Pandey and Mann 2000). Future work should investigate how probiotic supplementation can affect the host using a systems biology approach (i.e. transcriptomics and proteomics). The proteome has only been investigated in a few fish studies after probiotic supplementation (Brunt *et al*. 2008; Abbass *et al*. 2010; Sveinsdóttir *et al*. 2009) but the number of proteomic studies in fish in general are increasing slowly (Rodrigues *et al*. 2012). The routine utilisation of proteomic approaches in fish studies is hampered by the fact that many proteins and their functions have not been characterised in fish, the costs involved and issues associated with data interpretation.

The probiotics investigated in the current work, whether supplemented as mono or multi species applications and regardless of feeding regime used, did not appear to have a great effect on immuno-haematological parameters. This is consistent with other studies which also report little or no effect on blood parameters after probiotic supplementation in tilapia (Abd El-Rhman *et al*. 2009; Mehrim 2009; Ridha and Azad 2012; Telli *et al*. 2014; Iwashita *et al*. 2015). Circulating WBC levels were significantly higher after five weeks of probiotic supplementation at 3g kg^{-1} (Chapter 5) indicating an improvement in the systemic immune response. It would be interesting to investigate whether this pattern continued after a longer feeding period.

Throughout the current research, multiple immune benefits after probiotic supplementation have been reported. This is suggestive that the host will be better equipped to defend itself against invading pathogens. However sound this conclusion might appear it would be necessary to carry out *in vivo* challenge trials to test this theory, using pathogens which are destructive in tilapia aquaculture. When conducting these trials, the pathogen should be administered through oral routes (immersion, oral intubation or through the feed)

since this accurately reflects the way an enteropathogen would naturally infect the fish (as opposed to IP injection which bypasses the intestinal tract).

On a final note, the current research focuses on probiotic use in juvenile tilapia. It would be pertinent to conduct further research to investigate whether similar effects, i.e. improved growth performance, intestinal morphology and immunity, could be observed in tilapia of different developmental stages e.g. in fry from first feeding or in adult fish. Furthermore, the trials described here lasted a relatively short period of time (maximum eight weeks). It is well known that the prolonged use of immunostimulants can lead to the immune system becoming de-sensitised and in extreme circumstances immune suppression in fish (Bricknell and Dalmo 2005). Considering an immuno-stimulatory role was exerted by the probiotics in the current research, it is important that longer term trials are conducted to investigate whether this effect is also true for probiotics.

In conclusion, Chapter 3 demonstrated that monospecies applications *B. subtilis*, *L. reuteri* and *P. acidilactici* and multispecies applications AquaStar® Growout could modulate the intestinal microbiota in a favourable manner. Chapter 4 investigated the effects of dosage and feeding regime of probiotics in tilapia diets. The results indicated that the continuous supplementation of AquaStar® Growout at 3 g kg^{-1} can improve growth performance, up-regulate the expression of genes involved in cell turnover and immunity and improve parameters relating to intestinal barrier function. These results were not observed when the probiotic was supplemented at a lower dose (1.5 g kg^{-1}), fed in a pulsed regime (also at 1.5 g kg^{-1}) or fed as an initial (first two weeks) probiotic feed. Chapter 5 demonstrated that the same dose used previously (3 g kg^{-1}), and supplemented in a continuous or pulsed manner, could improve the localised and systemic immune response of tilapia. The extent of these improvements was dependent on feeding duration, feeding regime and parameter investigated. This work adds to a growing body of knowledge surrounding probiotic usage in key fish

267 species, including tilapia. With an increasing demand for seafood it is important that research
268 is also conducted on other commercially important species, and indeed novel aquaculture
269 species. Considering the probiotic concept is a relatively new one in aquaculture, when
270 compared to its terrestrial counterpart, research to date has provided data on how probiotics
271 may interact with the host fish including the indigenous microbiota, and how these
272 interactions may bring benefits in the form of improved growth or health. Having said this,
273 there is still a great deal of work to be conducted but the usage of probiotics has a promising
274 future.

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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

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).

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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.

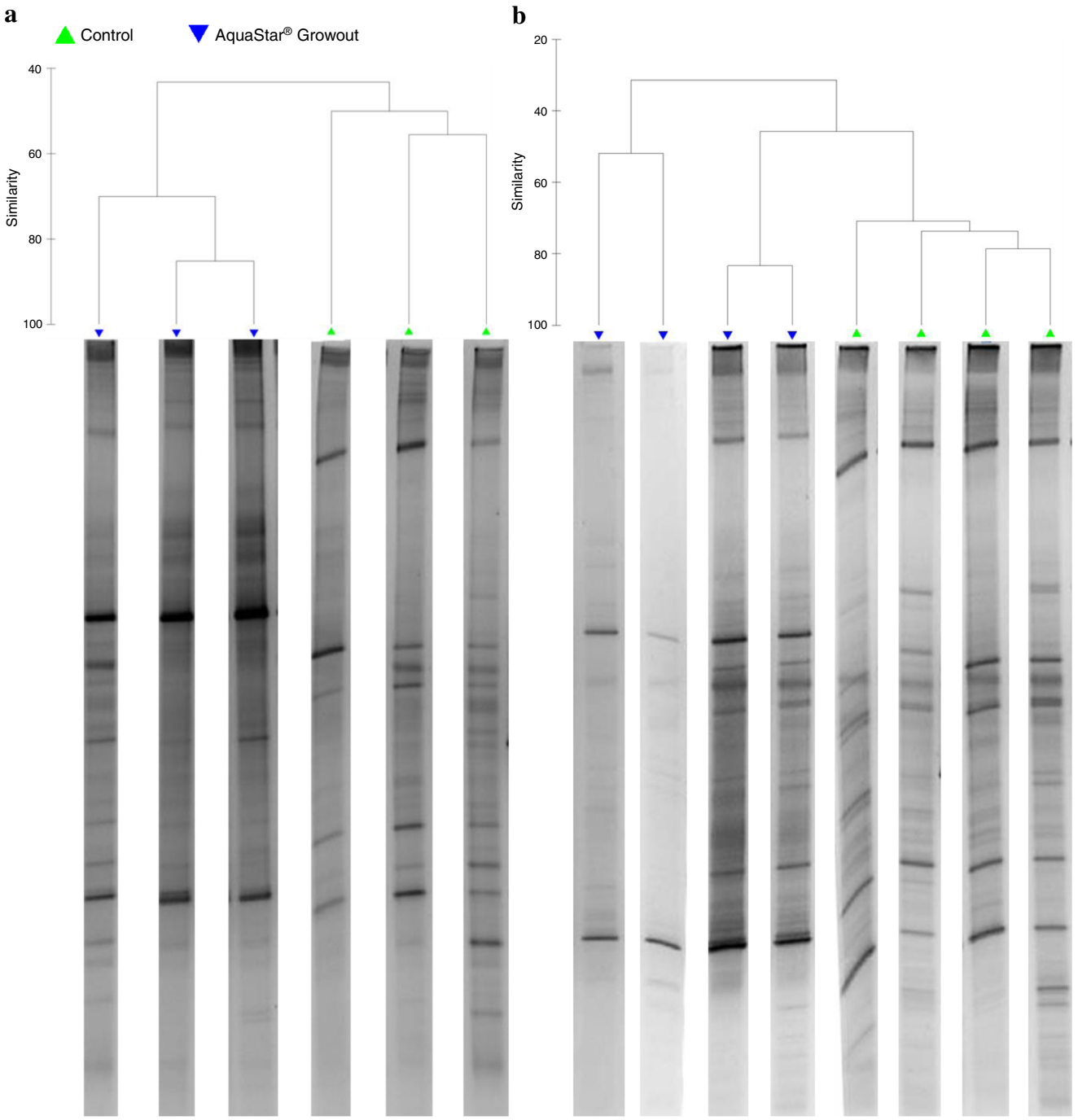


Fig. 1 Dendrograms representing the relatedness of the microbial communities in the digesta of tilapia after 4 weeks (**a**) and 8 weeks (**b**) of feeding with a control diet (*green triangles*) and probiotic diet (*blue triangles*). PCR-DGGE fingerprints below represent amplified products from the 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 ± 4.81per 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 ($P < 0.05$)

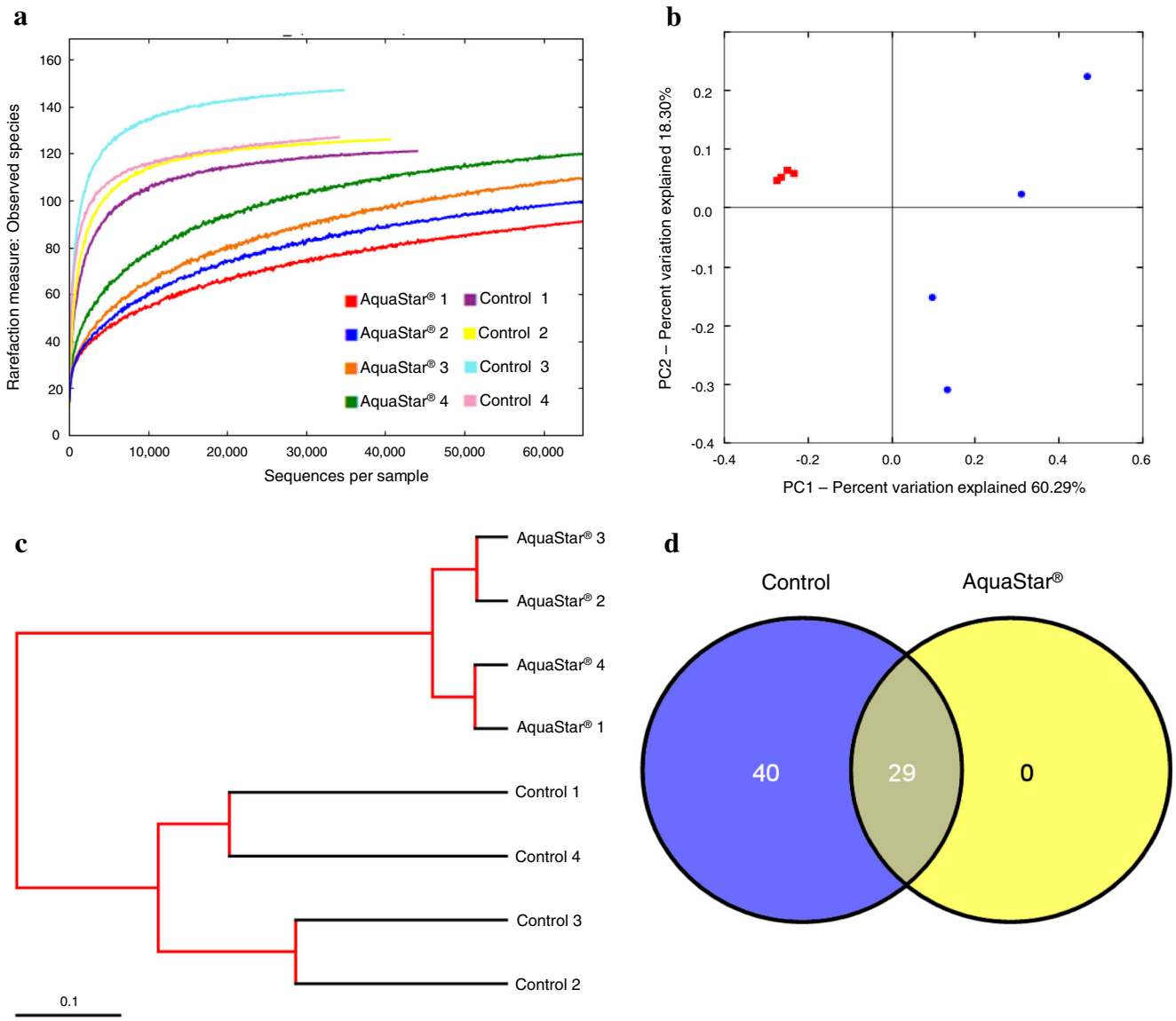


Fig. 2 Bacterial community composition and relatedness in the digesta of tilapia fed either a control or probiotic supplemented diet for 8 weeks. **a** Comparison of rarefaction curves between allochthonous intestinal microbiota composition between fish fed a control diet or probiotic diet. **b** PCoA 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

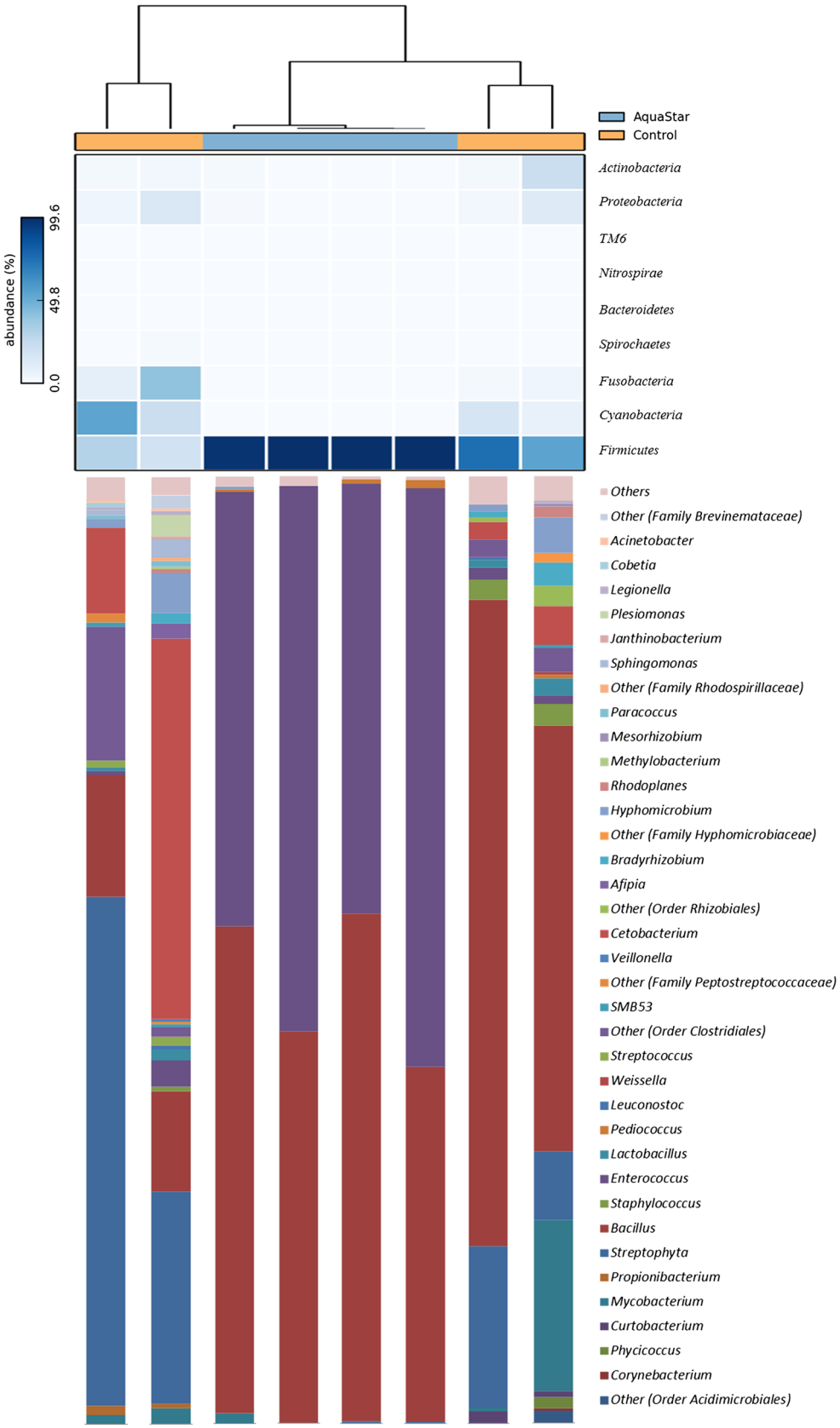
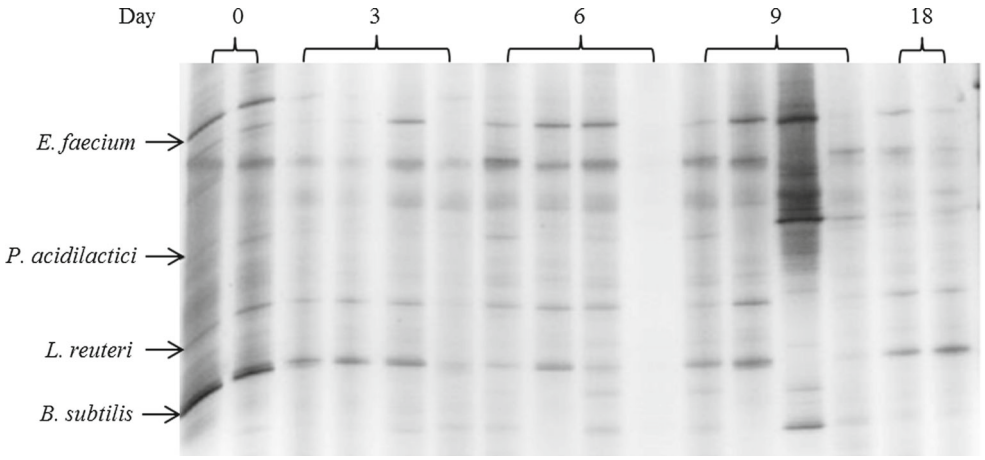


Fig. 3 Comparison of allochthonous intestinal microbiota composition between fish fed a control or probiotic diet after 8 weeks of experimental feeding. *Heatmap* shows bacterial OTUs assigned at the phylum level and *bars* show OTUs assigned at the genus level (showing genera accounting for >0.25 % of total reads)



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 $_i$th species.

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

n.d = not detected

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*)

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.

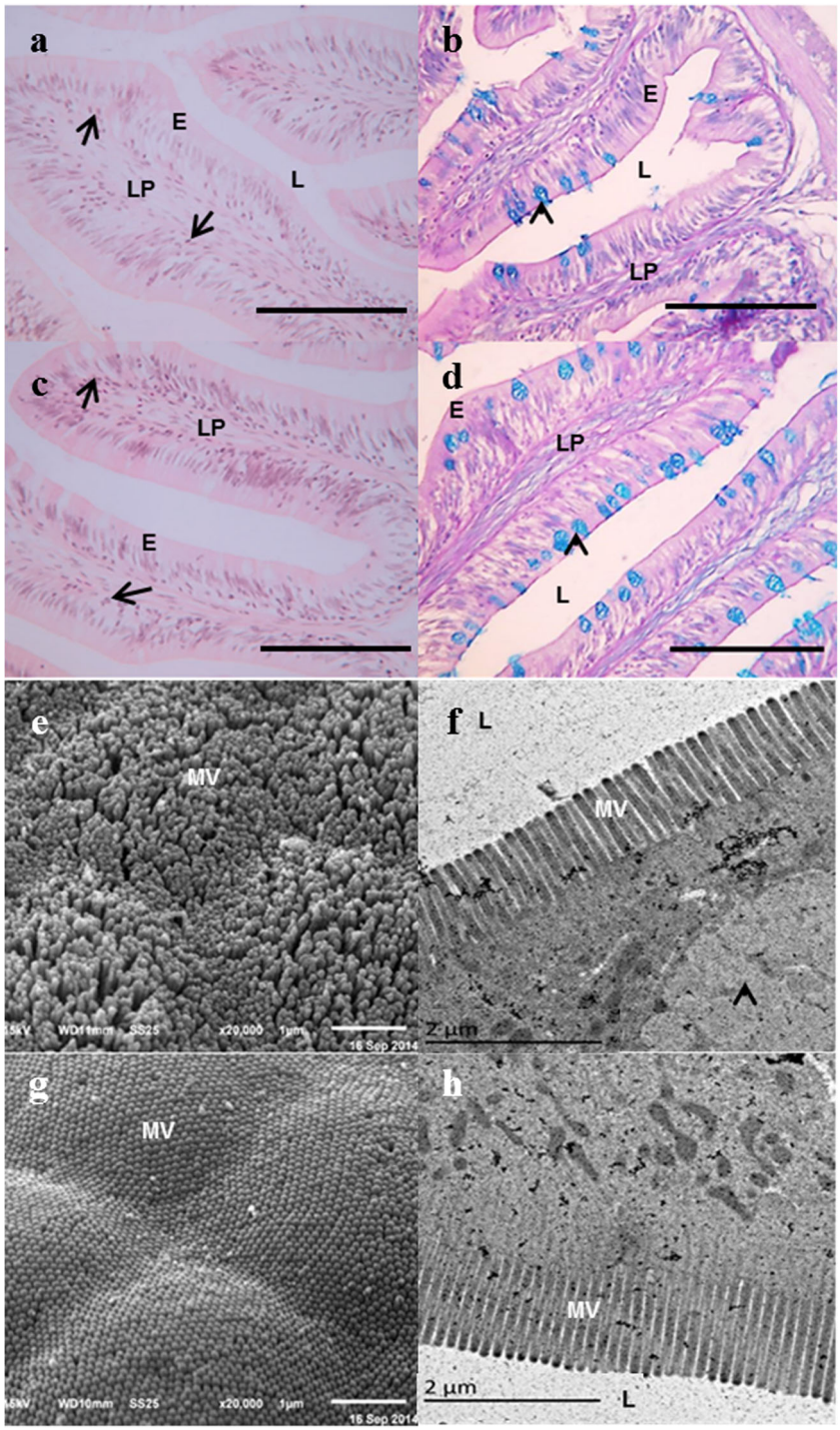


Fig. 5 Light (**a**–**d**), scanning electron (**e** and **g**) and transmission electron (**f** and **h**) micrographs of the mid-intestine of Nile tilapia fed either the control (**a**, **b** and **e**, **f**) or probiotic (**c**, **d** and **g**, **h**) diet at the end of the experimental period. 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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Conflict of interest The authors declare that they have no competing interests.

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