Effects of pre- and probiotics on pond production, growth and disease susceptibility of channel catfish *Ictalurus punctatus* and Nile tilapia *Oreochromis niloticus*

by

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Abstract

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 United States dollars and have been identified as a threat to the sustainability of the industry. Costs, regulations, certification protocols and consumer preferences are driving the industry away from the use of therapeutic drugs towards the use of prebiotics and probiotics. This dissertation evaluated the effects of a prebiotic and probiotics under pond production conditions as well as the effects on growth and disease susceptibility under laboratory conditions. Four investigations were conducted. The first two examined the effects of two commercially-available probiotic products Lymnozyme® and PondToss™ as water additives in reducing mortality due to columnaris and improving pond water quality and pond production of channel catfish *Ictalurus punctatus*. Results from these two investigations lead to the evaluation of a prebiotic Previda® and some probiotic strains of *Bacillus subtilis* as feed additives in diets of Nile tilapia, *Oreochromis niloticus*. Hence, the third and fourth studies evaluated the effects of the prebiotic and probiotics and their combinations as feed additives on growth, immunity parameters and survival from *Aeromonas hydrophila* and *Streptococcus iniae* challenges in Nile tilapia *Oreochromis niloticus*.

The effectiveness of the commercially-available aquatic probiotic product Lymnozyme® as a water additive to reduce mortality due to *Flavobacterium columnare*

in juvenile channel catfish was assessed. Two experiments were conducted. In the first experiment, Lymnozyme® treatment was applied for 2 hours daily and in the second 8 hours daily. Mean cumulative mortalities from the first experiment were 77±15.4%, 76±8.9%, 69±11.2% and 64±7.6%, respectively, for control, and 3-, 7-, and 14-days of treatment with no significant difference between treatments (p=0.3179). In the second experiment, control fish had a significantly higher (p=0.0041) mean cumulative mortality (80±12.5%) than all treatments for 3, 7 and 14 days with mean mortalities of 61±14.5%, 50±13.0%, and 44±14.6%, respectively.

The efficacy of using PondToss™ in ponds to improve water quality and pond production of channel catfish was evaluated over two production seasons (2011 and 2012). There was no significant treatment effects in mean total ammonia-nitrogen (TAN) and nitrite-nitrogen levels for untreated and treated ponds in both seasons (p=0.9130 in season 1, p=0.2131 for season 2). Water temperature, dissolved oxygen, pH, alkalinity, hardness and chloride levels were similar in all ponds (untreated and treated) for the two production years. Mean growth rate, percent survival, feed conversion ratio and mean weight at harvest in both production years showed no significant difference between treated and untreated ponds. Mean standing crops of 7,890±983kg/ha and 6,768±1294 kg/ha were obtained in 2011 with respect to treated and untreated ponds, while in 2012, mean standing crops were 9,060±620 kg/ha and 8,512±928 kg/ha for treated and untreated ponds, respectively. In either year, there was no significant treatment effects (p=0.2617 and 0.5604, respectively).

The individual and combined effects of an 8-week feeding of diets containing two probiotic *Bacillus subtilis* strains (Aqua NZ and AP193) and the prebiotic Previda® on mean growth performance, immune parameters and *Aeromonas hydrophila* disease

challenge in juvenile Nile tilapia were evaluated under laboratory conditions. None of the diets significantly improved growth performance ($p=0.6972$), respiratory burst activity ($p=0.14$) or lysozyme activity ($p=0.32$) of Nile tilapia. Except for the diet containing the prebiotic Previda® only ($p=0.17$), all other diets resulted in significantly lower mean cumulative fish mortality compared to the control ($p<0.05$). The combined effect of the prebiotic and probiotic strains showed a significant reduction in mortality from the prebiotic only diet.

The 3-week (21 days) feeding effects of the probiotic strains SB3086, SB3295, SB3615, SB3086 + SB3615 and AP193 on mean growth, immune parameters and susceptibility to *Streptococcus iniae* infection in juvenile Nile tilapia produced no significant improvement in growth performance. Results from serum bactericidal activity showed a significant difference between treatment and control groups ($p=0.0002$), except the group fed probiotic strain SB3295 ($p=0.5823$). Lysozyme activity was also significantly higher in all treatments than control ($p<0.0001$). Mean cumulative mortalities were significantly lower in probiotic fed fish as compared to control ($P\leq0.0170$). The combination of probiotic strains SB3086 and SB3615 did not appear to have any significant advantage in reducing mortality due to *S. iniae* in juvenile Nile tilapia.

In conclusion, the prebiotic and probiotic strains used as water or feed additives in combination or individually effectively reduced disease mortality in channel catfish and Nile tilapia under laboratory conditions. Under production conditions in channel catfish ponds; however, PondToss™ was not significantly effective in improving water quality and growth.

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CHAPTER I

Introduction and Literature Review

1. Introduction

Aquaculture is the fastest growing sector of food animal production in the world and supplies an increasing percentage of the total production of fish and shellfish for human consumption. It is steadily expanding both in terms of total world production and the diversity of cultured species. In 2010, global production of farmed food fish was 59.9 million tons, up by 7.5 percent from 55.7 million tons in 2009 and estimated at US$119.4 billion (FAO 2012). The number of finfish species and species groups recorded in FAO aquaculture production statistics in 2010 was 327 which included channel catfish and tilapia.

Channel catfish farming is important to the United States (U.S) aquaculture industry. Catfish production is the largest sector of the U.S. aquaculture industry, accounting for approximately half of the total aquaculture production in the country (Quagraine 2006). Farm-raised catfish was sixth in the 2010 “Top 10” fish and seafood consumption list for Americans (Hanson and Sites 2012).

Unlike the channel catfish industry, tilapia aquaculture is worldwide and only second to carps by volume of production. Recently, farmed tilapia represents more than 75% of world tilapia production and about 5% of global finfish aquaculture (FAO 2009) and this contribution has been increasing exponentially.

As both catfish and tilapia aquaculture industries have advanced and production increased, further improvement of productivity and expansion of production scales have led to price pressures, deterioration of water quality and outbreak of diseases. These challenges demand cost-effective input alternatives that are based on readily available, but less expensive products. However, any unreasonable push to reduce costs, especially of feed, could result in sub-lethal effects of potential nutritional deficiencies or anti-nutritional factors which could impair water quality, lower fitness, and increase susceptibility of cultured fish to stress and pathogen infections. Thus, as the culture of these species intensifies, problems involving water quality and bacterial infections, particularly *Aeromonas hydrophila* and *Streptococcus iniae* in tilapia and *Flavobacterium columnare* in channel catfish have been reported (Wakabayashi 1991; USDA 1997; Cipriano 2001; Wagner et al. 2002; Austin and Austin 2007; Tellez-Banuelos et al. 2010; Shoemaker et al. 2010).

Traditionally, water quality improvement for increased production efficiency has been by good management practices such as appropriate stocking and feeding rates, while bacterial diseases are treated with chemicals and antibiotics including potassium permanganate, salt, oxytetracycline, sulfadimethoxine and aquafluor. However, management and treatment expenses and regulatory problems associated with water quality impairment and the use of chemicals and antibiotics (FAO 2006; USDA 2003a; McEwen and Fedorka-Cray 2002) have contributed to an increased demand for cost-effective and environment-friendly alternatives that can improve water quality and confer nutritional and health benefits. Furthermore, as aquaculture development intensifies, both governmental and non-governmental scrutiny of the industry is increasing due to environmental and

health concerns. New regulations and certification protocols as well as consumer preferences are driving the industry away from the use of antibiotics and other synthetic additives towards the use of cost-effective natural alternatives. Both catfish and tilapia aquaculture industries are no exception to these challenges.

In recent years, prebiotics and probiotics are under extensive investigation for their potential beneficial effects on fish health, growth and survival (Verschuere et al 2000; Irianto and Austin 2002; Mussatto and Mancilha 2007; Grisdale-Helland et al. 2008; Yousefian and Amiri 2009). Consequently, a number of natural and biological products, such as live bacterial suspension and dry concentrates, enzyme preparations, and extracts of plant products are being promoted for use as water and soil quality conditioners in aquaculture ponds (Defoirdt et al. 2011; Cruz et al. 2012) and additives to fish feed (El-Rhman et al 2009; Honsheng 2010). Although an increasing volume of scientific research from aquatic animals suggests that the establishment, maintenance and enhancement of health promoting bacteria in the intestine can have wide ranging beneficial effects on growth and survival of the host (Fuller 1989; Gatesoupe 1999; Gibson 1999; Moriarty 1999; Verschuere et al 2000; Irianto and Austin 2002; Mussatto and Mancilha 2007; Zhou et al. 2007; Burr et al. 2008; Grisdale-Helland et al. 2008; Yousefian and Amiri 2009; El-Rhman 2009), results have been conflicting (Merrifield et al. 2010). While some researchers have shown that inclusion of prebiotic and/or probiotics in the diet of fish or direct inoculation into the aquatic environment changes gut microbiota, improves growth and survival, or improves water quality (Aly et al 2008; Wang et al 2008; Merrifield et al. 2010), Others have reported no beneficial effects (Shelby et al 2006; Marzouk et al. 2008; El-Rhman et al. 2009; Ferguson et al. 2010). A complex interaction exists between

prebiotic-probiotic-fish-disease-environment and a lack of understanding of all the components of this interaction could affect the capabilities of prebiotics, probiotics and/or their combinations to improve water quality or control disease outbreaks in a real world situation.

The overall goal of this research was to ascertain the effects of pre- and probiotics on growth and survival and disease control in channel catfish and Nile tilapia, two commercially important aquaculture species in the U.S. and the world and two potential models for related genera or species. This dissertation evaluated the effectiveness of two commercially-available probiotic products, Lymnozyme® and PondToss™, as live bacterial inocula to respectively control columnaris infection under controlled conditions and pond production of channel catfish in the real environment. Some proprietary probiotic strains of *Bacillus subtilis* and the prebiotic Previda® were formulated into practical diets and their prebiotic, probiotic and combined effects on growth performance, survival and disease challenge of Nile tilapia investigated under laboratory conditions.

The specific objectives were to:

a. Evaluate the effectiveness of Lymnozyme® in reducing mortality in juvenile channel catfish when challenged with *Flavobacterium columnare* using different lengths of application.
b. Carry out growth trials in earthen ponds to explore the effects of PondToss™ on the water quality and production of channel catfish from juvenile to market size.

c. Conduct growth trial in a flow-through laboratory system for Nile tilapia to explore longer-term feeding effects of probiotic strains of *Bacillus subtilis*, the prebiotic Previda® and their combinations on their growth and survival.

d. Evaluate the potential effects of pre-feeding of probiotic strains of *Bacillus subtilis*, the prebiotic Previda® and/or their combination on the survival of juvenile Nile tilapia challenged with *Aeromonas hydrophila* and *Streptococcus iniae*.

2. Literature Review

2.1. The channel catfish aquaculture industry

The United States catfish industry has grown rapidly since it began in the 1960s. Channel catfish, *Ictalurus punctatus,* is the most important commercially cultivated catfish species constituting 99% of cultured catfish in U.S. (Li et al. 2008). In 2003, 72,034 hectares of water were used to produce 300,000 metric tons (live weight) of farm-raised catfish (USDA 2003b). In recent years, however, the industry has been declining since the high mark in 2003. Production levels, which were worth a market value of 480 million dollars in 2004 (USDA 2006), have dwindled during the last decade to a value of 423 million dollars in 2011 (USDA 2012). In 2011, 151,500 metric tons were processed, down 62,596 metric tons (-29%) from 214,096 metric tons processed in 2010, and a 49% decrease from the peak value in 2003 (300,000 metric tons). The per capita consumption of farm-raised catfish in 2010 was 0.36 kg (Hanson and Sites 2012).

According to Hanson and Sites (2009), the sustainability of the industry is seriously threatened by increasing import of frozen catfish fillets from Vietnam and China. To

remain competitive, U.S. catfish producers recognize the need to improve on their efficiency in production given that their foreign competitors consistently offer lower prices. However, water quality deterioration and mortality due to diseases are major limiting factors that contribute to reduced outputs and higher production costs.

Water quality deterioration in commercial channel catfish ponds has been recognized as a major limiting factor for further intensification of the industry (Boyd and Tucker 1998). According to Mischke (2003), microbial communities in ponds are essential for the maintenance of optimum water quality. They transform inorganic and organic wastes into less toxic forms and thereby maintain suitable water quality for fish production. Boyd and Tucker (1998) stated that disruptions of bacterial communities in ponds may cause increases in potentially toxic ammonia or nitrite concentrations. Many attempts have been made to improve organic matter degradation in aquaculture ponds through direct additions of bacterial suspensions to pond water (Boyd et al. 1984; Tucker and Lloyd 1985; Chiayvareesajja and Boyd 1993; Queiroz and Boyd 1998). The addition of large numbers of specific bacteria to ponds helps with the remediation of water quality. Consequently, several companies have marketed various bacterial suspensions for use in aquaculture. Although positive results have been recorded in recirculating systems (Ehrlich et al. 1989) under controlled conditions, improvement of water quality in ponds has not been successful (Mischke 2003). The lack of treatment effects has been attributed to the fact that many bacterial suspensions are mixtures of bacteria already present in the pond environment and also that the quantity of bacteria applied is insignificant compared to the existing population of bacteria (Boyd et al. 1984). Furthermore, the physical and chemical characteristics of the pond water, such as alkalinity, hardness, DO and temperature, as well

as light intensity and photo-period, etc. may affect the function of the bacterial suspension introduced into the pond. The existing water quality characteristics will also determine the size and species composition of the bacterial population in the pond (Tucker and Lloyd 1985). Rather than physically adding bacterial suspensions, a different approach to improving water quality is to add vitamins, nutrients, and enzymes to accelerate natural degradation by the existing microbial community in the pond. Queiroz et al. (1998) stated that this approach has been used with some success for a variety of applications, which has led companies to recommend using their products for aquaculture.

Disease outbreak is also a major constraint in channel catfish production. The industry experiences substantial losses annually (>US$50 million) due to high mortality rates caused by infectious diseases. Over sixty percent of the total catfish losses are the result of single or mixed bacterial infection, thirty percent result from parasitic infestations, nine percent are due to fungal infections, and one percent are of viral etiology (Wagner et al. 2002; USDA 2003a). A number of bacterial diseases, such as enteric septicemia of catfish (ESC), columnaris, motile aeromonad septicemia (MAS), fish gangrene and mycobacteriosis among others, have been reported to cause losses in commercial production of channel catfish. However, ESC and columnaris have been cited as the major causes of catfish mortality (Wagner et al. 2002; USDA 1997, 2003a; Shoemaker et al. 2011; Declercq et al. 2013).

2.2. *Flavobacterium columnare* infection in channel catfish

Flavobacterium columnare is the causative agent of columnaris disease in diverse fish species worldwide (Declercq et al. 2013). Columnaris disease previously referred to as

myxobacterial infection and reported by Davis in 1922 (Mohamed and Refat 2011) remains one of the most frequently encountered and devastating bacterial diseases of freshwater fishes, such as salmonids, eels, carp, goldfish, tilapia and channel catfish (Rehulka and Minarık 2007; Soto et al. 2008; Suomalainen et al. 2009). This disease also poses a problem for many freshwater tropical aquarium fishes (Post 1987; Decostere et al. 1998).

It is an aerobic, gliding, gram-negative, rod-shaped aquatic bacterium about 2–10 μm in length (Bernardet and Grimont 1989). This microorganism is sensitive to vibriostatic compound O/129 and generates hydrogen sulfide and cytochrome oxidase (Bernardet et al. 1996).

The pathogen causes a combination of external and systemic infections (Hawke and Thune 1992). The bacterium can be a primary pathogen, but more commonly it is a secondary pathogen that affects hosts predisposed by stress or trauma. The environment and the condition of the fish are important in determining the rate and severity of infection. Channel catfish are susceptible to columnaris at temperatures from 15 to 30 ^{o}C and young fish are more severely affected than adults (Griffin 1992; Plumb 1999).

Acute disease is characterized by an incubation period of less than 24 hrs and the resulting mortalities are seen two to three days post exposure (Holt et al. 1975). Columnaris is contagious and can be transmitted horizontally through direct contact and skin wounds as well as through the orofecal route (Welker et al. 2005; Austin and Austin 2007). Due to the ubiquitous nature of the *F. columnare* in freshwater, an injury to the skin or gills of fish with elevation of water temperature may quickly initiate the infection. In scale less fishes, the lesions start with simple ulcers which predominately end with extensive saddleback-

like ulcers exposing the underlying musculatures (Morrison et al. 1981). Fin and gill rot is another lesion of the progressive infection in fishes (Eissa et al. 2010).

Treatment for columnaris infection involves the use of chemical agents and antibiotics. Most chemical treatments used for columnaris disease are external disinfectants used as baths or indefinite when applied to ponds. Wakabayashi (1991) reported the use of the herbicide 6, 7-dihydrodipyrido (1.2a: 2^1, 1^1-c) pyrazidinium bromide (Diquat®) and the disinfectant benzalkonium chloride (Roccal; Upjohn Co., Kalamazoo, MI, USA and Hyamine 3500; Rohm and Haas Co., Philadelphia, PA, USA) as effective in treating columnaris. Potassium permanganate ($KMnO_4$) at 4 mg L^{-1} also reduced mortality due to columnaris disease in fathead minnows *Pimephales promelas,* from 86–100% to 62–65% (Jee and Plumb 1981). Although Rogers (1971) suggested that 1 mg L^{-1} of copper sulfate ($CuSO_4$) was effective in controlling columnaris infection, Phelps et al. (1977) reported that it was not effective in treating a mixed infection of columnaris and MAS in bluegills. Treatment of juvenile rainbow trout, *Oncorhynchus mykiss* with hydrogen peroxide (H_2O_2) at 200 mg L^{-1} for 1 h, twice a week by Speare and Arsenault (1997) was effective in limiting the degree of fin and epithelial damage in thermally induced columnaris infection. Riley (2000) showed that prophylactic treatment of channel catfish with 15 mg L^{-1} chloramine-T decreased mortality due to *F. columnare* from 84–100% to 6–14%. Success has also been reported for treating external and internal columnaris infections with Terramycin® (oxytetracyline HCl) medicated feed. Better results were obtained when affected fish were treated immediately after the disease was detected. Romet 30® and Romet B® medicated feeds have also been used in treating columnaris infections (Durborow et al. 1998). A commercially available rifampicin-resistant live vaccine (AQUAVAC-COL, Intervet) has

been approved by FDA for use by farmers (Shoemaker et al. 2005; Bebak et al. 2009). As a result of the growing awareness of the negative effects of antibiotics, there is a trend towards stricter regulations on the use of antibiotics in the aquaculture sector and on the presence of antibiotic residues in aquaculture products (FAO 2006; Smith 2008). In some countries especially, in the EU, US and Japan, regulations on the use of antibiotics are strict and only a few antibiotics are licensed for use in aquaculture (FAO 2002). Currently, alternative methods to prevent and cure fish disease such as the use of probiotics are being developed and tested (Defroidt et al. 2011).

2.3. The tilapia aquaculture industry

Tilapia is the generic name for three economically important genera and species of fish in the family Cichlidae, namely *Oreochromis, Sarotherodon* and *Tilapia* (Fitzsimmons 2000). Tilapias are native to Africa, the Mediterranean and the Middle East, but have been distributed throughout the world. The highest production occurs in tropical and subtropical areas in developing countries but they can also be cultured in temperate climates, where production is carried out in indoor tanks. The species that are most important for aquaculture are in the genus *Oreochromis*, and these include the Nile tilapia, *O. niloticus*; the Mozambique tilapia, *O. mossambicus*; the Blue tilapia, *O. aureus*; the Zanzibar tilapia, *O. urolepis hornorum* and hybrids (Popma and Masser 1999; Fitzsimmons 2000; Lim and Webster 2006). However, among all these species, the Nile tilapia is the most widely cultured tilapia in the world because of its rapid growth, late age at sexual maturity, high tolerance to environmental conditions, and its planktivorous to omnivorous feeding habits (Redner and Stickney 1979; Winfree and Stickney 1981; Twibell and Brown 1998).

The culture of Nile tilapia is believed to have started in ancient Egyptian times dating over 4000 years ago. According to Gupta and Acosta (2004), the first recorded scientifically-oriented culture of tilapia was conducted in Kenya in 1924 and soon spread throughout Africa. While significant worldwide distribution of tilapias, primarily Mozambique tilapia occurred during the 1940s and 1950s, distribution of the much preferred Nile tilapia occurred during the 1960s up to the 1980s. Nile tilapia from Japan was introduced to Thailand in 1965, and from Thailand they were sent to the Philippines. Nile tilapia from Ivory Coast was introduced to Brazil in 1971, and from Brazil they were sent to the United States in 1974. In 1978, Nile tilapia was introduced to China, which currently leads the world in tilapia production and has consistently produced more than half of the global production since 1992 (Xia 2000; Mjoun et al. 2010).

Tilapias are of increasing importance in aquaculture globally and are only second to carps by volume of production. The total world tilapia production has increased annually from 2.5 million tons in 2004 to 3.5 million tons in 2011 and projected to reach 3.6 million tons in 2012 (Fitzsimmons et al. 2012) while global sales have also increased from US$1.7 billion to over US$5.0 billion in 2010. Of the total world production of tilapias by weight and value, the Nile tilapia alone represents approximately 84%. Its production has accelerated from around 200,000 tons in 1990 to about 2.8 million tons in 2010 (FAO 2009; Josupeit 2010; Fitzsimmons et al. 2012).

Production of tilapias has a wide distribution, and seventy-two percent (72%) are raised in Asia, 19% in Africa, and 9% in America (FAO 2012). China is the single largest producer, consumer and exporter, with a total production of 1.16 million tons in 2011. Thailand, Indonesia and the Philippines each increased their production significantly and

have roughly equal production of around 0.3 million tons per year. Several developing countries in Asia and Africa have, in recent years, either increased production or introduced programs that will support commercial tilapia development (Fitzsimmons et al. 2012).

Tilapia was until recently a low-valued fish but has gained higher acceptance by the consuming populace. Its consumption now transcends the traditional markets in Africa and Asia to the international market in the US, Japan and the European Union (Josupeit 2005). In the US, for instance, tilapia is now the fourth most popular seafood, after shrimp, tuna and salmon. The per capita consumption has reached 0.68 kg, which is approximately 1.5 kg of whole fish, essentially two fish or four fillets per person. More than 95% of the tilapia consumed in the US is imported. Imports of farmed tilapia recorded greater increase, up 23.3% from 153,248 metric tons in 2011 to 189,014 metric tons in 2012 (Ramsden 2013). The value of the imports was $838 million dollars in 2011. Even though domestic production in 2011 was only 12,000 tons, it earned producers around $80 million in farm gate sales (Fitzsimmons et al. 2012).

2.4. *Aeromonas hydrophila* and *Streptococcus iniae* infections in tilapia

The current trend in tilapia aquaculture development is towards increased intensification and commercialization (Goncalves et al. 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 and Austin 2007). At the local level, disease can have a serious impact on the livelihoods and food security of many individual small farmers and their families, particularly in poorer countries (Hill 2005). The incidence of microbial pathogens, particularly those of bacterial origin, is one of the

most significant factors affecting fish culture (Zorilla et al. 2003). Fish are constantly exposed to bacteria and will normally only succumb to an infection when exposed to prolonged periods of sub-acute stress or shorter periods of acute stress (Shayo et al. 2012). Despite concerted efforts by tilapia farmers to manage overall health and well-being of their fish, some bacterial pathogens, particularly *Aeromonas hydrophila* and *Streptococcus iniae*, continue to plague the culture of the Nile tilapia, resulting in decreased survivability and profitability.

Aeromonas hydrophila is a ubiquitous, free-living, rod-shaped, Gram-negative bacterium prevalent in aquatic habitats with wide distribution. It causes disease in fish known as "Motile Aeromonad Septicemia (MAS)", "Hemorrhagic Septicemia", "Ulcer Disease" or "Red-Sore Disease" depending on the lesions caused by this bacterium which include septicemia within numerous organs of the fish and ulcers of the fish's skin (Swann and White 1989). It is an opportunistic pathogen that has resulted in heavy mortalities in farmed and feral fishes (Harikrishnan and Balasundaram 2005). *A. hydrophila* is a major bacterial pathogen affecting warmwater fishes including the Nile tilapia (Tellez-Banuelos et al. 2010). In the US, *Aeromonas* primarily causes disease in cultured warmwater fishes like minnows, baitfishes, carp, channel catfish, striped bass, largemouth bass and tilapia (Cipriano et al. 2001).

Aeromonas hydrophila causes MAS in Nile tilapia, both in commercial production systems and in natural waters, and it is also associated with a number of other diseases of Nile tilapia such as epizootic ulcerative septicemia (EUS) as a secondary pathogen. Although usually considered as a secondary pathogen associated with disease outbreaks, *A. hydrophila* could also become a primary pathogen, causing outbreaks in fish farms with

high mortality rates, resulting in severe economic losses to the industry (Fang et al. 2004). For example, in the Philippines, *A. hydrophila* was consistently isolated from diseased Nile tilapia during disease outbreaks in several farms at different locations (Yambot 1998). Faisal et al. (1989) also reported consistent recovery of *A. hydrophila* from wild and cultured Nile tilapia in Lower Egypt during disease outbreaks. In West Alabama, a MAS disease outbreak caused by a more virulent strain of *A. hydrophila* (ML-09-119) in 2009 resulted in an estimated loss of more than 1.4 million kg of food size channel catfish (Pridgeon and Klesius 2011).

Infection of *A. hydrophila* is mainly by ingestion through contaminated feed and also through lesions or injury on the skin of the fish in contaminated water. Pathological conditions attributed to *A. hydrophila* infections may include dermal ulceration, tail or fin rot, ocular ulcerations, erythrodermatitis, hemorrhagic septicemia, red sore disease, red rot disease and scale protrusion disease (Cipriano et al. 2001). When disease is acute, as with disease challenge studies, fatal septicemia may occur so rapidly that fish die before the development of anything but a few gross signs of the disease. Clinically, infected fish frequently exhibit small pinpoint hemorrhages at the base of fins, operculum or on the skin, distended abdomens, and protruding eyes. Internal signs include fluid in the abdomen, swollen liver and spleen, and distended and fluid-filled intestines (Plumb 1999). Yambot and Inglis (1994) described an acute mortality in the Nile tilapia where apparent clinical signs included opaqueness in one or both eyes, accompanied by exophthalmia and finally bursting of the orbit. Bach et al. (1978) observed pathological changes in the spleen of fish injected with virulent *A. hydrophila*; whereas, fish infected orally showed little or no splenic involvement.

Although *Aeromonas* species are of ill repute as pathogens of fish, they constitute part of the normal external and internal environments of healthy fish (Trust et al. 1974). Their simple presence is not indicative of disease but stress is often a contributing factor in the outbreaks of disease caused by these bacteria. Severity of the disease is influenced by a number of interrelated factors, including bacterial virulence, the kind and degree of stress exerted on a population of fish based on the production system, the physiologic condition of the fish, and the degree of genetic resistance inherent within the specific population of fish. They also differ interspecifically and intraspecifically in their relative pathogenicity or their ability to cause disease. Eissa et al. (1994) found the prevalence of motile aeromonad septicemia in cultured and wild Nile tilapia was 10.0% and 2.5%, respectively, and concluded that environmental and physiological stressors had significant adverse effects on fish under intensive culture. Under controlled laboratory conditions, De Figueredo and Plumb (1977) found that strains of motile aeromonads isolated from diseased fish were more virulent to channel catfish than were those isolated from pond water. Culture conditions, such as poor water quality, overcrowding and rough handling, accelerate the susceptibility of fish to these bacteria. The bacteria are adapted to environments that have a wide range of conductivity, turbidity, pH, salinity, and temperature. Although the optimum temperature for the growth of *Aeromonas* species is strain-specific, generally, growth is best at 28°C and the maximum tolerated temperature goes up to 41°C (Shayo et al. 2012).

Streptococcus iniae is a hemolytic, gram-positive, spherical-shaped bacterium that has been associated with outbreaks of disease in freshwater and marine fish species in various parts of the world (Eldar and Ghittino 1999). It was originally isolated from

freshwater dolphins (Pier and Madin 1976), but has now gained prominence as an etiological agent of streptococcosis in farmed finfish (Agnew and Barnes 2007).

The first documented streptococcal infection in farmed fish occurred in 1958 in rainbow trout in Japan (Agnew and Barnes 2007) and later more streptococcal infections were identified as causes of mortality in farmed and wild fish species, with most of the reports coming from Japan and the US (Kitao et al. 1981). Currently, *S. iniae* is known to affect more than 30 species of farmed and wild finfish, including the Nile tilapia with concomitant economic losses (Agnew and Barnes 2007; Cheng et al. 2010).

The losses in aquaculture production systems of tilapia and other species have been reported by various researchers as ranging from 30% to 75% at a value that exceeds US $150 million annually (Eldar et al. 1994; Stoffregen et al. 1996; Shoemaker and Klesius 1997; Shoemaker et al. 2010). In 1997, the estimated annual impact of infection by this bacterium on the aquaculture industry in the US alone was US$ 10 million (Shoemaker et al. 2010; Salvador 2012).

Streptococci spp. have been shown to gain entry into fish by ingestion of moribund fish or contaminated feed (Shoemaker et al., 2000; Bromage and Owens, 2002), injured skin from contaminated water (Rasheed and Plumb, 1984), and through intraperitoneal and intramuscular injection for experimental purposes (Shoemaker et al. 2000, 2001; Bromage and Owens, 2002). Disease progression is variable and dependent upon the virulence of the isolate, the host species affected, route of infection, fish age, and other environmental and water quality factors. The median lethal dose (LD50) has been shown to generally range from 3×10^4 to 10^8 CFU/fish for tilapia (Eldar and Ghittino 1999).

Stress often plays a significant role in outbreaks of infectious disease in fish populations. Some stressors that have been associated with streptococcosis outbreaks include high water temperatures, high stocking densities, harvesting or handling, and poor water quality, such as high ammonia or nitrite concentrations. The optimum temperature for the growth of *S. iniae* has been shown to range between 25 and 28°C. Temperatures outside of that range result in decreased mortalities attributed to *S. iniae* (Bromage and Owen 2009). Streptococcus infections in fish can cause high mortality rates greater than 50% over a period of 3 to 7 days. Sometimes outbreaks may be chronic in nature and mortalities may extend over a period of several weeks. Clinical signs may include exophthalmia, corneal opacity, melanosis, lethargy and loss of orientation, swimming erratically, dorsal rigidity, vertebral deformity, tachypnoea, anorexia, emaciation, or sudden death with few accompanying signs (Bromage and Owens 2002; Eldar and Ghittino 1999).

2.5. The use of prebiotics and probiotics in fish culture

Chemical additives, such as anabolic steroids, growth promoters and some antibiotics (e.g. oxytetracycline, sulfadimethozine and ormetoprim), are commonly administered in feed to improve growth performance and to control the outbreak of diseases in aquaculture (Gaunt et al. 2010; Defoirdt et al. 2011). However, new regulations, certification protocols and consumer preferences are pushing the industry away from the use of antibiotics and other synthetic additives. The use of antibiotics in aquaculture has received considerable attention because their abuse has led to the development of drug-resistant bacteria, thereby, reducing drug efficacy. Moreover, the accumulation of

antibiotics both in the environment and in fish can pose potential risk to consumers and the environment (Carrias et al. 2012). 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 alternatives such as natural, cost-effective feed formulations that will decrease the effects of bacterial pathogens on farm profitability. In recent years, prebiotics, probiotics and their combination are under extensive investigation for their potential beneficial effects on fish health and growth.

2.5.1. Prebiotics

A prebiotic is defined as a non-digestible dietary ingredient that beneficially affects the host by selectively stimulating the growth of and/or activating the metabolism of health-promoting bacteria in the gastrointestinal tract (Gibson and Roberfroid 1995; Manning and Gibson 2004). FAO (2007) proposed a recent definition for prebiotics as non-viable food components that confer health benefit on the host associated with modulation of the microbiota. They are dietary carbohydrates that escape digestion in the upper gastrointestinal tract but alter the bacterial composition of the gut by changing the type of substrate provided to the existing gut microbiota (Gibson and Roberfroid 1995; Mei et al. 2011). The effects of a prebiotic, according to Wood and Gorbach (2001), are characterized by an increase in beneficial bacteria and/or a decrease in harmful bacteria in the gut of the host, a decrease in intestinal pH through the production of short chain fatty acids (SCFAs) and changes in bacterial enzymes concentrations.

Compounds which have been shown to have prebiotic characteristics include mannanoligosaccharides (MOS), trans-galactooligosaccharide (TOS), fructo-

oligosaccharides (FOS), galactooligosaccharides (GOS), lactose, and inulin (Hoffmann 2012). Results from several studies have indicated that prebiotics can improve growth performance and feed utilization of various fish species (Li and Gatlin 2005; Mahious et al. 2006; Staykov et al. 2007; Torrecillas et al. 2007; Zhou et al. 2007; Burr et al. 2008; Grisdale-Helland et al. 2008; Samrongpan et al. 2008; Yousefian and Amiri 2009), enhance their non-specific immune responses and resistance to bacterial infections (Li and Gatlin 2005; Staykov et al. 2007; Buentello et al. 2010), improve gut function and health by improving the ultrastructure of the intestine mucosa (Salze et al. 2008), and also activate health promoting bacteria in the intestine (Zhou et al. 2007).

Yousefian and Amiri (2009) stated that most reports on the effects of prebiotics on growth parameters in fish and cetaceans are contradictory. According to these authors, supplementation of the diet of the beluga whale diet with 1, 2 and 3% inulin showed negative relationships between some growth performance indices, which included weight gain, specific growth rate, protein efficiency ratio, energy retention, feed efficiency, protein retention and supplementation level of inulin.

When the Atlantic salmon was fed with diets supplemented with 10 g kg^{-1} of mannanoligosaccharide, fructooligosaccharide and galactooligosaccharide, these prebiotics did not show any effects on growth and digestibility (Grisdale-Helland et al. 2008). In a 7-week experimentation where the commercial prebiotic Grobiotic®-AE was fed to the hybrid striped bass at 10-20 g kg^{-1}, feed efficiency improved significantly but differences in the growth rates were not significant (Li and Gatlin 2004). Dietary supplementation of Grobiotic®-AE according to Li et al. (2007) improved survival of Pacific white shrimp cultured in low-salinity of 2‰. Growth rate, feed efficiency and

survival were improved in two experiments involving rainbow trout that were fed a diet containing 2 g kg^{-1} mannanoligosaccharide when compared with those fed the basal diet (Staykov et al. 2007; Grisdale-Helland et al. 2008).

Results from an 8-week feeding trial conducted by Zhou et al. (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®. 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.

The effects of different levels of the prebiotic Immunogen® (0, 0.5, 1, 1.5 and 2.5 g prebiotic/kg diet) on feed utilization, body composition, immunity and resistance to *A. hydrophila* infection in the common carp *Cyprinus carpio* fingerlings were evaluated by Ebrahimi et al. (2011) in an 8-week feeding trial. Weight gain showed no differences among the groups fed different Immunogen® levels. Both feed efficiency ratio and protein efficiency ratio significantly increased with increasing Immunogen® levels from 0.5 to 1.5 g/kg diet. The highest protein content was found in the fish fed a diet containing 2.5 g/kg prebiotic. Haematological parameters and plasma total protein concentration were also significantly higher in the fingerlings fed diets containing 1.5 and 2.5 g/kg prebiotic relative to control.

In a study conducted by Hui-Yuan et al. (2007) with hybrid tilapia (*Oreochromis niloticus* x *O. aureus*) fed FOS, mean specific growth rate, daily feed intake and feed

conversion ratio were significantly improved with increasing levels of the prebiotic. Survival rate and condition factor were however not affected. Genc et al. (2007) showed no significant differences between the treatment groups (0, 1.5, 3.0 or 4.5 g MOS kg^{-1}) on weight gain, specific growth rate, feed conversion ratio, protein efficiency ratio or organosomatic indices (hepatosomatic and viscerosomatic) of hybrid tilapia (*Oreochromis niloticus* x *O. aureus*).

Prebiotics can modify the microbial community within the gastrointestinal tract to boost non-specific immune responses (Bailey et al. 1991). The microflora in the colon ferments the prebiotic and causes significant modification of the colonic microflora. This is because the oligosaccharides serve as substrate for growth and proliferation of anaerobic bacteria, which inhibit the growth of putrefactive and pathogenic bacteria present in the colon (Mussatto and Mancilha 2007). They produce substances that stimulate the immune system, thus, enhancing the host's protection against infections (Yousefian and Amiri 2009).

The prebiotic FOS did not affect growth in hybrid tilapia but increased survival and enhanced activity of innate defense mechanisms, especially, lysozyme activity (He et al. 2003). They also reported improved survival and enhanced lysozyme activities in hybrid tilapia fed 0.6% MOS relative to control diet. Sado et al. (2008) in their study with juvenile Nile tilapia (13.6 ± 0.7 g) fed different levels of MOS (0, 2, 4, 6, 8 and 10 g kg^{-1}) found no significant effect on hematological parameters (red blood cell, white blood cell, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration and total plasmatic protein). However, daily feed consumption decreased with increasing levels of MOS. They concluded by speculating that

prebiotic dose, duration of administration, and population status, such as age, sex and gonad maturation of the fish could affect the results. Based on the results that 4 and 6 g inclusion levels of MOS significantly improved weight and resistance against *Streptococcus agalactiae* in comparison with the control group, Samrongpan et al. (2008) suggested that MOS was a beneficial feed supplement for juvenile Nile tilapia.

The gastrointestinal tract microbiota of farmed animals plays important roles in affecting the nutrition and health of the host organism (Pineiro et al. 2008; Salminen and Isolauri 2008; Possemiers et al. 2009). Thus, manipulation of the intestinal microbiota to achieve favorable effects, such as enhancing growth, digestion, immunity and disease resistance of the host organism, have been investigated in various terrestrial livestock, fish and humans (Gomez and Balcazar 2008; Reid 2008).

2.5.2. Probiotics

Probiotic is a Greek word that means “for life”. This term was initially proposed by Lilly and Stillwell in 1965 for the factors produced by a microbe to promote the growth of another microbe. It was coined to contrast with antibiotic, which is a substance produced by one microbe to counter another (Schrezenmeir and de Vrese, 2001). In 1971, Sperti used the term for tissue extracts that stimulate microbial growth. Parker (1974) was the first to use the term in the sense as it is today by defining probiotics as organisms and substances which contribute to intestinal microbial balance. This definition, however, appears misleading since the word ‘substances’ connotes the inclusion of antibiotics. The most generally accepted and widely used definition was by Fuller (1989), who improved Parker’s definition when he defined a probiotic as a live microbial feed supplement which

beneficially affects the host animal by improving its intestinal microbial balance. Fuller's definition emphasizes the requirement of viability for probiotics and introduces the aspect of a beneficial effect on the host, which according to his definition was an animal (Welker and Lim 2011). Havenaar et al. (1992) broadened the definition of probiotics with respect to host and habitat of the microflora as a viable mono- or mixed culture of microorganisms, which when applied to animal or man beneficially affects the host by improving the properties of the indigenous microflora. Gram et al. (1999) proposed that a probiotic is any live microbial supplement, which beneficially affects the host animal by improving its microbial balance. Their definition had no association with feed. Also, Salminen et al. (1999) considered a probiotic as any microbial (living or dead) preparation or the components of microbial cells with a beneficial effect on the health of the host. The need for live cells in association with feed has been ignored in this definition. Schrezenmeir and de Vrese (2001) reviewed the definition and redefined a probiotic as a preparation of or a product containing viable, defined microorganisms in sufficient numbers which, by implantation or colonization, alter the microflora in a compartment of the host and by that exert beneficial health effects in the host. It has also been defined as live microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO 2001). Based on the observation that organisms are capable of modifying the bacterial composition of water and sediment, irrespective of how temporal that may be, Moriarty (1999) suggested that the definition of a probiotic in aquaculture should include the addition of live naturally occurring bacteria to tanks and ponds in which animals are raised. A probiotic is, thus, broadly defined as a live microbial additive which has a beneficial effect on the host by modifying the host-associated or ambient microbial community by

ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease or improving the quality of its ambient environment. Based on this broad definition, probiotics may include microbial adjuncts that prevent pathogenic microbes from proliferating in the intestinal tract and in the culture environment of the cultured species. They also secure the optimal use of the feed by aiding digestion, improving water quality and/or stimulating the immune system of the host.

According to Gatesoupe (1999), the first application of probiotics in aquaculture was in the mid-1980s (Kozasa, 1986) and since then interest in such environment-friendly treatments has increased rapidly. The use of probiotics has been accompanied by a concomitant reduction in the levels of antibiotics used in aquaculture and in improved appetite and/or growth performance of the farmed species. Hence, they are becoming increasingly accepted as valuable alternative to antibiotic therapy to control or prevent infection from pathogenic bacteria (Irianto and Austin 2002).

Probiotic properties have been ascribed to several micro-organisms, mainly lactic acid bacteria (*Lactobacillus* and *Bifidobacterium* species) and few non-lactic acid bacteria (*Bacillus* spp.) due to the fact that these bacteria often produce bacteriocins and other chemical compounds that may inhibit the growth of pathogenic bacteria (Hidalgo et al. 2006). Examples of probiotics include gram-positive bacteria such as *Bacillus* sp., *Carnobacterium inhibens* and *Lactobacillus* sp. as well as gram-negative bacteria such as *Aeromonas hydrophila, Pseudomonas fluerescens* and *Vibrio fluvialis*. Other probiotics are bacteriophages and yeasts (*Saccharomyces cerevisiae, Phaffia rhodozoma, Debaryomyces hansenii*) as well as microalgae (*Tetraselmis suecica*) (Irianto and Austin 2002; Pond et al. 2006).

The use of probiotics for disease prevention and improved nutrition in aquaculture has gained prominence in recent times due to an increasing demand for environment-friendly aquaculture (Vine et al. 2006). Common probiotic products used in aquaculture, such as *Bacillus species*, can improve water quality by reducing the number of microbial pathogens in ponds (Wang et al. 2008). Probiotic inclusion in fish feed can also build up the beneficial bacterial flora in skin and intestine while they grow competitively over pathogenic bacteria (El-Rhman et al. 2009). Since *F. columnare* grows by stacking to the fish skin and gills and, consequently, cause damage through lesions, manipulation of the composition of the bacterial community on the skin and gills of fish may be used in prevention of the disease. A number of studies have reported their effectiveness in improving soil and water quality, immunity, health status, feed efficiency and growth performance of fish species while others also reported no beneficial effects (Boyd and Gross 1998, Verschuere et al. 2000, Shelby et al. 2006; Aboagye 2008, Denev et al. 2009).

Most studies concerned with the effects of probiotics on cultured aquatic animals have emphasized reduction in mortality (Moriarty 1998; Irianto and Austin 2002), improved resistance against disease (Gatesoupe 1994), ability to adhere to and colonize the gut (Joborn et al. 1997), ability to antagonize other organisms, notably putative pathogens (Joborn et al. 1997), ability to reduce the number of bacterial cells in kidneys (Park et al. 2000), production of polyamines and digestive enzyme activity (Tovar et al. 2002), and development of the non-specific immune system by means of cellular systems, such as increased phagocytic, lysozyme and respiratory burst activities (Irianto and Austin 2002).

Improved growth performance in tilapia fed probiotic diets has been reported by many researchers. Probiotics may improve digestion by stimulating production of digestive

enzymes or through other alterations in the gut environment (Merrifield et al. 2010; Nayak 2010). Digestive enzymes such as carbohydrases, phosphatases, esterases, lipases, peptidases, cellulases, and proteases are all produced by gut microbes in fish, including some commonly used probiotic species (Nayak 2010). Production of some of these enzymes has been enhanced by the inclusion of *Bacillus subtilis* in tilapia diets according to Honsheng (2010), who further attributed improved weight gain and feed efficiency to the increased enzyme production. Improved growth performance of Nile tilapia fed diets with *B. subtilis, Lactobacillus plantarum*, a mixture of *B. subtillis* and *L. plantarum*, and *Streptococcus cerevisiae* have been reported by Essa et al. (2010). Aly et al. (2008a) compared the potential effect of two doses of *B. 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 or 8 weeks. Mean body weight and survival rates of all treatment groups showed statistically significant increases as compared to the control group. *S. faecium* + *L. acidophilus* or *S. cerevisiae* supplementation in tilapia diets containing 27% or 40% crude protein produced significantly higher weight gain and feed utilization efficiency compared to the control diet (Lara-Flores et al. 2010). Tilapia fed *S. cerevisiae* (Lara-Flores et al. 2010), *B. subtilis* + *S. cerevisiae* ((Lara-Flores et al. 2010; Marzouk et al., 2008), *Micrococcus luteus* (El-Rhman et al. 2009), *Bacillus subtilus, Lactobacillus plantarum, B. subtilis* + *L. plantarum,* (Aly et al. 2008b; Essa et al. 2010), *Bacillus pumilus* (Aly et al. 2008b), *Lactobacillus acidophilus, Streptococcus faecium* (Lara-Flores et al. 2003), the commercial probiotic mixtures Organic Green™ (Aly et al. 2008c), Biogen® (El-Haroun et al. 2006; Ghazala et

al. 2010; Mehrim 2009), and Premalac® (El-Haroun et al. 2006) have all been shown to increase growth performance in tilapia.

However, other researchers reported no effect of some dietary probiotics on growth. Non-viable *S. cerevisiae* (Marzouk et al. 2008), *Pseudomonas spp.* (El-Rhman et al. 2009), *Pediococcus acidilactici* (Ferguson et al. 2010), *and Enterococcus faecium* (Biomate SF-20®), *B. subtilis + B. licheniformis* (Bioplus 2B®), and *P. acidilactici* (Bactocell PA10 MD®), and viable *S. cerevisiae* (Levucell SB 20®) (Shelby et al. 2006) have been shown to not affect growth of tilapia.

The effectiveness of probiotics in terms of protection against infection is often attributed to enhanced immunity (Welker and Lim 2011). Merrifield et al. (2010) noted that although probiotic use can enhance the immune response of tilapia and improve disease resistance, there has been reportedly mixed successes. The effectiveness of a variety of probionts against a number of different bacterial pathogens, especially the major ones like *S. iniae, A. hydrophila, F. columnare* and *Edwardsiella tarda*, in fish culture has not been completely satisfactory.

Pirarat et al. (2006) suggested that *L. rhamnosus* GG protection against *E. tarda* is accomplished by enhancing the alternative complement system, thereby, increasing phagocytic cell aggregation and phagocytic activity. They found an increase in the number of mucous cells in the distal portion of the intestine and a greater abundance of intraepithelial lymphocytes and acidophilic granulocytes in Nile tilapia fed diets containing *L. rhamnosus* GG. Although Ferguson et al. (2010) did not find any changes in the number of leucocytes in the intestinal epithelium, blood leucocyte numbers and serum lysozyme activity were enhanced in Nile tilapia given the probiotic *Pediococcus acidilactici. L.*

rhamnosus GG supplemented in the tilapia diet caused an increase in serum complement activity and enhanced phagocytosis and killing ability of head kidney leukocytes (Pirarat et al. 2011). A number of other systemic, non-specific immune functions have been shown to be enhanced by dietary probiotic supplementation, including lysozyme activity, peripheral blood immune cell counts, alternative complement activity, phagocytic ability of leucocytes, neutrophil migration and adherence, plasma bactericidal activity, respiratory burst, myeloperoxidase, and superoxide dismutase activities.

According to Shelby et al. (2006), probiotics can also be ineffective in preventing disease in tilapia. These authors after feeding the Nile tilapia with a commercial probiotics for 94 days observed that it did not prevent streptococcal disease infection. These authors 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*. There is far less evidence available suggesting that dietary probiotics influence the humoral immune response in tilapia. Shelby et al. (2006) again did not find an effect of dietary probiotic supplementation on the antibody response to *S. iniae*.

Gut microbiota perform a variety of functions that benefit the health of the host species by promoting nutrient supply, enhancing immune function, preventing colonization of pathogenic microbes, energy homeostasis, and maintenance of normal mucosal integrity and function (Welker and Lim 2011). Maintenance of a healthy gut microbiota is a likely benefit to the development of the gut epithelial architecture, and because many fish pathogens can disrupt the integrity of the intestinal epithelium, a healthy gastrointestinal

(GI) microbial population may reduce mucosal damage, increase absorptive area, and prevent pathogenic disease (Merrifield et al. 2010).

2.5.3. Combined effects of pre- and probiotics

A mixture of prebiotics and probiotics can beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the GI tract by selectively stimulating the growth and/or by activating the metabolism of one or a limited number of health-promoting bacteria and, thus, improving the host welfare (Gibson and Roberfroid 1995). An effective pairing would allow alteration of the gut environment by a prebiotic that would select for preferential growth conditions of known beneficial probionts. The benefits of this approach is that fish farmers are able to control and provide favorable conditions in the colon as well as ensure that a beneficial probiont is present in sufficient numbers.

A study was conducted by Mehrabi et al. (2012) to evaluate the influence of (Biomin IMBO) on serum parameters and feeding efficiency in rainbow trout (*Oncorhynchus mykiss*) fingerlings. After two months, all treatments supplemented with the product showed significant increase in final mean weight, percent weight gain, specific growth rate, condition factor, food conversion efficiency and survival rate compared to the control group. The highest growth factors and survival were observed in the treatment supplemented with 1 g of Biomin per kilogram of diet. Also, supplementation with 1 and 1.5 g/kg significantly increased the total serum protein, but there were no significant differences in globulin content, albumin/globulin ratio, and triglyceride contents among experimental treatments. In terms of body composition, carcass protein content of fish fed

a combined diet of pre- and probiotic significantly increased compared to the control. These results revealed that a feeding regime with the mixed pre- and probiotic for two months led to a significant increase in growth performance, survival rate and feeding efficiency in rainbow trout fingerlings.

A prebiotic is thought to give a probiotic a competitive advantage by providing a fermentable energy source enabling it to out-compete endogenous microbial populations (Gibson and Roberfroid 1995; Merrifield et al. 2010). When the Japanese flounder was fed a diet containing *B. clausii* or in combination with the prebiotics fructo- or mannanoligosaccharides, there was an improvement of the non-specific immune function (Ye et al. 2011). Although the diet containing either of the prebiotics with *B. clausii* exhibited the highest immune function, activity was not significantly different compared to flounder fed *B. clausii* alone. Feeding a combined mannanoligosaccharide and *Enterococcus faecalis* diet improved survival of rainbow trout challenged with *Vibrio anguillarum* compared to trout fed the individual prebiotic or probiotic (Rodriguez-Estrada et al. 2009).

Prebiotics, probiotics and/or their combination have all been demonstrated to positively modulate the intestinal microflora and could promote fish health. The use of these products in aquaculture is now widely accepted; however, some results on their efficiency from a few studies have been conflicting (Gatesoupe 2005; Grimoud et al. 2010) and several factors have been attributed to this. The type and dose concentration (dietary concentration and duration of feeding) of these products could affect their efficacy on disease prevention in fish, although in tilapia, short-term (2 weeks) and long-term (2 months or greater) feeding were observed to be effective in enhancing disease resistance

(Welker and Lim 2011). The form of prebiotic and probiotic administration can also impact effectiveness in affecting fish health. Viability of probionts during the feed making process and during feed storage can be maintained or improved by encapsulation in non-nutritive matrices, such as calcium alginate. Encapsulation of *Shewanella putrefaciens* in calcium alginate improved viability of the bacterium during feed storage, and its presence was found in the gastrointestinal track of Sengalese sole (*Solea senegalensis*) fed encapsulated but not non-encapsulated *S. putrefaciens* (Rosas-Ledesma et al. 2011). The route of administration can also affect the success of probiotic application. Addition of *Lactococcus lactis* RQ516 to rearing water was found to increase significantly the resistance of Nile tilapia to *A. hydrophila* (Zhou et al. 2010). Further research on the effects of dose dependency and form and route of probiont administration on disease resistance are needed for all fish species, including tilapia, in order to provide effective feeding and treatment regimens.

Variation among studies is likely due to differences in the choice of prebiont, probiont, dietary concentration, species strains and age/size of fish, feeding management and duration, dosage and virulence of challenge pathogens, and methods of challenge. Merrifield et al. (2010) noted that the success or potential of probiotics in many studies to prevent disease may be greater than the results showed due to the use of intraperitoneal (IP) 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. The IP challenges do not reflect the effect of probiotics on resistance to infection but rather demonstrate the effect of probiotics on infected fish (Merrifield et al. 2010). In studies where disease resistance is improved, it suggests that the probiotic may be providing

immune stimulation outside the GI tract. This is an important point to highlight in the application of probiotics to boost immunity of tilapia, because the vast majority of challenges performed in tilapia research studies are done by IP injection, especially with *Streptococcus*. Streptococcal disease, caused predominantly by *S. iniae*, is the biggest disease problem in tilapia culture (Shoemaker et al. 2006); however, it is difficult to reproduce reliably by bath immersion. So researchers have had to rely on IP injection to produce reliable, consistent infection to achieve the desired mortality rate.

Other factors, such as environmental conditions, handling practices, and stocking densities, may also affect results. All these factors can influence the success or failure of prebiotics, probiotics and their combination in the enhancement of growth, immunity and/or disease resistance in fish.

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CHAPTER II

Evaluation of the probiotic Lymnozyme® as an effective control of columnaris infection in juvenile channel catfish *Ictalurus punctatus*

1. Abstract

Catfish farming is a major component of the U.S. aquaculture industry. However, a major constraint to the industry is the incidence of bacterial diseases like columnaris that cause high fish mortalities. Columnaris is also a global disease problem affecting many cultured species. To reduce disease mortalities and improve catfish production efficiency, this study examined the effectiveness of applying the commercially-available aquatic probiotic product Lymnozyme® (Keeton Industries Inc., Wellington, CO), a dry water-soluble probiotic concentrate, to the culture water to reduce mortality due to *Flavobacterium columnare*. Two experiments were conducted. In the first experiment, naïve channel catfish of average size 7.0±0.2 g were used and daily exposed to Lymnozyme® treatment for 2 hours under static aerated condition. In the second experiment, catfish of average size 5.9±0.1 g were exposed to 8 hours of Lymnozyme® treatment daily. In both experiments, 15 fish were placed into each tank and maintained for 3 days prior to the challenge with three treatments receiving 5.0 g of Lymnozyme® (2.5 x 10^9 CFU/g) dissolved in 32 L of water daily and a control receiving none. The following treatments occurred post-challenge: control-received no Lymnozyme®; 1- received no Lymnozyme®; 2-received Lymnozyme® daily for the next 7 days; and 3- received

Lymnozyme® daily for the next 14 days. Fish were challenged by placing them in a plastic bucket containing 2 L of water with aeration. Ten (10) milliliters of *F. columnare* strain ALG 00-530 (1.6×10^8 CFU/ml and 1.8×10^8 CFU/ml in experiments 1 and 2, respectively) were inoculated into the water to obtain final dosages of 8.0×10^5 CFU/ml and 9.0×10^5 CFU/ml, respectively, for 30 minutes before returning with water into the tanks. Mean cumulative mortalities from the first experiment were 77±15.4%, 76±8.9%, 69±11.2% and 64±7.6% for control and treatments 1, 2 and 3, respectively. There was no effect of treatment ($p=0.2370$). In the second experiment, control fish had a mean cumulative mortality of 80±12.5%, while treatments 1, 2 and 3 had mean mortalities of 61±14.5%, 50±13.0%, and 44±14.6%, respectively. There were significant differences between the control and all treatments ($p=0.0041$) but no significant differences among treatments ($p>0.05$). Results indicated that Lymnozyme®, when administered daily for 8 hours under static conditions, effectively reduced mortality rates with more desirable effects achieved over longer application periods. The use of Lymnozyme® may have potential impacts on other fishes in reducing mortality from columnaris infection.

2. Introduction

Disease outbreaks remain a significant constraint to aquaculture production, trade and economic development of the aquaculture sector in many countries (Verschuere et al 2000; Welker and Lim 2011). In the U.S., channel catfish farmers continue to incur huge losses estimated to be over US$50 million annually due to diseases, which reduce the margins of profitability (Wagner et al. 2002; Shoemaker et al. 2011). A number of bacterial diseases, such as enteric septicemia of catfish (ESC), columnaris, motile aeromonad

septicemia (MAS), fish gangrene and mycobacteriosis among others have been reported to cause losses in commercial channel catfish production. However, columnaris (caused by *Flavobacterium columnare*) has been cited as a major cause of catfish mortality during production (Wagner et al. 2002; USDA 1997, 2003; Darwish et al. 2009; Shoemaker et al. 2011; Declercq et al. 2013).

F. columnare causes both external and systemic infections in diverse fish species worldwide (Hawke and Thune 1992; Declercq et al. 2013). The bacterium can be a primary pathogen, but more commonly, it is a secondary pathogen that affects hosts predisposed by stress or trauma. The environment and the condition of the fish are important in determining the rate and severity of infection. Channel catfish are susceptible to columnaris at temperatures from 15 to 30^{o}C, and young fish are more severely affected than adults (Griffin 1992; Plumb 1999). Therefore, minimizing production losses due to columnaris is an important component of the overall objective of achieving production efficiency on the part of U.S. catfish farmers.

Globally, fish farmers apply antibiotics, vaccines and chemicals in the treatment of diseases. However, in the U.S., a limited number of approved drugs, strict certification protocols and high cost of treatment with chemicals and vaccines, as well as concerns over development of antibiotic-resistant pathogens and their consequential threat to human health and the environment are increasingly limiting their use by fish farmers (Toranzo et al. 1984; McEwen and Fedorka-Cray 2002; USDA 2003; FAO 2006; Welker and Lim 2011). These issues underscore the importance of introducing probiotics, which is a biological control measure, to U.S. catfish farmers to prevent and manage the disease burden that plagues the channel catfish industry.

The use of probiotics for disease prevention and improved nutrition in aquaculture has gained prominence in recent times due to an increasing demand for environment-friendly aquaculture (Verschuere et al. 2000, Vine et al. 2006; Shelby et al. 2006; Wang et al. 2008; Cruz et al. 2012). A number of studies have reported the effectiveness of probiotics applied as water or feed additives in improving the immunity, health status, feed efficiency and growth performance of fish species with conflicting results (Boyd and Gross 1998; Verschuere et al. 2000; Aboagye 2008; Denev et al. 2009). The probiotic effect of the commercially-available product Lymnozyme® has been evaluated with naive juvenile channel catfish challenged with enteric septicemia of catfish (ESC) under laboratory conditions (Aboagye 2008), but there has not been any known report of its efficacy on channel catfish challenged with columnaris, the second most important channel catfish disease in the Southeastern United States.

This study was conducted to evaluate the effectiveness of Lymnozyme®, a water-soluble aquatic probiotic product on the market, in reducing mortality in channel catfish when challenged with *F. columnare* under controlled conditions in the laboratory.

3. Materials and Methods

3.1. Colony forming unit counts of bacteria in Lymnozyme®

A sample of the commercial probiotic Lymnozyme® used in both experiments was analyzed to quantify the number of bacteria present in one gram. One gram of the product was dissolved in a 150-ml tube containing 99 ml of PBS (phosphate buffered saline). Serial dilutions of 10^{-2} (primary dilution), 10^{-4}, 10^{-6} and 10^{-8} were made from four replicate

samples and 100 µl of each dilution was spread on TSA plates and incubated at 30°C overnight. After incubation, plates with colonies between 30 and 300 were counted.

3.2. Preparation of *F. columnare* for the challenge

F. columnare strain ALG 00-530 used for the challenge was obtained from the Southeastern Cooperative Disease Laboratory, Auburn University. Frozen isolate of *F. columnare* (-80ºC) was defrosted and 200 µl inoculated into two tubes each containing 5 ml Hsu-Shotts broth and incubated for 24 hours at 30ºC with shaking at 150 rpm. Each tube was then inoculated into 150 ml of fresh broth and incubated overnight with shaking. The culture was centrifuged at 3600 x g for 30 minutes. The bacterial culture was quantified using standard plate count methodology to verify challenge dose. The isolate was passed through juvenile channel catfish prior to challenge to confirm virulence.

3.3. Experimental design and *F. columnare* challenge protocol

This study was carried out over a 21-day post-challenge period at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA, under controlled temperature conditions. Naïve (never previously exposed to *F. columnare*) juvenile channel catfish of average sizes of 7.0±0.2 g (experiment 1) and 5.9±0.1 g (experiment 2) were provided by Dr. Joe Newton, School of Veterinary Medicine, Auburn University and acclimated for 1 week. For each experiment, random groups of 15 fish were transferred to twenty (20) 57-L aerated, flow-through aquaria containing 32 L of well water heated and blended with cold water to maintain a water temperature of about 28ºC for the duration of the experiment. Dissolved oxygen (DO) levels in the aquaria were kept above

5.0 mg/l through aeration. During the 1-week acclimation period, fish were maintained in a static system with incorporated daily 100% water exchange. Dead fish were replaced and salinity levels in the tanks raised to 1.5‰ and gradually decreased to 0 ‰ before the challenge to avoid any potential external infection of experimental fish. Prior to challenge, fish were fed at 3% of their wet body weight with slow sinking commercial catfish feed (40 % protein, Zeigler Bros Inc., Gardners, PA, USA). Post-challenge feeding was done ad libitum. Temperature and DO were measured randomly from sample tanks twice daily (morning and late afternoon) using YSI 85 DO meter (YSI Corporation, Yellow Spring, OH, USA). Total ammonia-nitrogen (TAN) was determined twice a week using YSI 9100 photometer (YSI Corporation, Yellow Spring, OH, USA) and a pH meter (Oakton pH 112 series, Oakton Instruments, Vernon Hills, IL) was used for pH measurements.

After the 1-week acclimation, treatment groups received 5.0 g of Lymnozyme® to obtain a final dosage of 3.95×10^5 CFU/ml daily (treatment groups 1-3) and the control group received none for 3 days. During treatment with Lymnozyme®, water flow was stopped for 2 hours in the first experiment and 8 hours during the second experiment in both treatment and control tanks. Water flow resumed after the 2- or 8-hour static exposure period. The following treatments occurred post-challenge: control-received no Lymnozyme®; 1-received no Lymnozyme®; 2-received Lymnozyme® daily for the next 7 days; and 3- received Lymnozyme® daily for the next 14 days.

The challenge was done by placing fish from each aquarium into a bucket containing 2 L of water with aeration. Ten (10) milliliters of *F. columnare* strain ALG 00-530 (1.6×10^8 CFU/ml in experiment 1 and 1.8×10^8 CFU/ml in experiment 2) were inoculated into the water to obtain a final dosage of 8.0×10^5 CFU/ml and 9.0×10^5

CFU/ml, respectively. Fish remained in the bucket for 30 minutes, and the fish and bucket contents were then returned to the appropriate aquaria under static water conditions for 2 additional hours. Water flow was then resumed. Probiotic treatment was continued in treatment groups the same day after the challenge. Fish mortality after challenge was monitored until no mortality occurred for 7 consecutive days in all the aquaria. All moribund and dead fish were removed and bacteria isolated from the skin, gills, kidneys and livers were plated on Hsu-Shotts's agar plates for pathogen recovery. At the end of each experiment, the mean number of mortalities in treatments was compared with the control.

3.4. Statistical analysis

Mortality data collected were analyzed by one-way analysis of variance using the SAS software (SAS 9.2, SAS Institute Inc., Cary, NC). The mixed linear model procedure (Wolfinger et al. 1991) was used to make contrasts of treatments. Randomized block design was incorporated to minimize variation due to location of aquarium units in three different banks. Significance was set at 5% level ($p<0.05$).

4. Results

4.1. CFU counts of bacteria in Lymnozyme® and cultured *F. columnare*

The colony-forming unit counts of the probiotic bacteria in the commercial probiotic product Lymnozyme® and cultured *F. columnare* strain ALG 00-530 used for the challenge in experiments 1 and 2 are presented in Table 1. The average colony counts

of the probiotic prior to its use for the study was 2.5 x 10^9 CFU/g, which falls within the theoretical targeted range of 6.0 x 10^8 – 6.0 x 10^{10} CFU/g on the label. The average count of *F. columnare* strain ALG 00-530 used for the challenges in experiments 1 and 2 were 1.6 x 10^8 CFU/ml and 1.8 x 10^8 CFU/ml, respectively (Table 1).

4.2. Water quality

Mean water temperature ranged from 26.8±1.1^{o}C during static hours (morning) to 28.2±0.7 ^{o}C during flow-through phase (afternoon) in experiment 1. During the second experiment, mean water temperature ranged from 26.6±0.8 ^{o}C to 28.3±0.4 ^{o}C in the morning and afternoon, respectively. Mean dissolved oxygen levels were 5.7±0.4 mg/l (morning) and 6.1±0.5 mg/l (afternoon) during experiment 1. For experiment 2, DO ranged from 5.8±0.3 mg/l to 5.9±0.4 mg/l, respectively, for morning and afternoon. Mean total ammonia nitrogen levels were 0.13±0.05 mg/l and 0.10±0.02 mg/l, while pH was 7.1± 0.1 and 7.2±0.1 for experiment 1 and 2 respectively (Table 2).

4.3. Columnaris challenge

In the first experiment, there were no significant differences in the mean percent cumulative mortality among the control group and the treatment groups ($p=0.2370$). The mean percent cumulative mortalities as shown in Table 3 were 77±15.4%, 76±8.9%, 69±11.2% and 64±7.6% for control, and treatments 1, 2 and 3, respectively. The second experiment showed statistically significant differences between control and all treatments ($p=0.0041$). However, there were no significant differences among treatments ($p>0.05$).

Mean percent cumulative mortality rates of the control and treatment groups in the second experiment were 80±12.5% for the control, 61±14.5% for treatment 1, 50±13.0% for treatment 2 and 44±14.6% for treatment 3 (Table 3).

During the first experiment, 50% cumulative mortality occurred for control and all the treatments between 5 and 7 days post-challenge (Fig. 1). In the second experiment, 50% mortality occurred about 3 days post-challenge in the control group and 6 days in the 3-day treatment group (treatment 1) while the 7-day treatment group (treatment 2) attained 50% mortality 17 days after the challenge (Fig. 2). The 14-day treatment group, however, recorded a mean cumulative percent mortality less than 50% at the end of the 21 days.

Colony morphology of bacterial isolates from the gills, kidney, liver and external lesions on the skin of moribund fish from all treatments and control as observed from Hsu-Shotts culture plates showed numerous rhizoid yellowish colonies. Gram staining of pure cultures revealed the typical long and slender pinkish-red *F. columnare* morphology (Bullock et al. 1986; Durborow et al. 1998; Declercq et al. 2013).

5. Discussion

F. columnare causes a combination of external and systemic infections (Hawke and Thune 1992) and may result in skin lesions, fin erosion and gill necrosis (Declercq et al. 2013). *F. columnare* grows externally by stacking or adhesion to the fish skin and consequently causes skin damage through lesions. Large numbers of bacteria can also be isolated from internal organs during systemic infection without any external lesions (Hawke and Thune 1992; Lafrentz and Klesius 2009). In the current study, the recovery of

F. columnare from the skin, gills, liver and kidney of fish challenged with the pathogen confirms their infection both externally and internally.

According to Griffin (1992), Plumb (1999) and Darwish et al. (2009), channel catfish are susceptible to columnaris at temperatures ranging from 15 to 30^{o}C, and young fish are more severely affected than adults. Water quality was maintained at an acceptable range for the healthy growth of channel catfish (Chapman 1992) during this study. The experimental conditions (i. e. water temperature, DO, pH and TAN levels) maintained and small size of fish used were, thus, suitable for columnaris infection.

The application of a probiotic to manipulate the composition and size of pathogenic bacterial community on the skin, gills, gut and the water environment of a fish may be used in preventing or reducing the severity of a disease. The application of Lymnozyme® at a dosage of 3.95×10^5 CFU/ml for 8 hours daily resulted in significant reduction in mortality rates when challenged with *F. columnare*. Lymnozyme® application at the same dosage for 2 hours daily, however, did not show any significant effect in reducing fish mortality. The lack of treatment effect in the case of the latter could possibly be due to the inability of the probiotic strains to proliferate in substantial numbers within the two hours of application to adhere to and colonize the skin, gills and gut of the catfish before they were flushed out of the tanks in the flow-through system when water flow was resumed. Even with the discontinuation of Lymnozyme® application after the pre-challenge period of 3 days (8 h daily), the probiotic significantly reduced mortality due to *F. columnare* as compared to the control. It is reasonable to propose that the probiotic bacteria possibly attached themselves to the skin and gills of the fish depriving the pathogens of such substrates as attachment sites. FAO (2001) noted that a probiotic confers health benefits on

the host when administered in adequate amounts by modifying the host-associated or ambient microbial community of the host. Joborn et al. (1997) also associated the efficacy of a probiotic with its adhesion to and colonization of the gut of the host. Furthermore, continuous application of the probiotic at 8 h/day for a longer duration of 14 days resulted in over 50% reduction in fish mortality as compared to the 2 h/day experiment. Thus, the length of time the probiotic was administered within a day and the number of days of its application (post-challenge) appeared to have an influence on the reduction in channel catfish mortality. Various studies on the effects of probiotics on cultured aquatic animals have emphasized reduction in mortality (Moriarty 1998; Irianto and Austin 2002), improved resistance against disease (Gatesoupe 1994) and the ability to adhere to and colonize the gut (Joborn et al. 1997) among others. In this study, the probiotic effect of Lymnozyme® probably prevented *F. columnare* from attachment and subsequent proliferation in the intestinal tract, gills or on the skin to overwhelm the fish or possibly helped boost the level of resistance in the fish to withstand systemic infection through enhanced immunity. According to Moriarty (1999), a major role of a probiotic is to prevent pathogenic microbes from proliferating in the intestinal tract and/or in the culture environment of the cultured species. Evaluating the effectiveness of the probiotic *Bacillus subtilis* in water or diet against intramuscular challenge with *F. columnare* infection in Nile tilapia fingerlings, Mohamed and Refat (2011) observed that *B. subtilis*, which is also present in Lymnozyme®, when administered in water or diet at a rate of 0.1 g/L was effective in ameliorating *F. columnare* infection.

Results of the second experiment were consistent with a similar challenge study done by Aboagye (2008) following the same procedure. He challenged naive channel

catfish fingerlings with *Edwardsiela ictaluri* after 8 hours daily bath exposure for 3 days (pre-challenge) and continued 7 and 14 days (post-challenge) treatment with a liquid Lymnozyme® product. Results from his study showed that, when administered daily for 8 hours under static condition, Lymnozyme® effectively reduced mortality rates with more desirable effects achieved over longer application periods.

In recent years, probiotic bacteria have been added to diets to increase immunity and disease resistance as well as improve growth performance in fish (Welker and Lim 2011). The inclusion of some probiotics in fish feed has been demonstrated to improve the beneficial bacterial flora in the intestine of the host fish as they multiply to out-compete the pathogenic bacteria (Shelby et al. 2006; Ziaei-Nejad et al. 2006; El-Rhman et al. 2009; Mohamed and Refat 2011). Shelby et al. (2006) observed a higher survival after 39-63 days of feeding Nile tilapia fry with Bioplus probiotic, *Bacillus* spp. against *S. iniae* infection. Aly et al. (2008) compared the potential effect of two doses of *B. pumilus* and the commercial probiotic product Organic Green™ in improving immune response, survival, growth and resistance in Nile tilapia to *Aeromonas hydrophila* infection after feeding for 4 or 8 weeks. In their study, survival rates of all treatment groups showed statistically significant increases as compared to the control group. Probably, to avoid 2 or 8 hours of stagnant condition, which could lead to the build-up of ammonia in the system, an alternative would be to administer Lymnozyme® as a feed additive, if this can have the same effect on the gills and skin as the water based.

In general, the use of probiotics is becoming increasingly accepted as valuable alternative to antibiotic therapy to control or prevent infection from pathogenic bacteria (Irianto and Austin 2002). Their use poses no threats to humans and the environment, and,

thus, while commercial farmers are encouraged to use these products following recommendations, manufacturers of the products on the other hand need to collaborate with researchers to conduct more trials with these products in real world situations.

6. Conclusions

Lymnozyme® reduced mortality due to *F .columnare* infection in juvenile channel catfish when applied as a water additive for 8 hours daily at a dosage of 3.95×10^5 CFU/ml for at least 3 days prior to challenge by immersion under tank conditions. Applying daily over longer periods appeared to further reduce mortalities. It is recommended, however, that additional studies be conducted to investigate any colonization of these bacteria in the gastro-intestinal tract of fish as a means to establish its competitive advantage over pathogenic bacteria in the gut as well as on gills and skin. Lymnozyme® holds some prospect in minimizing mortality and improving catfish production efficiency in the United States but more detailed studies need to be conducted to evaluate its effectiveness in pond production. It may also be applicable to other freshwater fish impacted by columnaris infection globally.

Table 1. Colony-forming unit counts of bacteria in Lymnozyme® dry product sample and the pathogen *F. columnare* strain ALG 00-530 culture used for the two challenge experiments.

No. of Samples	Lymnozyme® (CFU/g)	*F. columnare* (CFU/ml)	
		Exp. 1	Exp. 2
1	2.1×10^9	1.7×10^8	1.5×10^8
2	1.9×10^9	1.5×10^8	2.2×10^8
3	3.1×10^9	1.3×10^8	2.0×10^8
4	2.8×10^9	1.9×10^8	1.6×10^8
Average	2.5×10^9	1.6×10^8	1.8×10^8

Table 2. Mean water quality parameters during columnaris challenge experiments 1 and 2 in a flow-through system under laboratory conditions using well water.

Parameter	Experiment 1	Experiment 2
Water temperature (a.m.) ^oC	26.8 ± 1.1	26.6 ± 0.8
Water temperature (p.m.) ^oC	28.2 ± 0.7	28.3 ± 0.4
Dissolved oxygen (a.m.) mg/l	5.7 ± 0.4	5.8 ± 0.3
Dissolved oxygen (p.m.) mg/l	6.1 ± 0.5	5.9 ± 0.4
pH	7.1 ± 0.1	7.2 ± 0.1
TAN	0.13 ± 0.05	0.10 ± 0.02

Table 3. Mean percent cumulative mortality of treatment groups in experiment 1 (2 h) and experiment 2 (8 h) that did or did not receive Lymnozyme® inoculum for 3 days and then were challenged with *F. columnare* strain ALG 00-530 at a dosage of 9.0 x 10^5 CFU/ml by immersion. Significant differences among treatments ($p < 0.05$) are indicated by different letters within the same column.

Treatment	Mean cumulative percent mortality	
	2 h	8 h
Control	77.3 ± 15.4[a]	80 ± 12.5[a]
1	76.0 ± 8.9[a]	61 ± 14.5[b]
2	69.3 ± 11.2[a]	50 ± 13.0[b]
3	64.0 ± 7.6[a]	44 ± 14.6[b]

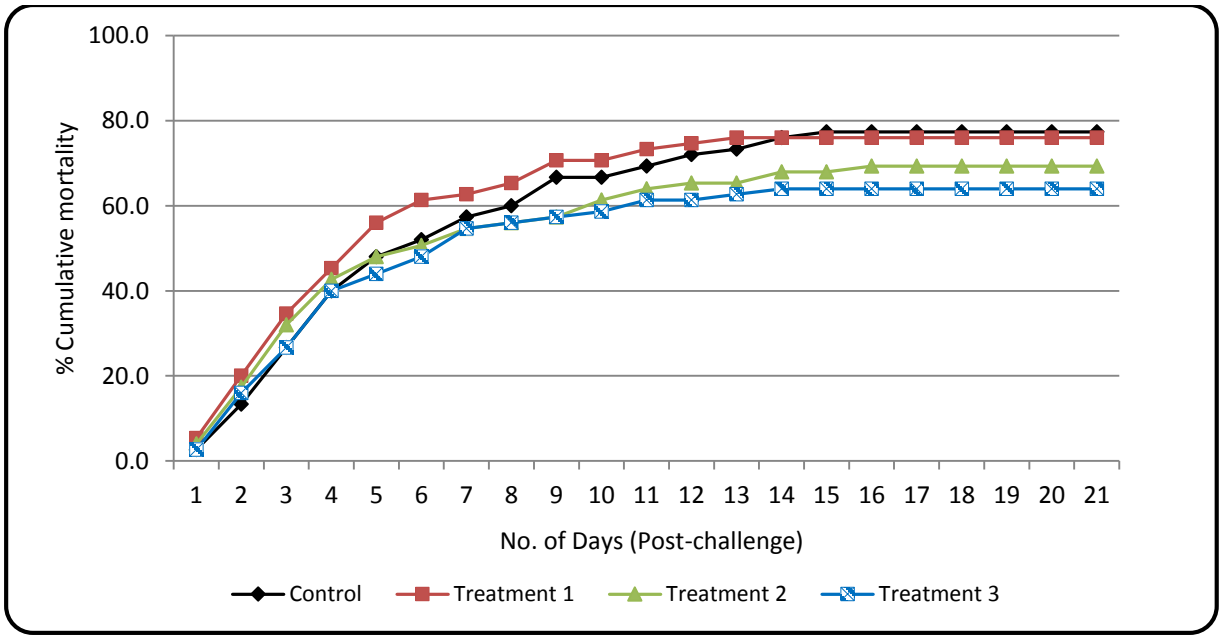

Fig. 1. Mean percent cumulative mortality of naïve channel catfish treated with or without Lymnozyme® for 2 h daily and then challenged with *F. columnare* by immersion.

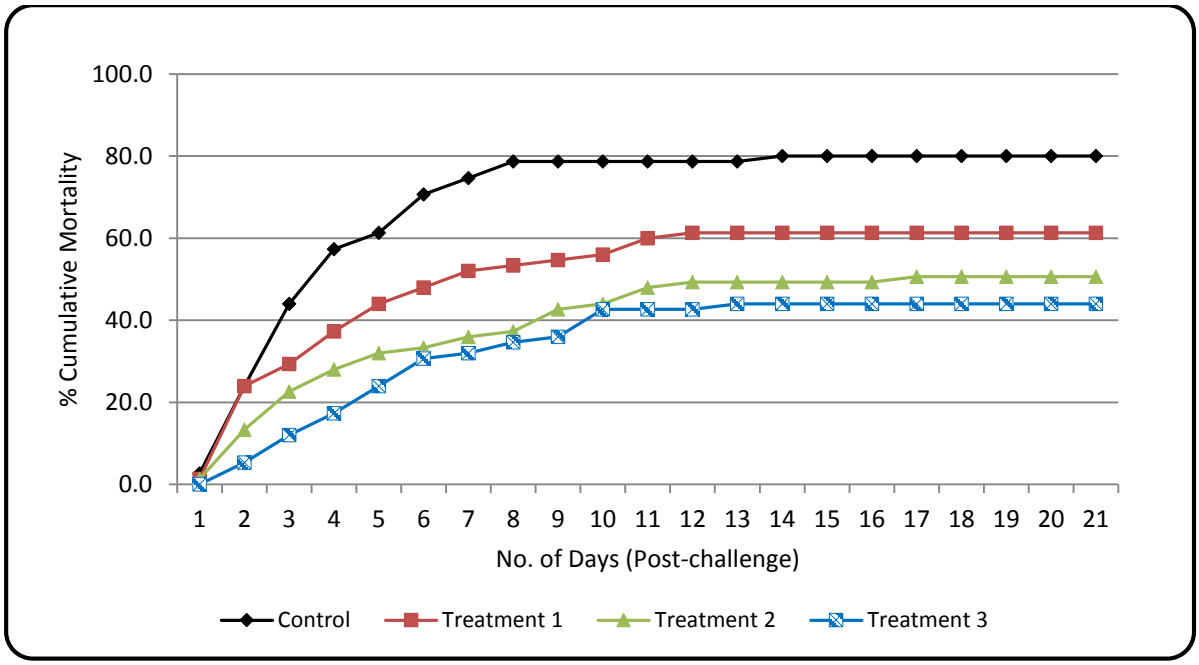

Fig. 2. Mean percent cumulative mortality of naïve channel catfish treated with or without Lymnozyme® for 8 h daily and then `challenged with *F. columnare* by immersion.

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CHAPTER III

Effects of the commercially-available probiotic product PondToss™ on water quality, growth and production of channel catfish from juvenile to market size in earthen ponds

1. Abstract

In recent times, many probiotic products are promoted and sold for use as water quality conditioners for the improvement of growth and health of fish in aquaculture, although the products are broadly marketed with limited field efficacy trials having been conducted. This study evaluated the commercially-available product PondToss™ (Keeton Industries Inc., Wellington, CO) to determine the efficacy of its use in controlling water quality and improving growth and survival of channel catfish *Ictalurus punctatus* in ponds. The study was conducted over two production years, May-October, 2011 and April-September, 2012. Channel catfish of average size of 21.0±0.5 g were stocked into six 0.04-ha earthen ponds at a rate of 34,595 fish/ha in the first production year. During the second year, fish harvested from the first year of average size 297.0±13.7 g were stocked into the ponds at a rate of 19,768 fish/ha. For each production year, three ponds were designated as treatment ponds and each received the microbial product PondToss™ twice a week at a dosage of 2.4×10^2 CFU/ml of pond water while the remaining three ponds were designated untreated controls and received no PondToss™. There was no significant treatment effects in mean total ammonia nitrogen (TAN) levels of 0.55±0.5 mg/l and 0.49±0.5 mg/l for untreated and treated ponds, respectively, in 2011 ($p=0.9130$). The result was similar in

2012 as mean TAN did not differ significantly for untreated (1.10±1.0 mg/l) and treated (1.30±1.2 mg/l) ponds (p=0.2131). Nitrite concentrations averaged 0.48±0.4 mg/l and 0.36±0.4 mg/l for untreated and treated ponds (p=0.0991) in 2011, and 0.50±0.4 mg/l each for untreated and treated ponds (p=1.0000) in 2012. Mean water temperature, dissolved oxygen, pH, alkalinity, hardness and chloride levels were similar in all ponds (untreated and treated) for each of the production years. Mean specific growth rate, percent survival, feed conversion ratio and weight at harvest were 1.10±0.2 % day^{-1} and 1.20±0.1 % day^{-1}, 75±13.5% and 84±3.5%, 1.8±0.3 and 1.5±0.03, 263.2±31.5 g and 292.6±9.3 g for untreated and treated ponds, respectively, in 2011. The values for untreated and treated ponds in 2012 were 0.49±0.01 % day^{-1} and 0.52±0.01 % day^{-1}, 97.9±2.0% and 98.2±1.4%, 2.7±2.8 and 2.7±0.18, 743.3±56.9 g and 756.7±47.3 g, respectively. In each production year, there were no significant differences between untreated and treated ponds. Mean final standing crops of 8,616±494 kg/ha and 7,051±2,024 kg/ha were obtained in 2011 in respect of treated and untreated ponds, while in 2012, mean final standing crops were 15,007±912 kg/ha and 14,442±1,247 kg/ha for treated and untreated ponds, respectively. In either year, mean final standing crops were not significantly different in treated and untreated ponds ($p>0.05$). The microbial product PondToss™ did not show any significant effect in improving water quality, growth and production of channel catfish in earthen ponds under the conditions of the present studies.

2. Introduction

Channel catfish *Ictalurus punctatus* (Rafinesque, 1818) production is the largest sector of the US aquaculture industry (Quagraine 2006; Hanson and Sites 2012),

accounting for approximately half of total aquaculture production in the country. However, production levels which were worth a market value of 480 million dollars in 2004 have declined over the last decade to a value of 423 million dollars in 2011 (USDA 2006, 2012). Furthermore, the industry experiences stiff competition from frozen catfish fillets imported from Vietnam, China and other foreign countries, which according to Hanson and Sites (2012) accounted for 74% of all U.S. sales of frozen catfish fillet products in 2011, since these foreign competitors consistently offer lower prices. In order for U.S. catfish producers to remain competitive, there is the need to improve their production efficiency to increase production beyond that which is currently achieved in commercial static water ponds. This could be reached if production systems can be managed to increase their waste treatment capacities (Tucker et al. 2009).

Water quality, particularly dissolved oxygen and nitrogenous wastes, is a major limiting factor influencing growth and survival during the production of channel catfish in commercial earthen ponds (Boyd and Tucker 1998). Stress from poor water quality promotes disease outbreaks, especially the two main bacterial diseases, enteric septicemia of catfish (ESC) and columnaris, which cause direct losses estimated at over $50 million/year. Waste treatment may be accomplished in ponds by frequent water exchange or by discharging wastes to public waters. However, in the U.S. catfish industry, neither of these methods is economically and environmentally acceptable due to strict environmental regulations and the high cost of water.

Lately, increasing the capacity of in-pond microbial communities to transform nitrogenous wastes through bioremediation has gained recognition as an environmentally-friendly alternative approach to intensifying aquaculture production. According to Mischke

(2003), microbial communities in ponds are essential for the maintenance of optimum water quality. They transform inorganic and organic wastes into less toxic forms and thereby maintain suitable water quality for fish production. Boyd and Tucker (1998) stated that disruptions of bacterial communities in ponds may cause increases in potentially toxic ammonia or nitrite concentrations. Growth and biodegrading activity of naturally occurring microorganisms in ponds can be stimulated by improving environmental conditions in ponds; however, as this is difficult to accomplish, an alternative is to supplement naturally occurring microflora with organisms produced in culture. Application of spore-forming bacteria as biological agents for improving water quality and reducing disease offers a number of advantages (Sanders et al. 2003; Wolken et al. 2003; Lalloo et al. 2007) and a number of spore-forming biological agents are produced worldwide for animal use (Sanders et al. 2003). Many attempts have been made to improve organic matter degradation in aquaculture ponds through direct additions of bacterial suspensions to pond water (Boyd et al. 1984; Tucker and Lloyd 1985; Chiayvareesajja and Boyd 1993; Queiroz and Boyd 1998; Boyd and Gross 1998) and the addition of large numbers of specific bacteria to ponds is purported to help improve water quality. Although positive results have been recorded in recirculating systems (Ehrlich et al. 1989) under controlled conditions, improvement of water quality in ponds has been conflicting (Mischke 2003). In spite of the conflicting record of success, many probiotic products are promoted and aggressively marketed for use as water quality conditioners for the improvement of growth and health of fish in aquaculture (Boyd and Gross 1998). One such product is PondToss™, a dry water soluble concentrate mixture of probiotics and waste and sludge reducing bacteria produced

by Keeton Industries Inc., Wellington, CO, which is available commercially but has not been fully field tested.

This study evaluated the effect of PondToss™ on water quality and production characteristics of channel catfish *Ictalurus punctatus* cultured in earthen ponds from juvenile stage to market size in two consecutive growing seasons, May to October, 2011 and April to September, 2012.

3. Materials and methods

3.1. Experimental channel catfish

This study was carried out over a period of 2 years. In the first production year (May-October, 2011), channel catfish obtained from a producer in Mississippi and grown the previous year (2010) to an average weight of 21.0±0.5 g were graded and stocked into six 0.04-ha earthen ponds at the E. W. Shell Fisheries Center, Auburn, Alabama, U.S.A, and used for the pond production study. At the end of the first production year, surviving fish were mixed together and overwintered at the E. W. Shell Fisheries Center. They were then graded and randomly selected and re-stocked at an average size of 297.0±13.7 g for continuation of growth in the second production year, which spanned April to September, 2012.

3.2. Pond description, preparation and stocking

Six 0.04-ha earthen ponds of average and maximum depths of 1.0 m and 1.5 m, respectively, located at the E. W. Shell Fisheries Center were used in this study. The ponds

were rectangular in shape and had earthen bottoms that gradually sloped towards the point of maximum depth with a drain in a catch basin. The edges were vertical and supported by concrete or wooden walls. Pond bottoms were sun dried for 5 days to eliminate all weeds and unwanted organisms. The catch basin and parts of pond bottom that remained wet were treated further with rotenone at a rate of 8.0 ml/m^3. The rotenone was neutralized 24 hours after application with potassium permanganate at a rate of 15 ml/m^3. Ponds were limed with agricultural lime at a rate of 5,000 kg/ha to adjust alkalinity and hardness to values >60 mg/l using agricultural lime to increase the buffering capacity of the water and reduce problems associated with elevated pH typical with phytoplankton bloom. Prior to filling of ponds with water from a watershed reservoir, inlet pipes were covered with "sock" strainers made of fine netting material to prevent entry of wild fish, larvae and eggs into ponds. Rock salt was added after the ponds had been filled at 920 kg/ha to increase the chloride level above 22 mg/l. Each pond was provided with a 0.5-hp electric spray aerator (Aquarian model by Isolator Corporation, Kansas City, MO, U.S.A) for aeration as needed. Stocking of ponds were done 3 days after filling of ponds to prevent the emergence of nymphs and larvae of aquatic insects. Channel catfish were counted by recording the average wet weight and bulk weights, and were stocked into the six ponds at a rate of 34,595 fish/ha (1400 fish/pond; 735 kg/ha) during the first production year. In the second year, fish were stocked at a rate of 19,768 fish/ha (800 fish/pond; 5940 kg/ha). Sufficient water was added periodically to each pond to compensate for evaporation and seepage.

3.3. Feeding

Fish were observed twice daily during feeding and were fed ad libitum to ensure that fish were fed until consumption declined to a level signifying fish have consumed all they wanted. They were fed with a high quality floating commercial catfish feed containing 32% protein and 6% fat (3.5 mm pellet size in year 1 and 5.5 mm in year 2) produced by Alabama Catfish Feed mill, Uniontown, AL, U.S.A. This was also to ensure that fish were not overfed or fed when they were not eating. The amount of feed consumed was recorded and used as a guide for the next feeding schedule. Feeding rate was adjusted weekly based on the actual feed fed and an assumed feed conversion ratio (FCR) of 1.8.

3.4. Pond treatment with probiotic, water quality monitoring and management

Pond treatments were as follows: 1) untreated control ponds-received no PondToss™ and 2) treated ponds received PondToss™. All ponds were aerated only when it was needed. Treated ponds received batches of PondToss™ produced on-site twice weekly. The probiotic strains in PondToss™ were incubated by measuring 18 g into 18 L (1.0 g/L) of well water heated to 28^{o}C and aerated continuously for 36 hours in a 260-L capacity plastic container. Bacterial counts in PondToss™ before and after incubation were determined in the laboratory. Six liters of the incubated probiotic (9.0×10^{10} CFU/ml) was applied to each treatment pond to obtain a final dosage of 2.4×10^{2} CFU/ml of pond water. Ten (10) and five (5) grass carp (*Ctenopharyngodon idella*) of average sizes 50±2.0 g and 55±5.0 g, respectively, were put into each pond at the rate of 125 fish/ha and 250 fish/ha in year one and two, respectively, to control aquatic weeds. Dissolved oxygen (DO) levels were maintained in all ponds above 3 mg/l by either turning on aerators overnight when afternoon

DO levels were below saturation or turning them on during cloudy days. DO and temperature were monitored twice daily; early morning (0600 h) and late afternoon (1700 h) using YSI-85 digital temperature/DO meter (YSI Corporation, Yellow Spring, OH, USA). Measurement of pH was done daily in mid-afternoon (1300 h) using a pH meter (Oakton pH 112 series, Oakton Instruments, Vernon Hills, IL). Total ammonia nitrogen (TAN) and nitrite-nitrogen were determined weekly while alkalinity, hardness and chloride concentrations were measured biweekly with YSI 9100 photometer (YSI Corporation, Yellow Spring, OH, USA). Salt was added to any pond with chloride levels below 22 mg/l to maintain adequate chloride levels to reduce any potential negative impacts of elevated nitrite levels that could cause brown blood disease. Mortalities were recorded and some moribund fish were taken to the Fish Disease Laboratory, Auburn University to determine the cause of death. Fish were not sampled during the study periods to reduce additional handling stress.

3.5. Harvesting of ponds

During harvest, ponds were partly drained and seined from the deep to the shallow end with a seine net (5 cm stretched mesh size). Two to three seine hauls were completed on each pond before complete draining to collect the remaining fish by hand. Harvested fish were counted and weighed and data used to evaluate some production parameters including specific growth rate (SGR), feed conversion ratio (FCR), percent survival, and mean final standing crop as follows:

$$SGR\ (\%\ day^{-1}) = (\ln W_f - \ln W_i) \times 100 / t$$

Where, lnW_f is the natural logarithm of the individual harvesting weight, lnW_i is the natural logarithm of the individual stocking weight, and t is the time (days) between lnW_f and lnW_i.

FCR = feed intake (as fed) / (final wet body weight – initial wet body weight).

Percent survival = (total number of fish harvested / total number of fish stocked) x 100

Final standing crop = final total biomass per unit area (kg/ha).

3.6. Statistical analysis

All statistical analyses were done using the statistical analysis system (SAS Institute 1999) version 9.2 in windows 8. The mixed linear model procedure (Wolfinger et al. 1991) was used to determine differences between treatments. A p-value less than 0.05 was taken to indicate statistical significance.

4. Results

4.1. Pond treatment and water quality

Incubation of the probiotic bacteria on-site yielded a significant increase in the average number of bacteria cells from 3.9 x 10^9 CFU/ml to 9.0 x 10^{10} CFU/ml, $p<0.0001$ (Table 1). This however, produced a very low final dosage of 2.4 x10^2 CFU/ml of pond total volume. During the first production year, which started in May and ended in October, 2011, weekly mean water temperature ranged from 15.0^{o}C in week 25 to 29.3^{o}C in week 13 in the mornings but was slightly higher in the afternoons and ranged from 17.4^{o}C in

week 25 to 31.6ºC in week 13 (Fig. 1). The second production year began in April, 2012 and ended in September, 2012 with mean weekly water temperature ranging from 19.9ºC week 1 to 28.9ºC week 15 and 22.1ºC week 1 to 31.1ºC in week 15, respectively, for morning and afternoon (Fig. 2). The overall mean water temperature did not vary significantly between ponds treated with probiotic and controls in the mornings and afternoons of either year (Table 2). Mean weekly dissolved oxygen (DO) ranged from 4.6 mg/l in week 11 to 7.2 mg/l in week 25 in the morning and 7.6 mg/l in weeks 22 and 23 to 10.5 mg/l in weeks 17 and 20 in the afternoon in 2011. In 2012, the lowest mean weekly DO levels recorded in the morning and afternoon were 4.6 mg/l (week 12) and 6.9 mg/l (week 1), respectively, while the highest mean weekly levels recorded were 6.5 mg/l (week 1) and 11.5 in week 22 (Figs 3 and 4). Overall mean DO values obtained did not differ significantly in control and treated ponds for both years (Table 2). Mean weekly total ammonia nitrogen (TAN) and nitrite levels were also not significantly different in control ponds and treated ponds in both years as shown in Table 2. Mean weekly TAN ranged from 0.02 mg/l in week 3 to 2.49 mg/l in week 15 for untreated ponds and 0.02 mg/l in week 3 to 1.95 mg/l in week 15 for treated ponds in 2011, and from 0.05 mg/l in week 1 to 3.85 mg/l in week 8 for both untreated and treated ponds in 2012 (Figs. 5 and 6). The mean weekly nitrite values recorded, respectively, for untreated and treated ponds in 2011 ranged from 0.01 mg/l in week 1 to 1.21 mg/l in week 15, and 0.01 mg/l in week 1 to 1.11 mg/l in week 15. In the second production year, recorded values ranged from 0.00 mg/l in week1 to 1.20 mg/l in week 10 (untreated ponds) and 0.00 mg/l in week 1 to 1.42 mg/l in week 12 (treated ponds). Mean bi-weekly total alkalinity, total hardness and chloride levels in ponds in 2011 and 2012 are shown in Figures 7 and 8, respectively. Mean bi-weekly total

alkalinity ranged from the lowest value of 35 mg/l in week 1 to the highest of 103.3 mg/l in week 9 in 2011 (Fig. 7) and from 45 mg/l (week 21) to 75 mg/l (week 13) in 2012 (Fig. 8). In either year, the overall total alkalinity did not show any significant difference between treated and untreated ponds (Table 2). Similarly, overall total hardness was not significantly different in treated and untreated ponds in both years (Table 2). It ranged from a mean bi-weekly value of 30 mg/l (week 1) to 71.7 mg/l (week 11) in 2011 and 45 mg/l (week 21) to 75 mg/l (week 13) in 2012. Mean bi-weekly chloride levels recorded increased from 33 mg/l to 45 mg/l and 22 mg/l to 41 mg/l in 2011 and 2012, respectively. There was no significant difference between treated and untreated ponds in either year (Table 2). Changes in overall mean afternoon pH in treated and untreated ponds were equally not significant as shown in Table 2 and the mean weekly values ranged from 6.7 to 9.0 and 7.1 to 9.2 in 2011 and 2012, respectively.

4.2. Growth and production characteristics

As shown in Table 3, fish were stocked in May 2011 at a rate of 34,595 fish/ha in the first production season at average sizes of 21.3±0.3 g and 20.7±0.5 g for untreated and treated ponds. During this growing season, fish increased in size with mean specific growth rates of 1.10±0.2 %/day (in untreated ponds) and 1.20±0.1 %/day (in treated ponds) and attained average weights of 263.2±31.5 g and 292.6±9.3 g, respectively, at harvest in October 2011. Although the average size of fish harvested from treated ponds were bigger than untreated, the difference was not statistically significant ($p=0.2081$). Fish were fed ad libitum up to a feeding rate of 854 kg/ha/week (122 kg/ha/day). There was not much variation in the weekly feeding rates between treated and untreated ponds (Fig. 13). The

overall mean feed conversion ratios and percent survival were slightly better in treated ponds (1.50±0.03 and 84±3.5%) as compared to untreated (1.80±0.3 and 75±13.5%) but did not differ significantly (Table 3). Mean standing crops in treated and untreated ponds were 8,616±494 kg/ha and 7,051±2,024 kg/ha, respectively, and were also not statistically significant (Table 3).

The second production season started with bigger fish harvested from the first season of average weights 297±14.5 g and 296±15.9 g, respectively, stocked at 19,768 fish/ha into treated and untreated ponds (Table 4). Mean weekly feed consumption rates were similar in treated and untreated ponds (Fig. 14). The maximum feeding rate recorded was 1050 kg/ha/week or 150 kg/ha/day. The mean specific growth rates 0.49±0.01 %/day and 0.52±0.01 %/day in untreated and treated ponds were not significantly different. Percent survival was very high in both untreated and treated ponds 97.9±2.0% and 98.2±1.4%, respectively. Feed conversion ratios were 2.70±2.8 and 2.70±0.18 in 2012 production season for untreated and treated ponds. At the end of the second production season, fish attained a cumulative mean weight of 743.3±56.9 g and 756.7±47.3 g with respect to untreated and treated ponds with no significant treatment effect (p=0.7709). Mean final standing crop was also not different statistically for untreated ponds 14,442±1,247 kg/ha and treated ponds 15,007±912 kg/ha (Table 4).

5. Discussion

Channel catfish production in static water pond systems results in waste accumulation and consequently disease proliferation with negative impact on the

environment and profitability. In the current study, bacterial inocula of 2.4 x 10^2 bacterial cells/ml were provided twice a week per pond, which were lower than inoculation rates of about 10^3 reported in studies with other bioremediation products (Chiayuvareesajja and Boyd 1993; Quieroz and Boyd 1998; Duvall and Anderson 2001; Duvall et al. 2001; Tucker et al. 2009). The modes of action for different probiotic products may vary, but typically they are purported to compete with phytoplankton and other autochthonous bacteria communities in the ponds for dissolved inorganic nitrogen and phosphorus, thereby, reducing the concentrations of these nutrients. Under the current conditions, the effect of PondToss™ on channel catfish pond water quality was inconsistent with the purported goal of reducing nitrogenous wastes, and improving growth possibly because the dosage used was too low. Furthermore, the high stocking densities and feeding rates had a major influence on water quality deterioration, especially increased accumulation of nitrogenous wastes. Some studies have shown that though bacteria communities in water are responsible for a significant portion of dissolved inorganic nitrogen uptake in oligotrophic waters, their role is much reduced in aquaculture ponds with higher nutrient loading rates. This is because in heavily fed ponds, phytoplankton uptake dominates inorganic nutrient budgets (Hargreaves 1997; Biddanda et al. 2001; Cotner and Biddanda 2002; Danger et al. 2007).

Feed was administered ad libitum and the maximum feeding rate of approximately 150 kg/ha/day far exceeded the range of 50 to 60 kg/ha/day fed by commercial catfish farmers in the southern United States. According to Hargreaves and Tucker (2003), during summer, organic matter loading rates from feeding of catfish ponds are high with maximum sustainable feeding rates ranging from 100 to 125 kg/ha/day with good fish

growth and relatively few problems with acute water quality deterioration. They noted, further, that short-term feeding rates can exceed 175 kg/ha/day. Schroeder et al. (1990) and Knud-Hansen et al. (1991) observed maximum feeding rates of 100-150 kg/ha/day to correspond with nitrogen loading rates of 500-750 mg N/m^2/day. The high feed consumption rate could be attributed to the high stocking densities used in this pond challenge study for the two growing seasons.

The high fish densities and feeding rates in the ponds were probably the basis for the current observation, where total ammonia nitrogen and nitrite levels were not significantly reduced in ponds treated with the probiotic as compared to untreated ponds. Total ammonia nitrogen exists as un-ionized ammonia (NH_3), which is quite toxic to fish, and ammonium ion ($NH4^+$) in a pH and temperature-dependent equilibrium. A rise in pH leads to an increase in the proportion of NH_3 and can cause stress, poor growth and even death of farmed fish. However, high alkalinity helps to buffer the systems to prevent wide fluctuations in pH. Wurts and Durborow (1992) noted that total alkalinity concentration in fish production ponds should not fall below 20 mg/L since pond pH can swing widely from 6 to 10 during the day when alkalinity concentrations are below this level. In either year of production, alkalinity, although not too high, never declined below 30 mg/l and this might have contributed to a mean afternoon pH not greater than 9.2. The mean values of alkalinity and hardness in the ponds in both years were lower than the recommended desirable range (100-200 mg/l), which is ideal for optimal growth of the probiotic bacteria in PondToss™ (Keeton, pers. comm). He stated that alkalinity and hardness levels in treated ponds are vital to the optimal function of the bacteria strains in the probiotic product and preferably should be greater than or equal to 100 mg/l. The overall mean concentrations of both

parameters were below 100 mg/l and could probably have affected the results of the study. The mean chloride levels recorded were above the 10:1 ratio with mean nitrite values needed to prevent the outbreak of brown blood disease triggered by high nitrite levels.

Water temperature and dissolved oxygen levels followed the normal pattern observed during the growing season (April to October). Mean morning and afternoon temperatures both increased from below 20^{o}C in April/May to the highest of about 31^{o}C in July/August, and declined below 20^{o}C again in October with mean afternoon temperatures slightly higher than morning. Morning DOs were higher during the cooler months (April/May and September/October) but were relatively lower than afternoon DOs. This is expected since water temperature generally has an inverse relationship with DO. It is worth noting, however, that in each production year, the highest TAN and nitrite readings occurred in weeks with relatively lower DO levels but higher water temperature and feeding rates.

Since there was no any infectious agents isolated in dead fish sent to the disease laboratory for diagnostics, especially during the first growing period, the relatively lower percent survival could be as a result of mortality due to predation by birds and other animals during feeding (due to their smaller size at stocking) as compared to their size in the second season. Also, the poor feed conversion rates in the second production year could be due to lower metabolism by the relatively bigger fish that were stocked in that season and possibly stress due to high TAN and nitrite levels. In this study, all water quality variables with the exception of TAN and nitrite were within suggested safe limits for channel catfish culture (Morris 1993).

Many attempts have been made to improve organic matter degradation in aquaculture ponds for further intensification of the industry through direct additions of bacterial suspensions to pond water (Boyd et al. 1984; Tucker and Lloyd 1985; Chiayvareesajja and Boyd 1993; Queiroz and Boyd 1998); however, improvement of water quality in ponds has not been successful. Mischke (2003) noted that this was due to the diverse array of environmental variables at play in open ponds since positive results have been recorded for their use in recirculating systems under controlled conditions (Ehrlich et al. 1989). Boyd et al. (1984) attributed the lack of treatment effects to the fact that many bacterial suspensions are mixtures of bacteria already present in the pond environment, and that the quantity of bacteria applied is insignificant compared to the already existing population of bacteria. Furthermore, the physical and chemical characteristics of the pond water may affect the function of the bacterial suspension introduced into the pond since these characteristics may determine the size and species composition of the bacterial population in the pond (Tucker and Lloyd 1985).

The application of PondToss™ twice a week at the rate of 2.4×10^2 cells/ml did not yield any significant advantage over untreated ponds with respect to water quality, growth rate, feed conversion ratio, percent survival, standing crop and average weight at harvest. The outcome of this study corroborates results by Tucker et al. (2009) who in a two year production study of the effects of a probiotic bacterial bioaugmentation product on water quality and production of channel catfish found no treatment effects as regards fish production and feed conversion ratios.

6. Conclusions

At a dosage of 2.4 x 10^2 CFU/ml and two times a week frequency of application, PondToss™ had no significant effect on water quality, growth and production of channel catfish from juvenile to market size. This dosage was probably too low to substantially reduce total ammonia nitrogen and nitrite levels in treated ponds. The high stocking densities and its associated high feeding rates used in the study resulted in increased TAN and nitrite levels, which might have influenced the outcome. Total alkalinity and hardness range of 100-200 mg/l required for optimal performance of the product could not be met under the conditions of the present study and this could have also affected its performance to reduce nitrogenous wastes in treated ponds. Therefore, within the limits of the study conditions, the application of PondToss™ did not help improve channel catfish production in earthen ponds.

Table 1. Average CFU counts (± SD) of PondToss™ before and after incubation at 28°C for 36 hours.

No	Before incubation (CFU/ml)	After 36 h incubation (CFU/ml)
1	$3.7x10^9$	$8.4x10^{10}$
2	$4.2x10^9$	$9.1x10^{10}$
3	$3.8x10^9$	$8.9x10^{10}$
4	$3.9x10^9$	$9.5x10^{10}$
Average	$3.9x10^9$	$9.0x10^{10}$

Table 2. Overall mean water quality parameters of treated and untreated channel catfish ponds in the first (May - October 2011) and second (April – September 2012) production seasons.

Variable	2011			2012		
	Untreated	Treated	p-value	Untreated	Treated	p-value
Water temperature (morning) (°C)	25.2±3.7	25.0±3.4	0.9106	25.7±2.4	25.8±2.3	0.9912
Water temperature (afternoon) (°C)	28.0±3.8	27.6±3.4	0.8975	28.6±2.3	28.5±2.3	0.9877
Dissolved oxygen (morning) (mg/L)	5.8±0.9	5.7±0.8	0.9956	5.6±0.7	5.6±0.6	0.9998
Dissolved oxygen (afternoon) (mg/L)	8.8±1.8	9.2±1.5	0.7799	9.4±1.7	8.8±1.4	0.7542
Total ammonia nitrogen (mg/L)	0.55±0.5	0.49±0.5	0.9130	1.10±1.0	1.30±1.2	0.2131
Nitrite (mg/L)	0.48±0.4	0.36±0.4	0.0991	0.50±0.4	0.50±0.4	1.0000
pH	7.9±0.5	7.9±0.5	1.0000	7.8±0.6	7.7±0.6	0.9899
Total alkalinity (mg/L)	65±21.4	75±28.3	0.2152	59±19.3	58±11.9	0.9997
Total hardness (mg/L)	55±18.7	57±18.3	0.7924	64±14.0	65±13.2	0.9651
Chloride (mg/L)	38±6.6	36±9.8	0.7575	32±8.4	34±10.3	0.8401

Values are means ± s. d. of three replicates and values within the same row with $p>0.05$ are not significantly different

Table 3. Channel catfish production variables in untreated ponds and in ponds treated twice a week with a commercial probiotic product PondToss™ in the first production season (May-October 2011).

Variable	Untreated	Treated	P-value
Average stocking weight per fish (g)	21.3±0.3	20.7±0.5	0.1939
Stocking rate (per hectare)	34,595	34,595	1.0000
Average harvest weight per fish (g)	263.2±31.5	292.6±9.3	0.2081
Specific growth rate (% day^{-1})	1.10±0.2	1.20±0.1	0.7986
Feed conversion ratio	1.8±0.3	1.5±0.03	0.1665
Percent survival (%)	75±13.5	84±3.5	0.2342
Final standing crop (kg ha^{-1})	7051±2024	8616±494	0.2617

Values are means ± s. d. of three replicates and values within the same row with p-value >0.05 are not significantly different.

Table 4. Channel catfish production variables in untreated ponds and in ponds treated twice a week with a commercial probiotic product PondToss™ in the second production season (April-September 2012).

Variable	Untreated	Treated	P-value
Average stocking weight per fish (g)	296±15.9	297±14.5	0.9421
Stocking rate (per hectare)	19,768	19,768	1.0000
Average harvest weight per fish (g)	743.3±56.9	756.7±47.3	0.7709
Specific growth rate (% day^{-1})	0.49±0.01	0.52±0.01	0.9286
Feed conversion ratio	2.7±2.8	2.7±0.18	0.7535
Percent survival (%)	97.9±2.0	98.2±1.4	0.9818
Final standing crop (kg ha^{-1})	14442±1247	15007±912	0.5604

Values are means ± s. d. of three replicates and values within the same row with p-value >0.05 are not significantly different.

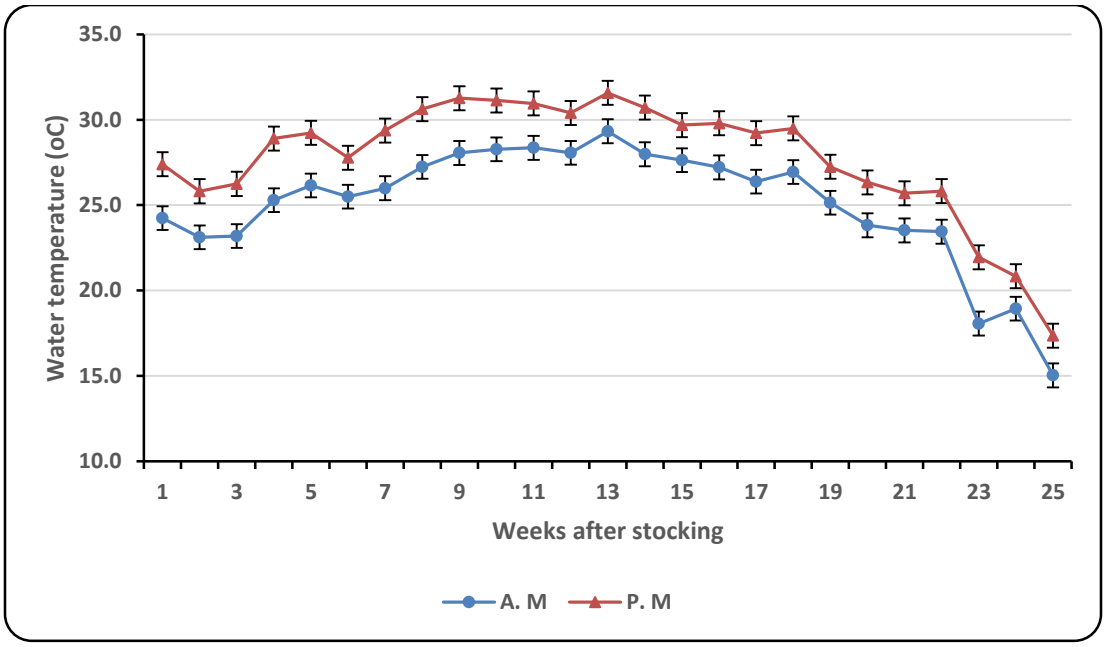

Figure 1. Overall mean weekly variation in morning and afternoon temperature in channel catfish ponds during the first production season (May-October 2011). Error bars indicate standard error.

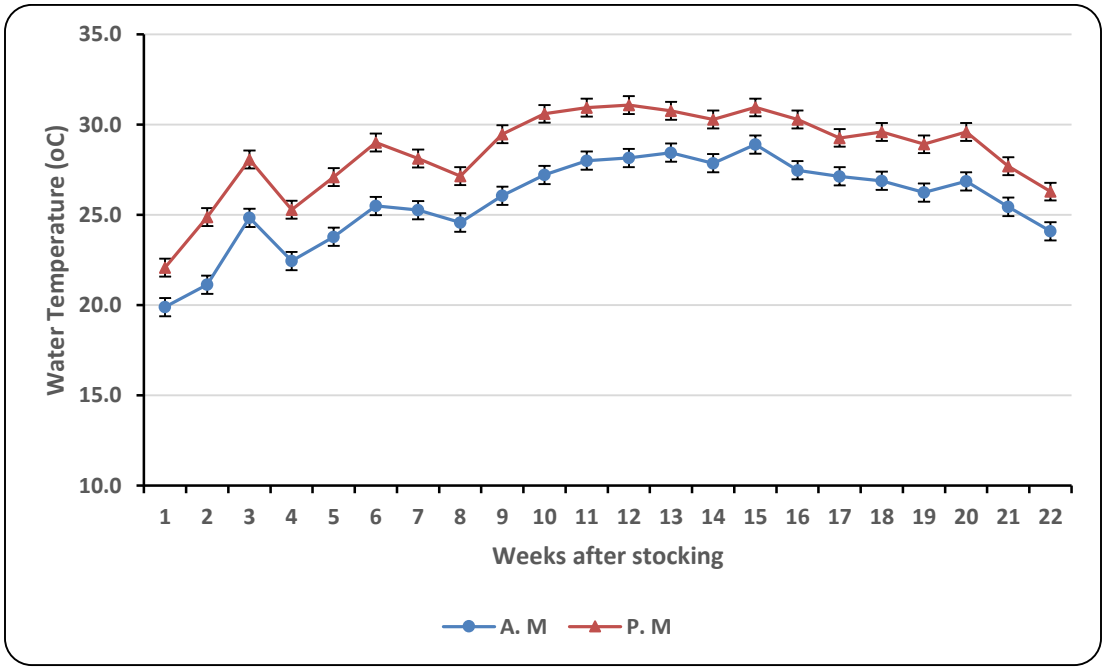

Figure 2. Overall mean weekly variation in morning and afternoon temperature in channel catfish ponds during the second production season (April-September 2012). Error bars indicate standard error.

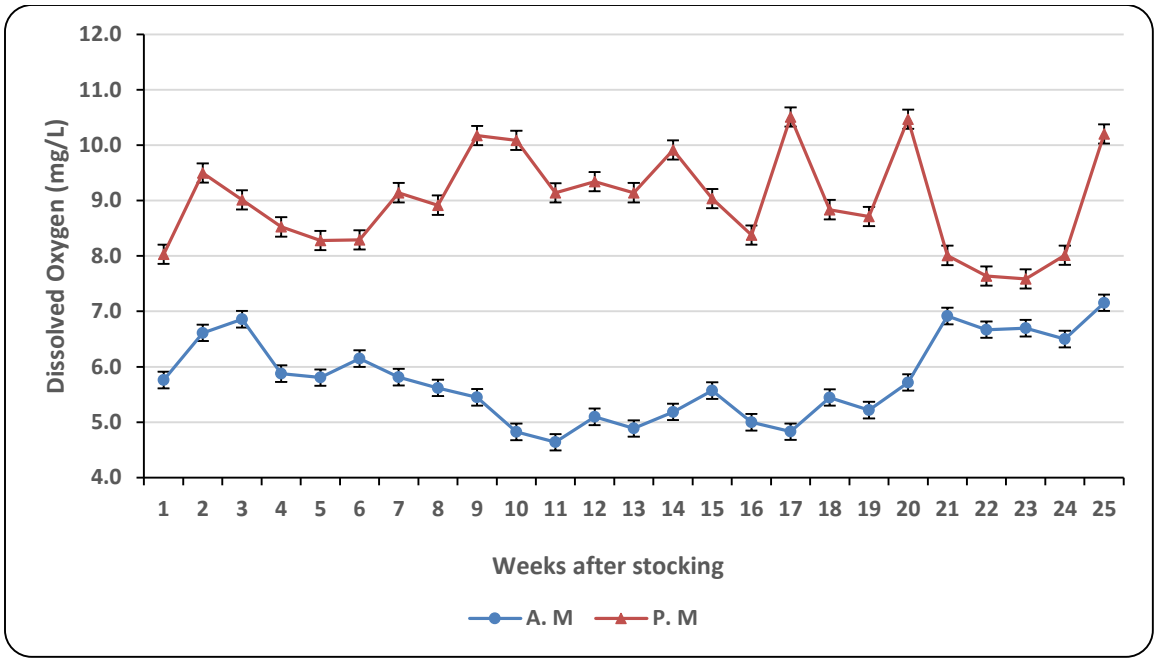

Figure 3. Overall mean weekly variation in morning and afternoon dissolved oxygen in channel catfish ponds during the first production season (May-October 2011). Error bars indicate standard error.

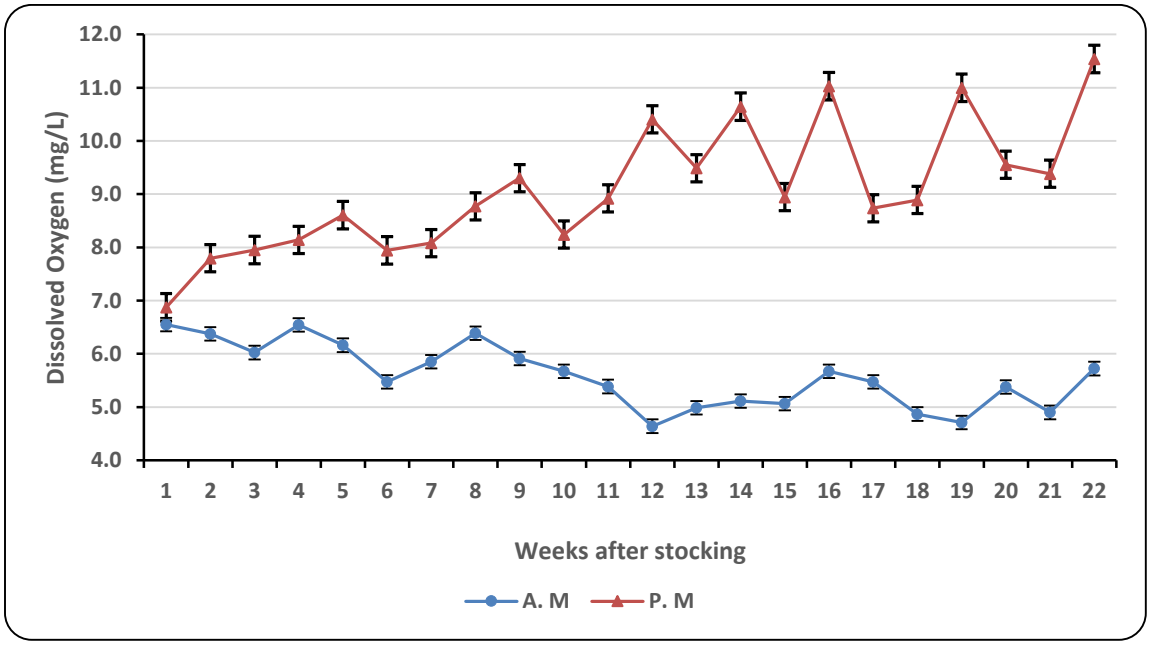

Figure 4. Overall mean weekly variation in morning and afternoon dissolved oxygen in channel catfish ponds during the second production season (April-September 2012). Error bars indicate standard error.

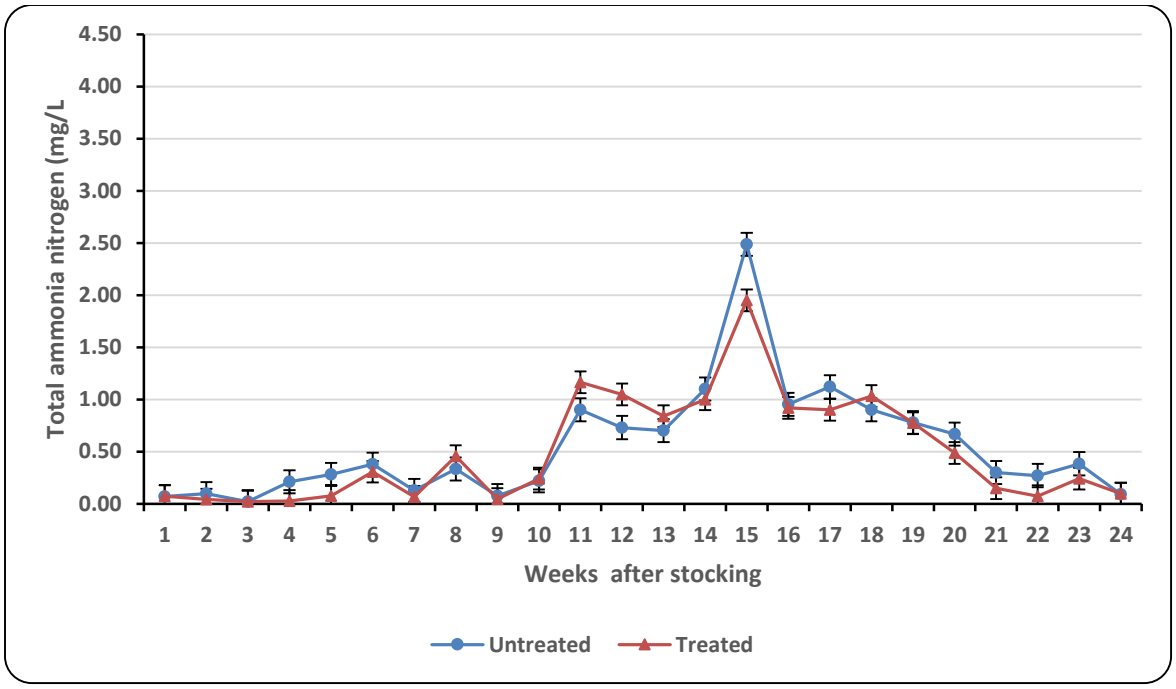

Figure 5. Mean weekly variation in total ammonia nitrogen in PondToss treated and untreated channel catfish ponds during the first production season (May-October 2011). Error bars indicate standard error.

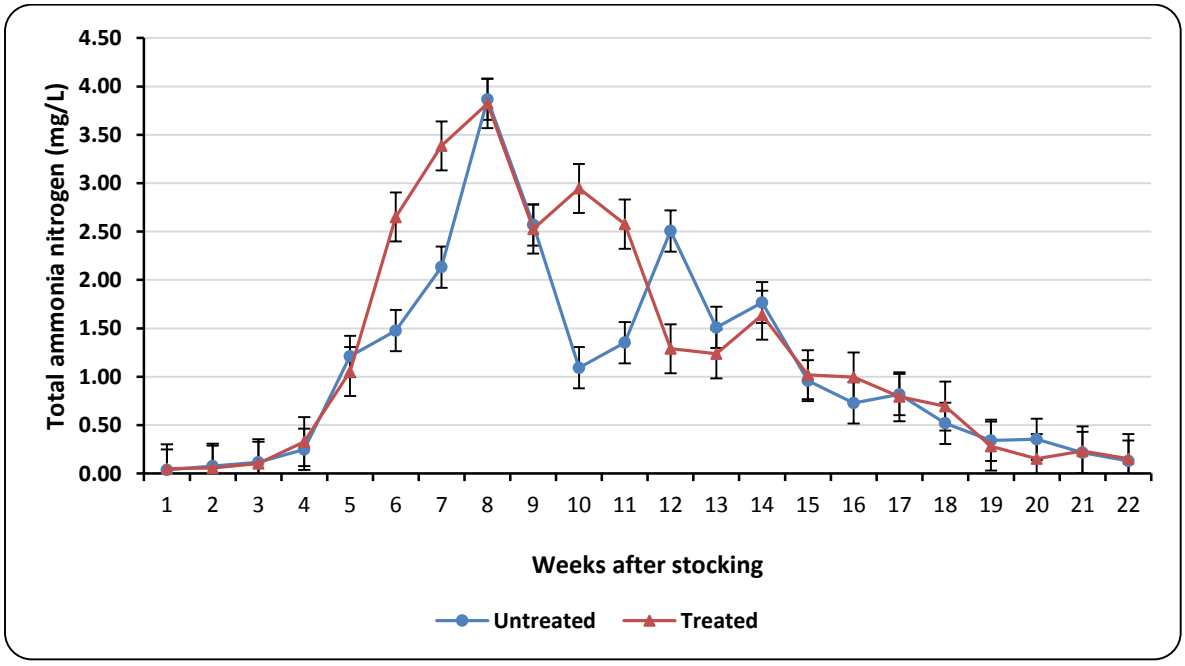

Figure 6. Mean weekly variation in total ammonia nitrogen in PondToss treated and untreated channel catfish ponds during the second production season (April-September 2012). Error bars indicate standard error.

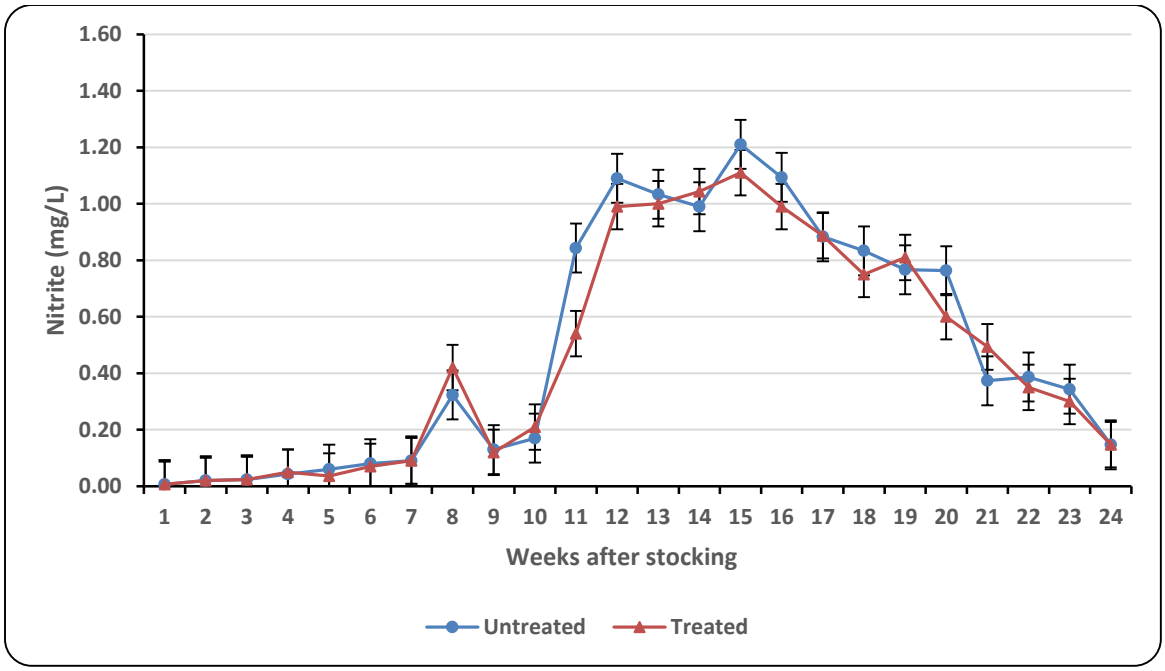

Figure 7. Mean weekly variation in nitrite level in PondToss treated and untreated channel catfish ponds during the first production season (May-October 2011). Error bars indicate standard error.

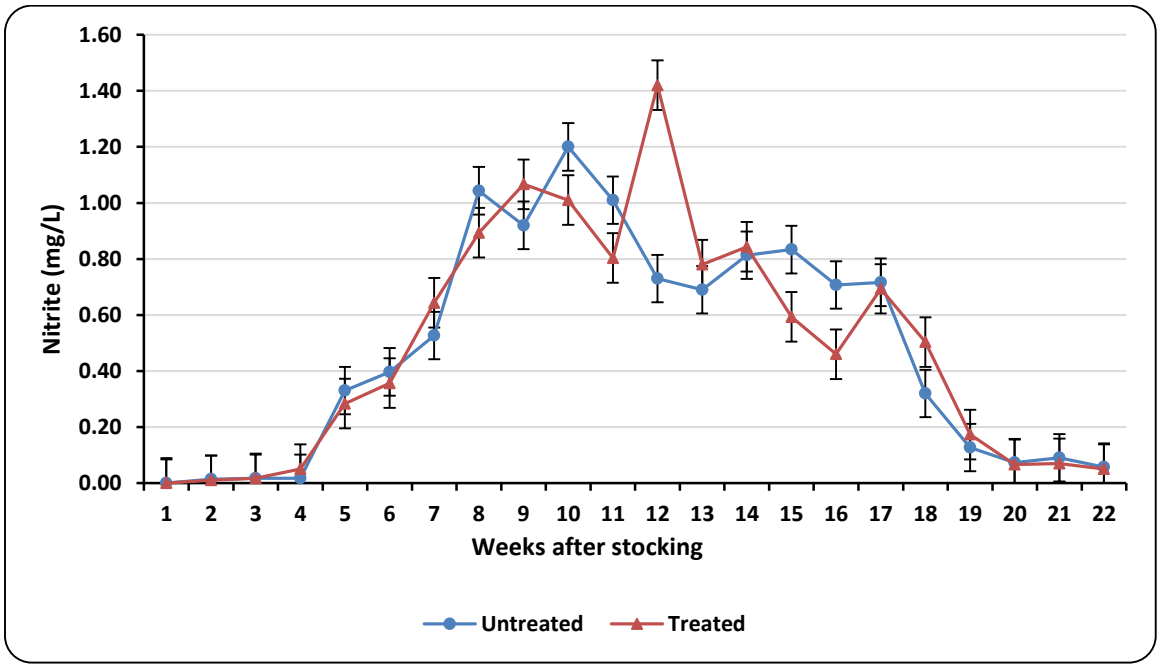

Figure 8. Mean weekly variation in nitrite level in PondToss treated and untreated channel catfish ponds during the second production season (April-September 2012). Error bars indicate standard error.

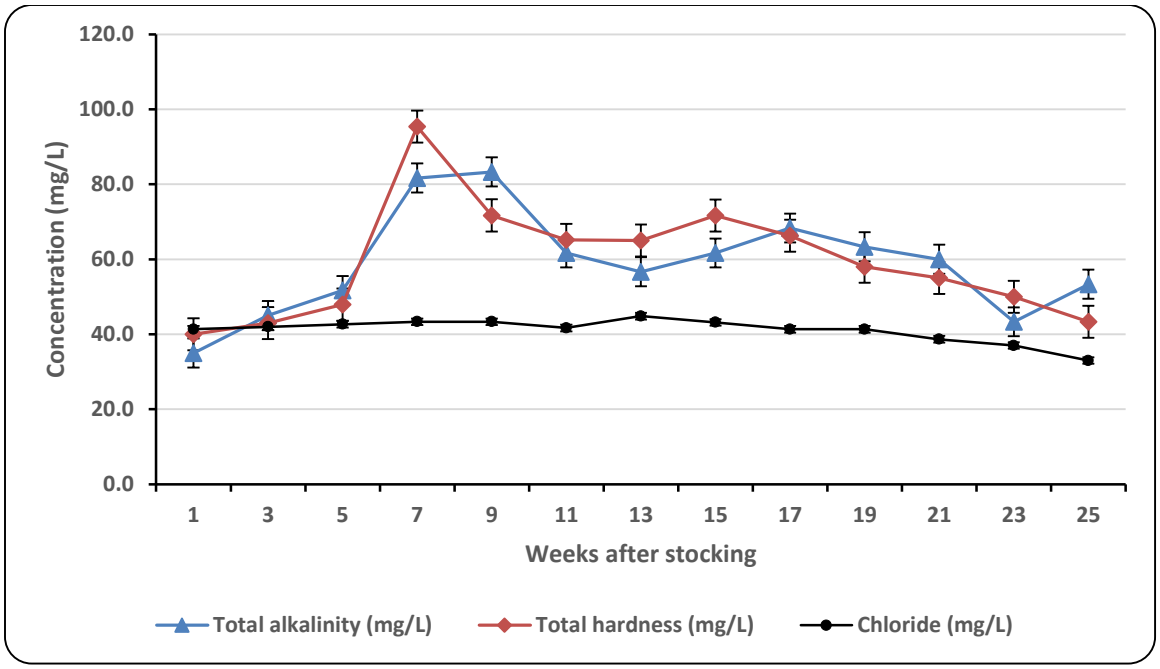

Figure 9. Overall mean bi-weekly variation in total alkalinity, hardness and chloride levels in channel catfish ponds during the first production season (May-October 2011). Error bars indicate standard error.

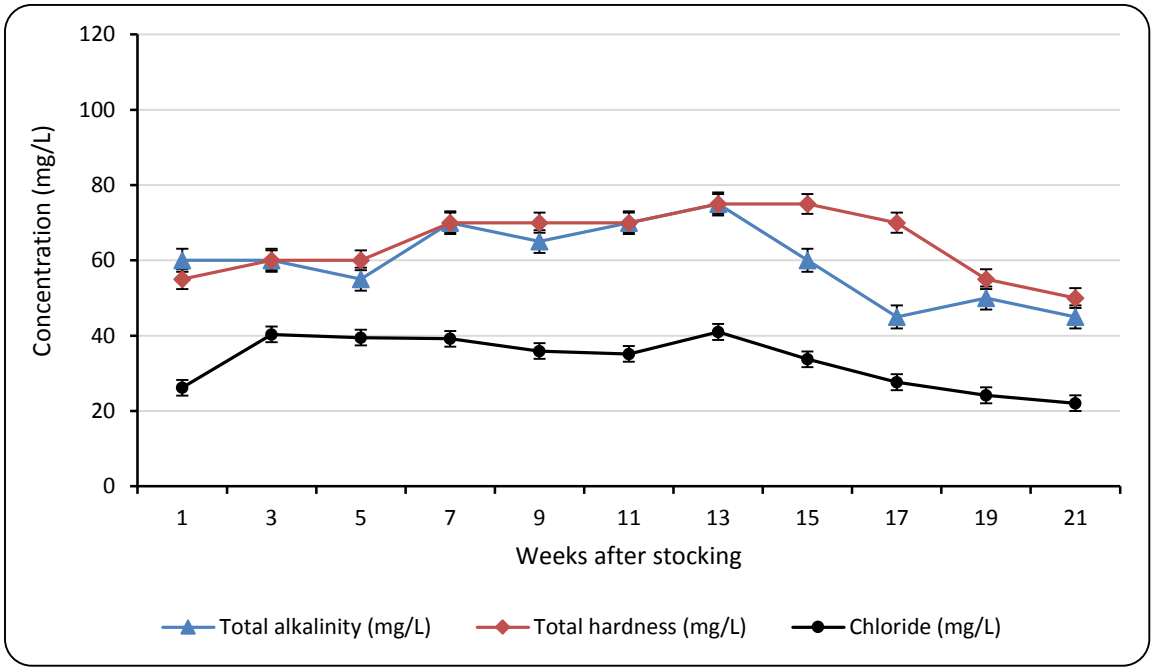

Figure 10. Overall mean bi-weekly variation in total alkalinity, hardness and chloride levels in channel catfish ponds during the second production season (April-September 2012). Error bars indicate standard error.

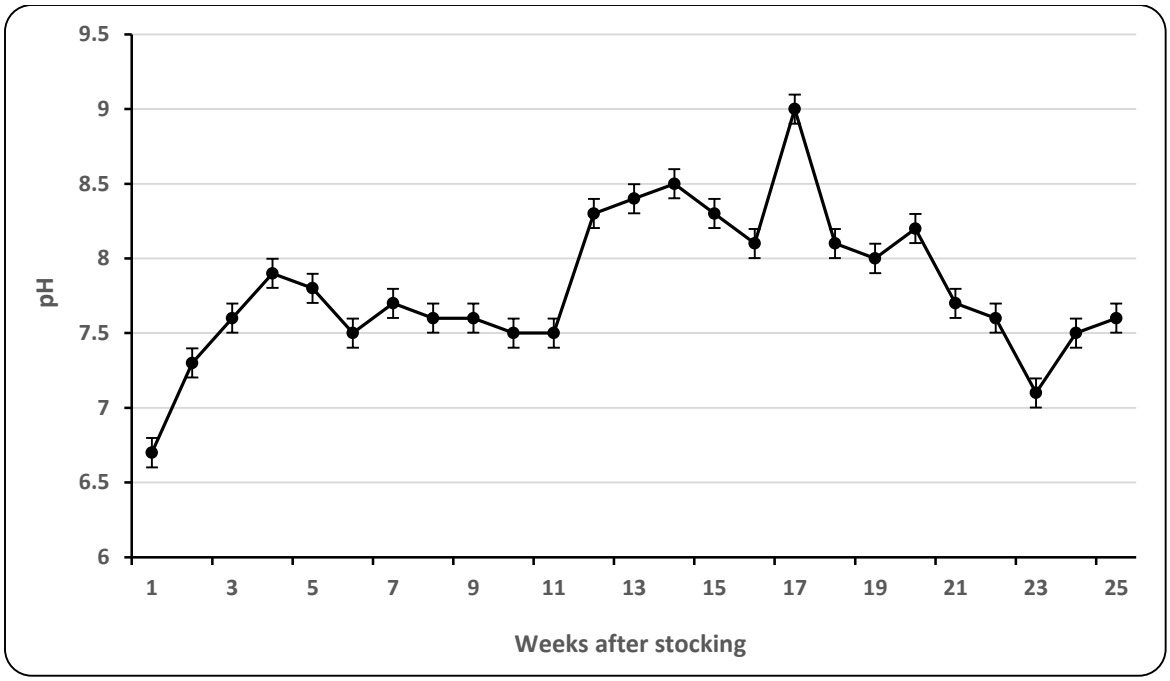

Figure 11. Overall mean weekly variation in afternoon pH in channel catfish ponds during the first season (May-October 2011). Error bars indicate standard error.

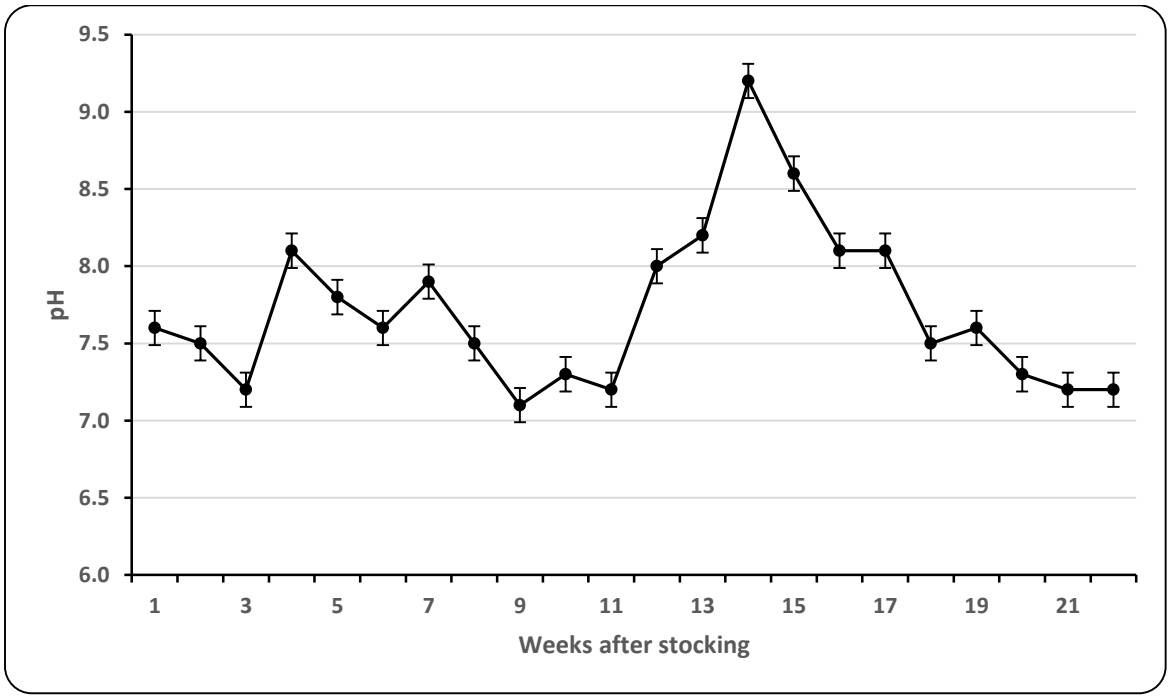

Figure 12. Overall mean weekly variation in afternoon pH in channel catfish ponds during the second season (April-September 2012). Error bars indicate standard error.

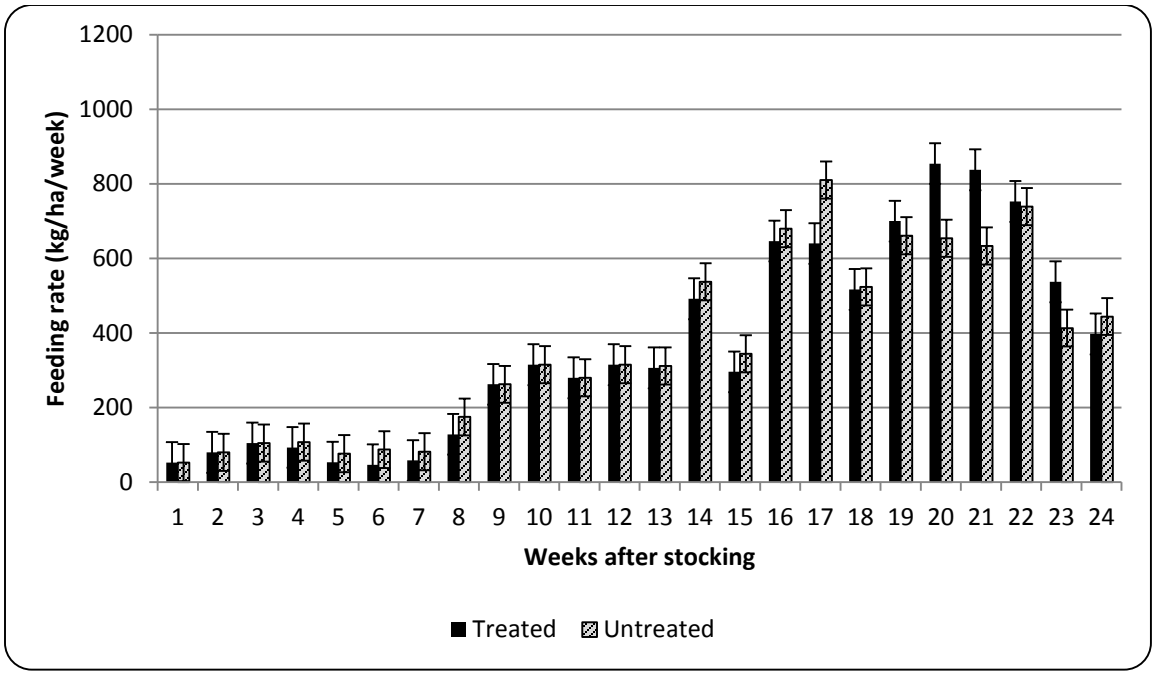

Figure 13. Mean weekly variation in feeding rate in PondToss treated and untreated channel catfish ponds during the first season (May-October 2011). Error bars indicate standard error.

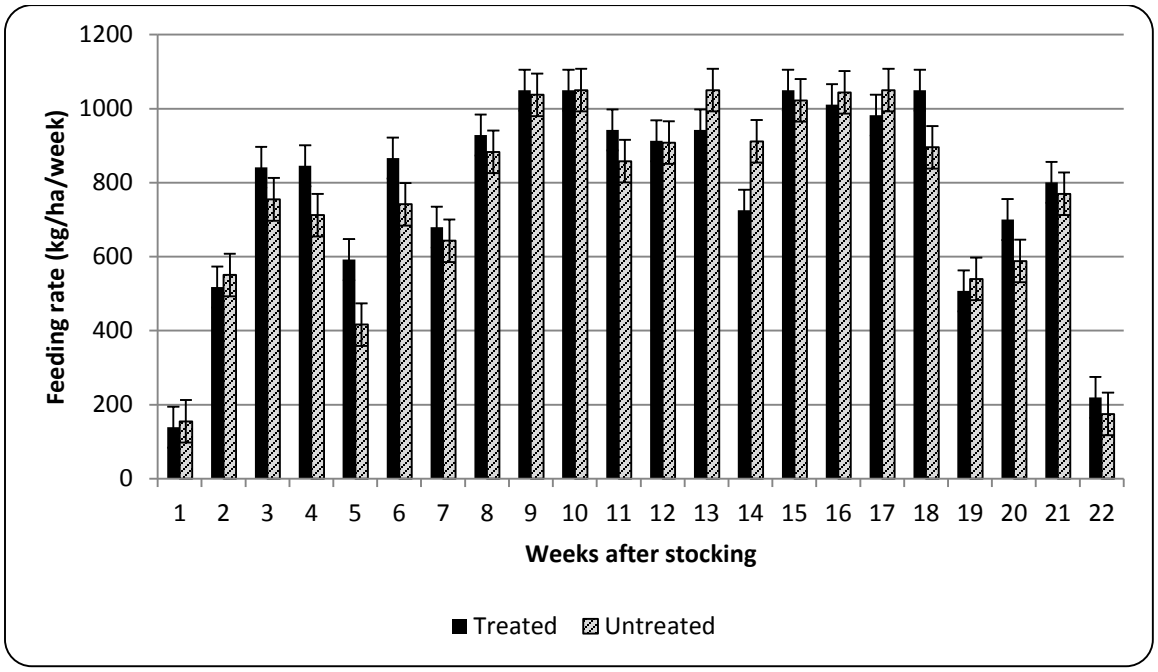

Figure 14. Mean weekly variation in feeding rate in PondToss treated and untreated channel catfish ponds during the second season (April-September 2012). Error bars indicate standard error.

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CHAPTER IV

Long-term feeding effects of two probiotic strains of *Bacillus subtilis* and of the prebiotic Previda® on growth, immune parameters and susceptibility to *Aeromonas hydrophila* infection in Nile tilapia *Oreochromis niloticus*

1. Abstract

The use of prebiotics, probiotics and/or their combinations in aquaculture is now gaining wide acceptance; however, results from a few studies on their efficacy have been conflicting. This study was conducted to evaluate the individual and combined effects of long-term feeding of diets containing two probiotic *Bacillus subtilis* 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 x 10^7 CFU/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 percent weight gain ($p=0.6972$), specific growth rate ($p=0.6205$) or feed conversion ratio ($p=0.8486$) 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 ($p<0.05$). Overall, the diet formulated with *Bacillus subtilis* strain AP193 and Previda® combined showed the lowest mean percent mortality (25.0 ± 15%) with the

highest mean mortality occurring in the control diet (71.0 ± 15%). The combined effect of the prebiotic and probiotic strains showed a significant reduction in mortality from the prebiotic only diet. However, relative to the individual probiotic treatments, the combinations did not appear to have any significant advantage in reducing mortality due to *A. hydrophila* in juvenile Nile tilapia. Mean lysozyme and respiratory burst activities also yielded no significant differences between treatments and control (p=0.14 and 0.32, respectively).

2. Introduction

Tilapias are of increasing importance in aquaculture globally and are only second to carps by volume of production. Among the several species of tilapia cultured commercially, Nile tilapia (*Oreochromis niloticus*) is the most abundant and important species. Global aquaculture production of this species has increased from around 200,000 metric tons in 1990 to about 2.8 million metric tons in 2010 (FAO 2012). The current trend in tilapia aquaculture development is towards increased intensification and commercialization (Goncalves et al. 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 and Austin 2007). Bacterial infectious diseases 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.

A. hydrophila causes motile aeromonad septicemia (MAS) and has also been associated with a number of other diseases of Nile tilapia, such as epizootic ulcerative

septicemia (EUS), as a secondary pathogen. Considered usually as a secondary pathogen, *A. hydrophila* could also become a primary pathogen, causing disease outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry (Fang et al. 2004). In recent years in West Alabama, U.S.A, a MAS disease outbreak caused by a more virulent strain of *A. hydrophila* (ML-09-119) resulted in an estimated loss of more than 1.4 million kg of food size channel catfish (Pridgeon and Klesius 2011).

Anabolic steroids, growth promoters and some antibiotics, such as oxytetracycline (OTC), sulfadimethozine and ormetoprim among others, are commonly administered in feed to improve growth performance and to control the outbreak of diseases in aquaculture (Defoirdt et al. 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. Further, accumulation of antibiotics both in the environment and in fish can pose potential risk to consumers and the environment (Carrias et al. 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. Consequently, 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 and Roberfroid 1995; Mei et al. 2011). The inclusion of common probiotic strains, such as *Bacillus* sp., in fish feed can also help build up beneficial bacterial flora on the skin and intestine to out-compete pathogenic bacteria (El-Rhman et al. 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 gastro-intestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of probiotic bacteria and improve the host welfare. Thus, an effective pairing of pre- and probiotics would allow alteration of the gut environment by the former for optimal growth and function of the latter. Although several studies have indicated that pre- and/or probiotics and their combinations can improve growth performance and feed utilization of various fish species including Nile tilapia (Mahious et al. 2006; Staykov et al. 2007; Torrecillas et al. 2007; Burr et al. 2008; Grisdale-Helland et al. 2008), enhance their non-specific immune responses and resistance to bacterial infections (Li and Gatlin 2005; Buentello et al. 2010), improve gut function and health by improving the ultrastructure of the intestine mucosa (Salze et al. 2008) and also activate health promoting bacteria in the intestine (Zhou et al. 2007), there has been reportedly conflicting results of their effectiveness for use in fish culture (Shelby et al. 2006; Merrifield et al. 2010).

This study was conducted to: (1) explore the individual and combined effects of feeding diets containing prebiotic and probiotics on growth performance of the Nile tilapia,

and (2) investigate the potential effects of pre-feeding of these diets on the survival of juvenile Nile tilapia when challenged with *A. hydrophila*.

3. Materials and Methods

3.1. Diet preparation

Two proprietary probiotic strains of *Bacillus subtilis* and Previda®, a commercial hemicellulose extract prebiotic product (Novus International Inc., St Charles, MO, USA), were added in single and in combination as additives to a basal diet. The first probiotic strain Aqua NZ Blend is a dry concentrate containing *Bacillus subtilis* (provided for testing by Novus International Inc.) and the second strain is a bacterial suspension (AP193) containing *Bacillus subtilis* proprietary of Auburn University, Auburn, AL, USA. The basal 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 probiotics. Both probiotic strains were added to their respective diets at the same 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 of feed. Diet 1 was the basal/control diet (no additives) while diet 2 was the prebiotic Previda® only, supplemented at 0.5% of the total diet (30.0 g). Diet 3 contained the proprietary strain of *Bacillus subtilis* Aqua NZ while diet 4 contained a mixture of the strain of *Bacillus subtilis* Aqua NZ and the prebiotic Previda®. Diet 5 contained the probiotic strain of *Bacillus subtilis* AP193 and diet 6, a mixture of the probiotic strain of *Bacillus subtilis* AP193 and the prebiotic 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 minutes. 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 to a moisture content of 8-10%, bagged, labeled and stored at 4^{o}C until feeding. In all, three batches of diets (6.0 kg/batch/diet) were prepared.

3.2. *Bacillus* quantification in experimental diets

Samples of the diets were analyzed to quantify the number of *Bacillus* probiotic bacteria present in a gram of feed. One gram of each diet was placed in a 15-ml tube containing 9 ml of phosphate buffered saline (PBS). Samples were left undisturbed for 30 minutes 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 TSA plates and incubated at 30°C overnight. After overnight incubation, colonies on plates with typical morphology characteristics of *Bacillus subtilis* Aqua NZ and *Bacillus subtilis* AP193 were counted.

3.3. Growth trial

The growth trial was conducted at the E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA, with *O. niloticus* fed the formulated diets (Diets 1 to 6) over a period of 8 weeks. Fingerlings of average size 7.47 ± 0.11 g were acclimated and then stocked at 40 fish/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/aquarium. Water temperature was maintained at 28^{o}C; however, from week 6-8 when temperatures began to

drop below 26^{o}C, the aquaria were placed on partial flow-through and re-circulation with water heated to 28^{o}C using a submersible heater. During this time, water was sterilized using Aqua Logic UV Sterilizer model ALUV-30, 0.6A (Aqua Logic, San Diego, CA, U.S.A). 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 6 replicate aquaria and fish fed throughout the experiment. Feeding was done twice a day, in the morning and late afternoon at a percent body weight, ranging from 5 to 10%. Feeding rates were adjusted every two weeks. Fish were removed, counted and weighed bi-weekly on a digital scale Ohaus Scout Pro 4000g (Ohaus Corporation, Parsippany, NJ, U.S.A) 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 with a photometer YSI 9100 (YSI Corporation, Yellow Spring, OH, USA). Photoperiod was set at 14 h light and 10 h dark. After 8 weeks, fish were weighed, counted and moved to the S-6 Fish Disease Diagnostics Laboratory at the E. W. Shell Fisheries Center.

3.4. Lysozyme and respiratory burst activity

For the determinations of respiratory burst and lysozyme activities, blood samples were collected from 3 fish per tank in each treatment (n=18 fish/treatment) a day before the disease challenge. Fish were anaesthetized to loss of equilibrium with MS222 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 activities, 50 µL of blood was placed into the wells of 96-well U-shaped 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 a further hour at room temperature. The supernatant was again removed and the cells were fixed with 100% methanol for 3 min and then washed three times with 30% methanol. The plates were then left to air dry 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.

The remaining blood samples after drawing sub-samples for the respiratory burst determination was prepared for the lysozyme activity test in blood serum. Blood serum as supernatant was extracted into sterile 1.7 ml microcentrifuge tubes after centrifuging blood samples stored at 4^{o}C overnight at 3000 x g for 15 min. On a flat-bottomed 96-well microtitre plate, 100 µL of 0.4 mg ml^{-1} suspension of *Micrococcus lysodeikticus* (obtained from Sigma) in 0.05 mol L^{-1} sodium phosphate buffer (SPB, pH 5.2) was added to 100 µL of the serum in serial dilutions of 1⁄5 to 1⁄40. The optical density (OD) reading was taken at 570 nm using a spectrophotometer 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.

3.5. Preparation of *A. hydrophila* for the challenge

A frozen stock (-80^{o}C) of *A. hydrophila* strain ML 09-119 was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. The isolate was tested on tilapia prior to the challenge to confirm virulence. *A. hydrophila* used for the challenge was prepared by inoculating 5 ml TSA broth with 200 µl of a frozen stock (–80 ^{o}C) of the bacteria. The 5 ml culture was incubated for 24 h at 30^{o}C while shaking at 150 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^{o}C while shaking at 150 rpm. Prior to use in challenges, the bacterial culture was centrifuged at 3600 x *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 of 1. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

3.6. *A. hydrophila* challenge system and conditions

Disease challenge in Nile tilapia (approx. 78 g) was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn, AL, U.S.A. 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. Prior to challenge, fish were acclimated for 1 week during which time a static water system in conjunction with daily 100% water exchange was incorporated. During this time, salinity levels in the tanks were maintained at 1.5‰ and then gradually decreased to 0‰ prior to challenge. Flow through water supply (0.4 L/min) and temperature of 30 ± 1 ^{o}C was maintained during and after *A. hydrophilla* challenge.

3.7. Experimental design and *A. hydrophila* challenge protocol

The challenge experiment maintained the same experimental design used in the 8-week feeding study. Briefly, the experiment included a control treatment (Treatment 1), which was a diet with no amendment, and the following five treatments composed of diets amended as follows: Treatment 2- Prebiotic Previda®, Treatment 3- *Bacillus subtilis* Aqua NZ, Treatment 4 *Bacillus subtilis* Aqua NZ Probiotic plus prebiotic Previda®, Treatment 5- *Bacillus* AP193, Treatment 6- *Bacillus* AP193 plus prebiotic Previda®. Each treatment was composed of six replicate aquaria each stocked with 25 fish obtained from the remaining fish after the 8-week feeding study. The challenge was performed 1 week after the termination of the 8-week feeding trial. Fish were challenged by administering 200 µL of *A. hydrophila* ML 09-119 by intragastric gavage obtaining a final dosage of 3.9×10^7 CFU/fish.

3.8. Feeding and husbandry activities during challenge

During the 1-week acclimation period prior to challenge, tilapia were maintained on their treatment diet as in the growth trial for the previous 8 weeks. During the challenge, feed was offered to fish; however, fish stopped feeding 1 day post-challenge and did not feed while mortalities occurred. Un-eaten feed and waste material 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 daily for behavioral changes and for gross signs of disease. Moribund and dead fish were removed twice daily and counted. Moribund/freshly dead fish, 18 from each treatment 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. At the end of the experiment, all surviving fish were counted, euthanized with 300 mg/L MS-222, and properly disposed.

3.9. Statistical analysis

Data collected were analyzed by one-way analysis of variance using the mixed linear model procedure (SAS 9.2, SAS Institute Inc. Cary, NC). The mixed procedure (Wolfinger et al. 1991) was used to compare differences among treatment means in this study. For the disease challenge experiment, the model used in the analysis included a complete randomized block design to minimize variation due to location of aquarium units in three different banks. Significance was set at 5% level ($p<0.05$).

4. Results

4.1. Bacteria quantification in diets

Bacterial concentrations present in the diets are presented in Table 2. The doses of both *Bacillus* strains present in the amended feed were determined to be very similar, approximately 4.7×10^7 CFU/g 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 of feed.

4.2. Growth parameters

In the growth trial, overall mean water temperature ranged from 27.8±1.2 ^{o}C in the morning to 28.4±0.9 ^{o}C in the afternoon. Dissolved oxygen readings in the morning

averaged 5.47±0.58 mg/l while the average measurement in the afternoon was 5.38±0.59 mg/l. Average total ammonia-nitrogen and nitrite-nitrogen were 0.27±0.22 mg/l and 0.12±0.04 mg/l, respectively (Table 3).

No fish mortalities, behavioral abnormalities, and external or internal abnormal gross signs were observed during the 8-week growth trial suggesting the safety of the prebiotic and the two probiotic strains as feed additives. 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, specific growth rate, 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. The prebiotic, however, emerged as the diet with the lowest mean FCR of 1.22.

4.3. Lysozyme and respiratory burst activity

Pre- and probiotic treatments did not significantly influence mean serum lysozyme activity compared with the control ($p = 0.14$, Table 6). 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 Aqua NZ probiotic. Respiratory burst activity, which is an important innate defense mechanism of fish, also did not change significantly between treatments and control ($p=0.32$). This activity ranged from 0.059 ± 0.006 to 0.062 ± 0.004 at OD_{540}.

4.4. *Aeromonas hydrophila* challenge

In the challenge experiment, mean water temperature was 30 ± 1°C while dissolved oxygen levels were maintained at 5.0 ± 0.5 mg/L across all treatments. Mean percent

mortalities of treated and control Nile tilapia fingerlings challenged with *A. hydrophila* are presented in Table 5. With the exception of the prebiotic diet, which did not differ significantly from the control diet ($p=0.17$), all other treatment groups showed significantly lower fish mortality compared to the control group ($p<0.05$). Overall, diet formulated with *Bacillus subtilis* strain AP193 and Previda® combined had the lowest mean percent mortality (25 ± 15%) with the highest mean percent mortality (71 ± 15%) occurring in fish fed the control diet. Specific comparison between the two probiotic strains used in this study: Aqua NZ and AP193, *Bacillus subtilis* strains indicated no significant difference ($p=0.17$). 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 against *A. hydrophila* infection in Nile tilapia.

5. Discussion

In aquaculture, probiotics can be applied either as feed additives or as additives to the water (Moriarty 1998; Taoka et al. 2006). The form and duration of prebiotic and probiotic administration can influence their effectiveness in affecting fish health (Welker and Lim 2011). The supplementation of pre- and probiotics through feed has been documented as a better method of ensuring the efficiency of the probiotic bacteria in the gastro-intestinal tract of fish. However, their use in commercial fish feed production is still uncommon.

In the current work, the prebiotic, probiotic strains, and/or their combinations were formulated with the feed and fed to Nile tilapia for 8 weeks prior to the challenge with *A. hydrophila*. The results indicate their safety for use as feed additives. The concentration of

probiotic strains obtained in the amended feed (4.7 x 10^7 CFU/g of feed) was not significantly different from the theoretical targeted dose (4.2 x 10^7 CFU/g of feed) suggesting that the process of feed preparation and storage did not negatively affect the viability of the bacteria while their similar concentrations in all 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*. Nonetheless, under the conditions of the growth trial, none of the diets significantly improved growth of the fish ($p=0.69$) 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 et al. 2006; Grimoud et al. 2010). Results from an 8-week feeding trial conducted by Zhou et al. (2010a) 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®. 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 et al. (2007) with hybrid tilapia (*Oreochromis niloticus* x *O. 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 inclusion level of the prebiotic in the diets in this study from

the 0.5% level might have improved growth performance; however, only testing different levels would verify any growth benefits. The effects of different levels of the prebiotic Immunogen® (0, 0.5, 1, 1.5 and 2.5 g prebiotic/kg diet) fed to common carp fingerlings by Ebrahimi et al. (2011) in an 8-week feeding trial did not show 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 et al. (2010) reported improved growth performance of Nile tilapia fed diets with *Bacillus subtilis*, *Lactobacillus plantarum*, a mixture of *B. subtillis* and *L. plantarum*, and *Streptococcus cerevisiae*. Aly et al. (2008) compared the potential effects of two doses of *B. 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 and corroborate findings of this study (Shelby et al. 2006; Marzouk et al. 2008; El-Rhman et al. 2009; Ferguson et al. 2010).

Although the study did not show any significant treatment effects with respect to growth performance, there was enough evidence to conclude that the probiotic strains and their combination with the prebiotic had a significant effect on resistance to *A. hydrophila* infection. Results from the combined effects of the pre- and probiotic strains showed a significant reduction in mortality compared to the prebiotic only and control diets, which

indicate the relevance of their pairing. 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 et al. 2009). When the Japanese flounder was fed a diet containing *B. clausii* or in combination with the prebiotics fructo- or mannanoligosaccharides, there was an improvement of the non-specific immune function (Ye et al. 2011). Although the diet containing either of the prebiotics with *B. clausii* exhibited the highest immune function, activity was not significantly different compared to flounder fed only *B. clausii*. Prebiotics are known to modify the microbial community within the gastrointestinal tract to boost non-specific immune responses. The microflora 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 inhibit the growth of putrefactive and pathogenic bacteria present in the colon (Bailey et al. 1991; Mussatto and Mancilha 2007; Yousefian and Amiri 2009; Mei et al. 2011). Thus, the prebiotic used in this study probably created an ideal condition for proliferation of the probiotic strains, which produced substances that stimulated the immune system to enhance the host's protection against *A. hydrophila* 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 treatments 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 defense mechanisms of fish (Irianto and Austin 2003; Nayak et al. 2007; Sharifuzzaman

and Austin 2009), other researchers have identified specific probiotics like *B. subtilis* and some members of LAB group to significantly stimulate respiratory burst activity in fish (Nikoskelainen et al. 2003; Salinas et al. 2005; Salinas et al. 2006; Zhou et al. 2010b). Dietary supplementation of probiotics like *Lactobacillus sakei* in *Salmo trutta* (Balcazar et al. 2007a), *L. sakei, L. lactis* ssp. *lactis*, *L. mesenteroides*, and *L. rhamnosus* in *Oncorhynchus mykiss* (Panigrahi et al. 2004; Panigrahi et al. 2005; Balcazar et al. 2007b); *A. sobria* in *O. mykiss* (Brunt et al. 2007) as well as water supplementation of *B. coagulans*, *B. subtilis* and *Rhodopseudomonas palustris* and *Enterococcus faecium* in *O. niloticus* (Pieters et al. 2008; Wang et al. 2008; Zhou et al. 2010b) failed to elevate lysozyme level.

It has also been suggested that variations in research 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 and Lim 2011). Merrifield et al. (2010) noted that the success or potential of probiotics in many studies to prevent disease may be greater than the results showed due to the use of intragastric and/or intraperitoneal (IP) method of disease challenge. Other factors, such as environmental conditions, handling practices, and stocking densities, may also affect 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.

6. Conclusions

Under the conditions of the current study, Previda®, Aqua NZ *Bacillus subtilis* strain, and AP193 *Bacillus subtilis* strain and their combination did not improve growth performance in juvenile Nile tilapia fed diets formulated with these products. However, varying the conditions of the current study, such as concentration/dosage of the pre- and probiotics in the diets in a similar study with the same fish species might be worth considering. The probiotics administered individually and in combination with Previda® proved significant in reducing *A. hydrophila* infection in juvenile Nile tilapia and might have significance when applied to other bacterial diseases of the Nile tilapia and/or other fish species of aquaculture importance. These products probably have a greater potential to prevent *A. hydrophila* outbreak in juvenile Nile tilapia than depicted by the results obtained in this study since the intragastric method of infection employed might be harsher than what could pertain in the culture and natural environment.

Table 1. Composition (g/100g as is) of test diets designed to contain 32% protein and 6% lipid for Nile tilapia.

Ingredients	Diet					
	1	2	3	4	5	6
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
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} using 25%	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
Corn gluten meal	4.65	4.65	4.65	4.65	4.65	4.65
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

Table 2. Mean concentrations of *Bacillus*-like colonies recovered from prebiotic and probiotic-supplemented diets. Values are means of four replicates and values with same letters are not significantly different (p =0.40).

Treatment	Mean (CFU/g)
Diet 1	0
Diet 2	0
Diet 3	4.75×10^{7a}
Diet 4	4.75×10^{7a}
Diet 5	5.5×10^{7a}
Diet 6	4.5×10^{7a}

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 (± S.D)
Water temperature (A.M) ^{o}C	27.8±1.2
Water temperature (P.M) ^{o}C	28.4±0.9
Dissolved oxygen (A. M) mg/L	5.47±0.58
Dissolved oxygen (P.M) mg/L	5.38±0.59
Total ammonia nitrogen (mg/L)	0.27±0.22
Nitrite nitrogen (mg/L)	0.12±0.04

Table 4. Prebiotic, probiotic and combined effects of formulated diets on the growth of juvenile *O. niloticus* under laboratory conditions. Values are means ± s. d. of six replicates and values within the same row with same letters are not significantly different (p>0.05).

Growth	Diets					
Parameter	1	2	3	4	5	6
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
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
%WG	602.0±57.4[a]	628.9±41.5[a]	603.8±50.4[a]	592.9±32.5[a]	570.9±38.2[a]	590.0±46.3[a]
SGR	3.71±0.13 [a]	3.77±0.09 [a]	3.72±0.11[a]	3.70±0.07[a]	3.65±0.09[a]	3.69±0.11[a]
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
FCR	1.25±0.08[a]	1.22±0.02[a]	1.25±0.09[a]	1.26±0.06[a]	1.24±0.06[a]	1.25±0.07[a]

IBW (g $fish^{-1}$), initial mean body weight
FBW (g $fish^{-1}$), final mean body weight.
%WG (percent weight gain) =100 x (final weight-initial weight)/initial weight.
SGR (specific growth rate) (%day^{-1}) = 100 x [(ln final weight-ln initial weight)/days]
FI (feed intake) = (g feed $fish^{-1}$ in 56 days)
FCR (feed conversion ratio) = feed intake / (FBW-IBW).

Table 5. Percent mortality of treatment groups that received feed amended with probiotics, prebiotic, or a mixture of both, and were challenged with *A. hydrophila*. Values are means ± s.d. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column.

Treatment	Mortality (%)
1(Control)	71 ± 15^{a}
2 (Prebiotic Previda®)	54 ± 18^{ab}
3 (Aqua NZ Probiotic)	46 ± 16^{bc}
4 (Aqua NZ Probiotic +Prebiotic Previda®)	29 ± 18^{c}
5 (AP193)	27 ± 9^{c}
6 (AP193 + Prebiotic Previda®)	25 ± 15^{c}

Pooled SEM = 9.7
P-value = <.0001

Table 6. Immune response (means ± s.d) of Nile tilapia fed different experimental diets with or without pre- or probiotics for 8 weeks. Means in the same column with a common letter were not significantly different ($p>0.05$)

Treatment	Lysozyme (ml^{-1})	Respiratory burst (OD_{540})
1 (Control)	620 ± 98.3^{a}	0.059 ± 0.006^{a}
2 (Prebiotic Previda)	590 ± 92.5^{a}	0.059 ± 0.005^{a}
3 (Aqua NZ Probiotic)	675 ± 92.9^{a}	0.061 ± 0.001^{a}
4 (Aqua NZ Probiotic +Prebiotic Previda)	598 ± 78.0^{a}	0.059 ± 0.003^{a}
5 (AP193)	591 ± 85.3^{a}	0.062 ± 0.004^{a}
6 (AP193 + Prebiotic Previda)	622 ± 118.3^{a}	0.062 ± 0.004^{a}

Lysozyme p value = 0.14
Respiratory burst p value = 0.32

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CHAPTER V

Short-term feeding effects of probiotic strains and their combination on growth, immune parameters and *Streptococcus iniae* susceptibility in Nile tilapia *Oreochromis niloticus*

1. Abstract

Streptococcal infections represent a major health and economic problem in fish species worldwide. Particularly, *Streptococcus iniae* has emerged as a significant finfish pathogen responsible for annual losses in aquaculture exceeding US$100 million. An alternative to control pathogenic bacteria is the use of probiotics, which when included as single or mixed in the diet of fish have become preferential to antibiotic therapy. The current study evaluated short-term (3 weeks) probiotic effects of practical 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 probiotic strains either individually or in combination (at a target concentration of approximately 4 x 10^7 CFU/g of feed), and a basal control diet with no additives for 3 weeks. The diets were: control (diet 1), and probiotic bacteria strains SB3086 (diet 2), SB3295 (diet 3), SB3615 (diet 4), SB3086 + SB3615 (diet 5) and AP193 (diet 6). The diets appropriately corresponded to treatments 1 to 6. After the 3-week growth period, no significant improvement in growth performance was observed under the current experimental conditions. Results from serum bactericidal activity showed a significant difference of all treatments from the control (p=0.0002), except treatment 3 (p=0.9020).

Lysozyme activity was also significantly higher in fish fed probiotic diets than those fed control diet (p=0.0001). Fish were challenged with *S. iniae* by intraperitoneal (IP) injection at a dosage of 8 x 10^6 CFU/fish. Results from the challenge showed significantly lower mortalities in all treatments as compared to the control ($P \leq 0.0170$). Overall, treatment 4 showed the lowest percent mortality (44.0 ± 7.2%) and the highest mortality occurred in treatment 1 (77.3 ± 7.0%). The synergistic effect of SB3086 and SB3615 (treatment 5) did not indicate any significant advantage in reducing mortality due to *S. iniae* infection in juvenile Nile tilapia as compared with the individual probiotic treatments in this study.

2. Introduction

Disease outbreak has 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 United States dollars 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 aetiological agent of streptococcosis in cultured finfish. It has gained recognition as the most 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), estimated economic impact of *S. iniae* outbreak 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 immuno-compromised humans (Weinstein 1997; Koh et al. 2004; Facklam et al. 2005; Lau et al. 2003; 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 growth and health of the host (Salminen et al. 1999; Lara-Flores et al. 2010). Due to 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 have become preferential to antibiotic therapy. 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, b; Marzouk et al. 2008; El-Rhman et al. 2009; Essa et al. 2010; Ferguson et al. 2010; Ghazala et al. 2010; Zhou et al. 2010; Pirarat et al. 2011). This makes it imperative for more studies to be carried out to ascertain specific probiotic strains and/or their combinations that can significantly control infections due to specific pathogenic bacteria in *O. niloticus*.

The present work examined short-term feeding effects of probiotic strains *Bacillus subtilis* individually and mixed on growth performance, non-specific immune activity and *Streptococcus iniae* susceptibility in juvenile *O. niloticus*.

3. Materials and methods

3.1. Diet preparation

Four proprietary probiotic strains of *Bacillus subtilis* and one combination were added as feed additives to a basal diet. Three probiotic strains (SB3086, SB3295 and SB3615) and a mixture of SB3086 and SB3615 were dry concentrates containing the probiotic strains blended with calcium carbonate (provided for testing by Novus International). The fourth strain was a bacterial spore suspension (AP193) containing *B. subtilis* proprietary of Auburn University, Auburn, AL, USA. The basal diet was formulated to meet the nutritional requirement of tilapia containing 32% protein and 6% lipid. The diets contained 3.3% menhaden fish oil to ensure palatability of the diets due to the addition of the probiotics. All the probiotic strains were added to their respective diets at 0.2% inclusion levels. The basal/control diet had no probiotic additives (Table 1). Test diets were prepared at the fish nutrition laboratory at the E. W. Shell Fisheries Center, Auburn University. Pre-ground dry ingredients and fish oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 minutes. 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^{o}C until feeding. Only one batch of diets (6.0 kg/diet) was prepared.

3.2. Bacteria quantification in experimental diets

Samples of the diets (n=4, 1 g of feed plus 9 ml PBS each) were analyzed to quantify the number of viable *Bacillus* cells present in a gram of feed. Samples were left undisturbed for 30 minutes and then homogenized. Ten-fold serial dilutions were made from each replicate sample and 100 µl of dilutions, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were spread on TSA plates, and incubated at 30°C overnight. After overnight incubation, colonies on plates with typical morphology characteristics of each of the *Bacillus* strains were quantified.

3.3. Serum bactericidal and lysozyme activity

Blood samples were collected from 3 fish per tank in each treatment (n=18 fish/treatment) after 3 weeks of feeding fish with the experimental diets and before the challenge. Fish were anaesthetized to loss of equilibrium with MS222 and blood samples 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^{o}C overnight. Blood serum was pipetted into sterile 1.7-ml microcentrifuge tubes from the blood samples as supernatant after centrifuging at 3000 x g for 15 min at 4^{o}C. Serum samples were stored at -80^{o}C and after 1 week of storage, samples were taken out and thawed for serum bactericidal and lysozyme activities determination.

In the serum bactericidal activity procedure, bacterial cultures of *A. hydrophila* were centrifuged and the pellet was washed and re-suspended in PBS. The optical density of the suspension was adjusted to 0.5 at 546 nm. The bacterial suspension was serially

diluted (1:10) with PBS 5 times. The serum bactericidal activity was determined by incubating 2 µl of the diluted bacterial suspension with 20 µl of the serum for 1 hr. at 37 ^{o}C. A control in which PBS replaced the serum was included. The numbers of viable bacteria was determined by counting the colonies after culturing on TSA plates for 24 h at 30 ^{o}C.

Lysozyme activity of serum was measured using the turbidity assay. On a flat-bottomed 96-well microtitre plate, 200 µl of 0.2 mg ml^{-1} suspension of *Micrococcus lysodeikticus* in sodium phosphate buffer (0.05 mol L^{-1}, 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 min. Chicken egg lysozyme (Sigma) was used as a standard.

3.4. Preparation of *S. iniae* for the challenge

Streptococcus iniae challenge strain was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. The bacteria isolate used was previously passed through tilapia to confirm virulence. Bacteria culture for *S. iniae* challenge was prepared by inoculating 5 ml tryptic soy broth (TSB) with 200 µl of a frozen stock (–80 ^{o}C) of the bacterium. The 5 ml culture was incubated for 36 h at 30 ^{o}C while shaking at 150 rpm, and then used to inoculate 100 ml of fresh TSB. The second inoculated culture was then incubated for an additional 15 h at 30 ^{o}C while shaking at 150 rpm. Prior to its use for the challenge, the bacterial culture was centrifuged at 3600 x *g* for 30 min, re-suspended in 100 ml of fresh TSB, allowed to grow an additional 3 h, and then standardized

to an OD of 1. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

3.5. *S. iniae* challenge system and conditions

S. iniae challenge was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn University. Fish were kept in 60-L aquaria containing approximately 45 L of well water. Each aquarium was equipped with aeration maintained at dissolved oxygen (DO) levels of 5.00 ± 0.5 mg/L. Prior to administering probiotic diets, fish were acclimated for 1 week at 28 ± 1^{o}C. During this time, salinity levels in the tanks were maintained at 1.5 ‰ and then gradually decreased to 0 ‰. During the 3 weeks administration of probiotics, fish were maintained in a static system with incorporated daily 100% water exchanges. During challenge, flow-through water supply (0.4 L/min) and temperature of 28 ± 1^{o}C were maintained.

3.6. Experimental design and *S. iniae* challenge protocol

The experimental design included a control treatment (Treatment 1), which was a basal diet with no amendment and the following five treatments composed of diets amended as follows: Treatment 2- SB3086, Treatment 3- SB3295, Treatment 4- SB3615, Treatment 5- SB3086+SB3615 mixture and Treatment 6- *Bacillus* strain AP193.

Each treatment was composed of six replicate aquaria each stocked with 28 fish. Aquaria were located in three different aquaria banks using a randomized block design (two replicate treatment tanks per bank). The challenge was performed 3 weeks after initial

treatment diet administration and on 25 fish/tank after the removal of 3 fish per tank for use in immunological analysis. Fish were challenged by administering 200 µL of *S iniae* suspension in PBS by intraperitoneal injection to obtain a final dosage of 8 x 10^6 CFU/fish. 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.

3.7. Feeding and husbandry activities during *S. iniae* challenge

During the 1-week acclimation period prior to feeding with experimental diets, tilapia was maintained on a commercial diet at 3% of their body weight. During the treatment phase, which spanned 3 weeks, feeding with the treatment diets and control diet was adjusted to 6% body weight. Fish were fed twice a day, in the morning and late afternoon. Any un-eaten 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, and disinfected in iodine solution after every use to avoid cross contamination. At the end of the 3-week feeding period, final mean body weight, percent weight gain [100 x (final weight-initial weight)/initial weight, specific growth rate [100 x (ln final weight-ln initial weight)/days] and feed conversion ratio (feed intake as fed / weight gain) were determined.

During disease 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/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.

3.8. Statistical analysis

Data collected on growth, immunology and disease challenge were analyzed using SAS (SAS Institute Inc., Cary, NC). 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. Differences between means were considered significant when probability values were less than 0.05.

4. Results

4.1. Experimental diets

The composition (g/100g as is) of all the experimental diets are shown in Table 1. The diets were prepared based on a standard basal diet (Diet 1) to which additives were supplemented consisting of equal concentrations of probiotic strains of *Bacillus*. Bacterial concentrations present in the diets are presented in Table 2. The dose of probiotic strains present in the amended diets were determined to be very similar among all treatments and were within target range of 10^7 CFU/g of feed.

4.2. Growth

During the 3 week period of growth, fish increased in biomass from a mean value of 16.5±0.2 g to 33.7±1.5 g. The mean percents weight gain, specific growth rates 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 phase of the experiment. Additionally, no gross external effects were observed.

4.3. Serum bactericidal and lysozyme activity

Results from immunological parameters are shown in Table 4. Mean serum bactericidal activities were significantly higher in all treatment groups ($p=0.0004$) as compared to the control except treatment 3 ($p= 0.9020$). Statistically, the mixed 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 of the control diet ($p<0.0001$).

4.4. *S. iniae* challenge

The mean percents cumulative mortality of Nile tilapia fingerlings challenged with *S. iniae* are presented in Table 5. They ranged from the lowest value of 44.0 ± 7.2% in treatment 4 to the highest value 77.3 ± 7.0% in the control treatment group. A significant difference by treatment was observed between treatment 1 (control) and all other treatments. In specific comparisons among treatments differences were observed only between treatment 3 and treatments 2, 4 and 5, as well as between treatments 4 and 6 (Table 5). The effect of the combination of strains SB3086 and SB3615 did not result in improved fish survival. The daily mortality pattern is shown in Figure 1. Mortalities began 24 hours post-challenge with higher numbers occurring between days 3 and 7. The experiment was

concluded at 16 days post-challenge when mortalities had ceased for two days in all treatments.

5. Discussion

Short-term (21 days) feeding of probiotic diets individually and mixed to juvenile Nile tilapia did not significantly improve growth in this study. The results may suggest that supplementation of diet with these probiotics probably needs to be done over a longer period of time to obtain a significant improvement in growth or feed conversion, although in an earlier longer-term (56 days) experiment with different strains of probiotic bacteria except for AP193 (which was repeated in this study), the results were not different from the present study. Apun-Molina et al. (2009) observed an apparent tendency towards improved growth in Nile tilapia fry (0.14 g) only after 75 days of feeding with diets composed of *Bacillus* or *Lactobacillus*. Honsheng (2010) attributed improved weight gain and feed efficiency to increased enzyme production due to the inclusion of *B. subtilis* in tilapia diets. Probiotics may improve digestion by stimulating production of digestive enzymes or through other alterations in the gut environment, which according to Ridha and Azad (2012) may require a certain period of time for the supplemented bacteria to colonize and establish in the fish gut in order to enhance the digestive processes by providing various enzymes that help in extracting more nutrients from the food and consequently improve feed utilization and assimilation.

Results on growth performance from this study nonetheless corroborate findings from other studies on probiotics. For instance, non-viable *S. cerevisiae* (Marzouk et al.

2008), *Pseudomonas spp.* (El-Rhman et al. 2009), *Pediococcus acidilactici* and *Enterococcus faecium* (Biomate SF-20®) (Ferguson et al. 2010), *B. subtilis* + *B. licheniformis* (Bioplus 2B®), *P. acidilactici* (Bactocell PA10 MD®) and viable *S. cerevisiae* (Levucell SB 20®) (Shelby et al. 2006) have all been reported as not having any significant effect on growth of tilapia. 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 weight gain of Nile tilapia after 4 or 8 weeks of feeding two doses of *B. pumilus* and the commercial probiotic product Organic Green™ as compared to the control group. According to Lara-Flores et al. (2010), supplementation of *S. faecium* + *L. acidophilus* or *S. cerevisiae* in tilapia diets containing 27% or 40% crude protein produced significantly higher weight gain and feed utilization efficiency compared to the control diet. Improved growth performance of Nile tilapia fed diets with *B. subtilis, L. plantarum*, a mixture of *B. subtillis* and *L. plantarum*, and *S. cerevisiae* have been reported by Essa et al. (2010). The contradicting reports on the effects of probiotics on tilapia may suggest that variations in 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.

Survival of Nile tilapia to *S. iniae* challenge was higher with the probiotic diets than the control. Serum bactericidal activity was higher in fish fed probiotics relative to the control except in fish fed the probiotic strain SB3295. There was also higher lysozyme activity in fish fed the probiotic diets than those fed the control diet. A number of systemic, non-specific immune functions including serum bactericidal activity, lysozyme activity,

respiratory burst, peripheral blood immune cell counts, alternative complement activity, phagocytic ability of leucocytes, and neutrophil migration and adherence can be enhanced by dietary probiotic supplementation (Nayak 2010; Pirarat et al. 2011). Although Ferguson et al. (2010) did not find any changes in the number of leucocytes in the intestinal epithelium, blood leucocyte numbers and serum lysozyme activity were enhanced in Nile tilapia given the probiotic *Pediococcus acidilactici.* In a study to evaluate the use of *Lactobacillus acidophilus* as a biocontrol agent against some common fish pathogenic bacteria including *Streptococcus* sp. in African catfish, *Clarias gariepinus*, Al-Dohail et al. (2011) observed a higher immunological response and concluded, based on the results, that *L. acidophilus* was useful as a probiotic agent in *C. gariepinus* against the pathogenic bacteria. Taoka et al (2006) investigated the effect of live and dead probiotic cells on the non-specific immune system of *O. niloticus* and found that the probiotics treatment enhanced non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of resistance to *Edwardsiella tarda* infection. 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 days with these commercial probiotics did not prevent streptococcal disease infection.

Various mechanisms have been proposed to explain the effects of probiotics. These include competition for adhesion sites on gut or other tissue surfaces, competition for nutrient and energy sources, release of chemical substances that have bactericidal effects on other microbial populations and enhancement of immune response of host. 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 the first probiotic effect since bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection (Verschuere et al. 2000a; La Ragione et al. 2003, 2004). Several studies have attributed a probiotic effect to competition for energy sources (Rico-Mora et al. 1999; Verchuere 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 (Williams and Vickers 1986; Bruno and Montville 1993; Vandenbergh 1993; Pybus et al. 1994; Vine et al. 2004; Servin 2004; Marden et al. 2008; Chaucheyras-Durand et al., 2008; Chaucheyras-Durand and Durand 2010). El-Rhman et al. (2009) noted that probiotic inclusion in fish feed can build up the beneficial bacterial flora in skin and intestine while they grow competitively over pathogenic bacteria. 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 likely that the positive results reported in the present work may be due to a combination of all or some of these mechanisms.

Merrifield et al. (2010) noted that the success of probiotics in many studies to prevent disease may be greater than the results showed due to the use of intraperitoneal (IP) 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 done by IP injection, especially with *Streptococcus*, which is difficult to reproduce reliably by bath immersion. In the current study, the challenge was done by IP, which does not reflect the mode of infection by *S. iniae* in the real culture environment.

6. Conclusions

Short-term feeding (21 days) of the probiotics used in this study, *Bacillus subtilis* strains SB3086, SB3295, SB3615, SB3086+SB3615 and AP193 did not improve growth of juvenile *O. niloticus* under the current conditions. The findings, however, demonstrated the potential and beneficial effect of in-feed supplementation of these probiotics in improving immune response and survival due to *Streptococcus 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. In addition, the degree of probiotic effects was different with the different probiotic strains, indicating that the best strains could be selected for further development and application in the real world situation for the ultimate benefit of the tilapia industry. Further studies to assess the effects of longer-term application of these probiotics on growth and survival to *S. iniae* and other important tilapia bacterial diseases, such as *A. hydrophila*

challenges, as well as their assessment in other cultured fish species prone to *S. iniae* infection are recommended.

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

Ingredients	1 Basal	2 SB3086	3 SB3295	4 SB3615	5 SB3086 + SB3615	6 AP193
Fishmeal	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal solvent extracted	46.50	46.50	46.50	46.50	46.50	46.50
Menhaden fish oil	3.31	3.31	3.31	3.31	3.31	3.31
Yellow corn	36.74	36.74	36.74	36.74	36.74	36.74
Corn starch	0.20	0.00	0.00	0.00	0.00	0.00
Trace mineral premix	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 250 mg kg^{-1} using 25%	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic	2.00	2.00	2.00	2.00	2.00	2.00
Corn gluten meal	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

Table 2. Mean concentrations of *Bacillus*-like colonies recovered from probiotic-supplemented diets. Values are means of four replicates and values with same letters are not significantly different

Treatment	*Bacillus* strain	Mean (CFU/g)
Diet 1 (Control)	---	0
Diet 2	SB3086	8.5×10^{7a}
Diet 3	SB3295	7.3×10^{7a}
Diet 4	SB3615	8.2×10^{7a}
Diet 5	SB3086+SB3615	7.0×10^{7a}
Diet 6	AP193	7.7×10^{7a}

Table 3. Effects of experimental diets on the growth of juvenile *O. niloticus* grown for 21 days in flow-through aquaria. Values are means ± s. d. of six replicates and values within the same row with same letters are not significantly different ($p>0.05$)

Growth	Diets					
Parameter	1	2	3	4	5	6
IBW	16.2±0.5[a]	16.6±0.8[a]	16.6±0.8[a]	16.6±0.8[a]	16.7±1.1[a]	16.7±0.8[a]
FBW	33.7±1.6[a]	30.6±1.5[a]	35.6±1.6[a]	34.1±2.6[a]	34.6±2.2[a]	33.3±1.3[a]
%WG	107.5±7.1[a]	101.6±10.7[a]	115.0±9.5[a]	106±14.3[a]	107.6±9.7[a]	100.2±6.7[a]
SGR	3.5±0.05[a]	3.0±0.07[a]	3.6±0.06[a]	3.4±0.08[a]	3.5±0.08[a]	3.3±0.05[a]
FCR	1.42±0.11[a]	1.41±0.09[a]	1.54±0.12[a]	1.42±0.12[a]	1.45±0.05[a]	1.35±0.18[a]

IBW (g $fish^{-1}$), initial mean body weight; FBW (g $fish^{-1}$), final mean body weight.
%WG (percent weight gain) =100 x (final weight-initial weight)/initial weight.
SGR (specific growth rate) (%day^{-1}) = 100 x [(ln final weight-ln initial weight)/days]
FCR (feed conversion ratio) = feed intake / (FBW-IBW).

Table 4. Mean serum bactericidal and lysozyme activities of juvenile *O. niloticus* fed probiotic diets for 21 days. Values are means ± s. d. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column

Treatment	Bactericidal Activity (CFU)	Lysozyme (ml^{-1})
1 (Control)	14.8 ± 6.8^{a}	838.3 ± 117.7^{a}
2 (SB3086)	3.3 ± 2.0^{c}	1015.0 ± 76.4^{b}
3 (SB3295)	12.0 ± 5.9^{ab}	1055.0 ± 70.4^{b}
4 (SB3615)	6.2 ± 4.3^{bc}	1048.3 ± 65.5^{b}
5 (SB3086 + SB3615)	6.2 ± 2.6^{bc}	1026.7 ± 110.8^{b}
6 (AP193)	4.3 ± 2.1^{bc}	1005.0 ± 54.7^{b}

Table 5. Mean percent mortality of juvenile *O. niloticus* fed probiotic diets and challenged with *S iniae*. Values are mean percentages ± s. d. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column.

Treatment	Mortality (%)
1 (Control)	77.3 ± 7.0^{a}
2 (SB3086)	47.3 ± 4.7^{cd}
3 (SB3295)	61.3 ± 8.6^{b}
4 (SB3615)	44.0 ± 7.2^{d}
5 (SB3086 + SB3615)	46.7 ± 9.7^{cd}
6 (AP193)	58.0 ± 10.0^{bc}

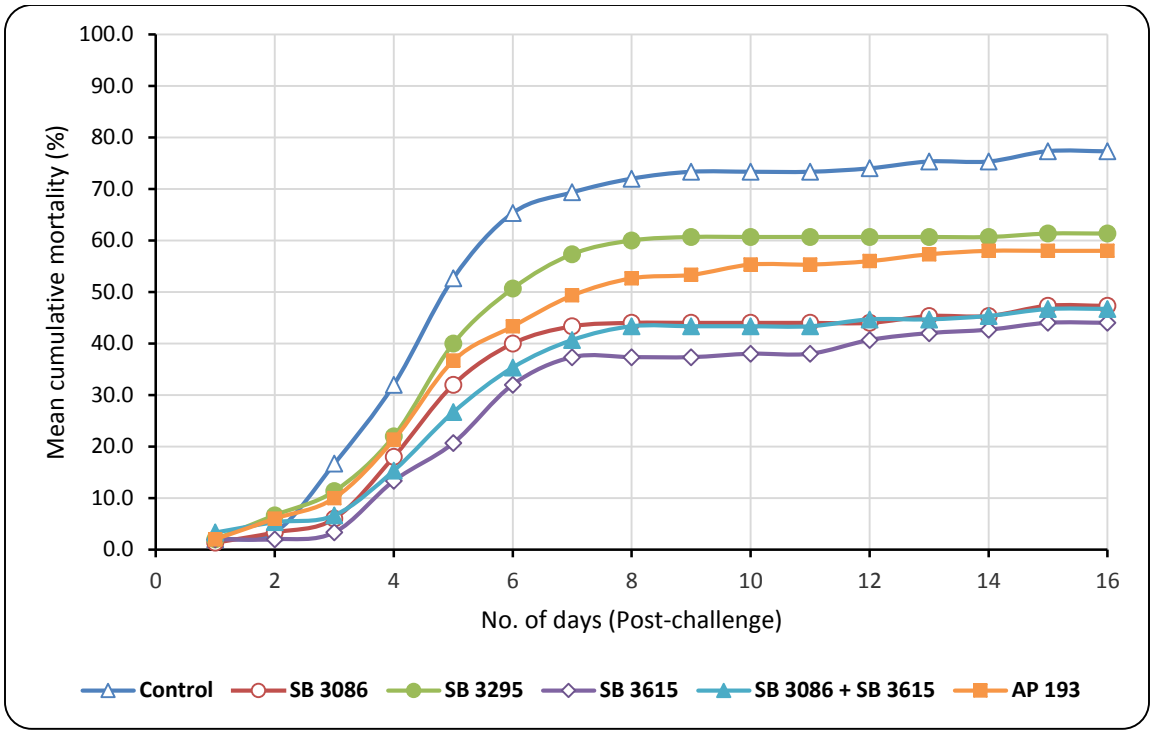

Fig. 1. Mean percent cumulative mortality of Nile tilapia fed a control and different strains of *Bacillus subtilis* diets for 3 weeks and challenged with *Streptococcus iniae* using IP injection.

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