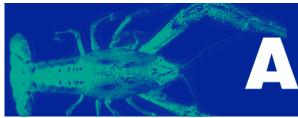
Aquaculture Research

Aquaculture Research, 2017, 1–13 doi: 10.1111/are.13300

Effects of *Bacillus subtilis* strains and the prebiotic Previda® on growth, immune parameters and susceptibility to *Aeromonas hydrophila* infection in Nile tilapia, *Oreochromis niloticus*

Samuel Addo[1], Abel A Carrias[1,2], Malachi A Williams[2], Mark R Liles[2], Jeffery S Terhune[1] & Donald A Davis[1]

[1]School of Fisheries, Aquaculture and Aquatic Sciences, Auburn University, Auburn, AL, USA

[2]Department of Biological Sciences, Auburn University, Auburn, AL, USA

Correspondence: D Allen Davis, Aquatic Animal Nutrition, School of Fisheries, Aquaculture and Aquatic Sciences, 203 Swingle Hall, Auburn, AL 36849-5419, USA. E-mail: davisda@auburn.edu

Abstract

This study was conducted to evaluate the individual and combined effects of long-term feeding of diets containing two probiotic *Bacillus subtilis* group strains (Aqua NZ and AP193) and the prebiotic Previda®, a commercial hemicellulose extract, on growth performance, immune parameters and *Aeromonas hydrophila* susceptibility of juvenile Nile tilapia, *Oreochromis niloticus*. Nile tilapia of average weight 7.47 ± 0.11 g were fed diets formulated with the probiotics and/or the prebiotic, or a control diet for 8 weeks and, subsequently, challenged with *A. hydrophila* by intragastric gavage at a dosage of 3.9×10^7 CFU per fish. Fish attained a mean weight of 59.5 ± 0.99 g at the end of the growth period. Under the conditions of the present trial, none of the diets significantly improved mean per cent weight gain ($P = 0.70$), thermal growth coefficient ($P = 0.88$) or feed conversion ratio ($P = 0.87$) of Nile tilapia. Except for the diet containing the prebiotic Previda® only ($P = 0.17$), all other diets resulted in significantly higher fish survival compared to the control ($P < 0.05$). The combined effect of the prebiotic and probiotic strains emerged as the most important diet with respect to mortality reduction. The mean lysozyme and respiratory burst activities did not show any significant differences between treatments and control ($P = 0.14$ and 0.32, respectively). Thus, these probiotic strains used in this study have the potential to prevent disease due to *A. hydrophila* in *O. niloticus*.

Keywords: prebiotic, probiotic, diets, disease challenge, Nile tilapia, immunity

Introduction

Tilapia production is of increasing importance in aquaculture globally and is second only to carp by production volume estimates (FAO 2012). Among the several species of tilapia cultured commercially, Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758) is the most abundant and important species. Global aquaculture production of tilapia has increased from around 200 000 metric tons in 1990 (FAO 2012) to about 4.2 million metric tons in 2012 and forecasted to reach a total production value of 5.0 million metric tons in 2015 (FAO 2016). The trend in tilapia aquaculture development is towards increased intensification and commercialization (Goncalves, Maita, Futami, Endo & Katagiri 2011); however, disease is a primary constraint to the growth of the industry and severely impedes both economic and socio-economic development in many producer countries (Austin & Austin 2007). Diseases caused by bacterial pathogens are responsible for heavy mortalities and annual losses. Among the major bacterial pathogens is *Aeromonas hydrophila*, which continues to plague the culture of this animal resulting in decreased survivability and profitability.

© 2017 John Wiley & Sons Ltd

Aeromonas hydrophila causes motile aeromonas septicaemia (MAS) and has also been associated as a secondary pathogen with a number of other diseases of Nile tilapia, such as epizootic ulcerative septicaemia. Primarily, however, *A. hydrophila* is usually a primary opportunistic pathogen, causing disease outbreaks in fish farms with high mortality rates, thus resulting in severe economic losses to the aquaculture industry (Fang, Ge & Sin 2004). In recent years in the western region of Alabama, USA, a MAS disease outbreak caused by a highly virulent strain of *A. hydrophila* has resulted in the loss of millions of kilogram of food size channel catfish (USDA, 2010; Pridgeon & Klesius 2011). This virulent *A. hydrophila* genotype corresponds to sequence-type 251, and is affecting farmed fish in China and the United States (Hossain, Sun, McGarey, Wrenn, Alexander, Martino, Xing, Terhune & Liles 2014; Pang, Jiang, Xie, Wu, Dong, Kwok, Zhang, Yao, Lu, Leung & Liu 2015).

Anabolic steroids, growth promoters and some antibiotics, such as oxytetracycline (OTC), sulfadimethoxine and ormetoprim among others, are commonly administered in feed to improve growth performance and to control the outbreak of diseases in aquaculture (Defoirdt, Sorgeloos & Bossier 2011). However, abuse of these chemicals, especially antibiotics, has led to the development of drug-resistant bacteria, which has reduced the efficacy of the drugs. Furthermore, accumulation of antibiotics both in the environment and in fish can pose potential risk to consumers and the environment (Carrias, Ran, Terhune & Liles 2012). Hence, to meet the increasing consumer demands for animal products that have not been treated with antibiotics whilst maintaining good health and growth, fish farmers are turning to cost-effective feed formulations that will decrease the negative effects of bacterial pathogens on farm profitability. Prebiotics, probiotics and their combinations are under extensive investigation for their potential beneficial effects on fish health and growth. Whilst a prebiotic is a non-viable food component that confers health benefit on the host associated with modulation of the microbiota (FAO 2007), a probiotic has been defined as live microorganisms which, when administered in adequate amounts, confers health benefits on the host (FAO 2001). Prebiotics are dietary carbohydrates that escape digestion in the upper gastrointestinal tract but alter the bacterial composition of the lower gut by changing the type of substrate provided to the existing gut microbiota (Gibson & Roberfroid 1995; Mei, Carey, Tosh & Kostrzynska 2011). The inclusion of common probiotic strains, such as *Bacillus* spp., in fish feed can also help promote beneficial bacterial taxa on the skin and intestine to outcompete pathogenic bacteria (El-Rhman, Khattab & Shalaby 2009). A mixture of prebiotics and probiotics, according to Gibson and Roberfroid (1995), can beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastrointestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of probiotic bacteria to improve host welfare. Thus, an effective pairing of pre- and probiotics would potentially allow alteration of the gut environment for optimal host growth and disease resistance. Several studies have shown that pre- and/or probiotics and their combinations can improve growth performance and feed utilization of various fish species including Nile tilapia (Mahious, Gatesoupe, Hervi, Metailler & Ollevier 2006; Staykov, Spring, Denev & Sweetman 2007; Torrecillas, Makol, Caballero, Montero, Robaina, Real, Sweetman, Tort & Izquierdo 2007; Burr, Hume, Neill & Gatlin 2008; Grisdale-Helland, Helland & Gatlin 2008). For instance, studies conducted by Li and Gatlin (2005) and Buentello, Neill and Gatlin (2010) indicated that prebiotics can enhance the non-specific immune responses and resistance to bacterial infections in hybrid striped sea bass and red drum. Other studies have also associated prebiotics with improvement of gut function and health, improvement of the ultrastructure of the intestinal mucosa of cobia (Salze, McLean, Schwarz & Craig 2008) and also the activation of health-promoting bacteria in the intestine of shellfish (Zhou, Ding & Huiyuan 2007). Some researchers have, on the contrary, noted that both pre- and probiotics have not been effective in their application in fish culture (Shelby, Lim, Aksoy & Klesius 2006; Merrifield, Dimitroglou, Foey, Davies & Baker 2010).

This study was conducted to: (i) explore the individual and combined effects of feeding diets containing a prebiotic and two probiotic strains on growth performance of juvenile Nile tilapia, and (ii) investigate the potential effects of pre-feeding of these diets on the survival of the Nile tilapia when challenged with *A. hydrophila*.

 © 2017 John Wiley & Sons Ltd, *Aquaculture Research*, 1–13

Materials and methods

Diet preparation

Two proprietary probiotic *Bacillus subtilis* strains and Previda®, a commercial hemicellulose extract prebiotic product (Novus International, St Charles, MO, USA), were added singly and in combination as additives to a basal diet. One of the probiotic strains, Aqua NZ Blend, is a dry concentrate containing *B. subtilis* (provided for testing by Novus International) and the other strain, AP193, is a bacterial suspension containing *Bacillus amyloliquefaciens* subsp. *plantarum* previously identified as having the capacity to reduce disease due to the enteric fish pathogen *Edwardsiella ictaluri* (Priest, Goodfellow, Shute & Berkeley 1987; Ran, Carrias, Williams, Capps, Dan, Newton, Kloepper, Ooi, Browdy, Terhune & Liles 2012). The basal/control diet was formulated to meet the nutritional requirements of tilapia containing 32% protein and 6% lipid (Table 1). The diet contained 3.3% menhaden fish oil to ensure palatability of the diets due to the addition of the pre- and/or probiotic. The probiotic was added to the diet at a weight of 1.7 g (0.028% of feed) by replacing corn starch to obtain a final concentration of approximately 4.2×10^7 CFU g^{-1} of feed. Diet 1 was the basal/control diet (no additives), whereas diet 2 was the prebiotic Previda® only, supplemented at 0.5% of the total diet (30.0 g). Diet 3 contained the probiotic Aqua NZ only, diet 4 was a mixture of Aqua NZ and Previda®, diet 5 contained AP193 only while diet 6 was a mixture of AP193 and Previda® (Table 1). Test diets were prepared at the fish nutrition laboratory of E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA. Briefly, pre-ground dry ingredients and fish oil were mixed in a 6.0 kg capacity food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was blended into the mixture for consistency and pelleted through a 3-mm die using the food mixer equipment. Pelleted diets were dried in an oven at temperature not exceeding 45°C to a

Table 1 Composition (g 100 g^{-1}) of test diets designed to contain 32% protein and 6% lipid for Nile tilapia

	Diets					
Ingredients	Control	Previda	AquaNZ	AquaNZ + Previda	AP193	AP193 + Previda
Fishmeal†	3.97	3.97	3.97	3.97	3.97	3.97
Soybean meal solvent extracted‡	46.5	46.5	46.5	46.5	46.5	46.5
Corn gluten meal§	4.65	4.65	4.65	4.65	4.65	4.65
Menhaden fish oil†	3.31	3.31	3.31	3.31	3.31	3.31
Yellow corn‡	36.0	36.0	36.0	36.0	36.0	36.0
Corn starch¶	0.97	0.47	0.94	0.44	0.97	0.47
Trace mineral premix††	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix‡‡	1.8	1.8	1.8	1.8	1.8	1.8
Choline chloride¶	0.2	0.2	0.2	0.2	0.2	0.2
Stay C 250 mg kg^{-1} §§	0.1	0.1	0.1	0.1	0.1	0.1
CaP-dibasic§	2.0	2.0	2.0	2.0	2.0	2.0
Prebiotic¶¶	0.00	0.50	0.00	0.50	0.00	0.50
Probiotic†††	0.00	0.00	0.028	0.028	0.028	0.028
Total %	100	100	100	100	100	100

†Omega Protein, Reedville, VA, USA.
‡Faithway Feed, Guntersville, AL, USA.
§Grain Processing Corporation, Muscatine, IA, USA.
¶MP Biochemicals, Solon, OH, USA.
††Trace mineral (g per 100 g Premix): cobalt chloride 0.004, cupric sulphate pentahydrate 0.25, furrous sulphate 4.0, magnesium sulphate anhydrous 13.862, manganous sulphate monohydrate 0.65, potassium iodide 0.067, sodium selenite 0.01, zinc sulphate hepahydrate 13.193, cellulose 67.964.
‡‡Vitamin (g kg^{-1} Premix): Thiamine HCl 0.44, Riboflavin 0.63, Pyridoxine HCl 0.91, D pantothenic acid 1.72, Nicotinic acid 4.58, Biotin 0.21, Folic acid 0.55, Inositol 21.05, Menadione sodium bisulphite 0.89, Vitamin A acetate (500 000 IU g^{-1}) 0.68, Vitamin D_3 (400 000 IU g^{-1}) 0.12, DL-alpha-tocopherol acetate (250 IU g^{-1}) 12.63, cellulose 955.59.
§§Stay-C® (L-ascorbyl-2-polyphosphate); Roche Vitamins, Parsippany, NJ, USA.
¶¶Prebiotic, Previda® (hemicellulose extract), Novus International.
†††Probiotics (Aqua NZ and AP193), *Bacillus subtilis* strains provided by Novus International and Auburn University, Auburn, AL USA, respectively.

moisture content of 8–10%. Diets were bagged, labelled and stored at 4°C until feeding. In all, three batches of diets (6.0 kg per batch per diet) were prepared.

Bacteria quantification in experimental diets

Samples of the diets were analysed to quantify the number of *Bacillus* spp. probiotic bacteria present in a gram of feed. One gram of each diet was placed in a 15-mL tube containing 9 mL of sterile phosphate-buffered saline (PBS). Samples were left undisturbed for 30 min and then homogenized. Dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were made from four replicate samples and 100 μL of each dilution was spread on tryptic soy agar (TSA) plates and incubated at 30°C overnight. After overnight incubation, colonies on plates with typical morphology characteristics of *B. subtilis* Aqua NZ and AP193 were counted.

Growth trial

The growth trial was conducted at the E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA, with tilapia fed the formulated diets (Diets 1–6) over a period of 8 weeks. Fingerlings of average size 7.47 ± 0.11 g were acclimated and then stocked at 40 fish per tank into 36 aquaria (132 L volume containing 100 L of water) supplied with flow-through water from a reservoir at a flow rate of 1.3 L min^{-1} per aquarium. Water temperature ranged from 27°C to 28°C during the first 5 weeks; however, from weeks 6–8 when temperatures began to drop below 26°C, the aquaria were placed on partial flow-through and re-circulation with water heated to 28°C using a submersible heater. During this time, water was UV treated using Aqua Logic UV Sterilizer model ALUV-30, 0.6A (Aqua Logic, San Diego, CA, USA). Dissolved oxygen (DO) levels in the aquaria were kept near saturation using air stones in each aquarium from a common airline connected to a regenerative air blower. Each diet treatment was randomly assigned to six replicate aquaria and fish fed throughout the experiment. Feeding was done twice a day, in the morning and late afternoon, at a per cent body weight ranging from a total of 5–10%. Feeding rates were adjusted every 2 weeks. Fish were removed, counted and weighed bi-weekly on a digital scale (Ohaus Scout Pro 4000g; Ohaus Corporation, Parsippany, NJ, USA) during which time the aquaria were cleaned. Temperature and DO were measured twice a day (early morning and late afternoon) using YSI-85 digital temperature/DO meter (YSI Corporation, Yellow Spring, OH, USA). Total ammonia nitrogen (TAN) and nitrite-nitrogen were determined twice a week from randomly selected aquaria and the photoperiod was set at 14-h light and 10-h dark. After 8 weeks, fish were counted, weighed and moved collectively by treatment approximately 6 km to the S-6 Fish Disease Challenge Laboratory at the E. W. Shell Fisheries Center.

Lysozyme and respiratory burst activity

For the determinations of respiratory burst and lysozyme activities, the assays used were adapted from Kumar, Sahu, Pal, Choudhury, Yengkokpam and Mukherjee (2005) and Lange, Gudmundsdottir and Magnadottir (2001), respectively. Blood samples were collected from three fish per tank in each treatment ($n = 18$ fish per treatment) a day before the disease challenge. Fish were anaesthetized with 100 mg L^{-1} Tricaine methanesulfonate (MS-222; Western Chemical, Ferndale, WA, USA) and blood samples collected with sterile syringes from the caudal vein around the caudal peduncle into 1.7 mL microcentrifuge tubes. For the respiratory burst activity, 50 μL of blood was placed into the wells of 96-well microtitre plates and incubated for 1 h at room temperature to assist cell adhesion. The supernatant was gently removed and the adhered cells were washed three times with PBS. After washing, 50 μL of 0.2% (w/v) nitroblue tetrazolium in PBS was added to the wells and incubated for 1 h at room temperature. The supernatant was removed and the cells were fixed with 100% methanol for 3 min and then washed three times with 30% methanol. The plates were air-dried before 60 μL of 2 mol L^{-1} potassium hydroxide and 70 μL dimethyl sulfoxide were added to each well to dissolve the formazan blue crystals. The optical density (OD) of the resulting solution was read in a spectrophotometer at 540 nm (Kumar *et al.* 2005). The remaining blood samples after drawing sub-samples for the respiratory burst determination were prepared for the lysozyme activity test in blood serum. Blood serum as supernatant was extracted into sterile 1.7 mL microcentrifuge tubes after subjecting the blood samples to centrifugation at 3000 *g* for 15 min, and the serum was stored at 4°C

© 2017 John Wiley & Sons Ltd, *Aquaculture Research*, 1–13

overnight. In a 96-well microtitre plate, 100 μL of 0.4 mg mL^{-1} suspension of *Micrococcus lysodeikticus* (Sigma Chemical, St Louis, MO, USA) in 0.05 mol L^{-1} sodium phosphate buffer (pH 6.2) was added to 100 μL of the serum in serial dilutions of 1:5 to 1:40. The OD_{590} reading was recorded at 0, 15, 30, 45 and 60 min. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of 0.001 units per min (Lange *et al.* 2001).

Preparation of *A. hydrophila* for the challenge

A frozen stock (−80°C) of *A. hydrophila* strain ML09-119 was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University (Tekedar, Waldbieser, Karsi, Liles, Griffin, Vamenta, Sonstegard, Hossain, Schroeder, Khoo & Lawrence 2013). The isolate was tested through LD50 assessment on tilapia prior to the challenge to confirm virulence at the dosage used. *A. hydrophila* used for the challenge was prepared by inoculating 5 mL TSA broth with 200 μL of a frozen cryostock of the bacteria. The 5-mL culture was incubated for 24 h at 30°C while shaking at 200 rpm and then used to inoculate 100 mL of fresh TSA. The second inoculated culture was then incubated for an additional 15 h at 30°C while shaking at 200 rpm. Prior to use in challenges, the bacterial culture was centrifuged at 3600 *g* for 30 min, re-suspended in 100 mL of fresh TSA, allowed to grow an additional 3 h and then standardized to an OD_{600} of 1. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

A. hydrophila challenge system and conditions

Disease challenge in tilapia (78 g mean weight) was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn, AL, USA under controlled temperature conditions. Fish were maintained in 60 L aquaria containing ~45 L of well water. Each aquarium was equipped with aeration and maintained at average DO of 5.00 ± 0.5 mg L^{-1}. Prior to the challenge, fish were acclimated for 1 week and fed the appropriate treatment diets. This experiment was conducted in a flow-through water supply system (0.4 L min^{-1}) with water temperature maintained at 30 ± 1°C during and after *A. hydrophilla* challenge. The laboratory was equipped with an efficient water heating system fitted with regulating valves and thermometers to maintain the water temperature.

Experimental design and *A. hydrophila* challenge protocol

The challenge experiment maintained the same experimental design used in the 8-week feeding study. Briefly, the treatments (1–6) corresponded with the diets (1–6). Each treatment was composed of six replicate aquaria each stocked with 25 fish obtained from the remaining fish after the 8-week feeding study. Fish were challenged by administering 200 μL of *A. hydrophila* ML09-119 strain (initial concentration of 1.95×10^8 CFU mL^{-1}) by intragastric gavage obtaining a final dosage of 3.9×10^7 CFU $fish^{-1}$.

Feeding and husbandry activities during challenge

During the 1-week acclimation period prior to challenge, tilapia were maintained on their treatment diets as in the growth trial for the previous 8 weeks. During the challenge, feed was offered to fish; however, fish stopped feeding a day after the challenge and did not feed while mortalities occurred. Un-eaten feed and faecal wastes were siphoned out of each aquarium as needed. Each treatment had its own set of equipment, such as nets and siphoning hose, and was disinfected after every use to avoid cross contamination. Fish were observed twice daily and, moribund and dead fish were removed and counted during each observation period. Fish were observed twice daily and, moribund and dead fish were removed and counted during each observation period. For each treatment, 18 moribund were necropsied and samples from trunk kidney, liver, skin and gills were streaked on TSA plates for bacterial isolation. Isolated colonies were identified using specialized M9 media containing *myo*-inositol (Hanson, Liles, Hossain, Griffin & Hemstreet 2014). At the end of the experiment, all surviving fish were counted, euthanized with 300 mg L^{-1} MS-222, and properly disposed.

Statistical analysis

Data collected were analysed by one-way analysis of variance using the mixed linear model procedure in SAS (SAS Institute, Cary, NC, USA). The

mixed procedure (Wolfinger, Tobias & Sall 1991) was used to identify differences among treatment means and pairwise comparisons made using Tukey's *post-hoc* test. For the disease challenge experiment, the experiment was set up in a block design to minimize variation due to location of aquarium units in three different banks. The model used in the analysis included a generalized complete block design to test for block × treatment interaction. Significance was set at 5% level ($P < 0.05$).

Results

Bacteria quantification in diets

Bacterial concentrations present in the diets are presented in Table 2. The doses of both *Bacillus* spp. strains present in the amended feed were determined to be very similar, approximately 4.7×10^7 CFU g^{-1} of feed, and were not significantly different from each other ($P = 0.40$). The counts coincided with the theoretical targeted dose of 4.2×10^7 CFU g^{-1} of feed.

Growth parameters

In the growth trial, overall mean water temperature ranged from 27.8 ± 1.2°C in the morning to 28.4 ± 0.9°C in the afternoon. Dissolved oxygen readings in the morning averaged 5.47 ± 0.58 mg L^{-1} while the average measurement in the afternoon was 5.38 ± 0.59 mg L^{-1}. Average total ammonia-nitrogen and nitrite-nitrogen were 0.27 ± 0.22 and 0.12 ± 0.04 mg L^{-1}, respectively (Table 3). No fish mortalities, behavioural abnormalities and external or internal abnormal gross signs were observed during the 8-week growth trial suggesting the safety of the prebiotic and the probiotic strain as feed additives.

Table 2 Mean concentrations of *Bacillus subtilis*-like colonies recovered from prebiotic and probiotic-supplemented diets

Treatment	Mean (CFU g^{-1})
Control	0
Previda	0
Aqua NZ	4.75×10^7
Aqua NZ + Previda	4.75×10^7
AP193	5.5×10^7
AP193 + Previda	4.5×10^7

Table 3 Overall mean water quality levels during the Nile tilapia growth trial in flow-through aquaria under laboratory conditions using water from a reservoir

Parameter	Mean levels (±SD)
Water temperature (A.M) °C	27.8 ± 1.2
Water temperature (P.M) °C	28.4 ± 0.9
Dissolved oxygen (A.M) mg L^{-1}	5.47 ± 0.58
Dissolved oxygen (P.M) mg L^{-1}	5.38 ± 0.59
Total ammonia nitrogen (mg L^{-1})	0.27 ± 0.22
Nitrite nitrogen (mg L^{-1})	0.12 ± 0.04

Table 4 shows an increase in biomass of juvenile Nile tilapia over the 8-week period of growth trial for all treatments and control although the overall % mean weight gain, thermal growth coefficient feed intake and feed conversion ratio for the treatments were not significantly different ($P > 0.05$) from the control diet at the end of the experiment.

Lysozyme and respiratory burst activity

From Table 5, pre- and probiotic treatments did not significantly influence mean serum lysozyme activity compared with the control ($P = 0.14$). The lowest mean activity of 590 ± 92.5 mL^{-1} was recorded for Previda® while the highest mean activity 675 ± 92.9 mL^{-1} occurred in the treatment group fed the Aqua NZ probiotic. Respiratory burst activity, which is an important innate defence mechanism of fish, also did not change significantly between treatments and control ($P = 0.32$).

A. hydrophila challenge

In the challenge experiment, mean water temperature was 30 ± 1°C while DO levels were maintained at 5.0 ± 0.5 mg L^{-1} across all treatments. The initial analysis showed a significant effect in both the treatment and block terms but no interaction effect because the interaction effect was not significant, the analysis was re-run as a randomized complete block removing the interaction effect from the model (Table 6). From the analysis of variance involving the randomized complete block design, both the block and treatment effects were significant at the 95% confidence level (Table 6). With the exception of the prebiotic diet, which did not differ significantly from the control diet ($P = 0.17$), all other treatment groups showed

 © 2017 John Wiley & Sons Ltd, *Aquaculture Research*, 1–13

Table 4 Prebiotic, probiotic and combined effects of formulated diets on the growth of juvenile *Oreochromis niloticus* L. under laboratory conditions

Growth parameter	Diet						
	Control	Previda	Aqua NZ	AquaNZ + Previda	AP193	AP193 + Previda	*P*-value
IBW	7.49 ± 0.07	7.39 ± 0.17	7.45 ± 0.14	7.49 ± 0.06	7.50 ± 0.06	7.48 ± 0.07	0.94
FBW	60.1 ± 4.6	60.5 ± 2.5	60.2 ± 4.3	59.4 ± 2.6	57.8 ± 3.1	59.1 ± 3.8	0.68
%WG	602.0 ± 57.4	628.9 ± 41.5	603.8 ± 50.4	592.9 ± 32.5	570.9 ± 38.2	590.0 ± 46.3	0.70
TGC	1.26 ± 0.02	1.28 ± 0.01	1.26 ± 0.03	1.25 ± 0.02	1.23 ± 0.01	1.25 ± 0.02	0.88
FI	65.3 ± 2.04	65.3 ± 2.14	65.4 ± 5.30	65.00 ± 1.99	62.4 ± 2.06	64.4 ± 1.85	0.77
FCR	1.25 ± 0.08	1.22 ± 0.02	1.25 ± 0.09	1.26 ± 0.06	1.24 ± 0.06	1.25 ± 0.07	0.87

Values are means ± SD of six replicates.
IBW (g $fish^{-1}$), initial mean body weight; FBW (g $fish^{-1}$), final mean body weight.
%WG (per cent weight gain) = 100 × (final weight − initial weight)/initial weight.
TGC (Thermal Growth Coefficient) = $[(\sqrt[3]{W_t} - \sqrt[3]{W_0})/(T \times t)] \times 1000$; where W_t = final weight, W_0 = initial weight, T = mean temperature, t = duration of growth.
FI (feed intake) = (g $fish^{-1}$ in 56 days).
FCR (feed conversion ratio) = feed intake/(FBW − IBW).

Table 5 Immune response (means ± SD) of Nile tilapia fed different experimental diets for 8 weeks

Treatment	Lysozyme (mL^{-1})	Respiratory burst (OD_{540})
Control	620 ± 98.3	0.059 ± 0.006
Previda	590 ± 92.5	0.059 ± 0.005
Aqua NZ	675 ± 92.9	0.061 ± 0.001
Aqua NZ + Previda	598 ± 78.0	0.059 ± 0.003
AP193	591 ± 85.3	0.062 ± 0.004
AP193 + Previda	622 ± 118.3	0.062 ± 0.004
P-value	0.14	0.32

Table 6 Per cent mortality of treatment groups that received feed amended with probiotics, prebiotic or a combination of both, and were challenged with *Aeromonas hydrophila*. Statistical analysis was performed using an ANOVA with a randomized complete block design. Analysis was followed by a Tukey's test to determine differences between treatments. Equal numbers of replicate treatment aquaria (six replicate aquaria total; two replicate aquaria per block; three blocks)

Treatment			% Mortality
Control			71 ± 15^{a}
Previda			54 ± 18^{ab}
Aqua NZ			46 ± 16^{bc}
Aqua NZ + Previda			29 ± 18^{c}
AP193			27 ± 9^{c}
AP193 + Previda			25 ± 15^{c}
Treatment effect	$n = 6$	F value = 11.64	$P < 0.0001$
Block effect	$n = 3$	F value = 15.62	$P < 0.0001$

Values are mean percentages ± SD. Significance between treatments ($P < 0.05$) is indicated by different letters within the same column.

significantly lower fish mortality compared to the control group (Fig. 1). Overall, the diet formulated with *B. subtilis* strain AP193 and Previda® combined had the lowest mean per cent mortality (25 ± 15%) with the highest mean per cent mortality (71 ± 15%) occurring in fish fed the control diet. Pairwise comparison showed that the two probiotic *B. subtilis* strains used in this study, Aqua NZ and AP193, were not significantly different ($P = 0.17$). Similarly, the combined prebiotic and probiotic treatments (AP193 and Previda®) and (Aqua NZ and Previda®) did not show any significant difference ($P = 0.97$) in their ability to reduce mortality due to *A. hydrophila* infection in Nile tilapia. Bacteria isolated from moribund fish showed growth in M9 minimal medium containing *myo*-inositol and this was presumptive indication of the presence of the ML09-119 strain of *A. hydrophila* used for the challenge (Hanson *et al.* 2014; Hossain *et al.* 2014).

Discussion

In aquaculture, probiotics can be applied either as feed additives or as additives to the water (Moriarty 1998; Taoka, Maeda, Jo, Kim, Park & Yoshikawa 2006). The form and duration of prebiotic and probiotic administration can influence their effectiveness in affecting fish health (Welker & Lim 2011). The supplementation of pre- and probiotics through feed has been documented as a better method of ensuring the efficiency of the probiotic bacterial colonization in the gastrointestinal tract

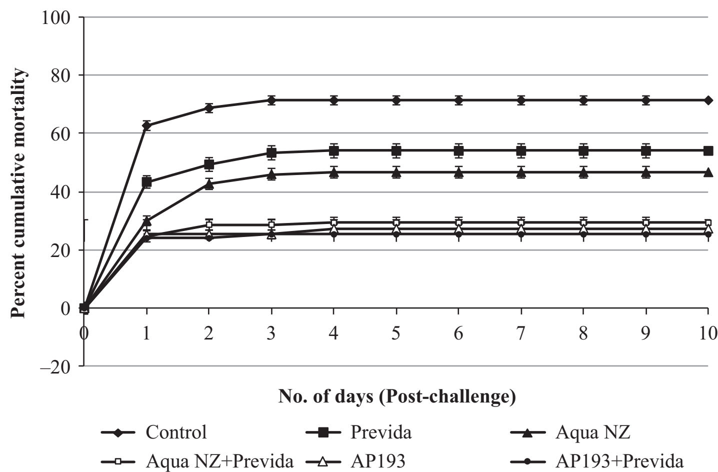

Figure 1 Per cent cumulative mortality of Nile tilapia fed probiotics, prebiotic and a mixture of both, and challenged with *Aeromonas hydrophila* by intragastric gavage.

of fish (Rurangwa, Laranja, Van Houdt, Delaedt, Geraylou, Van de Wiele, Van Loo, Van Craeyveld, Courtin, Delcour & Ollevier 2009; Merrifield *et al.* 2010; Mei *et al.* 2011). However, their use in commercial fish feed production is still relatively uncommon.

In the current work, a prebiotic was formulated separately and in combination with probiotic strains in feed and fed to Nile tilapia for 8 weeks prior to the challenge with *A. hydrophila*. The concentration of the probiotic strain obtained in the amended feed (4.7×10^7 CFU g^{-1} of feed), though not confirmed by molecular method, was not significantly different from the theoretical targeted dose (4.2×10^7 CFU g^{-1} of feed). This suggests possibly that the process of feed preparation and storage did not negatively affect the viability of the bacteria. The similar concentrations of the two probiotic strains in the bacteria-amended diets provided a controlled basis for comparison of their treatment effects. Also, the water quality parameters maintained during the study were in the range acceptable for the growth of *O. niloticus* (Mjoun, Rosentrater & Brown 2010). Nonetheless, under the conditions of the growth trial, none of the diets significantly improved nor negatively affected growth of the fish as compared to the control diet. Although prebiotics, probiotics and/or their combinations have been demonstrated to positively modulate the intestinal microflora and promote fish growth and health, results from some studies on their efficiency have been conflicting (Gatesoupe 2005; Shelby *et al.* 2006; Song, Wu, Cai, Zhang & Zheng 2006; Grimoud, Durand, Courtin, Monsan, Ouarné d, Theodoroue & Roques 2010). Results from an 8-week feeding trial conducted by Zhou, Alejandro Buentello and Gatlin (2010) with juvenile red drum to evaluate four different prebiotics, fructooligosaccharides (FOS) in the form of inulin, galactooligosaccharides (GOS), Bio-MOS® containing mannanoligosaccharides (MOS) derived from yeast and Previda® containing galacto-gluco-mannans from hemicellulose extract, showed that fish fed the diet containing Previda® had significantly higher weight gain than fish fed the basal diet or the one supplemented with Bio-MOS®. The feed efficiency and protein efficiency ratio of fish fed the various diets were not significantly different although fish fed the basal diet had the lowest values. In a study conducted by Hui-Yuan, Zhigang, Rudeaux and Respondek (2007) with hybrid tilapia, *O. niloticus* × *Oreochromis aureus* fed FOS, mean specific growth rates, daily feed intakes and feed conversion ratios were significantly improved with increasing levels of the prebiotic. Increasing the prebiotic concentration in the diets in this study from the 0.5% level may have improved growth performance. However, in a study that showed a similar lack of effect of prebiotic effects using different levels of the prebiotic Immunogen® (0, 0.5, 1, 1.5 and 2.5 g prebiotic kg $diet^{-1}$) fed to common carp fingerlings for 8 weeks, Ebrahimi, Ouraji, Khalesi, Sudagar, Barari, Zarei Dangesaraki and JaniKhalili (2011) did not observe any significant difference in growth among the groups fed different inclusion levels.

Various probiotic bacteria either singly or in combinations have been reported as important in improving growth and disease resistance in some fish species including Nile tilapia. Essa, El-Serafy, El-Ezabi, Daboor and Esmael (2010) reported

improved growth performance of Nile tilapia fed diets with *B. subtilis*, *Lactobacillus plantarum*, a mixture of *B. subtilis* and *L. plantarum* and *Saccharomyces cerevisiae*. Aly, Ahmed, Ghareeb and Mohamed (2008) compared the potential effects of two doses of *Basilus pumilus* and the commercial probiotic product Organic Green™ in improving immune response, survival, growth and resistance in Nile tilapia to *A. hydrophila* infection after feeding for 4 and 8 weeks. Mean body weight and survival rates of all treatment groups showed statistically significant increases as compared to the control group. Other studies conducted to evaluate the effects of some probiotic strains on growth of Nile tilapia, however, did not show any remarkable effects on growth performance similar to this study (Shelby *et al.* 2006; Marzouk, Moustafa & Mohamed 2008; El-Rhman *et al.* 2009; Ferguson, Merrifield, Harper, Rawling & Mustafa 2010). The differing impact of probiotics on growth performance in tilapia and other fish can relate to differences in the antibiosis activities of specific probiotic strains as well as the differing interactions between probiotics, gut microbiome, diet and the host in each study.

Although this study did not show any significant treatment effects with respect to growth performance, there was enough evidence to conclude that the probiotic strain and its combination with the prebiotic resulted in a significant reduction in mortality due to *A. hydrophila* infection. Results from the combined effect of the pre- and probiotic strain showed a significant reduction in mortality compared to the prebiotic only and the control diets, which indicates the importance of the probiotic strains in mediating disease resistance. Feeding a combined pre- and probiotic diet improved survival of rainbow trout challenged with *Vibrio anguillarum* compared to trout fed the individual prebiotic or probiotic (Rodriguez-Estrada, Satoh, Haga, Fushimi & Sweetman 2009). When the Japanese flounder was fed a diet containing *Bacillus clausii* or in combination with the prebiotics fructo- or MOS, there was an improvement of the non-specific immune function (Ye, Wang, Li & Sun 2011). Although the diet containing either of the prebiotics with *B. clausii* exhibited the highest immune function, this activity was not significantly different compared to flounder fed only *B. clausii*. Prebiotics are known to modify the microbial community within the gastrointestinal tract and to boost non-specific immune responses (Bailey, Blankenship & Cox 1991). The microbiota in the colon ferments the prebiotic and causes significant modification of the colonic microflora providing the substrate needed for growth and proliferation of probiotic bacteria, which may have the capacity to inhibit the growth of putrefactive and pathogenic bacteria present in the colon (Mussatto & Mancilha 2007; Yousefian & Amiri 2009; Mei *et al.* 2011). Thus, while the prebiotic used in this study was not observed to have any synergistic effect with the probiotic strains, it is possible that under different environmental conditions there might be beneficial interactions between pre- and probiotics in enhancing host protection against infection.

According to Welker and Lim (2011), the effectiveness of probiotics in terms of protection against infection is often attributed to enhanced immunity; however, in this study, lysozyme and respiratory burst activities were not influenced significantly by treatment effects. This agrees with the assertion that findings of lysozyme and respiratory burst activities following probiotics treatment in fish are often contradictory. While some studies have indicated probiotics do not have significant impact on these non-specific defence mechanisms of fish (Irianto & Austin 2003; Nayak, Swain & Mukherjee 2007; Sharifuzzaman & Austin 2009), other researchers have identified specific probiotics like *B. subtilis* and some members of *Lactobacillus* group to significantly stimulate respiratory burst activity in fish (Nikoskelainen, Ouwehand, Bylund, Salminen & Lilius 2003; Salinas, Cuesta, Esteban & Meseguer 2005; Salinas, Diaz-Rosales, Cuesta, Meseguer, Chabrillon & Morinigo 2006; Zhou, Tian, Wang & Li 2010). Dietary supplementation of probiotics such as *Lactobacillus sakei* in *Salmo trutta* (Balcazar, de Blas, Ruiz-Zarzuela, Vendrell, Calvo & Marquez 2007), *L. sakei*, *Lactobacillus lactis* ssp. *lactis*, *Leuconostoc mesenteroides* and *Lactobacillus rhamnosus* in *Oncorhynchus mykiss* (Panigrahi, Kiron, Kobayashi, Puangkaew, Satoh & Sugita 2004; Panigrahi, Kiron, Puangkaew, Kobayashi, Satoh & Sugita 2005; Balcazar, de Blas, Ruiz-Zazuela, Vandrell, Girones & Muzquiz 2007); *Aeromonas sobria* in *O. mykiss* (Brunt, Newaj-Fyzul & Austin 2007) as well as water supplementation of *Bacillus coagulans*, *B. subtilis* and *Rhodopseudomonas palustris* and *Enterococcus faecium* in *O. niloticus* (Pieters, Brunt, Austin & Lyndon 2008; Wang, Tian, Yao & Li 2008;

© 2017 John Wiley & Sons Ltd,

Zhou, Tian, *et al.* 2010) failed to elevate lysozyme level. Other innate immunity parameters such as phagocytic, cytokine, complement, and peroxidase and anti-protease activities in several fish species including *O. niloticus* have been stimulated through probiotic supplementation either in viable or inactivated form (Nayak 2010). Enhancement of phagocytic activity by LAB group of probiotics such as *L. rhamnosus, L. lactis* and *Lactobacillus acidophilus* has also been observed in several animals (Rutherfurd-Markwick and Gill 2004). Wang *et al.* (2008) observed that although *B. coagulans, B. subtilis, R. palustris* and *E. faecium* failed to elevate lysozyme level in *O. niloticus*, serum peroxidase level was elevated. Probiotics can also enhance natural complement activity of fish (Panigrahi *et al.* 2007; Salinas *et al.* 2005) and dietary as well as water treatment of many probiotics have been reported to stimulate the piscine complement components (Panigrahi *et al.* 2005; Wang *et al.* 2008). Thus, other innate immune parameters which were not considered in this study could be investigated in future studies.

It has also been suggested that variations in environmental conditions could be responsible for the conflicting results obtained in studies with pre- and probiotics due to differences in the choice of prebiotics, probiotics, pairing of pre- and probiotics, dietary concentrations, species strains, age/size of fish, feeding management and duration, dosage and virulence of challenge pathogens, and methods of challenge (Welker & Lim 2011). Merrifield *et al.* (2010) noted that the potential of probiotics to prevent disease may be greater than the results obtained under experimental conditions. This is because the use of intragastric and/or intraperitoneal (IP) method of disease challenge and the challenge dose may be harsher than what pertains in the natural environment of the fish. In this study, the challenge was done by intragastric gavage; hence, infection of *A. hydrophila* was very rapid compared to what would be observed in the commercial setting. This means that the potential reduction in mortality could be better than the results obtained. Other factors, such as environmental conditions, handling practices and stocking densities, may also affect the results. All these factors can influence the success or failure of prebiotics, probiotics and their combination in the enhancement of growth, immunity and disease resistance in fish.

Conclusion

Under the conditions of the current study, Previda®, Aqua NZ and AP193 strains and their combination did not improve growth performance in juvenile Nile tilapia fed diets formulated with these products. The probiotics administered individually or in combination with Previda® proved significant in reducing mortality due to *A. hydrophila* infection in juvenile Nile tilapia and might have significance when applied to other bacterial diseases of Nile tilapia and/or other fish species of aquaculture importance.

Acknowledgments

The authors would want to acknowledge Novus International Incorporated, St Charles, MO, USA for funding this work and also E. W. Shell Fisheries Center of the School of Fisheries, Aquaculture and Aquatic Sciences for the facilities used for this study.

References

Aly S.M., Ahmed Y.A.G., Ghareeb A.A.A. & Mohamed M.F. (2008) Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica*, *Oreochromis niloticus* to challenge infections. *Fish and Shellfish Immunology* **25**, 128–136.

Austin B. & Austin D.A. (2007) *Bacterial Fish Pathogens: Diseases of Farmed And Wild Fish* (4th edn). Springer-Praxis, Chichester, UK, 580 p.

Bailey J., Blankenship L. & Cox N. (1991) Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. *Poultry Science* **70**, 2433–2438.

Balcazar J.L., de Blas I., Ruiz-Zarzuela I., Vendrell D., Calvo A.C. & Marquez I. (2007) Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout, *Salmo trutta*. *British Journal of Nutrition* **97**, 522–527.

Balcazar J.L., de Blas I., Ruiz-Zazuela I., Vandrell D., Girones O. & Muzquiz J.L. (2007) Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout, *Oncorhynchus mykiss*. *FEMS Immunology and Medical Microbiology* **51**, 185–193.

Brunt J., Newaj-Fyzul A. & Austin B. (2007) The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **30**, 573–579.

Buentello J.A., Neill W.H. & Gatlin D.M.I.I.I., III (2010) Effects of dietary prebiotics on growth, feed efficiency and non-specific immunity of juvenile red drum,

 © 2017 John Wiley & Sons Ltd,

Sciaenops ocellatus fed soybean-based diets. *Aquaculture Research* **14**, 411–418.

Burr G., Hume M., Neill W.H. & Gatlin D.M.I.I.I. (2008) Effects of prebiotics on nutrient digestibility of soybean-meal-based diets by red drum, *Sciaenops ocellatus*. *Aquaculture Research* **39**, 1680–1686.

Carrias A., Ran C., Terhune J.S. & Liles M.R. (2012) Bacteria and bacteriophages as biological agents for disease control in aquaculture. In: *Infectious Disease in Aquaculture: Prevention and Control* (ed. by B. Austin), pp. 353–393. Woodhead Publishing Series in Food Science, Technology and Nutrition, No. 231, Philadelphia, PA, USA.

Defoirdt T., Sorgeloos P. & Bossier P. (2011) Alternatives to antibiotics for the control of bacterial disease in aquaculture. *Current Opinion in Microbiology* **14**, 251–258.

Ebrahimi Gh., Ouraji H., Khalesi M.K., Sudagar M., Barari A., Zarei Dangesaraki M. & JaniKhalili K.H. (2011) Effects of a prebiotic Immunogen®, on feed utilization, body composition, immunity and resistance to *Aeromonas hydrophila* infection in the common carp, *Cyprinus carpio* (Linnaeus) fingerlings. *Journal of Animal Physiology and Animal Nutrition* **96**, 591–599.

El-Rhman A.M.A., Khattab Y.A. & Shalaby A.M. (2009) *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. *Fish and Shellfish Immunology* **27**, 175–180.

Essa M.A., El-Serafy S.S., El-Ezabi M.M., Daboor S.M. & Esmael N.A. (2010) Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia, *Oreochromis niloticus*. *Journal of Arabian Aquaculture Society* **5**, 143–161.

Fang H.M., Ge R. & Sin Y.M. (2004) Cloning, characterization and expression of *Aeromonas hydrophila* major adhesion. *Fish and Shellfish Immunology* **16**, 645–658.

FAO (Food and Agriculture Organization) (2012) *The State of World Fisheries and Aquaculture 2012*. FAO, Rome, Italy, 209 pp.

FAO (Food and Agriculture Organization) (2016) GLOBEFISH – Analysis and information on world fish trade: Tilapia - September 2015.

FAO (Food and Agriculture Organization) (2001) Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria.

FAO (Food and Agriculture Organization) (2007) FAO Technical meeting on prebiotics. FAO, Rome, Italy. September 15–16, 2007.

Ferguson R.M.W., Merrifield D.L., Harper G.M., Rawling M.D. & Mustafa S. (2010) The effect of *Pediococcus acidilactici* on the gut microbiota and immune status of on-growing red tilapia, *Oreochromis niloticus*. *Journal of Applied Microbiology* **109**, 851–862.

Gatesoupe F.J. (2005) Probiotics and prebiotics for fish culture, at the parting of the ways. *Aqua Feeds: Formulation and Beyond* **2**, 3–5.

Gibson G.R. & Roberfroid M.B. (1995) Dietary modulation of the human colon microbiota: introducing the concept of prebiotics. *Journal of Nutrition* **125**, 1401–1412.

Goncalves A.T., Maita M., Futami K., Endo M. & Katagiri T. (2011) Effects of a probiotic bacterial *Lactobacillus rhamnosus* dietary supplement on the crowding stress response of juvenile Nile tilapia, *Oreochromis niloticus*. *Fisheries Science* **77**, 633–642.

Grimoud J., Durand H., Courtin C., Monsan P., Ouarné d F., Theodoroue V. & Roques C. (2010) In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. *Anaerobe* **16**, 493–500.

Grisdale-Helland B., Helland S.J. & Gatlin D.M. (2008) The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or galactooligosaccharide on the growth and feed utilization of Atlantic salmon, *Salmo salar*. *Aquaculture* **283**, 163–167.

Hanson L.A., Liles M.R., Hossain M.J., Griffin M.J. & Hemstreet W.G. (2014) 1.2.9 Motile Aeromonas Septicemia.

Hossain M.J., Sun D., McGarey D.J., Wrenn S., Alexander L.M., Martino M.E., Xing Y., Terhune J.S. & Liles M.R. (2014) An Asian origin of virulent *Aeromonas hydrophila* responsible for disease epidemics in United States-Farmed Catfish.

Hui-Yuan L., Zhigang Z., Rudeaux F. & Respondek F. (2007) Effects of dietary short chain fructo-oligosaccharides on intestinal microflora, mortality and growth performance of *Oreochromis aureus* × *O. niloticus*. *Chinese Journal of Animal Nutrition* **19**, 1–6.

Irianto A. & Austin B. (2003) Use of dead probiotic cells to control furunculosis in rainbow trout, *Onchorhynchus mykiss* (Walbaum). *Journal of Fish Diseases* **26**, 59–62.

Kumar S., Sahu N.P., Pal A.K., Choudhury D., Yengkokpam S. & Mukherjee S.C. (2005) Effect of dietary carbohydrate on haematology, respiratory burst activity and histological changes in *L. rohita* juveniles. *Fish Shellfish Immunology* **19**, 331–344.

Lange S., Gudmundsdottir B.K. & Magnadottir B. (2001) Humoral immune parameters of cultured Atlantic halibut, *Hippoglossus hippoglossus* L. *Fish Shellfish Immunology* **11**, 523–535.

Li P. & Gatlin D.M. (2005) Evaluation of the prebiotic Grobiotic®-A and brewers yeast as dietary supplements for sub-adult hybrid striped sea bass, *Morone chrysops* × *M. saxatilis* challenged in situ with. *Aquaculture* **248**, 197–205.

Mahious A.S., Gatesoupe F.J., Hervi M., Metailler R. & Ollevier F. (2006) Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). *Aquaculture International* **14**, 219–229.

Marzouk M.S., Moustafa M.M. & Mohamed N.M. (2008) The influence of some probiotics on the growth

performance and intestinal microbial flora of *Oreochromis niloticus*. Proceedings of 8th International Symposium on Tilapia in Aquaculture, Cairo, Egypt, pp. 1059–1071.

Mei G.-Y., Carey C.M., Tosh S. & Kostrzynska M. (2011) Utilization of different types of dietary fibers by potential probiotics. *Canadian Journal of Microbiology* **57**, 857–865.

Merrifield D.L., Dimitroglou A., Foey A., Davies S.J. & Baker R.T.M. (2010) The current status and future focus of probiotic and prebiotic applications for salmonids. *Aquaculture* **302**, 1–18.

Mjoun K., Rosentrater K.A. & Brown M.L. (2010) Tilapia: environmental biology and nutritional requirements. South Dakota Cooperative Extension Service, SDSU, USDA. http://pubstorage.sdstate.edu/agBio_publications/articles/Fs963-02.pdf.

Moriarty D.J.W. (1998) Control of luminous *Vibrio* sp. in penaeid aquaculture ponds. *Aquaculture* **164**, 357–358.

Mussatto S.I. & Mancilha I.M. (2007) Non-digestible oligosaccharides: a review. *Carbohydrate Polymers* **68**, 587–597.

Nayak S.K. (2010) Role of gastrointestinal microbiota in fish. *Aquaculture Research* **41**, 1553–1573.

Nayak S.K., Swain P. & Mukherjee S.C. (2007) Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham). *Fish and Shellfish Immunology* **23**, 892–896.

Nikoskelainen S., Ouwehand A.C., Bylund G., Salminen S. & Lilius E. (2003) Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria, *Lactobacillus rhamnosus*. *Fish and Shellfish Immunology* **15**, 443–452.

Pang M., Jiang J., Xie X., Wu Y., Dong Y., Kwok A.H., Zhang W., Yao H., Lu C., Leung F.C. & Liu Y. (2015) Novel insights into the pathogenicity of epidemic *Aeromonas hydrophila* ST251 clones from comparative genomics. http://www.ncbi.nlm.nih.gov/pubmed/26014286

Panigrahi A., Kiron V., Kobayashi T., Puangkaew J., Satoh S. & Sugita H. (2004) Immune responses in rainbow trout, *Oncorhynchus mykiss* induced by a potential probiotic bacteria, *Lactobacillus rhamnosus* JCM 1136. *Veterinary Immunology and Immunopathology* **102**, 379–388.

Panigrahi A., Kiron V., Puangkaew J., Kobayashi T., Satoh S. & Sugita H. (2005) The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout, *Oncorhynchus mykiss*. *Aquaculture* **243**, 241–254.

Panigrahi A., Kiron V., Satoh S., Hirono I., Kobayashi T. & Sugita H. (2007) Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus-mykiss* upon probiotic feeding. *Developmental and Comparative Immunology* **31**, 372–382.

Pieters N., Brunt J., Austin B. & Lyndon A.R. (2008) Efficacy of in-feed probiotics against *Aeromonas bestiarum* and *Ichthyophthirius multifiliis* skin infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Applied Microbiology* **105**, 723–732.

Pridgeon J.W. & Klesius P.H. (2011) *Molecular identification and virulence of three Aeromonas hydrophila isolates cultured from infected channel catfish during a disease outbreak in West Alabama in 2009. Diseases of Aquatic Organisms*, **94**, 249–253 Open access journal.

Priest F.G., Goodfellow M., Shute L.A. & Berkeley R.C.W. (1987) *Bacillus amyloliquefaciens* sp. nov. norn. rev. *International Journal of Systematic Bacteriology* **37**, 69–71.

Ran C., Carrias A., Williams M.A., Capps N., Dan B.C.T., Newton J.C., Kloepper J.W., Ooi E.L., Browdy C.L., Terhune J.S. & Liles M.R. (2012) Identification of *Bacillus* strains for biological control of catfish pathogens. *PLoS One* **7**, e45793.

Rodriguez-Estrada U., Satoh S., Haga Y., Fushimi H. & Sweetman J. (2009) Effects of single and combined supplementation of *Enterococcus faecalis*, mannanoligosaccharide and polyhydrobutyric acid on growth performance and immune response of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture Science* **57**, 609–617.

Rutherfurd-Markwick K.J. & Gill H.S. (2004) Probiotics and immunomodulation. In: *Diet and human immune function* (eds. by by D. A. Hughes, L.G. Darlington & A. Bendich,), pp. 327–344. Humana Press, Totowa, NJ.

Rurangwa E., Laranja J.L., Van Houdt R., Delaedt Y., Geraylou Z., Van de Wiele T., Van Loo J., Van Craeyveld V., Courtin C.M., Delcour J.A. & Ollevier F. (2009) Selected nondigestible carbohydrates and prebiotics support the growth of probiotic fish bacteria mono-cultures in vitro. *Journal of Applied Microbiology* **106**, 932–940.

Salinas I., Cuesta A., Esteban M.A. & Meseguer J. (2005) Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. *Fish and Shellfish Immunology* **19**, 67–77.

Salinas I., Diaz-Rosales P., Cuesta A., Meseguer J., Chabrillon M. & Morinigo M.A. (2006) Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). *Veterinary Immunology and Immunopathology* **111**, 279–286.

Salze G., McLean E., Schwarz M.H. & Craig S.R. (2008) Dietary mannanoligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture* **274**, 148–152.

Sharifuzzaman S.M. & Austin B. (2009) Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. *Fish and Shellfish Immunology* **27**, 440–445.

 © 2017 John Wiley & Sons Ltd, *Aquaculture Research*, 1–13

Shelby R.A., Lim C.E., Aksoy M. & Klesius P.H. (2006) Effect of probiotic diet supplements on disease resistance and immune response of young Nile tilapia, *Oreochromis niloticus*. *Journal of Applied Aquaculture* **18**, 23–34.

Song Z., Wu T., Cai L., Zhang L. & Zheng X. (2006) Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in *Miichthys miiuy*. *Journal of Zhejiang University of Science and Biotechnology* **7**, 596–602.

Staykov Y., Spring P., Denev S. & Sweetman J. (2007) Effect of a mannanoligosaccharide on the growth performance and immune status of rainbow trout, *Oncorhynchus mykiss*. *Aquaculture International* **15**, 153–161.

Taoka Y., Maeda H., Jo J.Y., Kim S.M., Park S. & Yoshikawa T. (2006) Use of live and dead probiotic cells in tilapia, *Oreochromis niloticus*. *Fishery Science* **72**, 755–766.

Tekedar H.C., Waldbieser G.C., Karsi A., Liles M.R., Griffin M.J., Vamenta S., Sonstegard T., Hossain M., Schroeder S.G., Khoo L. & Lawrence M.L. (2013) Complete genome sequence of a channel catfish epidemic isolate, *Aeromonas hydrophila* strain ML09-119. *Genome Announcements* **1**, e00755–13.

Torrecillas S., Makol A., Caballero M.J., Montero D., Robaina L., Real F., Sweetman J., Tort L. & Izquierdo M.S. (2007) Immune stimulation and improved infection resistance in European sea bass, *Dicentrarchus labrax* fed mannanoligosaccharides. *Fish and Shellfish Immunology* **23**, 969–981.

USDA (2010) *Catfish 2010 Part I: Reference of Catfish Health and Production Practices in the United States, 2009*. USDA-APHIS-VS, CEAH, Ft. Collins, CO, USA.

Wang Y.B., Tian Z.Q., Yao J.T. & Li W.F. (2008) Effect of probiotics, *Enteroccus faecium*, on tilapia, *Oreochromis niloticus* growth performance and immune response. *Aquaculture* **277**, 203–207.

Welker T.L. & Lim C. (2011) Use of probiotics in diets of Tilapia. *Journal of Aquaculture Research and Development*, S1:014. doi: 10.4172/2155- 9546.S1-014, 8. Open access journal.

Wolfinger R.D., Tobias R.D. & Sall J. (1991) Mixed models: a future direction. Proceedings of the Sixteenth Annual SAS Users Group Conference, pp. 1380–1388.

Ye J.-D., Wang K., Li F.-D. & Sun Y.-Z. (2011) Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder, *Paralychthys olivaceus*. *Aquaculture Nutrition* **17**, 902–911.

Yousefian M. & Amiri M.S. (2009) A review of the use of prebiotic in aquaculture for fish and shrimp. *African Journal of Biotechnology* **8**, 7313–7318.

Zhou Z.G., Ding Z.K. & Huiyuan L.V. (2007) Effects of dietary short-chain fructooligosaccharides on intestinal microflora, survival, and growth performance of juvenile white shrimp, *Litopenaeus vannamei*. *Journal of the World Aquaculture Society* **38**, 296–301.

Zhou Q.-C., Alejandro Buentello J. & Gatlin D.M.I.I.I. (2010) Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum, *Sciaenops ocellatus*. *Aquaculture* **309**, 253–257.

Zhou X., Tian Z., Wang Y. & Li W. (2010) Effect of treatment with probiotics as water additives on tilapia, *Oreochromis niloticus* growth performance and immune response. *Fish Physiology and Biochemistry* **36**, 501–509.