THE INFLUENCE OF THE PROBIOTIC PONDTOSS™ ON GROWTH PERFORMANCE AND HEALTH OF CHANNEL CATFISH, *ICTALURUS PUNCTATUS*, AND NILE TILAPIA, *OREOCHROMIS NILOTICUS*

by

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Abstract

In 2011, global aquaculture production of food fish comprising channel catfish and Nile tilapia reached 62.7 million tonnes with an estimated value of US$ 130 billion. As aquaculture technology intensifies to meet market demands, the economic losses in the aquaculture industry from bacterial diseases continue to grow. Of those bacterial diseases, columnaris has been reported as one of the major causes of massive economic losses in channel catfish and Nile tilapia farming. In the search for alternative environmentally-friendly treatments, probiotics have been suggested as possible alternatives to chemotherapeutants and antibiotics. Towards this goal, this research evaluated the effects of the commercially-available probiotics as a feed additive on channel catfish and Nile tilapia growth performance and protection against columnaris (*Flavobacterium columnare)* under laboratory conditions.

The first study was conducted to evaluate the influence of the probiotic product PondToss™ on growth performance and survival of the juvenile Nile tilapia *Oreochromis niloticus* and its potential to reduce mortality when challenged with *Flavobacterium columnare*. The study was carried out in two phases, growth phase and disease challenge phase. The growth phase was designed for feeding a control or probiotic (0.1% PondToss™ at 5.9×10^{7}CFU/g of feed or 0.2% PondToss™ at 1.2×10^{8} CFU/g of feed) diet in a flow-through system for 56 days. The results at the end of this study demonstrated that addition of PondToss™ to the feeds at these rates did not significantly improve mean final

body weight (p=0.4297) which was 35.3 ± 1.6 g, 35.2 ± 1.3g, and 34.5 ± 1.7g for control, .1% PondToss™ and 0.2% PondToss™, respectively. Mean percent weight gain (p=0.1159) was 930.4 ± 66.7%, 949.9 ± 41.6%, and 903.1 ± 49.5% for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Specific growth rates (p=0.0838) were 4.12 ± 0.09 % day^{-1}, 4.12 ± 0.06% day^{-1}, and 4.06 ± 0.05% day^{-1} for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Feed conversion ratio (p=0.7052) was 1.12 ± 0.04, 1.11 ± 0.01, and 1.12 ± 0.02 for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Mean survival rates (%) (p=0.1711) were 98.2 ± 1.5%, 96.2 ± 3.5% and 97.2 ± 3.5% for control, 0.1% PondToss™ and 0.2% PondToss™ respectively.

Fish from all replicates of a treatment were pooled and then randomly assigned to one of six treatments: C1 (Control fish fed Control feed), C2 (Fish from Control fed PondToss™ at 5.9×10^7 CFU/g), C3 (Fish from 0.1% PondToss™ now fed Control feed), C4 (Fish from 0.1% PondToss™ now fed 0.1% PondToss™ at 5.9×10^7 CFU/g), C5 (Fish from 0.2% PondToss™ now fed Control feed) and C6 (Fish from 0.2% PondToss™ now fed 0.2% PondToss™ at 1.2×10^8 CFU/g). All fish were fed their original diet until post-challenge when new feeds were fed. Fish were then challenged with *F. columnare* strain ALG-530 by immersion at a dosage of 1.4×10^6 CFU/mL. None of the fish were negatively impacted or experienced mortality. The failure to induce an outbreak of columnaris in all treatments could have been related to environmental conditions of the study, pre-exposure to *F. columnare,* or issues related to the *F. columnare* virulence or infection mode. Regardless, no conclusion could be made about potential benefits of PondToss™ in reducing mortalities related to columnaris in Nile tilapia fingerlings.

The second study was conducted to evaluate the influence of PondToss™ on growth performance and survival of channel catfish, *Ictalurus punctatus*, fingerlings and its potential effect on their survival when challenged with *F. columnare* ALG-530. The study was again carried out in two phases-growth phase and disease challenge phase. The growth phase was designed for feeding a control or probiotic (1% PondToss™ at: 4.1×10^7 CFU/g or 2% PondToss™ at 6.9×10^7 CFU/g of feed) diet in a flow-through system for 52 days. The results at the end of the study showed that under the conditions of the present trial, none of the diets significantly improved mean final body weight (FBW, CONTROL: 36.2 ± 2.5 g, 1% PONDTOSS™:34.5 ± 2.1 g, 2% PONDTOSS™: 35.0 ± 1.9 g), percent weight gain (%WG, CONTROL: 351.1 ± 30.3%, 1% PONDTOSS™: 334.9 ± 28.4%, 2% PONDTOSS™: 339.8 ± 22.2%), specific growth rate (SGR, CONTROL: 2.9 ± 0.1% day^{-1}, 1% PONDTOSS™: 2.82 ± 0.1% day^{-1}, 2% PONDTOSS™: 2.8 ± 0.1% day^{-1}), feed conversion ratio (FCR, CONTROL: 1.2 ± 0.2, 1% PONDTOSS™: 1.2 ± 0.1, 2% PONDTOSS™: 1.1 ± 0.1), and survival rate (SV: CONTROL: 76.4 ± 23.1%, 1% PONDTOSS™: 86.4 ± 24.6% and 2% PONDTOSS™: 87.2 ± 27.2%).

In the challenge phase, fish from each treatment in Phase 1 were combined and then randomly assigned to one of six treatments: C1 (Fish from Control fed Control feed), C2 (Fish from Control fed 1% PondToss™), C3 (Fish from 1% PondToss™ fed Control feed), C4 (Fish from 1% PondToss™ fed 1%PondToss™), C5 (Fish from 2% PondToss™ fed Control feed) and C6 (Fish from 2% PondToss™ fed 2%PondToss™). Fish were then challenged with *F. columnare* strain ALG-530 (3.4×10^6 CFU/mL) by immersion based upon doses determined during the pre-challenge study. The fish were exposed to the bacteria in static water for 6 hours and monitored for 20 days after the challenge. All dead

and moribund fish were removed and counted. Moribund and freshly dead fish were collected and isolated for bacterial recovery and confirmation. Under this study condition, the treatments had no significant effects on mean mortalities compared to the control. Because of the limited number of fish available for the challenge study, fish losses within each treatment replicate resulted in large changes in mortality rates leading to high within treatment variability and non-significant differences. Regardless, no significant benefit or general trends were observed when using PondToss™ as a feed additive in reducing mortalities related to columnaris infection.

In conclusion, PondToss™ used as a feed additive did not significantly enhance growth, FCR or survival of Nile tilapia or channel catfish fingerlings under the given experimental conditions. Likewise, challenges with *F. columnare* did not show any reduction in mortalities when using PondToss™ as a feed additive at the experimental dosages, either before or after challenge, for juvenile channel catfish and were inconclusive for juvenile Nile tilapia. Because columnaris typically infects fish from the outside through gills and skin and then becomes systemic, a better approach to using probiotics like PondToss™ might be via water application.

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CHAPTER I

Introduction and Literature Review

1. Introduction

As the world human population keeps increasing from year to year, the demand for food is also increasing. The food products that have been primarily produced in the world include both terrestrial and aquatic food products, such as poultry, beef, pork, sheep, vegetables, and freshwater and saltwater seafood comprising finfish, mollusks, crustaceans, seaweeds, etc. According to the Fisheries and Aquaculture Department of the Food and Agriculture Organization of the United Nations (FAO 2013), in 2011 global aquaculture production comprised 42 million tonnes for finfish, 21 million tonnes for seaweeds, 14 million tonnes for mollusks, and 6 million tonnes for crustaceans. Therefore, in total the world aquaculture production of food fish reached 62.7 million tonnes with an estimated value of US$ 130 billion according to the global aquaculture production statistics for the year 2011. In the same report, global aquaculture production in 2012 is estimated to be around 66.5 million tonnes. As for the major food fish species in 2011, 29 species were listed at production levels of 250,000 tonnes or more. These species included Nile tilapia, *Oreochromis niloticus*, and channel catfish, *Ictalurus punctatus*.

According to FAO (2013), in 2011, 2.79 million tonnes of tilapia were produced with a value of US$ 4.5 billion. Most importantly, tilapia have been cultured worldwide due to its high adaptability to the broad range of physical and environmental conditions.

Furthermore, it is also considered as one the most important freshwater aquaculture candidates. FAO (2009) reported that farm-raised Nile tilapia production accounted for 5% of the world finfish farming. More interestingly, Nile tilapia has been recognized as the second most produced species of farm-raised fish after carps (FAO 2014a).

Different from Nile tilapia, channel catfish has not became one of the widely globally farm-raised species, mainly because the adaptability of this species to the environment is more limited compared to tilapia and other major farm-raised species. Nevertheless, channel catfish is known as the primary farm-raised species in the United States, especially in the southeastern region comprising the producing states of Mississippi, Arkansas, Alabama, and Texas. Brown (2010) noted that in Alabama channel catfish is the most dominant farm-raised species and is among the biggest sources providing the employment and income in west Alabama.

As the demand for tilapia and channel catfish farm-raised products continues to increase, the aquaculture production technology has been developed and intensified to meet the demand, especially in China, USA, and the Russian Federation, etc. In this manner, aquaculture has been seen shifting day by day from conventional farming practices to more sophisticated, complicated and large-scale practices. Resultant high stocking densities lead to deterioration of water quality, including reduced dissolved oxygen levels, higher levels of suspended solids, and accumulation of ammonia and nitrite. Furthermore, crowding also can lead to fish injuries, which provide portals for harmful bacteria to easily infect them, especially if fish are being underfed. This facilitates and accelerates disease outbreaks. However, overfeeding also results in higher disease incidence due to higher buildup of toxic wastes. Likewise, Snieszko (1974) found that the occurrence of communicable

diseases is definitely possible when susceptible hosts and virulent pathogens are present in the environment where disease induction is favored. Diseases limit the potential of aquaculture production, trade and economic development in many countries (Verschuere et al 2000; Welker and Lim 2011). In recent years in the US, estimated losses to channel catfish farmers from diseases were estimated at over US$50 million annually, reducing profitability (Wagner et al. 2002; Shoemaker et al. 2011). Meyer (1991) emphasized that the most important diseases in all the numerous types of aquaculture production are bacterial infections. Hence, a number of bacterial diseases have been reported in channel catfish production; however, columnaris caused by the causative agent *Flavobacterium columnare* is a major cause of catfish mortality (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 globally, including tilapia (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 pre-disposed by stress or trauma. As mentioned earlier, the environment and the condition of the fish are both important in determining the rate and severity of infection. Channel catfish are susceptible to columnaris at temperatures from 15 to 30 C and young fish are more severely affected than adults (Griffin 1992; Plumb 1999). Decostere et al. (1999) found that adhesion of high virulence strains of *F. columnare* to gills of common carp (*Cyprinus carpio*) was enhanced by bivalent, ion-rich water, presence of nitrite or organic matter, and high temperatures.

To mitigate columnaris disease problems in aquaculture, antibiotics and other chemicals have traditionally been used. Unfortunately, there are many issues, mainly about human health and wellness of the environment, related to those antibiotics and

chemical applications. In the United States, few approved drugs exist and they are very expensive and restricted in use. Other concerns include development of antibiotic-resistant pathogens and potential consequences to human health and the environment (Toranzo et al. 1984; McEwen and Fedorka-Cray 2002; USDA 2003; FAO 2006; Marzouk et al 2008; Welker and Lim 2011). Vaccines used to prevent columnaris are not readily commercially available. Newman (1993) mentioned that there were some vaccination studies conducted with different types of administration; however, there were no consistent results. He also added that this might be due do the relationship of stress and the progression of diseases.

In response to the aforementioned issues, many researchers suggested that probiotics are possible alternatives to antibiotics. The use of probiotics for disease prevention and improved nutrition in aquaculture has gained increased attention as demand for environment-friendly practices has increased (Robertson et al. 2000; Verschuere et al. 2000, Vine et al. 2006; Shelby et al. 2006; Wang et al. 2008a; Cruz et al. 2012). A number of studies have reported conflicting results in the effectiveness of probiotics applied to the water or as feed additives in improving the immunity, health status, feed efficiency or growth performance of fish species (Boyd and Gross 1998; Verschuere et al. 2000; Aboagye 2008; Denev et al. 2009). The effect of the commercially-available probiotic product Lymnozyme® has been evaluated with specific pathogen free (SPF) juvenile channel catfish challenged with enteric septicemia of catfish (ESC) (Aboagye 2008) or *F. columnare* (Addo 2013) under laboratory conditions. They found significant positive effects of Lymnozyme® on their treated fish, reducing mortalities.

In this study, the probiotic commercially known as PondToss™ was used to evaluate its influence on growth performance and health of Nile tilapia*, Oreochromis*

niloticus, and channel catfish, *Ictalurus punctatus*. PondToss™ is a combination of Lymnozyme® and another product WSR™; the probiotic portion is from Lymnozyme® and the water conditioning bacteria from WSR™. It is a freeze-dried biological formula of natural microbes, enzymes, micronutrients and amino acids on a special carrier designed to improve microbial growth rates. Both are commercially-available aquatic products from Keeton Industries Inc., Wellington, CO, and are dry, water-soluble probiotic concentrates. To examine the ability of PondToss™ to improve fish growth, feed conversion ratio (FCR), and survival when used as a feed additive, research was conducted under two trials with the two main goals as follows:

a. To evaluate the efficacy of the probiotic product PondToss™ in enhancing growth, FCR and survival of Nile tilapia, *Oreochromis niloticus*, and channel catfish, *Ictalurus punctatus*, fingerlings when incorporated into a commercial diet.
b. To evaluate the effectiveness of the probiotic product PondToss™ as a feed additive in reducing mortality from columnaris in challenged tilapia and channel catfish fingerlings.

2. Literature Review

2.1. Global Channel Catfish Production

Based upon FAO (2014b), there are 8 channel catfish producing countries including the United States of America, the Russian Federation, China, Mexico, Cuba, Costa Rica, Brazil and Paraguay. Among these countries, China and Brazil have added large proportions of channel catfish production to the world production of channel catfish. Additionally, these two countries also exported their farm-raised catfish to the United States. In 2007, China

exported 260,000 kg of channel catfish to the United States (Stickney and Treece 2012). Channel catfish were introduced into China in 1984, but at that time the production was primarily made for the domestic consumption and very little was for exporting to the United States (FAO 2014b). In 2012, the global channel catfish production reached 394,179 tonnes with the value of US$638,748,000 (FAO 2014b). Channel catfish are raised in earthen ponds, cages, raceways or circular tanks in both the United States and China. Monoculture is predominantly practiced in the U.S. while both monoculture and polyculture with some indigenous species, such as carps, are practiced in China (FAO 2014b). In the U.S., the channel catfish industry is a relatively new commercial aquaculture enterprise even though its culture has been practiced for more than a century. According to Wellborn (1987), the channel catfish aquaculture industry began with the first efforts of culturing catfish practiced at a few federal and state fish hatcheries in the early 1900s. In the 1950s, Kansas and Arkansas were the first states in which commercial catfish farming started. Dr. H. S. Swingle and his co-workers at Auburn University provided much of the information on catfish farming to the farmers in the 1950s and 60s (Wellborn 1987; Stickney and Treece 2012). Since that time, channel catfish aquaculture industry grew rapidly to reach its annual sales of ~0.299 million tonnes in 2003, representing nearly half of the total US aquaculture production with the total value of US$425 million. In the same year, Mississippi was the largest catfish producing state with the value of US$243 million. In recent decades, channel catfish industry is the largest aquaculture industry in the U.S. The four major channel catfish producing states are located on the southern region of the United States, namely Mississippi, Alabama, Arkansas, and Texas. They shared about 95 percent of the US total sales in 2012. Between 2011 and 2013, Mississippi was the top producing state having 19,668 ha of catfish

farming areas (NASS 2013). In 2013, the US catfish growers had sales of 342 million dollars, which was up 1% from 341 million dollars from 2012 (NASS 2014). However, NASS (2014) reported that as of January 1, 2014, there was a 9 percent decline in water surface area being used for catfish production from the 33,710 ha used in the previous year. Current total water surface area for catfish production in the U.S. is 30,594 ha, 23,836 ha for food size fish, 4,452 ha for fingerling and 732 ha for brood fish production.

Although the US channel catfish industry grew rapidly, the stability of this business market has been seriously threatened by intense competition from imported catfish products, the increased production and investment costs due to the rise and fall of commodity prices, diseases, and environmental issues. The U.S. channel catfish market recently has been reportedly affected by the inflow of the Mekong catfish from Vietnam. In an attempt to alleviate the problem, legislation was passed to require the country of origin branding on the domestic products (i.e., COOL) while placing a tariff on the imported catfish product (FAO 2014b). Also, an increase in production costs, including feed and fuel costs, alongside volatile annual fish prices and lower demand for final products made it difficult for U.S. catfish producers to make profits. Consequently, many producers turned their pond area into soybean and corn fields between 2002 and 2012 (Hanson and Sites 2012).

In addition to the above situation, fish disease caused large economic losses in the U.S. catfish industry. Hawke et al. (1981) recognized that disease outbreaks, mainly bacterial diseases, were a major obstacle to economic growth in catfish farming. *Flavobacterium columnare*, the causative agent of columaris, and *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish (ESC), are the two bacterial diseases responsible for these economic losses. ESC accounts for more than US$20 to 30 million

losses in the channel catfish industry in the United States annually (Plumb and Vinitnantharat 1993). Beside these two highly infectious bacterial diseases, channel catfish are also prone to various kinds of diseases such as channel catfish virus (CCV), *Aeromonas septicaemia*, water molds, gill and/or external parasites, copepod parasites and other diseases (FAO 2014b).

Water quality limitations are also a big concern for catfish growth. Channel catfish aquaculture depends heavily on adequate supplies of ground water and that possibly leads to the deterioration of the environment when the polluted cultured water is discharged into the natural water bodies, such as streams or rivers, without proper and adequate treatment. US EPA (U.S. Environmental Protection Agency 1974) judged the effluents from channel catfish ponds as an impending point source of pollution. In fact, the water quality deterioration might or might not happen depending on how intensively the farming practices are adapted. Before the 1980s, the channel catfish pond water quality was better and stress on the farm-raised catfish populations was also lower, because at that time farming practices were not yet intensified. Conversely, nowadays the effluents released from the intensive channel catfish farms are reportedly environmentally problematic (MSU 2014). The nutrient rich water from the catfish farms is said to be low in dissolved oxygen, but high in ammonia and other nutrients. Boyd et al. (1979) stated that generally it is accepted that there are heavy phytoplankton blooms and some other low dissolved oxygen related issues as the restricting factors in channel catfish pond culture. Phytoplankton blooms, especially blue-green ones, cause a lot of issues in the catfish ponds, namely “off-flavor”. Off-flavor makes channel catfish undesirable. The taste of the catfish flesh contains earthy-muddy flavor (Stickney and Treece 2012). Tucker and Robinson (1990) explained that from a fish heath perspective,

off-flavor does not harm the fish, but it does affect the commercial channel catfish market seriously. Off-flavor occurs in fish flesh after the fish absorbs the odorous natural chemical products released into the water by the aquatic bacteria or algae, and it normally happens in high density ponds. According to Masser and Hyde (1996), it generally happens in the warmer months of the year from May to October, although it might occur in any month of the year. Stickney and Treece (2012) emphasized that once off-flavor is found in the fish, the fish are then rejected before they are sent to processing. So to avoid this, off-flavor tests need to be carried out.

2.2. Global Tilapia Production

Tilapia are freshwater fishes existing in the family Cichlidae, and they are now important in world fresh and saltwater aquaculture as the second most important food fish in the world (Fitzsimmons 2000b). Trewavas (1983) classified the fish into three major taxonomic groups, such as *Oreochromis* (the maternal mouth brooders), *Sarotherodon* (the paternal or biparental mouthbrooders) and *Tilapia* (the substrate spawners). However, because of the confusion in the 1970s and the arguments made by taxonomic "lumpers" and "splitters," Trewavas's classification has not yet been accepted by the American Fisheries Society (AFS) (Costa-Pierce and Doyle 1997).

Tilapias are reported to have originated from Africa, excluding Madagascar, but parts of the Middle East are included. To be specific, the central and eastern parts of Africa are dominated by *Oreochromis* species, whereas, the western part of Africa is endemic with *Tilapia* and *Sarotherodon* species (Suresh 2003). Even though they are originally from tropical fresh water of Africa, Lim and Webster (2006) stated that tilapia now are globally

distributed because they are now being farmed in tropical, sub-tropical and temperate climates, in both fresh and salt water, and in all types of farming systems. For instance, tilapia are being cultured in some U.S. states, such as California, Arizona, Hawaii, Florida, Nevada, Texas, Alabama, and North Carolina. Based upon Pillay (1990), tilapia were introduced to these states in the second half of the 20th century and were used for farming as food fish, sportfishing, controlling aquatic weeds, and for research purposes. It is documented that the worldwide distribution of tilapias, primarily Mozambique tilapia, occurred during the 1940s and 1950s while the distribution of the much preferred Nile tilapia occurred during the 1960s and the 1980s (Gupta and Acosta 2004). Therefore, nowadays, tilapias are focused in food fish aquaculture production.

There are some potential attributes that make tilapias one of the world's best aquaculture candidates, mainly in the third world. Those attributes are fast growth, high tolerance to a wide range of environmental conditions, disease and stress resistance, ability to grow and reproduce in captivity, acceptance of artificial feeds, etc. (El-Sayed 2006). Regarding tilapia species that are commercially significant for aquaculture, they are primarily from the genus *Oreochromis,* the maternal mouthbrooders comprising the Nile tilapia (*O. niloticus*), Mozambique tilapia (*O. mossambicus*), and blue tilapia (*O. aureus*) then secondarily important tilapias are *Sarotherodon galilaeus, S. melanotheron* (from West African lagoons), and hybridized red tilapia (*O. mossambicus* hybrids × *O. niloticus, O. aureus* and *O. urolepis hornorum*) (Schoenen 1982). According to Gupta and Acosta (2004), there are 9 out of 70 tilapia species that are used in farming.

Tilapia aquaculture production can be traced back to ancient Egyptian times. Historically, tilapia aquaculture has been documented to have originated about 4000 years

ago, and 1000 years before carp aquaculture was initiated in China (Balarin and Hatton 1979). According to El-Sayed (2006), from the Egyptian tombs, there are some references and painting depicting tilapia in the ponds; however, there is not much information describing the culture techniques in the early times in those references and illustrations. Now in the last few decades, tilapia culture has been greatly progressing. It is reported that tilapia aquaculture currently contributes 5% to the total fish farming production, which is second only to the group of carps (Lim and Webster 2006). Even so, with an annual increase of 12%, tilapia is predicted to become the leading cultured fish in the 21st century (Fitzsimmons 2000b). Likewise, annually, the global aquaculture production of tilapias, especially Nile tilapia, keeps increasing. Nile tilapia (*O. niloticus)* is the most important species of commercially significant tilapia species because it has a large contribution to global tilapia aquaculture production at 1.27 million metric tons, which was equal to 3.57% of the world aquaculture production in 2000 (Gupta and Acosta 2004). According to FAO (2014a), the world aquaculture production of Nile tilapia in 2012 reached 3,197,330 tonnes with a value of US$ 5.26 billion. In this regard, China, Egypt, Thailand, Philippines and Indonesia are the major producing countries (Gupta and Acosta 2004). FAO (2014a) reported that China is the biggest Nile tilapia producing country with 806,000 tonnes of 2003 annual farm-raised Nile tilapia production then followed by 200,000 tonnes shared by Egypt. In the same year, the Philippines, Thailand and Indonesia contributed 111,000 tonnes, 97,000 tonnes and 72,000 tonnes, respectively. As for American tilapia aquaculture, Watanabe et al. (2002) reported that in 1949 tilapias were introduced to America. Then in 2004, tilapia production in the U.S. was 9000 mt according to Fitzsimmons (2006). However, Fitzsimmons added that the production in the U.S. is mainly from domestically-

produced live fish for some Asian restaurants, groceries, local markets of some cities in some states, such as California, Idaho and Arizona. Zajdband (2012) reported that there is only 5% of tilapia produced domestically in the U.S. while the rest is imported from other countries. He added that in that 5% of domestically-produced tilapia, over 75% comes from recirculating aquaculture systems. Even so, Zajdband (2012) mentioned that the U.S. tilapia production statistics are frequently combined together, not updated, and generally not available even though there are some institutions conducting some research related to that. As for production techniques, tilapias are being reared using various kinds of techniques and production systems, which range from family to commercial-scale operations and from low to high technology. Fitzsimmons (2005) stated that those farming systems include ponds, cages, raceways, tanks, net pens, lake ranching, saltwater, brackish water, fresh water, aquaponics, plastic drums and computer-controlled intensive recirculating systems.

Tilapia culture has a long history with a variety of farming techniques practiced all over the world. Despite the long history, tilapia farms report to have some constraints affecting production. The expansion of tilapia farming is hindered by low production of fry due to low productivity of broodstock and asynchronous spawning cycles. The destruction of the genetic quality and little interest shown to its marketing and other business concepts are also believed to be major apprehensions for the future of industrial tilapia production and its growth (Gupta and Acosta 2004). In addition to these issues, environmental impacts mainly related to the consumption of water by tilapia aquaculture are important. Tilapia farmers need to be able to produce more fish with less water, food and time to minimize production costs and to diminish environmental pollution. Surprisingly, not different from channel catfish, off-flavor, which is caused by the chemical geosmin and blue-green algae,

is also another big issue in the tilapia earthen ponds and recirculating aquaculture systems (Stickney 1997). Last but not least, tilapias are also vulnerable to high losses from diseases even though they are known for resistance to many diseases compared to other cultured fishes. When the farming systems become more intensive with higher fish densities under more complicated management, disease outbreaks are likely. According to Plumb (1997), there are several bacterial species that cause high morality in the intensive tilapia industry, such as motile *Aeromonas* spp., *Pseudomonas* spp., *Vibrio* spp., *F. columnare, Edwarsiella tarda* and *Streptococcus* spp. In the Americas, *Streptococcus* spp. are the most serious bacteria infecting intensive tilapia culture.

2.3. Columnaris Disease in Channel Catfish and Tilapia

Columnaris disease is the second most commonly diagnosed infectious bacterial disease of channel catfish industry, behind enteric septicemia of catfish (ESC) caused by *Edwarsiella ictaluri* (Tucker and Robinson 1990). Tilapias are also infected by columnaris leading to economic losses. Plumb (1999) stated that columnaris, known as mouth fungus or cotton wool, is an acute to chronic infectious skin disease. This disease is able to infect fish externally on the skin or systematically resulting in dramatic losses, especially during warm summer temperatures (Pacha and Ordal 1970). At 15 C and above, columnaris can invade both external injured and uninjured tissues leaving lesions covered with yellowish white mucoid exudate (Wakabayashi 1993). Regarding the name “columnaris”, Snieszko and Bullock (1976) revealed that columnaris is the name of the disease first given by Davis based on her observation upon the column-like structure congregation of the organisms as seen in the wet mounts. According to Noga (2000) and Andrews et al. (1996), columnaris has been

referred to by different names, such as Myxobacterial disease, peduncle disease, saddle back, fin rot, cotton wool disease, black patch necrosis and mouth fungus. Snieszko and Axelrod (1970) and Durborow et al. (1998) stated that columnaris disease was first described by Davis in 1922; at that time, Davis defined columnaris as the disease of any animal that is infected by a disease agent existing in myxobacteria group. However, Noga (2000) mentioned that it is wrong to classify columnaris infection into myxobacterial infection. Throughout the 20th century, the causative bacterium of columnaris was known as *Flexibacter columnaris* (Wakabayashi 1993; Andrew et al.1996; Noga 2000). Finally, Bernardet et al. (1996) re-described this group of bacteria and eventually named the bacterium *Flavobacterium columnare* in 1996. Noga (2010) added that due to its worldwide presence, *F. columnare* possibly can infect most freshwater fishes and it can also cause serious infection with high mortality. Likewise, Plumb (1999) mentioned that no freshwater fish, including wild, farm-raised and ornamental fish, is completely resistant to columnaris. From 1987 to 1986, columnaris was the most infectious bacterial disease in the catfish industry covering 58% of all bacterial cases (Thune 1991). Shoemaker et al. (2011) mentioned that the economic losses to channel catfish farmers from columnaris diseases are estimated at US$30 million annually in the U.S.

Characteristics of the disease: Tucker and Robinson (1990) stated that columnaris can infect channel catfish at all sizes and ages; nevertheless, if the fish are not stressed, columnaris seldoms infects a catfish population. Therefore, some stress factors make fish susceptible to columnaris, such as poor water quality and rough handling of fish when water temperatures are warm. In this particular point, they claimed that the levels of disease severity are related to the water temperatures after the fish are stressed by the

aforementioned factors. They explained that at a high temperature range (approximately between 23 C and 29 C), columnaris outbreaks are acute and progress very fast making fish lose their appetite and die within a couple days. In contrast, when the water temperature range is low, the level of severity becomes chronic and there are fewer fish losses. Likewise, Noga (2010) confirmed that as the temperature increases, the acuteness and the mortality from columnaris also increases. Interestingly, Wakabayashi (1993) reported that in 1970, Pacha and Ordal studied the influence of water temperature on the pathogenicity of *F. columnare* strains of different levels of virulence. The results of this study revealed that at low temperature of 12.8 C, high-virulent strains were able to cause disease outbreaks and mortality by attacking susceptible fish. Different from high-virulent strains, low-virulent strains were only able to cause infections when the temperature was increased to 20 C. Besides temperature, Noga (2010) added that strain virulence is also the most essential element indicating the severity of the disease. Moreover, hardness of water also affects the pathogenesis, because *F. columnare* is found less virulent in soft water and avirulent in distilled water (Noga 2000).

Causative agent and its characteristics: *F. columnare* is reported as a ubiquitous soil and water-borne pathogen (Schachte 1983). Wakabayashi (1993) and Snieszko and Bullock (1976) described *F. columnare* as a gram-negative rod bacterium that has a long and slender body (0.5 to 0.7 × 4.0 to 8.0μm) having gliding motility by flexing or creeping movement. *F. columnare* is a facultative bacterium, which was isolated and characterized in 1944 from an outbreak of columnaris disease among sockeye salmon (Ordal and Rucker 1944). However, at that time, *F. columnare* was named *Chondrococcus columnaris* by Ordal and Rucker according to Plumb (1999). *F. columnare* can be isolated

from the most reliable tissues, such as internal organs, skin and gills of the fish (Bader et al. 2003), but the isolates appear to have weak growth when incubated on the media containing 1% sodium chloride (Plumb 1999).

Clinical signs and pathogenesis: Tucker and Robinson (1990) and Noga (2010) described that the fish that are infected by *F. columnare* as having erosive or necrotic skin as well as gill lesions that can begin from the surface to the internal parts of the fish causing systemic infections. On the gills, the necrotic areas affected by *F. columnare* normally appear yellowish and brownish with the progression of infections from the tips to the bases of the gill filaments and the gill arch. In addition, Tucker and Robinson (1990) emphasized that the infected areas on the gills or skin may be seen covered with swarms of yellowish bacteria and the skin might be completely damaged. As a result, the fish finally die due to extensive damage of the skin leading to massive loss of osmoregulation and electrolyte homeostasis in the fish's body (Tripathi et al. 2005). The fins become frayed and grayish, especially the dorsal fin, while the infected skin is seen with the occurrence of discolored areas that might progress to large ulcers surrounded by the redden areas or hemorrhaging at the margins of those ulcerations. These large ulcers normally appear on the head or back of the fish known as saddleback lesions when the water temperatures are lower than 15 C. These lesions are also susceptible to the invasion by fungi (Tucker and Robinson 1990). According to Noga (2000), a bacteremia (systemic infection) can happen when these ulcerations penetrate deeply into the tissues. However, the systemic infections might occur in channel catfish with no signs of external lesions, but the swelling posterior kidney might be seen (Hawke and Thune 1992). Schachte (1983) indicated that systemic infections with no obvious external signs are caused by *F. columnare* of low virulent strains. Nevertheless,

Schachte continued that most species of fish are more susceptible to cutaneous infections. On the other hand, the high virulent strains normally cause a "gill rot" by severely attacking the gill tissue (Wood 1974).

As for columnaris infection in tilapia, Plumb (1997) stated that systematic columnaris in tilapia occurs sporadically; however, the precursors involving death of tilapia is the injury of skin, fins and gills. In that situation, tilapia either swims slowly and tiredly or floats at the surface. Plumb added that uneven whitish to grayish discolored areas on the skin and/or on fins, frayed or ragged fins and different sizes of pale necrotic lesions on the infected gills are used to describe columnaris infection in tilapia. Since tilapia is a scaled fish, Amend (1983) pinpointed dorsal whitish-veiled erosions on the body of the fish are the preliminary signs of columnaris infection, which later on develops into proliferative ulcers. Furthermore, Amend explained that it is contrasting to other scale-less fishes, such as catfish, where the early signs of columnaris occur with normal ulcers and gradually progresses to serious saddleback-like sores. In addition, there might be some moldy or cottony lesions on the mouth while the gills appear necrotic as a result of the *F. columnare* invasion. In this manner, oxygen deprivation or hypoxia always occurs in the infected fish as they show gas piping behavior at the water surface. In case of acute severity, a so-called bacteremia might progress internally in the blood stream leaving dead fish with no external signs. Particularly, Plumb (1997) reported that juvenile fish are generally infected seriously by columnaris. In addition, the vulnerability and mortality of tilapia are influenced by pH; so, at more neutral pH, tilapia are less prone to columnaris (Marzouk and Baker 1991).

Modes of Transmission: During columnaris disease outbreaks, columnaris can be passed horizontally from one fish to another via direct physical contact or the water

column where *F. columnare* exists (Tucker and Robinson 1990). In addition, Schachte (1983) added that in an experiment, columnaris was spread from one fish to another directly when virulent strains of *F. columnare* were used. Likewise, it has been reported that virulent strains are able to infect fish when they are added to the water while low virulent strains infect fish only when they are injected into the fish (Snieszko and Bullock 1976). Suomalainen (2005) elaborated that the fish recovering from *F. columnare* infections normally come to be columnaris pathogen carriers that later on release *F. columnare* into the water column when they are stressed by some factors such as high water temperature, crowded conditions due to high stocking density, and rough handling, or when there is a serious concomitant parasitic infection. Therefore, the disease carrier fish at this point serves as a disease depot in a fish population. Besides the horizontal transmission, columnaris can be passed on from one culture unit or from one fish pond to another via *F. columnare*-contaminated equipment or dead fish harboring *F. columnare* (Tucker and Robinson 1990).

Diagnosis: Diagnosis of the causative agents of columnaris can be differentiated according to the types of infections. If it is an internal infection, *F. columnare* is only determined by laboratory isolation. If it is external infection, Noga (2010) and Plumb and Hanson (2011), suggested that a quick presumptive determination of *F. columnare* can be carried out by examining the wet mounts of external lesions on the body, fins and gills of the infected fish that appear with long slender rods (~0.50 to 1.0×4 to 10 μm) having flexing or gliding motion. They then added that the bacteria generally gather together to form a writhing mass that looks like a column or haystack after the wet mounts are allowed to stand for a few minutes. This diagnosis can be made under a microscope at magnifications of 200 x or larger based upon Tucker and Robinson (1990). Another *F. columnare* identification is

bacterial culture. With this regard, Noga (2010) stated that the result of the culture is not definitely assured if compared to the presumptive diagnosis, because possibly columnaris infections are predisposed to antibiotic or antiseptics. However, if cultivation and sensitivity are a must, he explained that using standard bacteriological media for those identifying methods is not effective, because *F. columnare* is reported to not have good growth on those media. Therefore, one must use fresh, poor nutrient content but rich in moisture media. Likewise, Wakabayashi (1993) and Plumb and Hanson (2011) suggested that the most often used media to culture *F. columnare* are cytophaga agar (CA) and Hsu-Shott agar. In addition, Ordal's and Hsu-Shotts media can also be used to prepare for broth. However, according to Bernardet and Bowman (2006) on trypticase soy agar (TSA) and nutrient agar or Marine 2216, there was no growth of *F. columnare.* Most importantly, growth of *F. columnare* does not happen in media containing sodium chloride concentrations more than 0.5% or a pH less than 6. Regarding incubation, *F. columnare* isolates can be incubated in 48 hours at temperatures of 25-30 C and most isolates after incubation appear as dispersal, rhizoid and detached colonies having yellow color in midpoints while strongly adhering to the media.

Treatment: Noga (2010) suggested that the effective ways to treat fish with early or mild infections of columnaris are antiseptic baths or long immersions in potassium permanganate or copper sulfate. On the other hand, if the infection is severe in which more than 5% of body surface is invaded, systemic antibiotics must be applied because normally isolates are susceptible to oxytetracycline and/or nifurpirinol. However, many of them were reported resistant to ormetoprim-sulfadimethoxine (Hawke and Thune 1992). According to Andrews et al. (1996), at the early stage, diseased fish must be treated with a

phenoxyethanol-based remedy while at the persistent or severe stage, antibiotic or antibacterial treatments must be applied. If diseased fish are anorexic due to severe infection, a potassium permanganate treatment is recommended for improving fish appetite prior to oral medication; however, there must be an improvement in environment along with any medical therapy (Noga 2010). Wood (1974) suggested that to reduce disease severity of columnaris, lowering the water temperature is also applicable. Raising salinity is also helpful although it is not yet validated in the clinical cases. Regarding chemotherapeutics and compounds used to treat columnaris, Wakabayashi (1993) stated that heavy metals, including copper sulphate and PMA (pyridylmercuricacetate) had been used before, but it is now curbed in some countries, such as Japan, UK, and USA, due to the buildup of the chemicals in the tissues of the diseased fish. For external columnaris, Durborow et al. (1998) suggested using potassium permanganate ($KMnO_4$) as an oxidizer of columnaris at 2 mg/l for the water having low organic load or at a higher concentration for the water having higher organic load. However, the 15-minute potassium permanganate requirement test must be conducted to determine the exact amount of potassium permanganate applicable to treat columnaris. As for systemic columnaris, Amend (1970) recommended adding sulphamerazine and oxytetracycline to the food to feed the fish in a two-phase administration, 220 mg/kg/day for 10 consecutive days then 50 to 75 mg/kg/day for other 10 consecutive days. Wood (1974) added that the usual antibiotic used in accordance with chemical bath treatment is oxytetracycline (Terramycin) combined with feed at the rate of 4 g/45 kg of fish fed 3% of fish body weight per day. According to the U.S. Fish and Wildlife Service (USFWS, 2011), oxytetracycline dihydrate (Terramycin 200® for fish) is being used nowadays as a feed additive to treat bacterial diseases, such as systemic columnaris. For a

standard therapeutic fish dose, USFWS (2011) suggested using 2.5-3.75 g oxytetracycline per 45 kg of fish per day for 10 days of treatment duration and 21 days for withdrawal period while 10 g oxytetracycline per 45 kg fish body weight per day for a high therapeutic fish dose for 14 days of treatment duration and 74 days for withdrawal period. MERCK Aquatic Animal Health (2014b) introduced AQUAFLOR®-COL®, an antibiotic which is claimed to be similar to florfenicol, for treating columnaris in catfish.

Control: As stated by Durborow et al. (1998), in order to diminish the outbreaks of columnaris, one needs to minimize stress factors on the cultured fish population via appropriate stocking and feeding. Furthermore, as recommended by Wood (1974), wild fish must be eliminated from the water supply and also minimize the number of other organisms that might exist in the environment where the fish are cultured. It is necessary to keep good environmental conditions in the culture units and handling must be stopped under high temperature (Tucker and Robinson 1990). Most importantly, excessive crowding must be avoided during fish transportation; also, seining fish in warm water must be done quickly. Vaccination is also another effective way to control or prevent columnaris infection. MERCK Animal Health (2014a) suggested using AQUAVAC-COL® as a vaccine to prevent columnaris in catfish.

2.4. Probiotics and the Use of Probiotics in Fish Culture

According to Schrezenmeir and Vrese (2001), probiotic simply is a Greek term combining *pro* and *bios* meaning “for life” or “supporting life”. Nowadays, probiotic refers to the bacteria that produce beneficial effects for both humans and animals (FAO and WHO 2001). In replacement to “probiotika”, the term “probiotic” was coined in the mid-1960s by

Lilly and Stillwell (1965) even though Metchnikoff and Tissier were reported as the first scientists who scientifically proposed the use of probiotic bacteria. With contradictory functions to antibiotics, probiotics at that time were referred to microbial-produced substances that extend the growth of other species. FAO (Food and Agriculture Organization) and WHO (World Health Organization. 2001) reported that Dr. Eli Metchnikoff, the Russian-born Nobel Prize recipient working at the Pasteur Institute, and Henry Tissier, a French pediatrician, were the early probiotic scientists who initially observed the beneficial roles of some selected probiotic bacteria. Metchnikoff advocated that since intestinal micro-flora rely on food, it is conceivable that the number of flora can be altered by adding useful microbes to replace the unfriendly gut microbes (Metchnikoff 1907). As for Henry Tissier, his observation revealed the abundance of irregular Y-shaped bacteria known as "bifid" bacteria in the gut of healthy children while scarce in the diarrhea-infected children. With this regard, he claimed that these bacteria could be used to restore a healthy gut flora of the diarrhea-infected patients (Tissier 1906). Hence, it is seen that probiotics were mainly studied for human and terrestrial animals in earlier times. For aquaculture, Gatesoupe (1999) reported that Kozasa was the person who firstly applied experimental probiotics in fish farming upon seeing the positive outcome resulting from the application of probiotics in human and poultry (Kozasa 1986). At that time, Kozasa used spores of *Bacillus toyoi* to mix with feed to increase the growth rate of yellowtail, *Seriola quinqueradiata.*

With regard to their definition, probiotics have been defined and redefined from time to time because the early definition was given based on various studies involving human and terrestrial animals, which is not appropriate for defining the probiotics used with aquatic

animals (Gomez-Gil et al. 2011). Sperti (1971) defined probiotic as the "tissue extracts that stimulate microbial growth". Then Parker (1974) defined the term probiotic as "organisms and substances that contribute to intestinal microbial balance". Fuller (1989) modified Parker's definition to "a live microbial food supplement that benefits the host (human or animal) by improving the microbial balance of the body". Verschuere et al. (2000) also agreed that Fuller's definition is precise and still widely used. However, they also redefined probiotics as "a live microbial adjunct 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 by improving the quality of its environment".

Concerning the types of probiotic bacteria, different probiotic bacteria exist in different organisms. *Bifidobacterium* is reported for its existence in human; *Lactobacillus* in swine, rodents and birds; *Enterococcus* in carnivores, etc.; however, *Lactobacillus* known as lactic acid-producing bacteria also exist in fishes, but they are just sub-dominant (Ringo and Vadstein 1998). As for the sources and types of aquatic probiotic strains in fishes, Balcázar et al. (2006) indicated that normally autochthonous and allochthonous microbiota of both saltwater and freshwater fishes have been isolated and used. To mention a few, *Vibrio sp.* and *Pseudomonas sp.* are gram-negative facultative anaerobic bacteria and the largest autochthonous gut microbes living in various kinds of saltwater fish species (Onarheim et al. 1994). On the other hand, Sakata (1990) described that in freshwater fishes, the dominant autochthonous microbiota are members of the genera *Aeromonas*, *Plesiomonas*, representatives of the family *Enterobacteriaceae*, and obligate anaerobic bacteria of the genera *Bacteroides*, *Fusubacterium*, and *Eubacterium*.

For the aquaculture sector, Irianto and Austin (2002) stated that some probiotics that have been used in aquaculture include both gram-negative and gram-positive bacteria, bacteriophages, yeasts (*Saccharomyces cerevisiae*) and unicellular algae (*Tetraselmis suecica*). They are available as commercial products or laboratory preparations. Both commercial and laboratory-generated probiotics have been used in artificial feed, live feed, i.e. *Artemia* and rotifers, and in the water. Wang et al. (2008a) emphasized that one of the popular aerobic, gram-positive probiotic species used in aquaculture is *Bacillus spp*. This probiotic species has the ability to minimize the number of bacterial pathogens to improve farm water quality. With this regard, Irianto and Austin (2002) explained that bacilli are found to act against serious pathogens in the aquatic environment. Besides *Bacillus spp.*, Gomez-Gil et al. (2011) described that lactic acid bacteria (LAB) and *Streptococcus phocae* are also gram-positive probiotic species, whereas gram-negative probiotic species are *Vibrios*, *Pseudomonadaceae*, *Aeromonadaceae*, and *Rhodobacterales* that are commonly used in aquaculture. However, mainly lactic acid bacteria have been found to be the most important group of probiotic bacteria due to the fact that LAB is the major component of fish intestinal microbiota (Ringo and Gatesoupe 1998).

So far, many researchers and farmers have tested and used various strains of probiotics in aquaculture in the hope of improving the growth, immune system (disease resistance) and survival of their cultured aquatic animals. Some researchers and aquaculturists claim that they are successful in using probiotics or probiotics are really effective in improving growth, health and survival rate of their aquatic animals or in improving water quality of the culture systems in their experiments or on their farms. In contrast, other researchers and farmers claim that probiotics have no significant effects on

their aquatic animals or their water quality. As evidence, Addo (2013) showed that the probiotic Lymnozyme® used as a water additive for 8 hours daily at a dosage of 3.95×10^5 CFU/ml for at least 3 days before challenge by immersion under tank conditions significantly reduced mortality in juvenile channel catfish from columnaris infection. However, when Addo (2013) used the probiotic PondToss™ as a water additive to study its effect on water quality, growth and production of channel catfish from juvenile to market size in earthen ponds over two years, he reported no significant differences in improvement in water quality, growth performance and production of the fish between untreated and treated ponds.

In recent decades, improved growth performance in tilapia fed probiotic diets has been reported by many researchers. Just to mention a few, a study done by EL-Haroun et al. (2006) to evaluate the effect of dietary probiotic Biogens® (*Bacillus sp.*) supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *O. niloticus* (L.) stated that after a 120-day experiment, mean weight gain, specific growth rate, protein efficiency ratio, protein productive value and energy retention were significantly higher in treatment groups of Nile tilapia fed with different levels of the probiotic Biogens® than Nile tilapia fed the control diet. Furthermore, a study about the effects of the probiotics *Enteroccus faecium* on tilapia *O. niloticus* growth performance and immune response conducted by Wang et al. (2008b) revealed that tilapia supplemented with probiotic *E. faecium* at a final concentration of 1×10^7 CFU mL^{-1} had significantly better final weight and daily weight gain (DWG) than those fed with the basal diet or control diet after 40 days. However, a study about the effects of probiotic bacteria as a dietary supplement on growth and disease resistance in young channel catfish, *Ictalurus punctatus*, (Shelby et al. 2007)

reported that no significant differences were observed for feed conversion among treated and control fish.

Besides improving growth performance of cultured fish, probiotics are also reported as a potential feed additive protecting farm-raised aquatic animals against infection by enhancing immunity (Welker and Lim 2011). Taoka et al. (2006) reported that viable probiotics fed to tilapia *O. niloticus* increased non-specific immune response, which was determined by some parameters such as lysozyme activity, neutrophile migration, and bactericidal activity, which improved the resistance of fish to infection by *E. tarda*. In contrast, having conducted 3 experiments, Shelby et al. (2007) found that there were no significant differences in levels of protein, immunoglobulin complement, lysozyme or anti-ESC antibody titers determined by ELISA noted between fish supplemented with the commercial probiotic (*Pediococcus* and *Enterococcus*) added diets and the control diet. As a result, they got high mortality of their experimental both probiotic treated and untreated young channel catfish after the fish were challenged by immersion with *E. ictaluri*, the causative agent of enteric septicemia (ESC) at the dosage of 6×10^6 CFU/mL for 30 minutes.

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CHAPTER II

The Potential of Pondtoss™ as a Control of Columnaris in Nile Tilapia *Oreochromis niloticus* and its Effects on Growth Performance

1. Abstract

This study was conducted to evaluate the efficacy of the probiotic product PondToss™ (a combination of Lymnozyme® and another product WSR™; Keeton Industries Inc., Wellington, CO) in enhancing growth and survival of Nile tilapia *Oreochromis niloticus* fingerlings when incorporated into the diet. Subsequently, the effectiveness of PondToss™ in reducing mortality from columnaris was tested in challenged Nile tilapia. To accomplish these objectives, the study was carried out in two phases. For the first phase, the experiment was conducted in a flow-through system receiving filtered pond water and located at the Auburn University E.W. Shell Fisheries Center, Auburn, AL, USA. This growth phase was designed for feeding control diet or probiotic diets (0.1% PondToss™ at 5.9×10^{7} CFU/g or 0.2% at 1.2×10^{8} CFU/g of diet) in the system for 56 days. The fingerlings were stocked at an average weight 3.42±0.14 g and were stocked at 30 fish/tank. During this phase, a commercial feed was top coated with PondToss™ (5×10^{10} CFU/g) and menhaden fish oil. To minimize exposure to columnaris and other diseases, a UV sterilizer was installed to treat incoming water. Temperature was maintained on average 25.4 C. Feeding rates were adjusted based upon actual biomass of each replicate, feeding response, actual mortalities, and feed inputs were re-adjusted every two weeks based upon

sampling of all fish per tank. The results at the end of this study showed that under the conditions of the present trial, none of the probiotic diets significantly improved growth parameters over those of the control. Mean final body weights were 35.3 ± 1.6 g, 35.2 ± 1.3g, and 34.5 ± 1.7g for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Mean percent weight gains were 930.4 ± 66.7%, 949.9 ± 41.6%, and 903.1 ± 49.5% for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Mean specific growth rates were 4.12 ± 0.09 % day^{-1}, 4.12 ± 0.06% day^{-1}, and 4.06 ± 0.05% day^{-1} for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Mean feed conversion ratios were 1.12 ± 0.04, 1.11 ± 0.01, and 1.12 ± 0.02 for control, 0.1% PondToss™ and 0.2% PondToss™, respectively. Last but not least, mean survival (%) of the treatment groups were also not significantly different from the control group and were 98.2 ± 1.5%, 96.2 ± 3.5% and 97.2 ± 3.5% for control, 0.1% PondToss™ and 0.2% PondToss™, respectively.

For the second phase, fish from each treatment in Phase 1 were combined and then randomly assigned to one of six treatments: C1 (Control fish now fed Control feed), C2 (Fish from Control now fed PondToss™ at 5.9×10^{7} CFU/g), C3 (Fish from 0.1% PondToss™ now fed Control feed), C4 (Fish from 0.1% PondToss™ now fed 0.1% PondToss™ at 5.9×10^{7} CFU/g), C5 (Fish from 0.2% PondToss™ now fed Control feed) and C6 (Fish from 0.2% PondToss™ now fed 0.2% PondToss™ at 1.2×10^{8} CFU/g) and then challenged with *F. columnare* strain ALG-530 by immersion at a dosage of 1.4 x 10^{6} CFU/mL None of the fish were negatively impacted nor experienced mortality. The failure to induce an outbreak of columnaris in all treatments could have been related to environmental conditions of the study, pre-exposure to *F. columnare,* or issues related to the *F. columnare* virulence or infection mode. Regardless, no conclusion could be made about

potential benefits of PondToss™ in reducing mortalities related to columnaris in Nile tilapia fingerlings.

2. Introduction

Having been documented to have been cultured around 4000 years ago and 1000 years before carps were farmed in China, tilapia, in family Cichlidae, nowadays are ranked as important species for both freshwater and saltwater aquaculture due to the fact that they are regarded as the second most important food fishes in the world and currently known as "aquatic chicken" (Fitzsimmons, 2000; El-Sayed 2006). Among tilapia species commercially cultured in almost 100 countries (Shelton and Popma 2006), Nile tilapia (*Oreochromis niloticus*) is the most cultured species because it has contributed 1.27 mmt, which is equal to 3.57% of the world aquaculture production in 2000 but increased to 5% in later years (Gupta and Acosta 2004; Shelton and Popma 2006). Regarding its world aquaculture production in 2012, FAO (2014) showed that around 3.2 mmt of Nile tilapia were produced with equivalent value of $US5.26 billion. In this regard, tilapia has become the shining star of aquaculture and surged further ahead of salmon and catfish aquaculture industries (Liping and Fitzsimmons 2011). Therefore, tilapia aquaculture production has increased rapidly due to increased demand for tilapia in both international and domestic markets. Thereby, tilapia production capacity has been intensified through promoting high stocking density and reusing water as being practiced by some intensive tilapia farming systems in the United States and other countries (Engle 1997). Unfortunately, water reuse systems and the intensified pond culture have resulted in a dramatic increase in the incidence and the severity of disease causative agents existing in tilapia (Shoemaker et al.

2006). Similarly, Plumb (1999) added that in contrast to those of the extensive farming systems, an overcrowded environment and poor water quality conditions under the intensified and water reuse systems do make tilapia more stressed and susceptible to a variety of highly infectious maladies including columnaris, a bacterial disease caused by *Flavobacterium columnare.*

Flavobacterium columnare is a gram-negative, aerobic, rod-shaped bacterial pathogen infecting the skin and gills of many freshwater fish species including tilapia. It is responsible for outbreaks in fish farms worldwide, causing high mortality rates (Frerichs and Roberts 1989). Marzouk and Baker (1991) showed that Nile tilapia were more prone to *F. columnare* resulting in high mortality when water pH was very acidic or alkaline, but less sensitive when water pH was near neutral. Plumb (1997) mentioned that with *F. columnare* advanced infections fish, the fish either swim slowly or float lethargically at the surface. Plumb added that uneven whitish to gray discolored areas on the skin and/or on fins, frayed or ragged fins and different sizes of pale necrotic lesions on the infected gills are characteristics used to describe columnaris infections in tilapia. It is reported that no freshwater fish including wild fish, farm-raised fish and ornamental fish are totally resistant to columnaris (Plumb 1999).

So far, columnaris infected fish have been treated in two different ways based on the types of infections, external infection or internal (systemic) infection. For external infections, potassium permanganate is used to combat columnaris at 2 mg/L and copper sulfate at 0.5-3 mg/L (Durborow et al. 1998; FAO 2014). As for systemic columnaris, Amend (1970) recommended adding sulphamerazine and oxytetracycline to the fish feed. According to the U.S. Fish and Wildlife Service (2011), oxytetracycline dihydrate

(Terramycin 200® for fish) is being used as a feed additive to treat bacterial disease such as systemic columnaris. However, generally, combined treatment of both internal and external infections are applied at the same time.

The use of such aforementioned therapeutic agents is now troublesome as a result of their gradual buildup in the environment and in fish that can be potentially dangerous to consumers and other organisms (Wakabayashi 1993; Aldermann and Hastings 1998). Additionally, lengthy use of these antibiotics could favor the development of antibiotic resistant strains, consequently, reducing drug efficacy (Aldermann and Hastings 1998; Marzouk et al. 2008). Because of these health threatening issues, greater emphasis has been placed on proper farming techniques through improving water quality, better nutrition, and proper sanitation, lowering stocking density, vaccination and using biological control agents (Robertson et al. 2000). For instance, the introduction of various strains of beneficial bacteria into fish gut to compete against groups of harmful bacteria is a type of biological control even though some researchers have still not yet agreed that it is a type of biological control. Those beneficial bacteria are known as probiotics that have an antimicrobial effect through improving the intestinal microbiota, releasing antibacterial substances (bacteriocins and organic acids), competing with pathogens to prevent them from attaching to the intestine, competing for nutrients essential for pathogen survival, modifying the immune system, and making an antitoxin effect. In recent decades, probiotics with different trade names have been used in both fresh and saltwater aquaculture. However, the effectiveness of these probiotics is still controversial.

In this study, the commercial probiotic PondToss™ was used as a feed additive to (1) evaluate its influence on the growth performance and survival of the Nile tilapia and (2)

explore its potential effects on the survival of juvenile Nile tilapia when challenged with *F. columnare*.

3. Materials and Methods

3.1 Experimental Design

The research was split into two main phases, the growth and disease challenge phases. The growth phase was conducted to study the growth performance of Nile tilapia after being fed with or without the probiotic PondToss™ while the disease challenge phase was designed for challenging the probiotic and non-probiotic (Control) fed fish to *F. columnare*, the causative agent of columnaris disease.

3.2 Phase 1-Growth Study

The growth study was conducted in a flow-through system consisting of 39 aquaria at the wet lab of Auburn University E.W. Shell Fisheries Center, Auburn, AL, USA for 56 days. Thirty-six aquaria were divided into 3 groups: control, treatment 1 (0.1% PondToss™ fed fish) and treatment 2 (0.2% PondToss™ fed fish). The other three aquaria were for the extra fish grown for the pre-challenge study. The aquaria for all the study groups were randomly assigned to treatments. Each 132-L aquarium contained 108 L of water and had a flow rate of 0.45 L/min, which was equal to 6 water exchanges per day. Each aquarium was aerated with an air stone receiving air from a regenerative blower to maintain the dissolved oxygen (DO) level near saturation. Pond water was used but was filtered by a filter system containing three sand filters (having sand inside) in a series (backwashed every week for 20-30 minutes per time) prior to flowing into the sump tank of the system. Since the study was done during some cooler months, the water was heated with a HTS5 Titan submersible heater

(PENTAIR 3,000 W, 240V, 1 ph, 12.5 amp) with a controller in an attempt to increase the temperature to at least 28 C. After being heated, the water was then pumped through a UV sterilizer (model ALQ60IL, Aqua Logic, San Diego, CA, U.S.A) with a maximum flow rate of 63.1 L/min (excess water returned to sump via release valve) before it was finally distributed into each aquarium via two main water distributing pipes on top of those aquaria. Flows to individual aquarium were adjusted with a small valve that was attached to the incoming end of each small hose connected to the main water distributing pipes. Regarding the experimental fish, 1170 Ivory Coast strain Nile tilapia fingerlings produced at the Auburn University E.W. Shell Fisheries Center were used for the study. The fingerlings were stocked at the average weight 3.42 ± 0.14 g and were stocked at 30 fish/tank. The fish were fed the control feed (Aquamax 200 and 300 having crude protein 50%) and acclimated for 5 days in water recirculated through the system at approximately 4 ppt salinity (through addition of rock salt) to limit incidental columnaris infections prior to the growth period. Prior to acquisition, the tilapia had been produced in pond hapas and then maintained in concrete raceways receiving recirculating pond water in the nursery facilities. These fish received a daily application of formalin to reduce the potential for any columnaris or other infection. Once the growth study started, the fingerlings were fed twice a day, in the morning and late afternoon, at a percent body weight ranging from 6 to 4%, decreasing as fish grew larger. Feeding rates were adjusted every two weeks after sampling. During sampling, the fish were removed from the tanks using soft mesh nets, counted and weighed in bulk in a tared bowl of water on a digital scale (Ohaus Scout Pro 4000 g, Ohaus Corporation, Parsippany, NJ, U.S.A) and then returned to the aquarium. Nets were dipped into a bucket with potassium permanganate and then a water bath in between sampling each aquarium. After all fish were

sampled, 2 mg/L potassium permanganate was applied to each aquarium to provide a 'bath' before feeding was resumed. Potassium permanganate was diluted gradually by the organic matter and the amount incoming water.

Water quality was monitored regularly to ensure good growing conditions. Temperature and dissolved oxygen (DO) were measured twice a day (early morning and late afternoon) with a YSI-85 digital temperature/DO meter (YSI Corporation, Yellow Spring, OH, USA). Total ammonia nitrogen (TAN) and pH were measured once a week from 2 randomly selected aquaria for each treatment using a YSI 9100 photometer (YSI Corporation, Yellow Spring, OH, USA). Any dead fish were counted, weighed and recorded. After 8 weeks, fish were weighed and counted prior to moving them to the S-6 Fish Disease Diagnostics Laboratory at the E. W. Shell Fisheries Center for the challenge study.

3.3. Diet Preparation

The probiotic and non-probiotic diets were prepared at the processing building in the complex of the E.W Shell Fisheries Center of Auburn University. Commercial feed Aquamax 300 and 400 (crude protein 50%), menhaden fish oil, distilled water and the probiotic PondToss™ (5.0×10^{10} CFU/g) were used to make 3 diets: diet 1 without PondToss™ (control), diet 2with 0.1% of PondToss™, and diet 3with 0.2% of PondToss™. The amount of feed for each treatment was weighed on a digital scale Ohaus Scout Pro 4000g (Ohaus Corporation, Parsippany, NJ, U.S.A) and placed into ziploc bags and put aside. Next, a 1000-ml flask was filled with 100 g of distilled water and 10 g of probiotics to make a total probiotic solution of 110 g based on 10/100 ratio being equivalent to 0.1%. The ingredients in the flask were then mixed using an electronic stirrer and mixed for 10 minutes

or longer. Afterward, the solution was poured into a spray bottle. Then it was sprayed onto the feed several times to add 11 g of solution by weight to yield the 0.1% inclusion rate of treatment 1 diets (0.1% probiotics). To ensure complete mixing, the feed in the ziploc bag was shaken thoroughly for 5 minutes for each time. Then it was top coated with 2% menhaden fish oil which was equal to 20 g of the total feed (1 kg). Oil top coating was also done by spraying oil for several times and followed by thorough shaking to ensure that the diets were well mixed. For the 0.2 % PondToss™ feed, the diet mixing procedure was done the same as that of the treatment 1, but the difference was that the amount of solution sprayed onto the feed was double (22 g/kg). As for the control diet, 10 g of distilled water and 20 g menhaden fish oil (2%) were added to the feed as previously described. Finally, diets were stored in the refrigerator and normally used within one week.

3.4. Bacterial Culture and Quantification

Samples of the first batch of diets were analyzed to quantify the number of probiotic bacteria present in a gram of each diet. To quantify the number of bacteria, four 1-g samples of each treatment diet were obtained. Each sample was then placed into a 15-mL tube containing 9 mL of phosphate buffered saline (PBS). Samples were left undisturbed for 30 minutes and then homogenized by careful stirring with cotton swaps. 20 µL of the homogenized samples were taken and added to 180 µl of PBS of each well on a 96-well plate in four replicates. Dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were made from the four replicate samples. After that, 100 µL of each dilution of each replicate was taken with an adjustable pipette and spread evenly on BHI (Brain-Heart Infusion) plates and

incubated at 30 C overnight. After incubation, the ideal plates for counting bacteria were those with bacterial CFU between 30 and 300 CFU.

3.5. Phase 2-Disease Challenge Studies

The disease challenge studies were held at the S6 Disease Laboratory, E.W. Shell Fisheries Center of Auburn University, AL, USA, for 15 days. Prior to the main challenge, two pre-challenge studies were carried out to determine the effective dosage and the virulence of *F. columnare* for the main challenge. The pre-challenge studies were conducted in six 47.6-L aerated, flow-through aquaria (0.43 L/min) with 40-L of well water heated and blended with cold water to maintain a water temperature and grouped into sets of two and then stocked with 10 naïve fish each from the three extra control aquaria (all control fish combined and then randomly assigned) associated with Phase 1. Then the fish were acclimatized for seven days prior to the pre-challenge studies beginning. The fish were fed at 3% of control feed and not fed 1-2 days prior to the challenge. *F. columnare* (strain ALG-530) were cultured and the number of colony forming units (CFU) determined by the dilution plate counting method. *F. columnare* strain ALG-530 were procured from the Auburn University Fish Disease Diagnostics Lab where they have been maintained at -80 C. Fish were challenged with *F. columnare* (strain ALG-530) at 3 different dosages 10^4, 10^5 and 10^6 CFU/mL. These were applied to each aquarium with different volumes of *F. columnare* for each set and fish then observed for 2 weeks. After inoculation, the fish were fed 1-2% biomass depending upon their feeding response. Fish were observed for any abnormal behaviors, lesions or disease clinical signs.

For the main challenge study, the fish were brought from the Phase 1 experimental units and acclimated for 7 days. During transportation, fish from each treatment were combined and treated with salt at 1.5‰ to avoid any potential external infections resulting from handling and transportation. Fish from each of the treatments in Phase 1 were then randomly assigned to the respective treatments: C1 (Fish from Control now fed Control feed), C2 (Fish from Control now fed 0.1% PondToss™ at 5.9×10^7 CFU/g), C3 (Fish from 0.1% PondToss™ now fed Control feed), C4 (Fish from 0.1% PondToss™ now fed 0.1% PondToss™ at 5.9×10^7 CFU/g), C5 (Fish from 0.2% PondToss™ now fed Control feed) and C6 (Fish from 0.2% PondToss™ now fed 0.2% PondToss™ at 1.2×10^8 CFU/g). They were grouped at 20 fish/aquarium and placed into the thirty six (36) 47.6-L aerated, flow-through aquaria (0.4 L/min) with 40-L of well water heated and blended with cold water to maintain a water temperature of 30 C for the duration of the experiment. Two replicates of each treatment were randomly assigned to each of 3 bays of aquaria to provide six replicates in a randomized block design. 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. Fish were maintained in the system 7 days prior to challenge and any mortalities replaced with equivalent size fish from their respective treatments. Prior to challenge, fish were fed at 2-1% of their wet body weight with the respective treatment feeds. Then fish were not fed for 2 days prior to challenge to induce stress. Post-challenge feeding rates were adjusted based upon response of fish to feed. Temperature was measured twice daily in each aquarium.

The challenge was done by placing fish from each aquarium into a bucket containing 3 L of water with aeration. Based upon pre-challenge research, 100 mL of *F. columnare*

strain ALG-530 of known CFU prepared at the School of Fisheries, Aquaculture and Aquatic Science disease laboratory was inoculated into the water to obtain the final dosage of 1.4×10^6 CFU/mL. Fish remained in the bucket for 60 minutes, and the fish and bucket contents were then returned to the appropriate aquarium under static water conditions for 3 additional hours. Water flow was then resumed. Fish mortality after challenge was to be monitored until no mortality occurred for 7 consecutive days in all aquaria. All moribund and dead fish were to be removed and observed for external clinical signs and the kidneys and livers sampled for pathogen recovery. The bacterial identification was to be carried out through bacterial isolation and wet mount observation under a microscope. At the end of each experiment, the number of mortalities in treatments were to be compared.

3.6. Growth Parameters

The mean final body weight of each treatment tank was determined by dividing the actual fish biomass by the number of fish of each tank at each sampling. Survival rate (SR), Specific growth rate (SGR), feed conversion ratio (FCR), and percent weight gain (%WG) were calculated using the following equations:

SR = (Total final number fish / Initial number of fish stocked) × 100

SGR (% body weight gain/day) = $[(\ln W_2 - \ln W_1) / (t_2 - t_1)] \times 100$

Where ln is the the natural logarithmic; W_1 and W_2 are body weights at the start and end of the growth period, respectively, and $t_2 - t_1$ is the length of the period in days.

FCR = Total wet weight of feed fed (g) / weight gain (WG) (g)

Where WG = Final body weight (g) – initial body weight (g).

%WG (percent weight gain) = 100 x (final weight-initial weight)/initial weight

3.7. Statistical Analysis

Data collected from the growth and challenge studies were analyzed by one-way analysis of variance (The GLM procedure) using SAS 9.3 (SAS Institute Inc. Cary, NC). Tukey-Kramer method was used for mean multiple comparisons. In the disease challenge experiment, the model used in the analysis included a completely randomized block design to minimize variation due to location of aquarium units in three different banks. Probabilities lower than 0.05 ($P<0.05$) were considered significant.

4. Results

4.1. Bacterial Quantification in Diets

The result of the bacterial quantification showed that there was no bacterial growth on the plates of the control feed. Diet 2 (0.1% PondToss™) contained 5.9×10^7 CFU/g, which was slightly more than the theoretical targeted dose of 5.1×10^7 CFU/g. In the 0.2% PondToss™ diet, there were 1.2×10^8 CFU/g, which is similar to the theoretical targeted dose of 1.02×10^8 CFU/g.

4.2 Growth Phase Water Quality Parameters

The temperatures in the morning and afternoon of each treatment are indicated in Table 1. In general, the mean temperature in the fish tanks in the morning across the 3 treatments was 25.2 ± 1.6 C and varied between 21.8 C and 28.3 C. On the other hand, in the afternoon, the temperature in the fish tanks averaged 25.6 ± 1.8 C and varied between 21.1 C and 28.5 C. Overall, the mean temperature across all three diets was 25.4 ± 1.7 C. The

overall mean temperatures across the 3 diets in the morning and afternoon were not significantly different (p= 0.9884).

Recorded dissolved oxygen (DO) levels were at acceptable levels throughout the experiment. Mean morning DO across the 3 treatments was 6.2 ± 0.5 mg/L. DO ranged between 4.4 mg/L and 7.8 mg/L. Overall afternoon DO was 5.7 ± 0.5 mg/L and ranged between 4.1 mg/L and 6.8 mg/L. Overall, the mean DO levels across all 3 groups were not significantly different (p= 0.3222) (Table 1). Mean DO across all treatment and control groups was 5.9 ± 0.5 mg/L.

Recorded pH and ammonia levels (Table 1) were also in acceptable ranges. The mean pH across the 3 treatments were not significantly different (p= 0.6743) and the overall mean was 7.3 ± 0.1, ranging between 7.2 and 7.5. Total ammonia nitrogen (TAN) was 0.6 ± 0.2 mg/L and ranged between 0.0 to 0.8 mg/L. Overall, mean TAN across treatments were not significantly different (p= 0.9784).

4.3 Growth Parameters

At the end of the growth study, growth parameters and survival were calculated and the means are presented in Table 3 and 4. The results at the end of this study showed that addition of PondToss™ to the feeds at these rates did not significantly improve mean final body weight (p=0.4297), which were 35.3 ± 1.6 g, 35.2 ± 1.3 g, and 34.5 ± 1.7 g for Control, 0.1% PondToss™, and 0.2% PondToss™, respectively. Mean percent weight gain (p=0.1159) were 930.4 ± 66.7%, 949.9 ± 41.6%, and 903.1 ± 49.5% for Control, 0.1% PondToss™, and 0.2% PondToss™, respectively. Mean specific growth rate (p=0.0838) were 4.12 ± 0.09 % day^{-1}, 4.12 ± 0.06% day^{-1}, and 4.06 ± 0.05% day^{-1} for Control, 0.1%

PondToss™, and 0.2% PondToss™, respectively. Mean feed conversion ratio (p=0.7052) were 1.12 ± 0.04, 1.11 ± 0.01, and 1.12 ± 0.02 for Control, 0.1% PondToss™, and 0.2% PondToss™, respectively. Mean survival (%) (p=0.1711) were 98.2 ± 1.5%, 96.2 ± 3.5% and 97.2 ± 3.5% for Control, 0.1% PondToss™, and 0.2% PondToss™, respectively.

4.4 Challenge Studies

During the challenge studies, mean morning temperature in the fish tanks across the 3 challenge treatments was 23 ± 2.0 C (Table 5) ranging between 20 and 26 C; the mean afternoon temperature was 23.4 ± 1.8 C ranging between 20 C and 26 C. Mean temperature across all the three systems was 23.2 ± 1.9 C. The mean temperatures of the treatment and control groups were not significantly different (p= 0.9651). After being challenged with *F. columnare* ALG-530 at a dosage of 1.4 x 10^6 CFU/mL, all Nile tilapia remained alive after 15 days and showed no external lesions.

5. Discussion

Probiotics have been reported to improve digestion by stimulating production of digestive enzymes, such as carbohydrases, phosphatases, esterases, lipases, peptidases, cellulases, and proteases or through other modifications in the gut environment, which could translate into improved growth performance (Nayak 2010a). Likewise, many researchers and farmers have tested and used various types of probiotics in aquaculture in the hope of improving growth, immune system (disease resistance) and survival rate of their cultured aquatic animals. Some researchers and aquaculturists claimed that they were successful in using probiotics to improve growth, health and survival rate of their aquatic animals or

improve water quality of the culture systems in their experiments and on their farms while others claimed that probiotics gave no improvement. EL-Haroun et al. (2006) evaluated the effects of dietary probiotic Biogens® (containing not less than 6×10^7 CFU/g of *Bacillus subtilis* Natto) supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *O. niloticus* ranging from 22.96 to 26.40 g. They showed that after a 120-day experiment, mean weight gain, specific growth rate, protein efficiency ratio, protein productive value and energy retention were significantly higher in treatment groups of Nile tilapia fed different levels of the probiotic Biogens® (0.5%, 1%, 1.5%, or 2%) than those of Nile tilapia fed the control diet. A 60-day study conducted by Essa et al. (2010) to determine the effects of four probiotic groups, *B. subtilis* NIOFSD017, *Lactobacillus plantarum* NIOFSD018, a mixture of *B. subtilis* NIOFSD017 and *L. plantarum* NIOFSD018 and a yeast, *Saccharomyces cerevisiae* NIOFSD019*,* isolated from healthy Nile tilapia, *O. niloticus*, at a dosage of 10^7 CFU/g of diets on growth performance, feed utilization and digestive enzymes activities of Nile tilapia also showed that all the diets containing different probiotic groups significantly ($p<0.05$) improved Nile tilapia (initial weight 24.55 ± 0.03g) growth and feed utilization compared to those of the control diet. Using PondToss™ at the dosages of 5.9×10^7 CFU/g and 1.2×10^8 CFU/g in Nile tilapia feeds for diets 2 and 3, respectively, for 56 days in the current study did not show any beneficial results. However, it should be noted that the commercial diet used probably exceeded many of the nutritional requirements of Nile tilapia; therefore, reducing potential benefits related to increased nutrient utilization. Under the conditions of the current growth study, PondToss™ as a feed additive at these rates did not provide any significant growth improvement or survival in the treated Nile tilapia compared to the non-treated.

Merrifield et al. (2010) and Welker and Lim (2011) suggested that the conflicting results obtained in probiotic studies might be due to variation in research conditions, such as the differences in the environmental conditions, handling practices, stocking densities, choice of probiotics, dietary concentrations, species strains, age/size of fish, feeding management and duration, dosage and virulence of challenge pathogens, and methods of challenge. Temperature is also known as one of the most influential factors affecting the physiology, growth, reproduction and metabolism of tilapia (El-Sayed 2006). In the current study, the mean temperature across all the treated and untreated groups was 25.4 ± 1.7 C. In the flow-through systems, DeLong et al. (2009) stated that tilapia are warmwater fish; therefore, they can grow best when the water temperature is approximately 27 to 29 C. Suresh and Bhujel (2007) stated that the optimum water temperatures for best growth are 28-30 C. Since Nile tilapia is a tropical species, FAO (2014) suggested a preferable temperature for best growth is 30-31 C. Therefore, compared to these suggested temperatures and those of the aforementioned studies by EL-Haroun et al. (26.9-28 C; 2006) and Essa et al. (28.2 C; 2010), the current study temperatures were below optimal growth conditions for Nile tilapia and possibly for the bacteria. Albeit growth may not had been maximized, all tilapia appeared healthy and in excellent condition. The temperatures, DO (5.9 ± 0.5 mg/l), pH (7.3 ± 0.1) and total ammonia nitrogen (TAN 0.6 ± 0.2 mg/l) were in acceptable and safe ranges for tilapia growth based on Suresh and Bhujel (2007). As for probiotic bacteria, the temperature and the pH of the present study were within the acceptable ranges for the bacteria to grow based on the PondToss™ manual by Keeton Industries.

Besides suboptimal temperatures, high stocking density might also have been a negative source affecting the growth performance of the experimental fish due to the fact that it could be an influential source of stress that could potentially affect fish appetite and feed uptake (small fish versus large fish). Likewise, Al-Dohail et al. (2009) added that lower stressor levels in fish fed with a probiotic-supplemented diet could possibly be connected to better growth performance and nutrient efficiency. A study about the effects of two different stocking densities (18.75 fish/m^3; 62.50 fish/m^3) on growth performance of male Nile tilapia *O. niloticus* fed *B. subtilis* at a dosage of 5×10^6 CFU/g of diet for 84 days by Telli et al. (2014) revealed that there were no significant differences in the growth performance between the treated and untreated fish. They found that the fish stocked at the higher stocking density showed lower mean individual feed intake, weight gain, individual final weight, and specific growth rate than those of the fish cultured at the lower stocking density. The current study's stocking density was 30 fish/tank containing 108 L of water, which is equal to 277 fish/m^3. Thus, the present study's stocking density is even higher compared to that of the previous study. This stocking density might not be an issue at the beginning of the study when the fish initial mean weight was 3.42 ± 0.14 g. Nonetheless, when the fish grew larger, this high density could have stressed the fish. Smaller fish might have had smaller chance of probiotic diet uptake. Slightly more than the current study, Addo (2013) stocked 40 fish (7.47 ± 0.11 g)/tank containing 100 L of water in a flow-through system for 56 days and found none of the diets significantly improved mean percent weight gain, specific growth rate or feed conversion ratio of the Nile tilapia at the end of the study.

The effectiveness of any probiotic on improving fish growth is also known to be correlated to dosage and feeding duration, because they could potentially affect viability of

the bacteria (Panigrahi et al. 2005; Merrifield et al. 2010; Welker and Lim 2011). In the recent study, Nile tilapia fingerlings were fed PondToss™ at the dosages of 5.9×10^7 CFU/g and 1.2×10^8 CFU/g of diet for 56 days, which is similar to the study conducted by Addo (2013) whose probiotic dosage and feeding period of *Bacillus* strains were 4.2×10^7 CFU/g and 56 days. At the end of the experiment, these two studies found growth parameters, such as mean percent mean weight gain, specific growth rate, feed intake, and feed conversion ratio for the treatments were not significantly different from those of fish fed the control diet. Two other experiments done by Shelby et al. (2006) using the probiotic Bioplus, *Bacillus spp*., at the dosages of 1×10^6 CFU/g and 1×10^8 CFU/g for 39 days and 63 days, respectively, did not find significant differences in growth performance among the treatment and the control Nile tilapia. Bisht and Pandey (2013) fed *B. subtilis* at three different dosages, 1×10^5, 1×10^8 and 1×10^{10} CFU/g of diet to *Labeo rohita* fingerlings. The results showed that the fish supplemented with *B. subtilis* for 90 days at the level of 1×10^8 CFU/g showed significant improvement in mean growth parameters (body weight gain, specific growth rate, condition factor and RNA DNA ratio, and feed conversion efficiency) in comparison to those of the other fish groups. It is possible that one limiting factor in obtaining positive effects in the fish growth in the current study may have been related to the dosages tested or possibly the feeding duration under the cooler temperatures. In agreement to that, Nayak (2010b) emphasized that the optimum concentration of probiotics is essential for the establishment and subsequent proliferation of the probiotics themselves in the fish gut.

Besides improving growth performance of cultured fish, probiotics also have been reported as a potential feed additive protecting farm-raised aquatic animals against infection

by elevating immunity (Welker and Lim 2011). Similarly, Cruz et al. (2012) stated that the use of probiotics is a feasible alternative for the inhibition of disease causative agents and disease control in cultured fish. They added that probiotic microbes are able to release chemical substances with bactericidal or bacteriostatic effects on pathogenic bacteria that reside in the intestine of the fish, hence, establishing a barrier against the multiplication of those opportunistic pathogens. Unfortunately, the current study was not able to confirm these claims due to the fact that challenge of Nile tilapia to columnaris at the dose of 1.4×10^6 CFU/mL did not induce a columnaris outbreak. *F. columnare* ALG-530 was not able to infect the experimental Nile tilapia leaving all of them alive with no clinical signs of columnaris infection.

There may have been factors influencing the results of this challenge study as well as the infection by *F. columnare* ALG-530. According to Snieszko's host-pathogen relationship (1974), environment is one potential factor influencing the results of infection which affects both the host and the disease causative agent. This relationship shows that infectious diseases can occur when vulnerable fishes are exposed to pathogens under certain environmental stress conditions, such as temperature changes, metabolic products of fishes, and pollution. In the current study, temperature and bacterial strains, stress and health of the fish prior exposure, concentration of *F. columnare* ALG-530, and method of disease challenge in relation to the fish species might be potential factors affecting the results of the study. Similarly, Merrifield et al. (2010) and Welker and Lim (2011) suggested that the conflicting results obtained in probiotic challenge studies might be the outputs of the variation in research conditions, such as dosage, virulence of challenge pathogens, and methods of challenge.

Schachte (1983) stated that generally temperature seems to be the main determining factor as to when infection may happen. In addition to Schachte, Wakabayashi (1993) added that generally when the conditions of the environment are suitable for *F. columnare* and stressful to fish, outbreaks can happen whenever the temperature of the water reaches 15 C and above. The mean temperature of the challenge study was 23.2 ± 1.9 C; so, it is higher than 15 C. In this regard, it could be assumed that the temperature of the study might not have been a potential factor preventing columnaris infection. However, Wakabayashi (1993) emphasized that *F. columnare* infects fish only when the temperature is comparatively high. Virulence of bacterial strain also influences the infection (Schachte 1983). Wakabayashi (1993) reported that in 1970, Pacha and Ordal studied the influence of water temperature on the pathogenicity of *F. columnare* strains of different levels of virulence in salmon. The results of their study revealed that at low temperature of 12.8 C, high-virulent strains were able to cause disease outbreaks and mortality by attacking susceptible fish. Different from high-virulent strains, low-virulent strains were only able to cause infection when the temperature was increased to 20 C. Therefore, *F. columnare* ALG-530, as either a high or low virulent strain, should have been able to infect Nile tilapia within the temperature of the current challenge study (23.2 ± 1.9 C).

Besides temperature and virulence of bacterial strains, dosage of *F. columnare* should be considered. Low or high concentrations of bacterial inoculum could be an influential factor on the success of the challenge. Indeed, the correlation of pathogen dosage to fish species is also a factor that should not be ignored. Nile tilapia under the current challenge study were challenged with *F. columnare* ALG-530 at a final dosage of 1.4×10^6 CFU/mL, and the challenge did not work. In contrast, Addo (2013) had a successful

challenge on channel catfish by inoculating *F. columnare* ALG-530 into the water with lower final dosages of 8.0 x 10^{5} CFU/ml and 9.0 x 10^{5} CFU/mL. So, different species of fish could have different susceptibility to pathogens, which could affect the effectiveness of the pathogenic dosage.

Schachte (1983) added that in an experiment, columnaris was spread from one fish to another directly when *F. columnare* of virulent strains were used. Likewise, it has been reported that virulent strains are able to infect fish when they are added to the water while low virulent strains infect fish only when they are injected to the fish (Snieszko and Bullock 1976). In this regard, *F. columnare* ALG-530 used in the current challenge study might not be a high virulent strain to the experimental Nile tilapia because the bacteria did not infect the fish when they were inoculated into the water column (immersion challenge). Therefore, they might be low virulent bacteria that should have been challenged via injection method. If the bacteria were not virulent while the fish were very healthy, it would be difficult for the infections to become established. Likewise, Amin et al. (1988) suggested that the fish must be stressed or injured in order to enhance the infections regardless if it is a low or high virulent bacterial strain.

Last, but not least, the quality of *F. columnare* and the immunity acquisition of the fish probably are also good factors to consider. The quality of the *F. columnare* used for challenge should be tested in order to confirm its pathogenicity. The present challenge study was not able to do so because the pre-challenge studies also did not work. Again, *F. columnare* ALG-530 virulence quality might have been degraded by prolonged storage in the media with 10% glycerol in the Revco-80C freezer having -80 C. On the other hand, since the experimental Nile tilapia were exposed to pond water at the hatchery facilities

prior to acquisition and during the growth study, albeit it run through a UV sterilizer, columnaris immunity acquisition of those fish might have been problematic. However, this assumption might not be true because all the fish at the end of the challenge study were alive and not all fish from the same batch would be expected to acquire immunity of a particular disease. That means there should have been at least some fish which would have been infected with columnaris resulting in at least some mortality or external signs of columnaris.

6. Conclusion

In the present study, the commercially-available probiotic PontToss™ applied at the dosages of 5.9×10^7 CFU/g and 1.2×10^8 CFU/g of diets for 56 days under the present research conditions did not produce significant effects on growth performance, survival or FCR among treated and non-treated Nile tilapia *O. niloticus* fingerlings. The lack of benefits achieved in this probiotic study might be a result of feeding probiotics under suboptimal temperatures, reduced growth under high stocking density, low application dosages (dietary concentrations), or short feeding duration. Likewise, this product might not work well when used as a feed additive for Nile tilapia. Additionally, the potential of PondToss™ to reduce mortality of Nile tilapia when exposed to *F. columnare* could not be confirmed due to the fact that the challenge was unsuccessful. The lack of infection under the experimental conditions with the Nile tilapia may have been related to the environmental conditions, bacterial virulence or dosage, method of challenge, or prior immunity acquisition. Therefore, additional studies may be needed with different naïve tilapia or different *F. columnare* strains to determine potential benefits of applying PondToss™ in feeds.

Table 1. Overall mean (± standard deviation) water quality parameters during the Nile tilapia growth trial in flow- through aquaria under laboratory conditions using water from a pond reservoir. Statistical analysis was done using One-way ANOVA, compared with Tukey-Kramer method ($p \leq 0.05$).

Water quality parameters	Mean ± S.D	Min	Max	P-value
Water temperature (A.M.) ^{O}C	25.2 ± 1.6	21.8	28.3	0.9981
Water temperature (P.M.) ^{O}C	25.6 ± 1.8	21.1	28.5	0.9821
Overall temperature ^{O}C	25.4 ± 1.7	21.2	28.5	0.9884
Dissolved oxygen (A.M.) mg/L	6.2 ± 0.5	4.4	7.8	0.2308
Dissolved oxygen (P.M.) mg/L	5.7 ± 0.5	4.1	6.8	0.7904
Overall dissolved oxygen mg/L	5.9 ± 0.5	4.1	7.8	0.3222
pH	7.3 ± 0.1	7.2	7.5	0.6743
Total ammonia nitrogen (mg/L)	0.6 ± 0.2	0.0	0.8	0.9784

Table 2. Diet conditions (compositions) and number of Colony Forming Units (CFU) in each diet of the three treatments fed to Nile tilapia fingerlings grown in flow-through aquaria for 56 days.

Treatments	Conditions	Formulated CFU/g
Diet 1 (Control, 0%)	Fed Commercial feed + 2% Menhaden fish oil	NA
Diet 2 (0.1% probiotic)	Fed PondToss™ (50 billon CFU/g) at 0.1% commercial feed + 2% Menhaden fish oil	5.9×10^{7}
Diet 3 (0.2% probiotic)	Fed PondToss™ (50 billon CFU/g) at 0.2% commercial feed + 2% Menhaden fish oil	1.2×10^{8}

Table 3. Mean survival (%)of juvenile Nile tilapia *O. niloticus* that were fed diets at 0 (control), 0.1 or 0.2% PondToss™ added to a commercial feed and grown for 56 days in flow-through aquaria receiving filtered, heated and UV-treated pond water . Values are means ± SD. and p-value of multiple comparisons of survival. Means in the same column with the same letter were not significantly different (p≥0.05) using ANOVA, and Tukey-Kramer method for mean multiple comparisons

Treatment	N Obs	Survival (%)
Diet 1 (control)	12	98.2 ± 1.5[a]
Diet 2 (0.1% PondToss™)	12	96.2 ± 3.5[a]
Diet 3 (0.2% PondToss™)	12	97.2 ± 3.5[a]

P-value = 0.1711

Table 4. Effects of two levels of the probiotic PondToss™ added to commercial fish feeds on the growth performance of juvenile Nile tilapia *O. niloticus* compared to a control diet in flow-through aquaria receiving filtered, heated and UV-treated pond water for 56 days. Values are means ± SD. Means within rows with the same letter are not significantly different (p ≤ 0.05) when analyzed using ANOVA and Tukey-Kramer method for mean multiple comparisons

Fish growth parameters*	Diet 1 (Control, 0%)	Diet 2 (0.1% PondToss™)	Diet 3 (0.2% PondToss™)	P-values > 0.05
IBW	3.4 ± 0.1 [a]	3.4 ± 0.1 [a]	3.5 ± 0.1 [a]	0.2135
FBW	35.3 ± 1.6 [a]	35.2 ± 1.3 [a]	34.5 ± 1.7 [a]	0.4297
%WG	930.4 ± 66.7 [a]	949.9 ± 41.6 [a]	903.1 ± 49.5 [a]	0.1159
SGR	4.12 ± 0.09 [a]	4.12 ± 0.06 [a]	4.06 ± 0.05 [a]	0.0838
FCR	1.1 ± 0.0[a]	1.1 ± 0.0[a]	1.1 ± 0.0[a]	0.7052

* IBW: initial mean body weight (g $fish^{-1}$)
FBW: final mean body weight (g $fish^{-1}$)
%WG (percent weight gain)
SGR (specific growth rate) (%day^{-1})
FCR (feed conversion ratio)

Table 5. Overall mean water temperatures in 3 flow- through aquaria systems during the Nile tilapia columnaris challenge trial using cold and hot blended well water.

Water quality parameters	Mean ± S.D	Min	Max	P-value
Water temperature (A.M.) C	23 ± 2.0	20	26	0.9842
Water temperature (P.M.) C	23.4 ± 1.8	20	26	0.9811
Overall temperature C	23.2 ± 1.9	20	26	0.9651

Table 6. Mean percent survival of Nile tilapia treatment groups and control 15 days post-challenge with *F. columnare* strain ALG-530 at a dosage of 1.4×10^6 CFU/mL by immersion. P-value is not available since all fish survived after the challenge.

Treatment	Treatment description	Survival (%)
C1-Control	Fish from Control now fed Control post-challenge	100
C2	Fish from Control now fed 0.1% PondToss™ at 5.9×10^7 CFU/g post-challenge	100
C3	Fish from 0.1% PondToss™ now fed Control feed post-challenge	100
C4	Fish from 0.1% PondToss™ now fed 0.1% PondToss™ at 5.9×10^7 CFU/g post-challenge	100
C5	Fish from 0.2% PondToss™ now fed Control feed post-challenge	100
C6	Fish from 0.2% PondToss™ now fed 0.2% PondToss™ at 1.2×10^8 CFU/g post-challenge	100
P-Value	NA	

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CHAPTER III

The Potential of PondToss™ as a Control of Columnaris in Channel Catfish, *Ictalurus punctatus*, and its Effects on Growth Performance

1. Abstract

This study was conducted to evaluate the efficacy of the probiotic PondToss™ product in enhancing growth of channel catfish *Ictalurus punctatus* fingerlings when incorporated into the diet. Subsequently, the effectiveness of PondToss™ in reducing mortality from columnaris was tested in challenged channel catfish. To accomplish these objectives, the study was carried out in two phases. The first phase or growth phase was designed for feeding a commercial feed (control, G1) with no added PondToss™ or the same commercial feed containing either 4.1×10^{7} CFU PondToss™/g of commercial feed (G2, 1% PondToss™) or 6.9×10^{7} CFU PondToss™/g of diet (G3, 2% PondToss™) in the flow-through system of 39 aquaria receiving filtered, UV-treated pond water, each 132 L containing 108 L of water with a flow rate of 0.76 L/min for 52 days. The fingerlings were stocked at 30 fish/aquarium at the average weight of 8.0 ± 0.18 g and cultured at an average temperature of 25.4 C. The results at the end of the study indicated that under the experimental conditions, none of the treatment diets significantly improved growth parameters ($P > 0.05$). Mean final body weight of fish maintained on the various diets were 36.2 ± 2.5 g, 34.5 ± 2.1 g, and 35.0 ± 1.9g for Control, 1% PondToss™ and 2% PondToss™, respectively. Percent weight gain were 351.1 ± 30.3%, 334.9 ± 28.4%, and 339.8 ± 22.2%

for Control, 1% PondToss™ and 2% PondToss™, respectively. Specific growth rate were $2.9 \pm 0.1\%$ day^{-1}, $2.82 \pm 0.1\%$ day^{-1}, and $2.8 \pm 0.1\%$ day^{-1} for Control, 1% PondToss™ and 2% PondToss™, respectively. Feed conversion ratio were 1.2 ± 0.2, 1.2 ± 0.1, and 1.1 ± 0.1 for Control, 1% PondToss™ and 2% PondToss™, respectively. Last, but not least, mean survival (%) of the treatment groups were also not significantly different from the control group after white spot (Ichthyophthiriasis) and columnaris disease outbreaks during the growth study. After termination of the ich infection, the mean survival rates of all treatments were $78.4 \pm 9.5\%$, $98.1 \pm 0.6\%$, and $95.0 \pm 3.8\%$ for the control fish, 1% PondToss™ fed fish, and 2% PondToss™ fed fish, respectively while after columnaris outbreak, mean survival rates of all group were 90.6 ± 5.6 %, 88.3 ± 7.4 %, and 91.9 ± 7.2 %, for the control fish, 1% PondToss™ fed fish, and 2% PondToss™ fed fish, respectively.

The disease challenge phase was conducted over a 20 day post-immersion challenge. Fish within each treatment in Phase 1 were combined and then randomly re-assigned to one of six treatments: C1 (fish from Control now fed Control feed), C2 (fish from Control now fed 1% PondToss™ at 4.1×10^{7} CFU/g), C3 (Fish from 1% PondToss™ now fed Control feed), C4 (Fish from 1% PondToss™ now fed 1% PondToss™ at 4.1×10^{7} CFU/g), C5 (Fish from 2% PondToss™ now fed Control feed) and C6 (Fish from 2% PondToss™ now fed 2% PondToss™ at 6.9×10^{7} CFU/g). Fish were then challenged with *F. columnare* strain ALG-530 by immersion based upon doses determined during the pre-challenge study. The challenge dosage was 3.4×10^{6} CFU/mL as a final dosage of each replicate. Post-challenge, all moribund and freshly dead fish were removed and observed for external clinical signs and the skin, gills, kidneys and livers sampled for pathogen recovery. Isolated colonies were identified based on their color and colony structure, yellowish rhizoid structure

on the Hsu-Shott agar and wet mount observation under a microscope at magnifications of 400x. The results at the end of the challenge study showed that there were no significant differences in mortalities among treatment and control groups (P=0.1397) under the given experimental conditions. Mean cumulative mortality rates were 52 ± 24.9%, 56 ± 16.7%, 72 ± 19.2%, 58 ± 14.8%, 52 ± 26.8%, and 82 ± 14.8% for C1, C2, C3, C4, C5 and C6, respectively. This result suggested that PondToss™ was not able to reduce mortalities of channel catfish from columnaris under the present study conditions.

2. Introduction

Although channel catfish *Ictalurus punctatus* (Rafinesque 1818) has not yet become one of the globally farm-raised species like tilapia; 376,352 tonnes of channel catfish were produced worldwide with a value of US$ 680,262,000 (FAO Year Book 2011). Most importantly channel catfish is a major aquaculture species farmed commercially in the United States (Wellborn 1988). The four major channel catfish producing states are located in the southeastern region of the United States, namely Mississippi, Alabama, Arkansas, and Texas. They shared about 95% of the US total sales in 2012. Between 2011 and 2013, Mississippi was the top producing state having 19,668ha of catfish farming areas (NASS 2013). In 2013, the US catfish growers had sales of US$ 342 million, which was up 1% from US$ 341 million from 2012 (NASS 2014). As for Alabama, channel catfish is well-known as the most dominant farm-raised species. According to Boyd et al. (2000), there were about 10,000 ha of channel catfish production ponds with 10.7% of the area for fry and fingerlings and 89.3% for food fish. Food fish production was about 37,014 tonnes in 1997.

Also, it is reported that channel catfish aquaculture is among the biggest sources providing the employment and income in west Alabama (Brown 2010).

Although the US channel catfish industry grew quickly, especially in the southern United States, the stability of the market has been seriously threatened by some big issues, such as the intense competition with the influx of imported catfish products (Mekong catfish, *Pangasius hypophthalmus*) from Vietnam, the increasing production and investment costs due to the rise and fall of commodity prices, and environmental concerns and diseases (FAO 2014). Therefore, diseases have been reported as one of the most concerning issues causing economic losses in the channel catfish aquaculture industry in the U.S. Likewise, Hawke et al. (1981) also agreed that disease outbreaks mainly caused by infectious bacteria were the most highly recognized obstacle in the channel catfish farming economic growth. For instance, annually the channel catfish industry loses more than US$ 50 million to catfish mortalities caused by the infectious bacterial diseases (Addo 2013). Basically, Hawke et al. (1981) pointed out that *Flavobacterium columnare*, the causative agent of columaris, and *Edwardsiella ictaluri*, the causative agent of enteric septicemia of catfish, are the two bacterial species that are responsible for most of the bacterial-related economic losses in the channel catfish farming. In agreement to Hawke et al. (1981), Tucker and Robinson (1990) mentioned columnaris disease was the second most infectious bacterial disease of farm-raised channel catfish industry behind enteric septicemia of catfish (ESC). Shoemaker et al. (2011) stated that the economic losses to channel catfish farmers from columnaris diseases were estimated at US$30 million annually in the U.S.

Columnaris has the ability to infect and cause disease in most freshwater fish, including farm-raised channel catfish and other ictalurids. To mitigate columnaris disease

problems in aquaculture, antibiotics and other chemicals have been used to treat diseases. Unfortunately, these treatments can cause many issues in human health and wellness of the environment. Similarly, the pathogens may become antibiotic-resistant and cause potential negative consequences to human health and the environment (Toranzo et al. 1984; McEwen and Fedorka-Cray 2002; USDA 2003; FAO 2006; Marzouk et al. 2008; Welker and Lim 2011).

Upon seeing the aforementioned issues and based on the outcomes of their research, many researchers suggested that probiotics are possible alternatives to these antibiotic-based treatments. Therefore, the use of probiotics for disease prevention and improved nutrition in aquaculture has gained increased attention as demand for environmentally friendly practices has increased (Robertson et al. 2000; Verschuere et al. 2000, Vine et al. 2006; Shelby et al. 2006; Wang et al. 2008; Cruz et al. 2012). However, research results are often inconsistent or contradictory. Some researchers and aquaculturists claim that they are successful in using probiotics to improve growth, health and survival rate of their aquatic animals from the diseases in their culture systems, while others claim that probiotics have no significant effects on growth and survival rate.

As a contribution to the research on probiotic efficacy in aquaculture, in this study, the commercial probiotic PondToss™ (a combination of Lymnozyme® and another product WSR™, the probiotic portion from Lymnozyme and the water conditioning bacteria from WSR). It is a freeze-dried biological formula of natural microbes, enzymes, micronutrients and amino acids on a special carrier designed to improve microbial growth rates. Both are commercially-available aquatic products from Keeton Industries Inc., Wellington, CO, and are dry water-soluble probiotic concentrates. PondToss™ was used as

a feed additive to (1) evaluate its influence on growth performance and survival of the channel catfish and (2) explore its potential effects on the survival of juvenile channel catfish when challenged with *F. columnare*.

3. Materials and Methods

3.1 Experimental Designs

There were two main phases in this study, growth and disease challenge phases. The growth phase was carried out to study the growth performance of channel catfish after being fed with or without the probiotic PondToss™ while the disease challenge phase was designed for challenging the fish from the growth study with *F. columnare*, the causative agent of columnaris disease.

3.2 Phase 1-Growth Study

The growth study was conducted in a flow-through system at the wet lab of Auburn University E.W. Shell Fisheries Center, Auburn, AL, USA for 52 days. The system consisted of 39 aquaria with 36 of them allocated to 3 treatments: control (no PondToss™), treatment 1 (1% PondToss™ fed to fish) and treatment 2 (2% PondToss™ fed to fish). The other 3 aquaria were added for the extra fish to be used for the pre-challenge study, but those were also fed the same diet as the control. Assignment of treatments to the aquaria was completely randomized. Each 132-L aquarium contained 108 L of water and had an approximate flow rate of 0.76 L/min, which was equal to 10 water exchanges per day. Each aquarium was aerated with an air stone to maintain the dissolved oxygen (DO) level near saturation. Pond water was used but was filtered by a filter system containing three sand

filters in a series (backwashed every week for 20-30 minutes per time) prior to flowing into the sump tank of the system. To stabilize and maintain temperature, the water was heated with a HTS5 Titan submersible heater (PENTAIR 6,000 W, 240V, 1 ph, 12.5 amp) to increase the temperature to at least at 28 C. However, during the study, the temperature was not steadily maintained at 28 C and it was lower than the desired temperature during the first week of the study. After being heated, the water was then pumped through a UV Sterilizer (model ALQ60IL, Aqua Logic, San Diego, CA, U.S.A) with a maximum flow rate of 63 L/min, and this maximum flow rate was cut to ~53.03% to flow back into the sump tank before it was finally distributed into each aquarium. So, the actual maximum flow rate was 29.6 L/min. 1170 naïve, never previously exposed to columnaris, channel catfish fingerlings were used for the study and were obtained from the S6 disease laboratory of Auburn University where they had been isolated and raised in uncontaminated well water. The fingerlings were stocked at the average weight 8.0 ± 0.18 g and were stocked at 30 fish/tank. The fish were acclimated for 21 days in recirculating water at ~2.4 g/L salinity through addition of rock salt (sodium chloride) to avoid incidental columnaris infections prior to the growth study and fed a commercial feed (Aquamax 300 and 400 having crude protein 50%-45%). Once the growth study started, the fingerlings were fed twice a day, morning and late afternoon, at a percent body weight ranging from 6 to 2.5%. Basically, feeding rates were adjusted every two weeks after sampling, but they were also adjusted based on fish feeding response. During sampling, the fish were removed from the tanks using soft mesh nets, counted and weighed in bulk in a tarred bowl of water on a digital scale (Ohaus Scout Pro 4000g, Ohaus Corporation, Parsippany, NJ, U.S.A) and then returned to the aquarium. Nets were dipped into a bucket with potassium permanganate (~10 mg/L) and then a water bath in

between sampling each aquarium. After all fish were sampled, 2 mg/L potassium permanganate was applied to each aquarium to provide a 'bath' before feeding was resumed.

Water quality was monitored regularly to ensure good growing conditions. Temperature and dissolved oxygen (DO) were measured twice a day, morning and late afternoon, with a YSI-85 digital temperature/DO meter (YSI Corporation, Yellow Spring, OH, USA). Total ammonia nitrogen (TAN) and pH were measured once a week from 2 randomly selected aquaria from each treatment using a YSI 9100 photometer (YSI Corporation, Yellow Spring, OH, USA). Any dead or moribund fish were removed daily, counted, weighed and data recorded. After 52 days, the growth study was terminated and the fish from each aquarium were weighed in bulk, counted and moved to the S-6 Fish Disease Diagnostics Laboratory at the E. W. Shell Fisheries Center.

3.3. Diet Preparation

The probiotic and non-probiotic diets were prepared at the nutrition processing building in the complex of the E. W. Shell Fisheries Center of Auburn University. The commercial feeds Aquamax 300 and 400 (crude protein 50-45%), menhaden fish oil, and the probiotic PondToss™ (5×10^{10} CFU/g of product) were used to make 3 diets, diet 1 without PondToss™ (control), diet 2 containing 1% PondToss™, and 3 diet containing 2% PondToss™. To prepare each diet, the amount of feed for each treatment was weighed on a digital scale (Ohaus Scout Pro 4000g, Ohaus Corporation, Parsippany, NJ, U.S.A) in a ziploc bag. Next, a 1000-ml flask was filled with 180 g of distilled water and 20 g of PondToss™ to make a total 1% PondToss™ solution of 200 g. Then the flask was put on an electronic stirrer with a stir bar so that a well-mixed solution could be achieved at the end of

stirring. The solution was stirred for at least 10 minutes and ended when well-mixed solution was obtained. Afterward, the solution in the flask was poured into a spray bottle for application to the feed that was being prepared. The solution was continually mixed manually while spraying it on the feed to add 100 g of solution to 970 g of feed to yield the 1% inclusion rate for treatment 1 diets (1% PondToss™). To ensure that all feed pellets were evenly contacted with the bacterial solution, the feed in the ziploc bag was manually shaken thoroughly for 5 minutes for each time. It was then top coated with 20 g/kg of menhaden fish oil, which was equal to 2% of the total feed. Oil top coating was also done with oil spraying for several times and thorough shaking of feed in the bag to ensure well mixing and even coating. As for treatment 2 (2 % probiotics), the diet mixing procedure was done the same as that of the treatment 1, but the amount of probiotics and the total solution to spray on the feed required 40 g probiotics to mix with 180 g of distilled water. 110 g of probiotic solution were then spray onto 960 g of feed to yield the 2% inclusion rate for treatment 2 diets. As for the control diet, 100 g of distilled water and 20 g of menhaden fish oil (2%) were sprayed onto 980 g of feed and top coated. The diets were then dried at the temperature between 40 and 50 C in the forced air dryer having an air heater (Titan 120V, 60Hz, 1500W). with the control diet on top rack, 1% PondToss™ diet on middle rack and 2% PondToss™ diet on bottom rack for an hour to remove the excess water used as a mean to spray the probiotic onto the feed. Finally, the diets were stored in freezer ziploc bags in the refrigerator. To avoid contamination and molding, the bags of the diets were put on the rack of the refrigerator that allowed enough air circulation and proper temperature.

3.4. Bacterial Culture and Quantification

After each diet preparation, samples of the diets were analyzed to quantify the number of probiotic bacteria present per gram of each diet. To quantify the number of bacteria, three diet samples (1 g each) of each treatment were obtained. Each sample 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 by careful stirring with cotton swaps. 20 µl of the homogenized samples were taken and added to 180 µl of PBS in each well of a 96-well plate in four replicates that was already prepared. Dilutions of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} were made from the four replicate samples. After that, 100 µl of each dilution of each replicate was taken with an adjustable pipette and spread evenly on BHI (Brain-Heart Infusion) plates and incubated at 30 C overnight. After the incubation, total plate counts performed with the ideal plates for counting bacteria having bacterial CFU between 30 and 300.

3.5. Diagnosis and Treatment of White Spot and Columnaris Diseases During Growth Study

During the growth phase, there were two disease events that were diagnosed and treated. The first was white spot disease or "Ich" (Ichthyophthiriosis) caused by *Ichthyophthirius multifilis*, which occurred in the middle of the third week of the growth study. It was diagnosed primarily by examining the grainy skin and gills of the fish with unaided eyes and examining under a microscope. Under a microscope, Ich appeared rounded with moving cilia and the C-shaped macronucleus, which was easily seen in large size trophonts. After diagnosis, all fish were treated by applying acidified copper sulphate to the

reservoir pond, which was the main source of the water for the experimental system. The UV light in the system was also removed and cleaned thoroughly. The system was also put on recirculating mode to cut off any new influx of Ich, which might come from the pond and the temperature was increased to maintain temperatures above 28 C to speed up the life cycle of Ich. Formalin was applied to the system at a dosage of 25 mL/m^3 for 3 consecutive treatments. The formalin was poured into the sump tank allowing the pump to distribute the formalin solution to each aquarium. Each treatment was applied once every two days for one hour. After an hour treatment, the water in the each aquarium was not discharged leaving the remaining formalin to leave the water by aeration. Dead fish were removed, counted and weighed. The fish were not fed for the first and second days of the Ich outbreak, then feeding was resumed. Thereafter, the feeding rate ranging from 1-4%, was applied based on fish feeding response. On the 7th day, after all three treatments were completed, white spots on the fish started to disappear and the fish were put back on flow-through system. It took 11 days to get the system back to its normal operation.

After white spot disease, columnaris outbreaks occurred at the end of week 5. To diagnose columnaris, all moribund and freshly dead fish were removed to observe the external clinical signs. Then, necropsies were conducted to isolate the bacteria from the external lesions, skin, gills, kidneys and liver on Hsu-Shott agar plates for bacterial identification. After 48 hours at 30 C incubation, *F. columnare* were identified based on their color and colony structure, yellowish rhizoid structure on the culture media. Additionally, small amounts of *F. columnare* isolates on each plate were smeared on the wet mounts to conduct further bacterial identification under a microscope at magnifications of 400x. No antibiotic treatment was applied to treat the fish, but potassium permanganate was

applied to the system at a dosage of 2 mg/L as a prolong bath in each aquarium for 2 times. The fish were not removed out of the aquarium. Any dead fish found were removed, counted and weighed quickly. Removing freshly dead columnaris-infected fish from the system helps minimize the infectious pressure of *F. columnare* on the fish in the whole system (Shoemaker and LaFrentz 2014)

3.6. Phase 2-Disease Challenge Studies

The disease challenge studies were conducted at the S6 Disease Laboratory, E. W. Shell Fisheries Center of Auburn University. Prior to the main challenge, a pre-challenge study was carried out to determine the effective dosage of *F. columnare.* The pre-challenge study was conducted in six aquaria grouped into sets of two and then stocked with 10 fish each. Due to the shortage of fish, a total of 30 extra fish from the growth study control tanks were moved to S6 in addition to another 30 naïve fish from the same original stock from S6. The fish were acclimated for 6 days. While the fish were being acclimated, *F. columnare* strain ALG-530 isolation procedure was conducted on other 6 S6 naïve catfish to obtain new isolates. Those new isolates were used for the pre-challenge study. Once the infections occurred, all freshly dead and moribund fish infected with *F. columnare* strain ALG-530 were necropsied to isolate the new bacteria on Hsu-Shott agar plates. To prepare for the pre-challenge, the new isolates were transferred to a flask containing Hsu-Shott broth for culture. Once they were ready for the challenge, 10 mL sample was kept to quantify the number of bacteria per milliliter by the dilution plate counting method so that the doses of bacteria used for pre-challenge study were known.

During the pre-challenge, six aquaria containing 10 fish were divided into 2 groups. Group 1 was 2 tanks stocked with 20 naïve fish from the S6 disease lab and 1 tank stocked with 10 fish from the control fish from the growth study. These were inoculated with 2 mL of *F. columnare* broth at a concentration of 4.3×10^5 CFU/mL. Group 2 had 1 tank stocked with 10 naïve catfish from the S6 disease lab and 2 tanks stocked with 10 fish from the control tanks from the growth study and these were inoculated with 20 mL (4.3×10^6 CFU/mL) of *F. columnare* broth. The fish from each aquarium were removed from the tanks and exposed to *F. columnare* for 1 hour in 5-L buckets, each containing 2 L of aerated water. Then, the contents were poured back into the aquaria for another 6 hours under aerated, static conditions before the water was flushed out by well water of the system for 1 hour. The fish were kept in static water in the aquaria except in the morning and afternoon that the water was continuously flushed out for one hour and 2 times/day as part of water quality management. The fish were observed for 9 days. All freshly dead and moribund fish were collected and recorded for diagnosis to confirm that they were killed by *F. columnare.*

The main challenge study was then carried out over a 20-day post-challenge period at the S6 Disease Laboratory under static, aerated water conditions. The fish were brought from the Phase 1 experimental units. Fish from each treatment in Phase 1 were combined together and then randomly re-assigned to one of six treatments: C1 (Fish from control now fed control feed post-challenge), C2 (Fish from control now fed 1% PondToss™ post-challenge), C3 (Fish from 1% PondToss™ now fed control feed post-challenge), C4 (Fish from 1% PondToss™ now fed 1% PondToss™ post-challenge), C5 (Fish from 2% PondToss™ now fed control feed post-challenge) and C6 (Fish from 2% PondToss™ now fed 2% PondToss™ post-challenge). Before the challenge started, the fish

were fed with their respective diets given in the growth study. Ten (10) fish were placed into each of thirty (30) 47.6-L aquaria, which were aerated and contained ~40-L of well water. Each treatment had 5 replicates. The replicates of each treatment were randomly assigned to each of 3 bays of aquaria to provide a randomized block design. 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. Fish were maintained in the system 7 days prior to challenge and any mortalities replaced with equivalent size fish from the respective treatments. To minimize pre-exposure to columnaris, salt was added to each tank after each 1-h water flushing at approximate salinity of 1-2 ppt. Despite prevention efforts during transportation and acclimation, several fish in the challenge system became infected with columaris. Therefore, two potassium permanganate prolonged bath treatments were applied at a rate of 2 mg/L for 4 hours in all tanks. Pink color was maintained within the four hours by adding additional potassium permanganate when the color deviated from pink. After potassium permanganate treatments, the water was flushed out with well water by opening the valve on top of each aquarium until the water was crystal clear. Then salt was added back into each aquarium. During acclimation, the fish were fed at 2% of their biomass with the respective treatment feed given during the growth study. The fish were not fed for 2 days prior to challenge. Post-challenge feeding rate was adjusted based upon response of fish to feed, but mainly it was assigned at 2%.

Due to mortalities occurring during the acclimation, a number of fish per tank were reduced. There were 6 treatments and each had 5 replicate tanks. Once the challenge started, the fish from the same treatment were removed out of the tanks and placed into one

container (~ 150 L) to randomize all the fish again and then 10 fish were counted and placed into a bucket containing 2 L of water with an air stone. Next, *F. columnare* broth was inoculated into the bucket at a rate of 15 ml (3.4×10^6 CFU/mL) based upon pre-challenge research. The fish were exposed to the bacteria for 2 hours. Then the contents were returned to the appropriate aquarium under static water conditions for 4 additional hours before the water was flushed out for 1 hour at a flow rate of ~0.7 L/min. Therefore, the fish were exposed to the bacteria under static, aerated conditions for 6 hours. Then, feeding and water quality checks resumed while fish mortality after challenge was monitored until no mortality occurred for 7 consecutive days in all the aquaria. All moribund and dead fish would be removed, observed and external clinical signs recorded. Necropsies were conducted to isolate and identify the bacteria as previously described. At the end of the experiment, the number of mortalities in treatments were compared.

3.7. Growth Parameters

The mean initial fish body weight and the mean final body weight of each treatment tank were determined by dividing the actual fish biomass by the number of fish in each tank. Survival rate (SR%), specific growth rate (SGR), feed conversion ratio (FCR), and percent weight gain (%WG) were calculated using the following equations:

SR% = (Total survival fish / Initial number of fish stocked) × 100

SGR (% body weight gain/day) = $[(\ln W_2 - \ln W_1) / (t_2 - t_1)] \times 100$

Where ln is the the natural logarithmic; W_1 and W_2 are body weight at the start and end of the growth period, respectively, and $t_2 - t_1$ is the length of the period in days.

FCR = feed intake (g) / weight gain (WG) (g)

Where WG = Final body weight (g) – initial bodyweight (g).

%WG (percent weight gain) =100 x (final weight-initial weight)/initial weight

3.8. Statistical Analyses

Data collected from the growth and challenge studies were analyzed by one-way analysis of variance (the GLM procedure) using SAS 9.3 (SAS Institute Inc. Cary, NC). Tukey-Kramer method was used for mean multiple comparisons of growth parameters. The survival rates during the growth study were analyzed by non-parametric one way ANOVA using Kruskal-Wallis test (In control group, a tank that had 0% survival rate was removed from analysis except for the White spot disease infection survival rate). In the disease challenge experiment, the model used in the analysis included a completely randomized block design to minimize variation due to location of aquarium units in three different banks. Probabilities lower than 0.05 ($P \leq 0.05$) were considered significant

4. Results

4.1 Growth Study Water Quality Parameters

Based upon Table 1, the mean morning temperature in the fish tanks across the control and treatment groups was 27.6 ± 2.0 C. The temperature varied between 21.5 C and 31.2 C while in the afternoon, the overall mean temperature was 28.5 ± 1.9 C and varied between 23.7 C and 31.6 C. In general, the temperature across all the three groups was 27.7 ± 2.0 C. The overall mean temperatures across the 3 diet treatments in the morning and afternoon were not significantly different (p= 0.9119).

The dissolved oxygen in the fish tanks (Table 1) across the 3 diet treatments in the morning was 5.8 ± 0.3 mg/L and ranged between 4.3 mg/L and 6.1 mg/L while afternoon DO was 4.9 ± 0.3 mg/L ranging between 4.3 mg/L and 5.8 mg/L. Mean DOs were not significantly different across treatments (p= 0.7086) and the overall mean DO was 5.9 ± 0.5 mg/L.

The mean pH values (Table1) across the 3 diet treatments were not significantly different (p= 0.9259). The overall mean pH across all diet treatments was 7.4 ± 0.2 and ranged between 7.2 and 7.8. Additionally, total ammonia nitrogen (TAN) was 0.6 ± 0.2 mg/L and ranged between 0.2 to 0.8 mg/L. TAN was not significantly different (p= 0.8565) across the whole system. Also, total alkalinity was 78.0 ± 8.1 mg/L and ranged between 65 and 102.5 mg/L. The results showed that there were no significant differences in total alkalinity among the treatment and control groups (p= 0.5452).

4.2 Bacterial Quantification in the PondToss™ and Experimental Diets

The results of the bacterial quantification can be found in Table 2 and indicate that the level of bacteria in a gram of PondToss™ product given by the company and used in calculating feed mixs was 5×10^9 CFU/g while the documented dose was 1.02×10^9 CFU/g using this quantification method. There were 12 batches of diets made during the whole study. Based on the bacterial quantification, group 1 (control diet) contained on average $2.5 \pm 0.63 \times 10^4$ CFU/g. The 1% PondToss™ diet contained an average of $4.1 \pm 0.26 \times 10^7$ CFU/g, which is close to the theoretical targeted dose of 5×10^7 CFU/g. As for the 2% PondToss™ diet, it contained $6.9 \pm 0.70 \times 10^7$ CFU/g on average, which is less than the theoretical targeted dose of 1×10^8 CFU/g.

4.3 White Spot and Columnaris Disease Outbreaks During the Growth Study

White spot disease or Ichthyophthiriasis caused by *Ichthyophthirius multifiliis* infected the experimental catfish in the middle of the third week of the growth study excluding the acclimation period (Fig.1). It happened when the temperature dropped to 26.9 C in the system but lower in the reservoir pond during a cold front, which provided optimal conditions for an ich outbreak. After termination of the ich infection, the mean survival rates of all treatments were 78.4 ± 9.5%, 98.1 ± 0.6%, and 95.0 ± 3.8% for the control fish, 1% PondToss™ fed fish, and 2% PondToss™ fed fish, respectively. Based on Table 4, PondToss™ had no significant influence among the treated and untreated probiotic fish (P= 0.4239). The lack of significance was partly due to the high within treatment variability.

At the end of week 5 after ich outbreak was over and also after the second sampling, there was a columnaris outbreak while the temperatures of the system went up between 28.6 and 29.6 C (Fig. 1). Finally, mortalities stopped in early week 7. Mean survival rates of all groups between week 4 and 6 (after accounting for mortalities from ich outbreak) were 90.6 ± 5.6 %, 88.3 ± 7.4 %, and 91.9 ± 7.2 %, for the control fish, 1% PondToss™ fed fish, and 2% PondToss™ fed fish, respectively. The statistical analysis results in Table 5 show that there were no significant differences among the treated and untreated probiotic fish resulting from columnaris (P=0.7718).

4.4 Growth Parameters

Some fish growth parameters in Table 5, such as mean final body weight, Control: 36.2 ± 2.5g, 1% PondToss™:34.5 ± 2.1g, 2% PondToss™: 35.0 ± 1.9g; percent weight gain

Control: 351.1 ± 30.3%, 1% PondToss™: 334.9 ± 28.4%, 2% PondToss™: 339.8 ± 22.2%; specific growth rate Control: 2.9 ± 0.1% day^{-1}, 1% PondToss™: 2.82 ± 0.1% day^{-1}, 2% PondToss™: 2.8 ± 0.1% day^{-1} and feed conversion ratio Control: 1.2 ± 0.2, 1% PondToss™: 1.2 ± 0.1, 2% PondToss™: 1.1 ± 0.1 show that the fish among treatments and control under the growth study conditions had no significant differences in growth performance from each other ($p > 0.05$).

4.5 Challenge Study Water Quality

In the pre-challenge study, temperature was the only water parameter that was checked in the two systems utilized. In system A, the temperature was 24.6 ± 0.5 C in the morning while in the afternoon the temperature was 24.5 ± 0.3 C. As for system B, the temperature was 24.7 ± 0.5 C in the morning while in the afternoon the temperature was 24.5 ± 0.4 C. As for the challenge study, Table 6 gives the mean temperatures and indicates that during the challenge study that the overall mean temperature in the fish tanks in the morning across all three challenge systems was 23.5 ± 1.2 C and varied between 21.3 C and 26.0 C. The afternoon temperature was 23.5 ± 1.2 C and ranged between 21.5 C and 26.0 C. Overall, mean temperature across all three systems was 23.5 ± 1.2 C. Across all the treatments, there were no significant differences ($p=0.9965$) in mean temperatures during morning ($p=0.9867$) or afternoon ($p=0.9988$). Table 6 also shows that throughout the challenge systems, the mean pH was near neutral and was 7.2 ± 0.1, ranging between 7.1 and 7.3 with no significant differences among systems ($p=0.2374$). Total ammonia nitrogen (TAN) was also not significantly different across the treatments ($p=0.4289$) with a mean of 0.7 ± 0.1 mg/L and range of 0.6 to 0.9 mg/L.

4.6 Pre- and Challenge Study and *Flavobacterium columnaris* Identification Results

There were 2 dosages of *F. columnare* ALG-530 used in the pre-challenge study to determine the effective dose for the challenge study, 4.3×10^5 CFU/mL and 4.3×10^6 CFU/mL. After 9 days post-challenge, it appeared that a dose of 4.3×10^5 CFU/mL could kill 50% of the fish between days 6 and 7 while a dosage of 4.3×10^6 CFU/mL killed 50% of the fish between days 3 and 4 (Fig. 2).

Using a dosage of 3.4×10^6 CFU/mL in the challenge study produced the mean percent cumulative daily mortality rate of each treatment and control as shown in Fig. 3. C2 (Control fish fed 1% probiotics) reached its 50% mortality between days 6 and 7. C3 (Fish from 1% PondToss™ fed Control feed) and C6 (Fish from 2% PondToss™ fed 2% probiotics) reach their 50% mortality between days 5 and 6. C4 (Fish from 1% PondToss™ fed 1% PondToss™) reached its 50% mortality on day 6 while C5 (Fish from 2% PondToss™ fed Control feed) reached its 50% mortality on day 7. In contrast, C1 (Control group) reached its 50% mortality on day 11, which was the last group to reach 50% mortality. At the end of the challenge study, mean cumulative mortality rates were 52 ± 24.9%, 56 ± 16.7%, 72 ± 19.2%, 58 ± 14.8%, 52 ± 26.8%, and 82 ± 14.8% for C1 (fish from Control now fed Control feed), C2 (fish from Control now fed 1% PondToss™ at 4.1×10^7 CFU/g), C3 (Fish from 1% PondToss™ now fed Control feed), C4 (Fish from 1% PondToss™ now fed 1% PondToss™ at 4.1×10^7 CFU/g), C5 (Fish from 2% PondToss™ now fed Control feed) and C6 (Fish from 2% PondToss™ now fed 2% PondToss™ at 6.9×10^7 CFU/g), respectively (Table 7). There were no dead fish on day 1, day 13, day 14 and from day 16 to day 20. On day 2, there were significant effects; however, from days 3 to day 12 and

on day 15 there were no significant effects post-challenge. Overall, there were no significantly different treatment effects on mean cumulative mortalities among treatment and control groups (p=0.1397).

The results of the bacterial identification observed on Hsu-Shotts culture plates indicated that the colony morphology of bacterial isolates from the external lesions, skin, gills, kidney, liver of the moribund and freshly dead fish from all treatments and control appeared to have numerous yellowish rhizoid structures. Furthermore, there were long slender rod bacteria as observed from the additional bacterial identification on wet mounts under a microscope at the magnifications of 400x (Snieszko and Bullock 1976; Tucker and Robinson 1990; Noga 2010; Plumb and Hanson 2011). So the results of both bacterial identification methods and the external clinical sign observations suggest that those fish were infected with *F. columnare*, which was assumed to be the source of mortality.

5. Discussion

FAO (2014) and Tucker (2012) noted that regardless of the culture system used, whether indoor or outdoor, or flow-through, raceway, tank culture or recirculating system, the ideal temperature for channel catfish growth was between 25 and 30 C. Wellborn (1988) emphasized that the temperature for the optimal growth of channel catfish is 29.4 C. However, Tucker and Hargreaves. (2004) showed a wider range of temperature in which the channel catfish can live and grow. He added that growth is slow at water temperatures below 20 and above 35 C. The mean temperature across the treatment and control tanks in the growth study, 27.7 ± 2.0 C, was in the range that channel catfish can grow well. During the

growth study, the channel catfish were provided oxygen near saturation (overall mean of 5.9 ± 0.5 mg/L). According to Wellborn (1988), both farm-raised and wild channel catfish start to slow down their growth and die whenever they are exposed to the culture water having oxygen concentrations of less than 4 ppm and 1 ppm, respectively. Therefore, DO was maintained at adequate levels for good growth. As for pH, TAN (total ammonia nitrogen) and total alkalinity for channel catfish, Chapman (2010) showed that the ideal pH, TAN and total alkalinity should be between 6.5-9, <0.5-1 mg/L, and 50-100 mg/L, respectively. Referring to the current growth study, pH, TAN and total alkalinity, 7.4 ± 0.2, 0.6 ± 0.2 mg/L and 78.0 ± 8.1mg/L, respectively, were within the above recommended ranges. Overall, the water quality parameters maintained during the growth study were in the range acceptable for good growth of channel catfish.

Generally in aquaculture, probiotics are administered by feed and/or as a water additive. Likewise, Irianto and Austin (2002) elaborated that probiotic products have been used as additives in artificial feed, live feed, and in water, and they are available as commercial products or laboratory preparations. Probiotics can be administered to the aquaculture organisms through feeding, injection or immersion. However, the addition of probiotics through feed has been reported as the best method for ensuring the effectiveness of the probiotic bacteria in the gastrointestinal tract of fish (Gildberg et al. 1997; Moriarty 1998; Rengpipat et al. 1998; Robertson et al. 2000; Verschuere 2000). In the present study, the PondToss™ was incorporated into a commercial feed and fed to channel catfish for 52 days prior to the challenge with *F. columnare* strain ALG-530. The results of bacteria quantification between the actual doses and targeted doses of both probiotic diets 2 (1% probiotics) and 3 (2% probiotics) were similar. Across all batches of feed, the standard

deviations of the amount of bacteria in each diets were not high, demonstrating consistency in diet preparation. Compared to the control diet, none of the treatment diets showed significantly different growth improvement under the growth study conditions over 52 days ($P > 0.05$). No significant improvement in growth performance among the treatments and control might be related to issues such as inappropriate dosages or low viability of probiotics, the duration of probiotics to colonize the gastrointestinal tract mucosa, suboptimal temperature or inappropriate water quality for the bacteria to grow and multiply in the fish gut (Bucio Galindo et al. 2009).

A 56-day study on the effects of *B. subtilis* strains on growth of Nile tilapia, *O. niloticus*, by Addo (2013) when applied at a rate of 4.2×10^7 CFU/g of diet reported that overall mean percent weight gain, specific growth rate, feed intake and feed conversion ratio for the treatments were not significantly different from the control diet at the end of the experiment. This dosage falls in the similar range of the dosages used in the current study and these two studies also had the same results although they used different fish species. Panigrahi et al. (2005) emphasized that viability of the bacterium was a vital consideration to obtain a probiotic effect. Another study was conducted by Bisht and Pandey (2013) using *B. subtilis* at three different dosages, 1×10^5, 1×10^8 and 1×10^{10} CFU/g of diet, and fed to *Labeo rohita* fingerlings for 90 days. The results showed that the fish supplemented with *B. subtilis* at the level of 1×10^8 CFU/g showed significant improvement in growth parameters (body weight gain, specific growth rate, condition factor and RNA DNA ratio, and feed conversion efficiency) in comparison to other fish groups. So, a lack of response using PondToss™ may have been related to the dosage or strains of bacteria used.

Besides sufficient dosages, Balcázar et al. (2006) emphasized that probiotic bacteria require a long period of time to adhere and multiply to colonize the intestinal mucosa of the gastrointestinal tract of the fish. This can happens effectively when probiotics are constantly added and body temperature and good water quality are maintained. In this regard, 52-day feeding duration of the current study might be categorized within the inappropriate ranges of duration for the probiotics, *B. subtilis* and *Lactobacillus spp*., to multiply and colonize in the gastrointestinal (GI) tract to produce their effects since most successful studies of these probiotic species took place longer than the present study. For instance, Saini et al. (2013) observed the growth of *L. rohita* was comparatively higher in all the treatments than control by feeding probiotic product containing *B. subtilis, B. licheniformis,* and *Lactobacillus spp.* for 60 days under the temperature of 26.8 ± 0.06 C and pH 7.81 ± 0.38. Similarly, Giri et al (2013) also found significant improvement in growth performance in *L. rohita* fed probiotic *L. plantarum* VSG3 at three dosages $1x10^6$, $1x10^8$ and 1×10^{10} CFU/g within 30 days and 60 days. After 30 days of feeding, they found the fish fed at the dose of 10^{10} CFU/g had significantly higher weight gain than the control group, but no significant effect on SGR and FCR. Then after 60 days of feeding, they found dietary supplementation of *L. plantarum* VSG3 at $1x10^8$ CFU/g was optimal for increasing the growth performance of *L. rohita* under the temperature of 28 ± 2 C and pH 7-8. Finally, a study on the effects of probiotic *L. acidophilus* as a dietary supplement on growth performance of African catfish, *Clarias gariepinus*, conducted for 84 days by Al-Dohail et al. (2011) reported growth performance parameters were significantly better in fish maintained on the probiotic-supplemented diet at a dosage of 3.01×10^7 CFU/g compared with those on the control diet. These studies indicate

that feeding duration and dosage may affect the efficacy of probiotics on growth performance of fish depending upon species and culture conditions.

Besides insufficient dosage and feeding duration, the discontinuation and fluctuation of feeding and feeding rates, stress from the ich and columnaris infections, and subsequent treatments using formalin and potassium permanganate during these outbreaks might have also been negative factors influencing the multiplication and competition for adhesion sites of the probiotic bacteria in the gastrointestinal (GI) tract and could have indirectly affected the ability of probiotics to stimulate appetite and enhance nutrient absorption of the fish. This assumption follows Fuller (1992) who stated that the probiotic population in the GI tract can decrease sharply within the days after the intake had stopped. However, this was not measured in this study.

In the current study, PondToss™ added to the feeds neither improved growth performance nor improved disease resistance of the experimental fish. The results of Ich and columnaris infections during the growth study showed that the probiotic strains had no significant effect on resistance to these two types of disease infections caused by *I. multifilis* and *F. columnare.* While this probiotic product under the current study application did not improve the mean final survival rates of treated groups at the end of the growth study, there is an indication that further studies looking at the potential of PondToss™ to reduce mortalities from Ich may be warranted. However, this could be a good lesson learned for creating new and further research on the effects of probiotic *B. subtilis* and *Lactobacillus spp.* on white spot disease because it is also an important fish disease and so far not many studies have been done on the efficacy of probiotics against Ich. There has been one study conducted by Pieters et al. (2008) on the efficacy of in-feed

probiotics, GC2 (*Aeromonas sobria*) and BA211 (*Brochothrix thermosphacta*) against *Aeromonas bestiarum* and *Ichthyophthirius multifiliis* skin infections in rainbow trout (*Oncorhynchus mykiss, Walbaum*). They applied the probiotics orally at the dosages of 10^8 cells per g feed for GC2 (*Aeromonas sobria*) and 10^{10} CFU/g of feed for BA211 (*B. thermosphacta*) for 14 days. Results showed that after challenge with *A. bestiarum*, probiotics GC2 and BA211 led to 76% and 88% survival, respectively, in contrast to 22% survival for controls. Fish fed with probiotic GC2 had 100% survival after challenge with Ich compared with 2% for probiotic BA211 and 0% for controls.

In addition to improving growth performance of cultured fish, probiotics have also been reported as a potential feed additive protecting farm-raised aquatic animals against infection by elevating immunity (Welker and Lim 2011). This claim was elaborated by Gomez et al. (2007) that probiotics can prevent or diminish the colonization of pathogenic bacteria by competition for the attachment sites on the mucosa, competition for nutrients, or production of inhibitory substances by the microbiota which prevents multiplication and destroys the challenging bacteria. As a result, fish treated with probiotics in some studies were reported to have higher survival rates than the control fish when they were challenged with the pathogenic bacteria. For instance, Nicoskelainen et al. (2001) claimed that after being challenged with *A. salmonicida* rainbow trout *(O. mykiss*) fed *L. rhamnosus* (ATCC 53103) at 2 different dosages (10^9 and 10^{12} CFU/ g feed) were found to have a significant reduction in mean mortality from 52.6% in the control (which was fed the dry feed without the *L. rhamnosus* bacteria) to 18.9% and 46.3% in the 10^9 CFU/ g and 10^{12} CFU/ g feed groups, respectively. Even though the dosage is lower and the mode of administration is different if compared with those of Nikoskelainen et al.

(2001), Addo (2013) also found that the probiotic Lymnozyme® containing lactic acid bacteria like *Lactobacilli* and *Bifidobacteria* used as a water additive for 8 hours daily at a dosage of 3.95×10^5 CFU/mL for at least 3 days before challenge by immersion under tank conditions reduced mortality in juvenile channel catfish from columnaris infection. Lymnozyme® is a component of PondToss™. In contrast to these two experimental results, in the current study, the columnaris challenge results showed that there were no significant differences in mean final mortality among treatment and control groups. These results suggest that fish that were fed probiotics incorporated into the feed at the dosages of 1% PondToss™ and 2% PondToss™ for 52 days during the growth study and then fed with the probiotics for another 20 days during the challenge study did not show any significant differences in mortality from the control fish during the challenge study. It is stated that the conflicting results obtained in probiotic studies might be the outputs of the variation in research conditions, such as the differences in the choice of prebiotics, dietary concentrations, species strains, age/size of fish, feeding management and duration, dosage and virulence of challenge pathogens, methods of challenge, environmental conditions, handling practices, and stocking densities (Merrifield et al. 2010; Welker and Lim 2011). Therefore, dosage and duration of probiotic administration in the current challenge study still remain in doubt given the insignificant differences in mean mortalities in both the growth and challenge studies. However, if compared to dosages used by Addo (2013) in his study, the probiotic dosages in the present study were higher. So, the question is why the lower dosage used by Addo could have reduced mortality in juvenile channel catfish from columnaris infection and not in the present study. Addo (2013) used Lymnozyme® as a water additive for 8 hours daily at a dosage of 3.95×10^5 CFU/mL by immersion under

tank conditions. As a water additive, Lymnozyme® may have had a better chance to directly outcompete the attachment sites on the mouth, skin, gills, and fins of a pathogenic bacteria like *F.columnare* whose infection mechanisms generally begins from the surface to the internal parts of the fish causing systemic infections (Tucker and Robinson 1990; Noga 2010). In contrast to Lymnozyme®, PondToss™ was used as a feed additive in the recent study. This type of administration requires fish to feed so that the probiotic bacteria are able to reach the GI tract of the fish. So the question is what would be the negative impact on feed uptake if fish got incidentally injured and stressed during and after samplings. The consequence is that fish may not take up the feed or at a reduced rate; therefore, the quantity of probiotics would also decrease in the GI tract of the fish.

Columnaris immunity acquisition of the infected fish might also have affected the challenge. Some fish from the growth study had been exposed and infected with columnaris during the growth study and the acclimation period prior to the challenge study. Channel catfish that survived the infection may have developed an acquired immunity, which will confer some protection against any subsequent exposure to this *F. columnare* in the challenge study. Because of being stressed by the challenge activities, such as netting and moving out of the tanks, those remaining fish from the previous infections might have been able to release *F. columnare* into the tanks which made other tank mates even more susceptible to columnaris while they themselves might not be affected. This could result in high within treatment variability. This assumption follows Suomalainen (2005) who stated that the fish recovering from *F. columnare* infections normally come to be columnaris pathogen carriers that later on release *F. columnare* into the water column when they are stressed by some stress factors, such as high water

temperature, crowded condition due to high stocking density, or rough handling, or when there is serious concomitant parasitic infection.

6. Conclusion

The commercially- available PondToss™ applied at the dosages of 4.1×10^7 CFU/g and 6.9×10^7 CFU/g under the present research conditions did not significantly affect the measured growth parameters and survivial among treated and non-treated channel catfish fingerlings. Furthermore, at the end of the growth study, these probiotics also did not significantly affect mean mortalities of juvenile channel catfish from white spot disease infection caused by *Icthyophthirius multifilis,* nor columnaris during the growth study and challenge study. It has been discussed that the results of the current studies may have been influenced by some factors such as feeding duration, probiotic dosage, stress caused by fish sampling and treatment of diseases during disease outbreaks, discontinuation of probiotic uptake and mode of probiotic administration. Therefore, further studies may be needed to find out the appropriate feeding duration and dosages since these two factors are mutually related. Research systems (flow-through using possibly disease-contaminated pond water) must be improved to minimize stress and chances of disease outbreak that can affect the constant probiotic uptake of the research animals and the whole research operation. Finally, modes of probiotic administration and modes of probiotic action in relation to the mechanisms of columnaris infections on improving growth and disease resistance are also worth considering.

Table 1. Overall mean water quality levels during the channel catfish growth trial in flow- through aquaria under laboratory conditions using filtered, UV-treated, heated pond water. Significant differences among treatments ($p \leq 0.05$).

Water quality parameters	Mean ± S.D	Min	Max	P-value
Water temperature (A.M.) C	27.6 ± 2.0	21.5	31.2	0.9505
Water temperature (P.M.) C	28.5 ± 1.9	23.7	31.6	0.9559
Overall temperature C	27.7 ± 2.0	21.5	31.2	0.9119
Dissolved oxygen (A.M.) mg/L	5.8 ± 0.3	4.3	6.10	0.9609
Dissolved oxygen (P.M.) mg/L	4.9 ± 0.3	4.3	5.8	0.5680
Overall dissolved oxygen mg/L	5.9 ± 0.5	4.1	7.8	0.7086
pH	7.4 ± 0.2	7.2	7.8	0.9259
Total ammonia nitrogen (mg/L)	0.3 ± 0.2	0.2	0.8	0.8565
Total alkalinity (mg/L)	78.0 ± 8.1	65	102.5	0.5452

Table 2. Actual bacterial numbers, average numbers and standard deviations of bacterial counts in each batch of 1 kg of experimental diet with a unit of CFU/g of feed and ± SD.

Feed Batch	Control	1% probiotic diet	2% probiotic diet
Batch 1	2.6×10^4	4.0×10^7	7.2×10^7
Batch 2	2.8×10^4	4.6×10^7	7.2×10^7
Batch 3	3.7×10^4	3.9×10^7	6.3×10^7
Batch 4*	3.6×10^4	3.8×10^7	5.9×10^7
Batch 5	2.3×10^4	4.2×10^7	7.5×10^7
Batch 6	1.5×10^4	3.7×10^7	6.2×10^7
Batch 7	2.4×10^4	3.7×10^7	6.0×10^7
Batch 8	1.9×10^4	4.1×10^7	6.3×10^7
Batch 9	2.7×10^4	4.1×10^7	8.1×10^7
Batch 10	2.6×10^4	4.0×10^7	7.3×10^7
Batch 11	2.1×10^4	4.1×10^7	7.3×10^7
Batch 12	2.3×10^4	4.4×10^7	7.3×10^7
Average	2.5×10^4	4.1×10^7	6.9×10^7
Standard deviation	$\pm 0.632 \times 10^4$	$\pm 0.268 \times 10^7$	$\pm 0.706 \times 10^7$

* when feed were changed Aquamax 300 to 400

Table 3. Mean survival rates channel catfish fingerlings fed diets with or without PondToss™ after Ich outbreaks for 11 days from week 2 to week 4 during the growth study. Values are means ± SE. Overall p-value of multiple comparisons of survival is at the bottom of the table. Means within columns with the same letter are not significantly different ($P > 0.05$).

Treatment	N Obs	Survival (%)
Group 1 (control)	15	78.4 ± 9.5 [a]
Group 2 (1% PondToss™)	12	98.1 ± 0.6 [a]
Group 3 (2% PondToss™)	12	95.0 ± 3.8 [a]

P-value = 0.4239

Table 4. Mean survival rates of channel catfish fed diets with or without PondToss™ after columnaris outbreaks at the end of week 5 to the end of the growth study. Values are means ± SE. Overall p-value of multiple comparisons of survival is at the bottom of the table. Means within columns with the same letter are not significantly different ($P > 0.05$).

Treatment	N Obs	Survival (%)
Group 1 (control)	11	90.6 ± 5.9 [a]
Group 2 (1% probiotics)	12	88.3 ± 7.4 [a]
Group 3 (2% probiotics)	12	91.9 ± 7.2 [a]

P-value = 0.7718

Table 5. Potential effects of probiotic and non-probiotic diets on the growth performance of juvenile channel catfish in a flow-through aquaria system receiving filtered, UV-treated, heated pond water. Values are means ± SD. Means within rows with the same letter are not significantly different ($P > 0.05$).

Fish growth parameters	(Control)	(1% probiotic)	(2% probiotic)	P-values > 0.05
IBW	8.0 ± 0.18[a]	7.9 ± 0.21[a]	7.9 ± 0.2[a]	0.4729
FBW	36.2 ± 2.5[a]	34.5 ± 2.1[a]	35.0 ± 1.9[a]	0.1408
%WG	351.1 ± 30.3[a]	334.9 ± 28.4[a]	339.8 ± 22.2[a]	0.3372
SGR	2.9 ± 0.1[a]	2.82 ± 0.1[a]	2.8 ± 0.1[a]	0.4226
FCR	1.2 ± 0.2[a]	1.2 ± 0.1[a]	1.1 ± 0.1[a]	0.2174

* IBW: initial mean body weight (g $fish^{-1}$)
FBW: final mean body weight (g $fish^{-1}$)
%WG (percent weight gain)
SGR (specific growth rate) (% day^{-1})
FCR (feed conversion ratio)

Table 6. Overall mean water quality levels during the channel catfish challenge trial in static well water aquaria under laboratory conditions in 3 systems. Significant differences among treatments ($p \leq 0.05$).

Water quality parameters	Mean ± S.D	Min	Max	P-value
Water temperature (A.M) C	23.5 ± 1.2	21.3	26	0.9867
Water temperature (P.M) C	23.5 ± 1.2	21.5	26	0.9988
Overall temperature C	23.5 ± 1.2	21.5	26	0.9965
pH	7.2 ± 0.1	7.1	7.3	0.2374
Total ammonia nitrogen (mg/L)	0.7 ± 0.1	0.6	0.9	0.4289

Table 7. Mean percent cumulative mortality of treatment groups and control in 20 days post-challenge with *F. columnare* strain ALG-530 at a dosage of 3.4×10^6 CFU/mL by immersion. Values are means ± SD. Overall p-value of multiple comparison of mortality is at the bottom of the table. Significant differences among treatments ($P \leq 0.05$). Means in the same column with the same letter were not significantly different ($P > 0.05$)

Treatment	Treatment description	Mortality (%)
C1-Control	Fish from Control fed Control feed post-challenge	52 ± 24.9[a]
C2	Fish from Control fed 1% PondToss™ post-challenge	56 ± 16.7[a]
C3	Fish from 1% PondToss™ fed Control feed post-challenge	72 ± 19.2[a]
C4	Fish from PondToss™ fed 1% PondToss™ post-challenge	58 ± 14.8[a]
C5	Fish from 2% PondToss™ fed Control feed post-challenge	52 ± 26.8[a]
C6	Fish from 2% PondToss™fed 2% PondToss™ post-challenge	82 ± 14.8[a]
P-Value	0.1397	

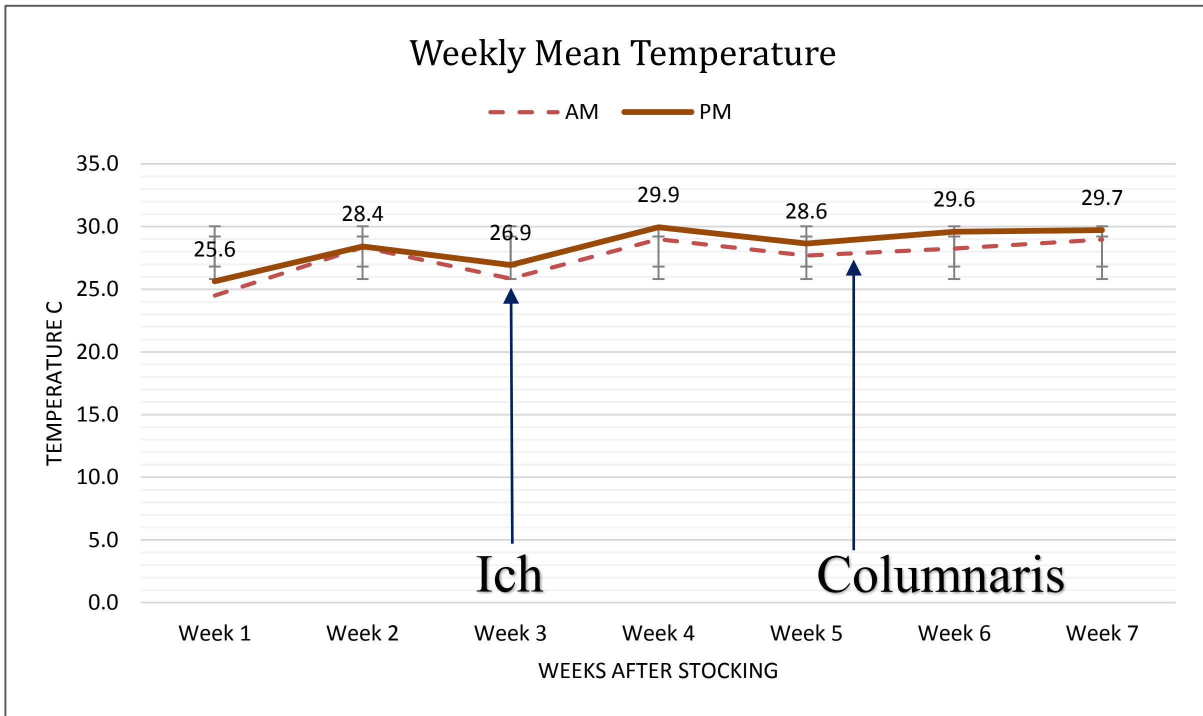

Fig. 1. Weekly mean temperature of the flow-through aquaria system receiving filtered, UV-treated, heated pond water in relation to white spot and columnaris outbreaks of channel catfish fingerlings being fed diets with or without PondToss™

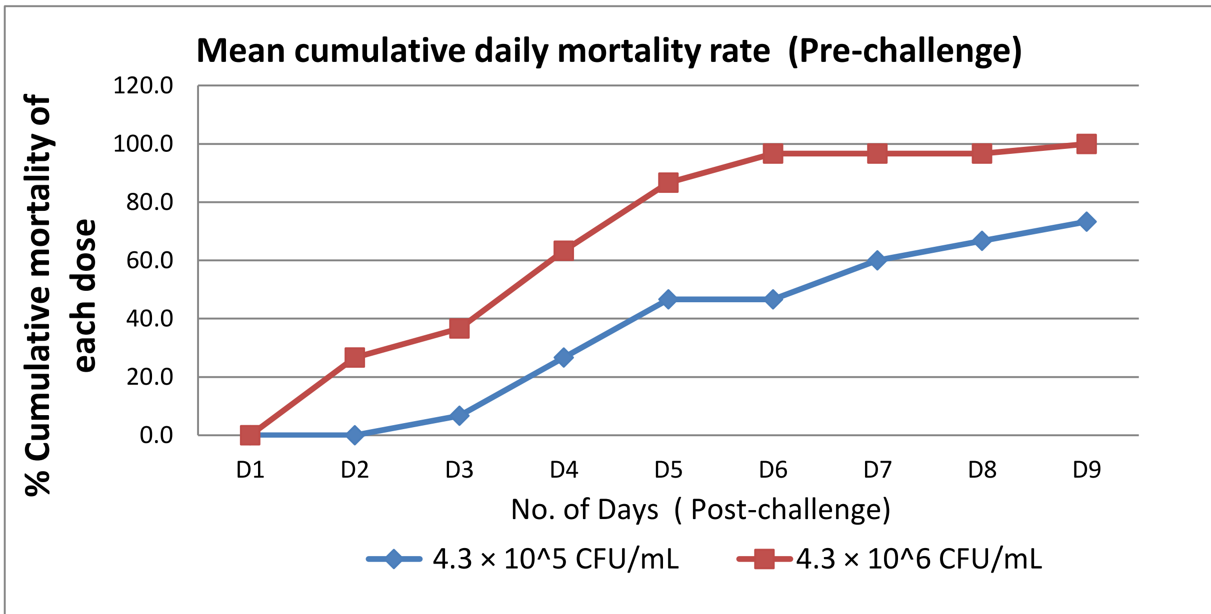

Fig. 2. Mean percent cumulative mortality of naïve channel catfish challenged with 2 different dosages in the pre-challenge study.

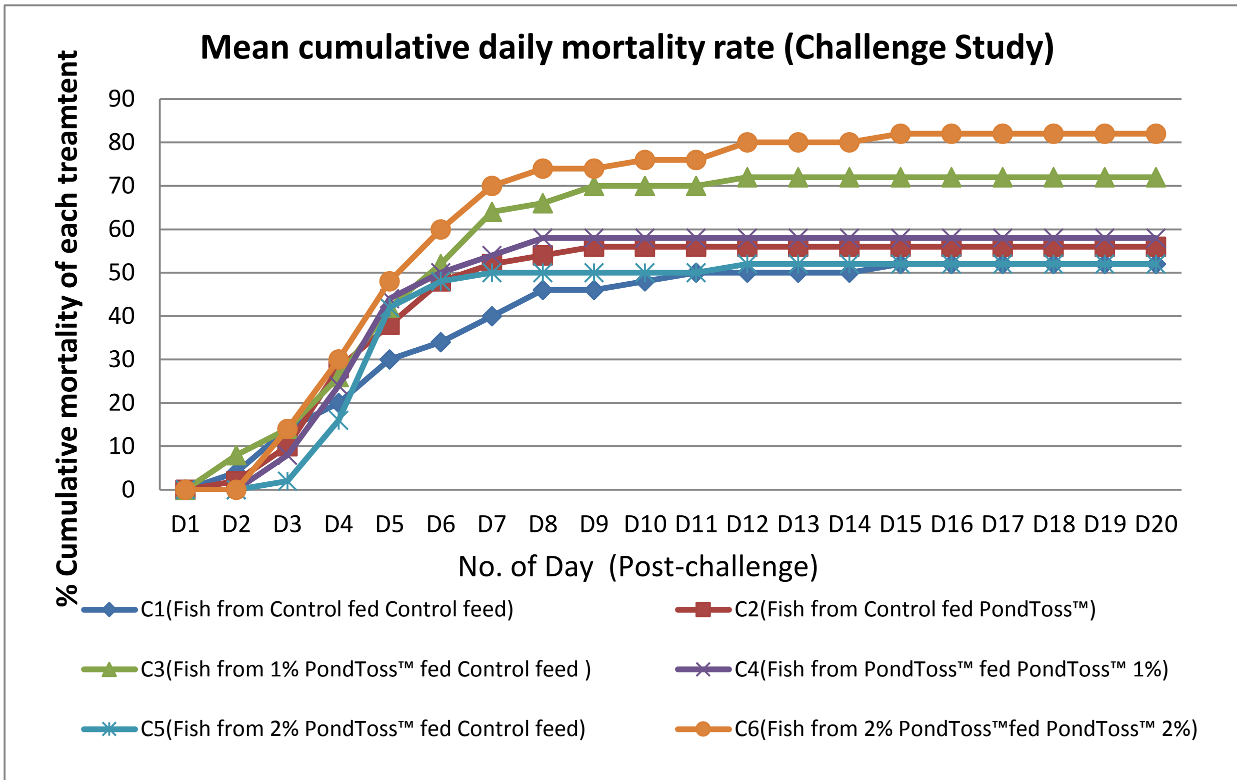

Fig. 3. Mean percent cumulative mortality rates of channel catfish in control and 5 different treatments.

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