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Effects of *Bacillus subtilis* Strains on Growth, Immune Parameters, and *Streptococcus iniae* Susceptibility in Nile Tilapia, *Oreochromis niloticus*

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Abstract

This study was conducted to evaluate the effects of probiotic-amended diets fed to juvenile Nile tilapia, *Oreochromis niloticus*, on growth and susceptibility to *Streptococcus iniae* infection. Fish (average weight 16.5 ± 0.2 g) were fed five diets formulated with *Bacillus subtilis* strains SB3086, SB3295, SB3615, or AP193 either individually or in combination of strains SB3086 and SB3615 at a targeted concentration of approximately 4×10^7 colony-forming units (CFU)/g of feed or with a basal control diet with no additives for 21 d. After the 21-d growth trial, no significant difference in growth performance was observed with any probiotic-amended diet. Results from serum bactericidal activity showed a significant difference between treatments and the control ($P = 0.0002$), except for the SB3295-amended diet ($P = 0.9020$). Lysozyme activity was also significantly different in fish fed probiotic diets from those fed control diet ($P = 0.0001$). After 21 d of feeding, fish were challenged with *S. iniae* by intraperitoneal injection at a dosage of 8×10^6 CFU per fish. Results from the challenge also showed a significant difference between treatments and control ($P = 0.0001$). Overall, fish fed with strain SB3615 showed the lowest percent mortality (44.0 ± 7.2%) and those fed the control diet showed the highest mortality (77.3 ± 7.0%). The combined feeding with strains SB3086 and SB3615 did not result in any significant difference in reducing mortality because of *S. iniae* infection in juvenile Nile tilapia when compared with the individual probiotic treatments.

Disease outbreaks have become a major challenge to the profitable culture of fish and shellfish as aquaculture operations intensify. Globally, total annual losses from disease outbreaks have reached billions of dollars (US) and have been identified as a threat to the sustainability of the industry (Pridgeon and Klesius 2011). Streptococcal infections in fish, particularly those caused by *Streptococcus iniae*, have increased markedly with intensification of aquaculture practices (Pier and Madin 1976; Buchanan et al. 2005). Although originally isolated from freshwater dolphins (Pier and Madin 1976), *S. iniae* has emerged as an important etiological agent of streptococcosis in cultured finfish. It has gained recognition as the most

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important bacterial disease of cultured Nile tilapia, *Oreochromis niloticus*, causing mass mortality and severe economic losses (Shoemaker et al. 2001). According to Shoemaker et al. (2010), the estimated economic impact of *S. iniae* outbreaks on the US aquaculture industry is approximately US$10 million and greater than US$100 million globally. This bacterium has also been discovered as a zoonotic pathogen, with the confirmation of a number of cases involving the elderly or immunocompromised humans (Weinstein et al. 1997; Lau et al. 2003; Koh et al. 2004; Facklam et al. 2005; Agnew and Barnes 2007). Thus, the need for an effective control method is not only limited to the economic loss in aquaculture but also to protect the health of fish farmers and processers. Conventionally, antibiotics are used to control *S. iniae* infection in aquaculture; however, reported cases of lack of efficacy and resistance of bacteria to antibiotics (Stoffregen et al. 1996; Shoemaker and Klesius 1997; Locke et al. 2008; Gaunt et al. 2010) have heightened the need for alternative disease control methods. An alternative to prevent and control pathogenic bacteria is the use of probiotics. These are biologically active components of single or mixed cultures of live microorganisms, which when administered in adequate amounts are capable of improving the growth and health of the host (Salminen et al. 1999; Lara-Flores et al. 2010). Because of their reported benefits, probiotics have been commercialized and sold in the aquaculture industry as feed additives. Prevention of disease by inclusion of individual probiotic bacteria strains and/or their mixtures in the diet of fish has become preferential to antibiotic therapy (Boyd and Gross 1998; Shelby et al. 2006; Welker and Lim 2011).

In Nile tilapia, the use of probiotics in feeds to improve growth and disease resistance has been investigated by many researchers with mixed results (Lara-Flores et al. 2003; El-Haroun et al. 2006; Shelby et al. 2006, 2009; Taoka et al. 2006; Aly et al. 2008a, 2008b; Marzouk et al. 2008; El-Rhman et al. 2009; Essa et al. 2010; Ferguson et al. 2010; Ghazalah et al. 2010; Zhou et al. 2010; Pirarat et al. 2011). Because of these mixed results, it is imperative for more studies to be carried out to ascertain specific probiotic strains and/or their combinations that can significantly control infections in *O. niloticus*.

This study examined 21-d feeding effects of four *Bacillus subtilis* strains, SB3086, SB3295, SB3615, and AP193, and a combination of SB3086 and SB3615 on growth performance, nonspecific immune activity, and *S. iniae* susceptibility in juvenile *O. niloticus*.

Materials and Methods

Diet Preparation

Three proprietary probiotic strains of *B. subtilis* and one strain identified from a collection at Auburn University, Auburn, AL, USA (Ran et al. 2012) were added as feed additives to a basal diet. The basal diet was formulated to meet the nutritional requirements of tilapia, containing 32% protein and 6% lipid. All the diets contained 3.3% menhaden fish oil to ensure palatability. The basal/control diet had no probiotic additives and contained 0.2% corn starch. The probiotic strains were added to their respective diets at 0.2% inclusion levels but did not contain corn starch (Table 1). Three of the probiotic strains, SB3086, SB3295, and SB3615, and a combination of SB3086 and SB3615 were dry concentrates containing the probiotic strains blended with calcium carbonate (provided for testing by Novus International, Inc., Saint Charles, MO, USA). The fourth strain was a bacterial spore suspension (AP193) containing *Bacillus amyloliquefaciens* subsp. *plantarum*. AP193 was isolated from a soybean rhizosphere and was part of a collection of plant growth-promoting rhizobacteria that were screened for activity against aquaculture pathogens (Hanson et al. 2014; Hossain et al. 2014). The test diets were prepared at the fish nutrition laboratory at the E. W. Shell Fisheries Center, Auburn University. Preground dry ingredients and fish oil were mixed in a 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 same equipment. Pelleted diets were dried in an oven to a moisture content of 8–10%, bagged, labeled, and stored at 4 C until feeding.

TABLE 1. *Composition (g/100 g as is) of experimental diets, with or without probiotics, formulated to contain 32% protein and 6% lipid and fed to Nile tilapia.*

Ingredients	1 Control	2 SB3086	3 SB3295	4 SB3615	5 SB3086 + SB3615	6 AP193
Fishmeal[a]	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal solvent extracted[b]	46.50	46.50	46.50	46.50	46.50	46.50
Menhaden fish oil[a]	3.31	3.31	3.31	3.31	3.31	3.31
Yellow corn[b]	36.74	36.74	36.74	36.74	36.74	36.74
Corn starch[c]	0.20	0.00	0.00	0.00	0.00	0.00
Trace mineral premix[d]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[e]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride[c]	0.20	0.20	0.20	0.20	0.20	0.20
Stay-C® 250 mg/kg[f]	0.10	0.10	0.10	0.10	0.10	0.10
Calcium Phosphate-dibasic[g]	2.00	2.00	2.00	2.00	2.00	2.00
Corn gluten meal[g]	4.65	4.65	4.65	4.65	4.65	4.65
Probiotic	0.00	0.20	0.20	0.20	0.20	0.20
Total %	100	100	100	100	100	100

[a]Omega Protein, Inc., Reedville, VA, USA.

[b]Faithway Feed Co., Guntersville, AL, USA.

[c]MP Biochemicals, Inc., Solon, OH, USA.

[d]Trace mineral (g/100 g premix): cobalt chloride 0.004, cupric sulfate pentahydrate 0.25, ferrous sulfate 4.0, magnesium sulfate anhydrous 13.862, manganese sulfate monohydrate 0.65, potassium iodide 0.067, sodium selenite 0.01, zinc sulfate heptahydrate 13.193, and cellulose 67.964.

[e]Vitamin (g/kg premix): thiamin 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 bisulfite 0.89, vitamin A acetate (500,000 IU/g) 0.68, vitamin D_3 (400,000 IU/g) 0.12, DL-alpha-tocopherol acetate (250 IU/g) 12.63, and cellulose 955.59.

[f]Stay-C (L-ascorbyl-2-polyphosphate), Roche Vitamins, Inc., Parsippany, NJ, USA.

[g]Grain Processing Corporation, Muscatine, IA, USA.

Bacteria Quantification in Experimental Diets

Samples of the diets ($n = 4$, 1 g of feed plus 9 mL phosphate-buffered saline [PBS] each) were analyzed to quantify the number of viable *Bacillus* cells present in a gram of feed. Samples were left undisturbed for 30 min and then homogenized. From each replicate sample, 10-fold serial dilutions were made, and 100 µL of dilutions, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}, were spread on tryptic soy agar (TSA) plates, and then incubated at 30 C overnight. After overnight incubation, colonies on plates with typical morphology characteristics of each of the *Bacillus* strains were quantified for colony-forming units (CFU) per gram of feed.

Growth

The 21-d feeding of fish with the experimental diets was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn University. Fish of average size 16.5 ± 0.2 g were kept in 60-L aquaria containing approximately 45 L of well water (28 fish per aquarium). Each aquarium was equipped with aeration maintained at dissolved oxygen levels of 5.00 ± 0.5 mg/L. Prior to administering the diets, fish were acclimated for 1 wk at 28 ± 1 C. During the acclimation period, tilapia were maintained on a commercial diet at 3% of their body weight. At the commencement of the feeding phase, treatment and control diets were adjusted to 6% body weight. Fish were fed twice a day, in the morning and late afternoon. Any uneaten feed and waste materials were siphoned out of each aquarium as needed. Each treatment had its own set of equipment, such as nets and siphoning hose, which were disinfected in iodine solution after every use to avoid cross-contamination. At the end of the feeding period, final mean body weight, percent weight gain (WG) ($100 \times$ [final weight − initial weight]/initial weight), specific growth rate (SGR) ($100 \times$ [ln final weight − ln initial weight]/d), and feed conversion ratio (FCR) (feed intake as fed/WG) were determined.

Serum Bactericidal and Lysozyme Activity

Blood samples were collected from three fish per tank in each treatment ($n = 18$ fish per treatment) after 21 d of feeding fish with the experimental diets and before the challenge. Fish were anesthetized to loss of equilibrium with MS-222 (Sigma-Aldrich, St. Louis, MO, USA) and blood samples were collected with sterile syringes from the caudal vein around the caudal peduncle into 1.7-mL microcentrifuge tubes without anticoagulants. Blood samples were allowed to clot for 30 min at room temperature and stored at 4 C overnight. Blood serum was pipetted into sterile 1.7-mL microcentrifuge tubes from the blood samples as supernatant after centrifuging at 3000 *g* for 15 min at 4 C. Serum samples were stored at −80 C, and after 1 wk of storage, samples were taken out and thawed for serum bactericidal and lysozyme activities determination.

In the serum bactericidal activity procedure, bacterial cultures of *S. iniae* were subjected to centrifugation, and the pellet was washed and resuspended in PBS. The optical density (OD) of the suspension was adjusted to 0.5 at 546 nm. The bacterial suspension was serially diluted (1:10) with PBS five times. The serum bactericidal activity was determined by incubating 2 µL of the diluted bacterial suspension with 20 µL of the serum for 1 h at 37 C. A control in which PBS replaced the serum was included. The numbers of viable bacteria were determined by counting the colonies after culturing on TSA plates for 24 h at 30 C. Lysozyme activity of serum was measured using the turbidity assay. On a flat-bottomed 96-well microtiter plate, 200 µL of 0.2 mg/mL suspension of *Micrococcus lysodeikticus* in sodium phosphate buffer (0.05 mol/L, pH 5.2) was added to 5 µL of serum. The reduction in the absorbance at 570 nm was determined 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 minute. Chicken egg lysozyme (Sigma, Sigma-Aldrich, St. Louis, MO, USA) was used as a standard (Kajita et al. 1990; Rainger and Rowley 1993; Lange et al. 2001).

Preparation of S. iniae *for the Challenge*

The *S. iniae* challenge strain ARS 98-60 (Iwashita et al. 2015) was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. The bacteria isolate used was previously passed through tilapia to confirm virulence. A bacterial culture for *S. iniae* challenge was prepared by inoculating 5 mL tryptic soy broth (TSB) with 200 µL of a frozen stock (−80 C) of the bacterium. The 5-mL culture was incubated for 36 h at 30 C while shaking at 150 rpm, and then inoculated into 100 mL of fresh TSB. The second inoculated culture was then incubated for an additional 15 h at 30 C while shaking at 150 rpm. Prior to its use for the challenge, the bacterial culture was subjected to centrifugation at 3600 *g* for 30 min, resuspended in 100 mL of fresh TSB, allowed to grow an additional 3 h, and then standardized to an OD_{600} of 1.0. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

The challenge was performed 21 d after initial-treatment diet administration with 25 fish per tank after the removal of three fish per tank for use in the immunological analysis. All fish were removed from aquaria and anesthetized in 100 mg/L of MS-222 and challenged by administering 200 µL of *S. iniae* suspension in PBS by intraperitoneal (IP) injection to obtain a final dosage of 8×10^6 CFU per fish. Fish were replaced into original aquaria after injection. For the negative control, fish were exposed to the same challenge conditions as those groups receiving challenge bacteria, except that buffer was administered instead of bacteria. During challenge, flow-through water

TABLE 2. *Concentrations of* Bacillus*-like colonies recovered from probiotic-supplemented diets.*

Diet	Mean ± SD (CFU/g)
Control	0
SB3086	$8.5 \pm 9.3 \times 10^7$
SB3295	$7.3 \pm 4.2 \times 10^7$
SB3615	$8.2 \pm 3.9 \times 10^7$
SB3086 + SB3615	$7.0 \pm 4.2 \times 10^7$
AP193	$7.7 \pm 5.1 \times 10^7$

CFU = colony-forming units.

TABLE 3. *Effects of experimental diets on the growth of juvenile* Oreochromis niloticus *grown for 21 d in flow-through aquaria.*

Growth	Diets						
Parameter	Control	SB3086	SB3295	SB3615	SB3086 + SB3615	AP193	*P* value
IBW	16.2 ± 0.5	16.6 ± 0.8	16.6 ± 0.8	16.6 ± 0.8	16.7 ± 1.1	16.7 ± 0.8	0.6205
FBW	33.7 ± 1.6	30.6 ± 1.5	35.6 ± 1.6	34.1 ± 2.6	34.6 ± 2.2	33.3 ± 1.3	0.1390
% WG[a]	107.5 ± 7.1	101.6 ± 10.7	115.0 ± 9.5	106 ± 14.3	107.6 ± 9.7	100.2 ± 6.7	0.3910
SGR[b]	3.5 ± 0.05	3.0 ± 0.07	3.6 ± 0.06	3.4 ± 0.08	3.5 ± 0.08	3.3 ± 0.05	0.0955
FCR[c]	1.42 ± 0.11	1.41 ± 0.09	1.54 ± 0.12	1.42 ± 0.12	1.45 ± 0.05	1.35 ± 0.18	0.1172

FBW = final mean body weight (g per fish); IBW = initial mean body weight (g per fish).
[a]% WG (percent weight gain) = 100 × (final weight − initial weight)/initial weight.
[b]SGR (specific growth rate) (%/d) = 100 × ([ln final weight − ln initial weight]/d).
[c]FCR (feed conversion ratio) = feed intake/(final mean body weight − initial mean body weight).

TABLE 4. *Serum bactericidal and lysozyme activities of juvenile* Oreochromis niloticus *fed probiotic diets for 21 d.*

Treatment	Bactericidal activity (CFU)	*P* value	Lysozyme (mL/L)	*P* value
Control	14.8 ± 6.8	0.0004	838.3 ± 117.7	0.0001
SB3086	3.3 ± 2.0	0.0010	1015.0 ± 76.4	0.0015
SB3295	12.0 ± 5.9	0.9020	1055.0 ± 70.4	0.0002
SB3615	6.2 ± 4.3	0.0065	1048.3 ± 65.5	0.0002
SB3086 + SB3615	6.2 ± 2.6	0.0205	1026.7 ± 110.8	0.0007
AP193	4.3 ± 2.1	0.0035	1005.0 ± 54.7	0.0031

CFU = colony-forming units.

supply (0.4 L/min) and temperature of 28 ± 1 C were maintained.

After challenge, fish were observed daily for behavioral changes and for gross signs of disease. Moribund and dead fish were removed and counted early morning and late afternoon each day. Samples of moribund or freshly dead fish were necropsied and samples from trunk kidney, liver, and brain were streaked on TSA plates for bacterial isolation. Isolated colonies were identified using biochemical tests. At the end of the challenge experiment, all surviving fish were counted, euthanized with 300 mg/L MS-222, and properly disposed.

Statistical Analysis

Data collected on growth, immunology, and fish mortality were analyzed using SAS (SAS Institute, Inc., Cary, NC, USA). The mixed procedure (Wolfinger et al. 1991) was used to make treatment contrasts. A complete randomized block design was incorporated in this study to minimize variation due to location of aquarium units in three different banks of aquaria. Differences between means were considered significant when probability values were less than 0.05.

Results

Experimental Diets

The diets were prepared based on a standard basal diet to which the 0.2% corn starch was substituted with equal concentrations (0.2%) of the probiotic *B. subtilis* strains (Table 1). Bacterial concentrations of the diets are presented in Table 2. The dose of the probiotic strains present in the amended diets was determined to be statistically indifferent among all treatments and was within the targeted range of 10^7 CFU/g of feed.

Growth

During the 21-d feeding trial, fish increased in biomass from a mean value of 16.5 ± 0.2 to 33.7 ± 1.5 g. The mean percent WG, SGRs, and FCRs for the treatments were not significantly different from the control diet at the end of the experiment (Table 3). No fish mortalities or abnormal behavior were observed during this

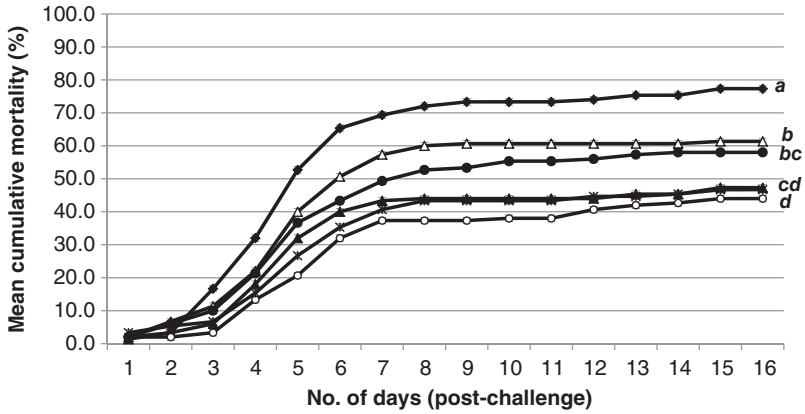

FIGURE 1. *Mean percent cumulative mortality of Nile tilapia fed probiotic diets for 21 d and challenged with* Streptococcus iniae *using intraperitoneal injection. Significance between treatments is indicated by different letters in the graph. (—◆—) Control; (—▲—) SB 3086; (—△—) SB 3295; (—○—) SB 3615; (—✳—) SB 3086 + SB 3615; (—●—) AP 193.*

phase of the experiment. Additionally, no gross external effects were observed.

Serum Bactericidal and Lysozyme Activity

Results from immunological parameters are presented in Table 4. Mean serum bactericidal activities were significantly higher in all treatment groups ($P = 0.0004$) when compared with the control except for strain SB3295 ($P = 0.9020$). Statistically, the combined probiotic treatment did not show any improved advantage over the individual groups. Mean lysozyme activities were significantly higher in fish fed the probiotic diets than that fed the control diet ($P < 0.0001$).

S. iniae *Challenge*

The mean percent cumulative mortality of Nile tilapia fingerlings challenged with *S. iniae* revealed a significant reduction in mortality associated with each of the probiotic strains, with differences in mortality being observed as early as Day 3 postchallenge and most mortalities occurring by Day 7 post challenge. The experiment was concluded 16 d post challenge when mortalities had ceased 2 d earlier in all the treatment groups. The mortalities ranged from the lowest value of $44.0 \pm 7.2\%$ in Treatment 4 to the highest value of $77.3 \pm 7.0\%$ in the control treatment group (Fig. 1 and Table 5).

TABLE 5. *Percent mortality of juvenile* Oreochromis niloticus *fed probiotic diets and challenged with* Streptococcus iniae.

Treatment	Mortality (%)	*P* value
Control	77.3 ± 7.0	0.0001
SB3086	47.3 ± 4.7	0.9768
SB3295	61.3 ± 8.6	0.0170
SB3615	44.0 ± 7.2	0.0001
SB3086 + SB3615	46.7 ± 9.7	1.0000
AP193	58.0 ± 10.0	0.2097

A significant difference by treatment was observed between the control treatment group and all other treatments. In specific comparisons among treatments, differences were observed only between diets with strain SB3295 (mean mortality = 61.3%) and strain SB3086 (mean mortality = 47.3%), as well as strain SB3615 (mean mortality = 44.0%) and strain AP193 (mean mortality = 58.0%). The effect of the combination of strains SB3086 and SB3615 did not result in improved fish survival relative to the use of these strains individually.

Discussion

Feeding trials with probiotics have produced mixed results when growth is measured. In this study, growth of juvenile Nile tilapia fed the selected probiotic diets (individually and one combination) for 21 d did not significantly

improve. These results are not unexpected given the short duration of this trial. This statement agrees with Apun-Molina et al. (2009) who observed a tendency toward improved growth in Nile tilapia fry (0.14 g) only after 75 d of feeding with diets composed of *Bacillus* or *Lactobacillus* probiotics. Honsheng (2010) attributed improved WG and feed efficiency to increased enzyme production because of the inclusion of *B. subtilis* in tilapia diets. According to Ridha and Azad (2012), probiotics may improve digestion by stimulating production of digestive enzymes or through other alterations in the gut environment of fish. The lack of significant results in growth performance from this short study nonetheless corroborates findings from other studies on probiotics. For instance, nonviable *Saccharomyces cerevisiae* (Marzouk et al. 2008), *Pseudomonas* spp. (El-Rhman et al. 2009), *Pediococcus acidilactici*, and *Enterococcus faecium* (Biomate SF-20®) (Chr. Hansen, Hørsholm, Denmark) (Ferguson et al. 2010), *B. subtilis* + *Bacillus licheniformis* (Bioplus 2B®) (Chr. Hansen, Hørsholm, Denmark), *P. acidilactici* (Bactocell PA10 MD®) (Lallemand Animal Nutrition, Milwaukee, WI, USA), and viable *S. cerevisiae* (Levucell SB 20®) (Lallemand Animal Nutrition, Milwaukee, WI, USA) (Shelby et al. 2006) have all been reported as not having any significant effect on tilapia growth. Contrarily, other studies conducted by different researchers using the same or different strains of probiotic bacteria have produced significant improvement in growth of Nile tilapia. Aly et al. (2008a) noted statistically significant increases in WG of Nile tilapia after 4–8 wk of feeding two doses of *Bacillus pumilus* and the commercial probiotic product Organic Green™ (Hangpoong Industry Co. LTD, Korea) when compared with the control group. According to Lara-Flores et al. (2010), supplementation of combined *S. faecium* and *Lactobacillus acidophilus* or *S. cerevisiae* singly in tilapia diets containing 27 or 40% crude protein produced significantly higher WG and feed utilization efficiency compared with the control diet. Improved growth performance of Nile tilapia fed diets with *B. subtilis*; *Lactobacillus plantarum*; or a mixture of *B. subtilis*, *L. plantarum*, and *S. cerevisiae* has been reported by Essa et al. (2010). The contradicting reports on the effects of probiotics fed to tilapia after both short and longer periods may suggest that variability in probiotic strain efficacy, research conditions, handling practices, and stocking rates among other factors might have affected the results, which consequently influenced the success or failure of probiotics and their combinations to improve growth. The lack of detectable growth enhancement in this study could be possibly due to the fact that the experimental conditions were ideal for optimal growth of the fish. The gastrointestinal (GI) tract colony present might have influenced results because intestinal microbiota have important and specific metabolic and trophic functions (Denev et al. 2009). Gutowska et al. (2004) noted that the bacterial flora in the GI tract of fishes, in general, has a very important and diversified enzymatic potential with the capacity to produce proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which is important for digestion of proteins, carbohydrates, cellulose, lipids, and chitin to enhance growth.

Serum bactericidal activity was higher in fish fed probiotics than those fed the control, except in fish fed the probiotic strain SB3295. There was also higher lysozyme activity in fish fed the probiotic diets than in those fed the control diet. A number of systemic, nonspecific immune functions, including serum bactericidal and lysozyme activities, have been observed to be enhanced by dietary probiotic supplementation (Nayak 2010; Pirarat et al. 2011). Ferguson et al. (2010) found that blood leukocyte numbers and serum lysozyme activity were enhanced in Nile tilapia fed the probiotic *P. acidilactici*. In a study to evaluate the use of *L. acidophilus* as a biocontrol agent against some common fish pathogenic bacteria, including *Streptococcus* spp. in the African catfish, *Clarias gariepinus*, Al-Dohail et al. (2011) observed a higher immunological response and concluded that *L. acidophilus* was useful as a probiotic agent in *C. gariepinus* against bacterial pathogens. Taoka et al. (2006) investigated the effect of live and dead probiotic cells on the nonspecific immune system of *O. niloticus* and found that probiotic administration enhanced nonspecific

immune parameters such as lysozyme activity, migration of neutrophils, and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection. However, the viable cells might not be stimulating the immune system, but rather their cellular products or components or just the high concentration of the viable cells might be causing the increased response. Shelby et al. (2006) did not find any effect on lysozyme activity, alternative complement, or total serum immunoglobulin in tilapia fed commercial probiotics containing *B. subtilis*, *B. licheniformis*, *P. acidilactici*, and *S. cerevisiae*. They concluded that feeding Nile tilapia for 94 d with these commercial probiotics did not prevent streptococcal infection.

Various mechanisms have been proposed to explain the effects of probiotics in fish disease resistance. These include competition for adhesion sites on the intestinal epithelium or other tissue surfaces, competition for nutrient and energy sources, release of secondary metabolites that have bactericidal effects on other microbial populations, and enhancement of the host immune response. It has been observed that the ability to adhere to enteric mucus and intestinal wall surfaces was indispensable for probiotic bacteria to become established in fish intestines (Onarheim and Raa 1990; Westerdahl et al. 1991; Olsson et al. 1992). Montes and Pugh (1993) proposed that competition for adhesion receptors with pathogens might be a critical probiotic phenotype because bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection (Verschuere et al. 2000a; La Ragione and Woodward 2003; La Ragione et al. 2004). According to FAO (2001), probiotics confer health benefits on the host when administered in adequate amounts. In this study, survival of Nile tilapia to *S. iniae* challenge was significantly higher with the probiotic diets than with the control diet, which means that the probiotic diets probably conferred some health benefits to the fish.

Several studies have attributed a probiotic effect to competition for energy sources (Rico-Mora et al. 1998; Verschuere et al. 1999, 2000a, 2000b) and the production and release of inhibitory substances such as antibiotics, bacteriocins, siderophores, lysozymes, proteases, and hydrogen peroxide, which constitute a barrier against the proliferation of pathogens (Chaucheyras-Durand et al. 2008; Marden et al. 2008; Chaucheyras-Durand and Durand 2010). El-Rhman et al. (2009) noted that probiotic inclusion in fish feed can stimulate the growth of beneficial bacterial taxa on skin and in the intestine, which could aid in the competitive exclusion of pathogens. The effectiveness of probiotics in terms of protection against infection has also been demonstrated to be as a result of enhanced immunity (Delcenserie et al. 2008; Johnson-Henry et al. 2008; Welker and Lim 2011). Merrifield et al. (2010) stated that probiotic use can enhance the immune response of tilapia and improve disease resistance. It is more likely that the positive results reported in this study may be due to enhanced immune response because the IP injection administered bypasses the defense system of the GI. Merrifield et al. (2010) noted that the capacity of probiotics to prevent disease may be greater than the results observed in many studies because of the use of IP injection as a method of disease challenge. The IP method bypasses competitive exclusion, which is one of the most important ways probiotics can prevent infection in the GI tract. These authors stated that IP challenges may not reflect the effect of probiotics on resistance to infection, but rather demonstrate the effect of probiotics on infected fish. According to Shoemaker et al. (2006), the majority of challenges performed in tilapia research studies are performed by IP injection, especially with *Streptococcus*, which is difficult to reproduce reliably by bath immersion. In this study, the challenge was performed intraperitoneally, which does not reflect the mode of infection by *S. iniae* under culture conditions. This means that the potential reduction in mortality could be better than the results obtained. Because of this limitation, it would be difficult to conclude which of the probiotic treatments was most effective.

Conclusions

A short-term feeding (21 d) of *B. subtilis* strains SB3086, SB3295, SB3615, or AP193 did

not significantly improve the growth of juvenile *O. niloticus*. The findings, however, did demonstrate beneficial effects of in-feed supplementation of these probiotics in improving the immune response and survival against *S. iniae* challenge in *O. niloticus*. Probiotic treatments, singly and combined, significantly enhanced serum bactericidal and lysozyme activities of tilapia and decreased mortality from *S. iniae* infection. Further studies to assess the effects of longer-term application of these probiotics on growth and survival against *S. iniae* and other important tilapia bacterial diseases, as well as their assessment in other cultured fish species prone to *S. iniae* infection, are recommended.

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