Establishment and Utilization of Tools for Enhancing Foodfish Health

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ACADEMIC ABSTRACT

Aquacultured products assist the human demands for seafood so that foodfish supplies can remain sustainable and consistent. Although the fish-farming industry has seen dramatic growth and intensification in recent years, the latter has led to an increase in bacterial diseases and fish health management problems, resulting in major economic losses around the world. In addition to the lack of understanding of fish physiology, these complications are exacerbated by the inappropriate and controversial use of antibiotics. This work addressed these issues in striped catfish (*Pangasius hypophthalmus*) and Nile tilapia (*Oreochromis niloticus*), two economically important foodfish, by investigating alternative, more cost-effective options to promote fish health. The first two studies established reference intervals for immunology, hematology and plasma chemistry analytes in striped catfish in a recirculating aquaculture system (RAS). In a third study, the immunomodulatory effects after directly feeding probiotic strains of *Bacillus subtilis* NZ86 and O14VRQ in Nile tilapia were ascertained. This last study revealed that supplementation with both of the probiotic strains for 51 days stimulated several local and systemic innate immune responses of tilapia. When these transient probiotic bacteria were present in the gut, a pro-inflammatory environment was developed as evidenced by the localized higher expression of the cytokines tumor necrosis factor (TNF) – α and interleukin (IL) – 1β. Significant increases ($p < 0.05$) were noted differentially by both probiotic strains throughout the trial in plasma lysozyme content, alternative complement activity, and in the peripheral blood leukocyte profiles. Additionally, there were trends for increased levels of phagocytosis and respiratory burst in leukocytes of the anterior kidney and spleen at the end of the trial, suggesting the potential use of these probiotic strains for improved immune-

competence. These findings help to understand and clarify the potential mechanism of action associated with the increased disease resistance recorded in preliminary studies with the same probiotic strains. Implementation of the tools established and validated in this work could be useful in evaluating fish welfare situations involving striped catfish grown in RAS conditions, and also show promise for a healthier foodfish supply where antibiotic applications practices could be minimized.

GENERAL AUDIENCE ABSTRACT

Aquaculture, or fish farming, is one of the most prosperous production sectors of animal-derived food. Despite the success story of aquaculture, the fish industry is heavily plagued by bacterial diseases, which cause losses in billions of dollars annually around the world, and directly contribute to increases in human food insecurity. Since the options to cost-effectively address diseases are limited, I explored alternative ways to more safely monitor and also ensure optimal health in striped catfish and tilapia, two globally important aquaculture fishes. I investigated the values of different cellular and chemical components of the blood to monitor the health of striped catfish when grown in indoor recirculating conditions, in order to understand normal catfish physiology. The values of these blood components were comparable to those of other freshwater fishes. As part of another study, I supplemented probiotics in the diet of the tilapia for 51 days, and assessed the effects of these on the immune system of the fish. Dietary supplementation of the probiotics resulted in the presence of the probiotics in the gut of the fish. Furthermore, the presence of these microbes was tightly linked to elevated values of numerous functions of the immune system. These functions included levels of lysozyme, alternative complement, and percentage of neutrophils, which are all related with a state of heightened immunity in the animal host. The tools that I established and validated in this study are promising alternatives to optimize the health of these two important foodfish. Moreover, they could be useful for the fish farmer because of their greater cost-effectiveness, and can potentially lead to a safer foodfish supply by decreasing the reliance on antibiotics.

DEDICATION

I dedicate this dissertation to my cousin and childhood friend Carlos Andrés Arias Angulo, who passed away before he completed his studies in human medicine a few years back. Requiescat in pace.

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LIST OF ACRONYMS

AK…….Anterior kidney

ANOVA…….Analysis of variance

BCR……B cell receptor

cDNA…….Complementary deoxirybonucleic acid

C_q ……..Quantification cycle

ELISA……Enzyme-linked immunosorbent assay

FCR……Feed conversion ratio

GIT……..Gastrointestinal tract

IEL……..Intraepithelial lymphocytes

Ig…….....Immunoglobulin

IL…….....Interleukin

IP……Intraperitoneal

LP……...Lamina propria

MCV.…….Mean corpuscular volume

mRNA……Messenger ribonucleic acid

MS222……Tricaine methanesulfonate

NTC……Non-template control

PBS…….Phosphate buffered saline

PCV…….....Packed cell volume

RAS…….....Recirculating aquaculture system

RBC…….....Red blood cell

RI…….Reference interval

RT-qPCR…….Real time quantitative polymerase chain reaction

SD…….Standard deviation

SEM……..Standard error of the mean

SGR…….....Specific growth rate (%/day)

TCR…….....T cell receptor

TLR……..Toll-like receptor

TNF…….....Tumor necrosis factor

WBC……White blood cell

CHAPTER I

Introduction

1.1 Importance of Aquaculture

According to a statistic reported earlier by the United Nations, the world human population currently grows at an average rate of 1.1% per annual basis, and reached 7.6 billion on 2017 (United Nations, 2017). Although positive, given its slightly lower growth rate than in previous years, the human population continues to increase, yielding an additional 83 million people annually. Moreover, the global growth is projected to reach 9.8 billion by 2050, and increase further in the following years (United Nations, 2017). An alarming part of this growth trend is that countries in the developing world continue to contribute more than 50% to the growth by year basis.

The increase in human population growth, among other factors such as urbanization, global warming, and change in diet lifestyles, is predicted to increase the likelihood of a serious food crisis. Historically, there have been many initiatives to confront the problem of food shortages such as improving crop yields, better livestock productivities, use of genetic engineering, better dissemination of relevant knowledge, and improved technologies. Despite all the good intentions to improve food security, agricultural productivity is anticipated to decline by 2050 in southern Asia and Africa (Knox et al., 2012).

Fish and fish products are a cheaper form of animal protein, and are often considered as a good alternative to the terrestrial animal derived protein. One advantage to growing fish consists in that, just like chicken, they have low feed conversion rates (FCRs). The low FCR results are attractive to farmers given the efficiency of the fish to turn plant protein into animal protein. In

developing countries, which are affected to a greater extend by food shortages, foodfish are an important commodity and are often an integral part of the local daily diet (FAO, 2016). Moreover, these aquatic foods can be easily digested and also contain a good protein profile, with all essential amino acids, and account for 17% of the global intake of animal protein (FAO, 2016). Additionally, fish products are a rich source of essential fats (e.g. omega 3 fatty acids), vitamins (e.g. A, B and D), and minerals (e.g. potassium, selenium, zinc, iodine and iron). Given their large nutrient composition, these food commodities are often regarded as a vital component in the human diet.

To ensure the food security of these staples, aquaculture, or fish farming, has been growing and evolving rapidly to meet the world demands of fish products. It contributes a large share of the total produced finfish and shellfish for human consumption. In 2014, aquaculture was the source of over 50% of the annual world fish production for human consumption (FAO, 2016). This highlights the potential of aquaculture to sufficiently augment fish supplies to feed the world, a task that would be unattainable for natural fisheries alone, where the fish stocks run at biologically unsustainable levels due to overfishing. The importance of aquaculture is further evidenced by the statistic of the growth of global fish production surpassing the rate of world population growth as of 2012 (FAO, 2014). This provides a hint of the potential of aquaculture to improve food security around the globe.

Fish farming is important in providing fish worldwide. The practice of aquaculture occurs in most regions around the world, but Asia provides a large share of the total production of fish (FAO, 2014). A key characteristic that contributes to the successful story of aquaculture lies in the diversity of cultured species. According to the FAO, there are 327 finfish species farmed to

date, including the commercially important striped catfish (*Pangasius hypophthalmus*) and tilapia (FAO, 2012).

1.2 Striped catfish, tilapia and foodfish health

The success in the aquaculture of striped catfish and tilapia is attributed to the exploitation of their excellent qualities for tropical aquaculture; such features include high adaptability to intensive culture, ease of reproduction, resistance to poor water quality, low input feeds, good survival rates, and rapid growth (Ali et al., 2013). It is also worth noting that both fish species stand out by aquaculture production numbers, and they are only behind those of carp species, and also Atlantic salmon in the case of striped catfish (FAO, 2012; Phu et al., 2015).

Striped catfish is an important freshwater finfish species in Southeast Asia. The catfish species is native to the Mekong River in Vietnam, and this country represents the primary producer of this fish. In 2015 Vietnamese production of striped catfish remained stable at 1.1-1.2 million tons, and the fish species is currently exported to more than 80 countries around the world, including the United States (FAO, 2016; Globefish, 2015). According to the National Fisheries Institute, striped catfish was the sixth most consumed fish in the US in 2015 (NFI, 2016), demonstrating the importance of this foodfish commodity in the American diet.

Unlike the striped catfish industry, farming of tilapia is more widespread in the world, with production being second only to carp species. Tilapia output from aquaculture contributes roughly 75% of the total production of this foodfish, and signifies about 7% of total fish farming around the world (FAO, 2014). Among the several species of tilapia, Nile tilapia (*Oreochromis niloticus*) and its hybrids are among the most frequently farmed, given several beneficial characteristics including rapid growth, high adaptability to intensive production systems, easy

acceptance of feed and relative disease resistance. Data from the National Fisheries Institute demonstrates that tilapia was the fourth most consumed food of aquatic origin in the US in 2015 (NFI, 2016), indicating the acceptance of the US consumer towards this fish as well.

As both industries of these freshwater fish have made advances to achieve intensification and sustainability, issues such as poor water quality and disease outbreaks remain problematic for their further expansion. Pathogens of particular attention include *Aeromonas hydrophila* and *Streptococcus iniae* in tilapia, and *Edwardsiella ictaluri* and *A. hydrophila* in striped catfish (Abd-El-Malek, 2017; Crumlish et al, 2002; Ferguson et al, 2001; Kitao, 1993; Shoemaker et al, 2001). Animal infections with such pathogenic bacteria are of great concern, to the fish sector, due to their massive economic impact in millions of dollars, with costs associated to the loss in productivity, labor and increased use of antibiotics. Therefore one of the major challenges for the success of both aquaculture industries lies in the search of fish culturing inputs that will promote fish fitness without compromising production costs.

Historically, water quality has been improved via good management practices (e.g. stocking densities, optimal water quality parameters, and feeding rates), whereas bacterial diseases can be treated with antibiotics. Although the management of these two variables has had a degree of success, the widespread use of antibiotics is concerning. While only oxytetracycline, Aquaflor® (florfenicol) and sulfadimethoxine are approved for use in aquaculture in the United States, regulations for using antibiotics in the rest of the world are not as strict, especially in developing nations, where the majority of aquaculture production occurs. A review of antibiotic usage in fish-farming across the world highlights how there is limited data on antibiotic usage in some developing nations due to the absence of tracking systems (Sapkota et al., 2008). This lack of readily available knowledge brings to attention the potential for antibiotics being misused, and

leading to the controversy of possible antibiotic residues in the foodfish. The central danger of antibiotic use is the development, or selection, of antibiotic resistant bacteria, which can occur even at the sub-therapeutic amounts (Pruden *et al*, 2013). Thus to meet the pressure exacted by consumer preferences on foodfish products, the aquaculture industry has to consider more cost-effective alternatives to overcome the challenges jeopardizing fish fitness.

In efforts to reduce the costs but still target optimal fish health, the fish industry has to accept that prevention can be more cost-effective than control of disease. One of the issues with the decreased effectiveness of antibiotics lies in that many fish farmers, especially in the developing world, lack the education to appropriately utilize these drugs. With the fish farmers in mind, intervention strategies should be tailored towards readily available options that will not result in increased costs.

One indispensable component to prevention encompasses knowledge of the normal physiology of an organism. In relation to the striped catfish, basically, nothing is known about its basic characteristics of multiple hematologic and blood plasma chemistry. There is only one study limited to describing hematologic analytes after exposure of the catfish to chemical agents (Hedayati and Tarkhani, 2014). However, work describing the normal plasma chemistry indices of this fish does not exist. An important thing to note is that basic physiology values, also known as reference intervals (RIs), are only known for a few fish species, including tilapia (Hrubec et al, 2000; Knowles et al, 2006; Tavares-Dias and Moraes, 2007a; Tavares-Dias and Moraes, 2007b). Establishment of RIs can be critical to closely monitoring and enhancing the optimal production of striped catfish; while also providing a database of normal physiology that could be utilized in future health studies of this fish species. Since blood analytes may be notably different between fish species, comparisons should not be considered as mean to assess fish health.

Considering the commercial relevance of striped catfish, it is necessary that knowledge of its baseline blood analytes is available for more facilitated farming.

In the last decade, the use of probiotics has increased in popularity as a "functional food" not only in the human food industry, but also for animals. Furthermore, many microorganisms have been extensively studied for use in aquaculture, given their potential benefits on fish health (Aly et al., 2008b; Brunt et al., 2007; Lara-Flores et al., 2003). Some of the positive effects reported in the literature include alteration of the gastrointestinal tract (GIT) morphology, stabilization of the gut microbiota, immunomodulation and disease resistance (Aly et al., 2008a; Bagheri et al., 2008; El-Ezabi et al., 2011; Harikrishnan et al., 2011; Merrifield et al., 2010).

Among the biological agents with potential for probiosis, members of the genus *Bacillus* offer an appealing solution to many of the animal health challenges of modern aquaculture (He et al., 2013). Of particular attention, *B. subtilis* possesses characteristics that make it stand out from other bacterial species; these traits include their omnipresence in nature, the ability for endospore formation, high antimicrobial production, general safety, and also it being one of the most characterized bacterium to date (Fajardo-Cavazos et al., 2006; Stein, 2005). The ability of endospore formation is ideal for feed application, being able to withstand the harsh conditions of both feed production processed and the animal gut. Multiples studies of various *B. subtilis* strains have shown that their incorporation in aquafeed exerts a differential degree of health benefits, particularly in immunostimulation in tilapia (Aly et al., 2008a; Apun-Molina et al., 2009; He et al., 2013; Liu at al., 2017; Ng et al., 2014). By carefully looking into these studies, it is evident that using different strains of *B. subtilis* does not yield the same measurable effects, even when the same animal model is considered; and also highlights the point that functional properties of probiotics are dependent on strain rather than species, making strain-to-strain generalizations

impossible. Moreover, there is still much work to be done to characterize possible mechanisms of action of strains of *B. subtilis*, and how those hold to be true in the tilapia model.

1.3 Research aims and objectives

The overall purpose of this research was to establish and assess a set of tools that can be useful for monitoring and promoting fish health in striped catfish and Nile tilapia farming operations. The work proposed here sought to first cultivate striped catfish in recirculating aquaculture systems (RAS), and evaluate different fundamental details on the hematology, blood chemistry and peripheral immunology of this poorly studied, yet important foodfish. Additionally, two probiotic candidates of *B. subtilis*, provided by Novozymes Biologicals, chosen for their disease protection properties in shrimp and striped catfish (unpublished data), were tested in tilapia. This study sought to investigate a possible mechanism of action of immune-enhancement that could explain the occurrence of disease protection.

The specific objectives were to:

I. Isolation and purification of Pangasius-derived immunoglobulins for the development of secondary antibody probes to determine total plasma immunoglobulin in striped catfish.
II. Establish reference intervals for blood hematologic and plasma chemistry parameters for striped catfish.
III. Explore the long-term effects of feed supplementation, with strains O14VRQ and NZ86 of *B subtilis,* on growth and innate immune parameters in Nile tilapia.

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CHAPTER II

Review of Literature

2.1 Striped catfish

Habitat and biological traits

Striped catfish (Figure 1.1) is a ray-finned fish that belongs to the family Pangasiidae of the order Siluriformes, which includes all catfish, and its members inhabit freshwater in southeast Asia. Members of this family are large (adult sizes ranging 0.2 - 3 m), and morphologically characterized by a laterally compressed body, with pairs of mandibular and maxillary barbels, and a long anal fin with 26-46 rays (Roberts and Vidthayanon, 1991). Within the Pangasiid members, striped catfish is of worth mention due to its high commercial importance in southeast Asia.

Striped catfish has been known by many scientific names including, *Helicophagus hypophthalmus* (Sauvage, 1878), *Pangasius sutchi* (Fowler, 1937), *Pangasius hypophthalmus* (Sauvage, 1878), to its now more accepted name *Pangasianodon hypophthalmus* (Vidthayanon and Hogan, 2011). In addition, it goes by many other vernacular synonyms such as swai, or tra catfish, or iridescent shark (due to the shiny color of the juvenile catfish).

It is a highly migratory species that moves up and down the streams, in the Mekong River in Vietnam, one of its natural origins (Phuong and Oanh, 2010). In the wild, this catfish species can reach a maximum length and weight of 90 cm and 44 kg, respectively (Robertson and Vidthanayon, 1991). It is an omnivorous fish that feeds on algae, plants, zooplankton, insects, crustaceans and smaller fish (FAO, 2015). This fish is also an facultative air breather, due to its modified swimbladder (Browman and Kramer, 1985; Roberts and Vidthayanon, 1991).

Interestingly, and despite the presence of this vascularized organ, striped catfish retains fully functional gills that can uptake oxygen from the water. This dual capacity of oxygen acquisition highlights their adaptability to environments with limited aeration and ability to use atmospheric oxygen as an auxiliary mode of respiration.

This fish species is a native to the Mekong Delta area in Vietnam, but also occurs naturally in the Chao Phraya River basin in Thailand (Roberts and Vidthanayon, 1991). Before its development as an economically important species, the catfish was traditionally farmed in small earth ponds near both of these rivers to feed the local communities. Striped catfish, along with one of its close relative *Pangasius bocourti* (Basa catfish), are two of the main finfish species cultured in Vietnam.

The distribution of this catfish is now more widespread in Asia. This has been due to introduction of this catfish species to other countries, including Indonesia, Cambodia, Malaysia, Myanmar, Bangladesh, China and Lao People's Democratic Republic, for aquaculture purposes (FAO, 2015; Sustainable Fisheries Partnership, 2015). This geographical location in the world is an indicator of its preference towards warm water (22-26 °C).

Farming of striped catfish dates back to the late 1900's, but intensive production did not start to gain popularity until the mid 2000's (Phuong and Oanh, 2010). Its production exceeds 1.6 million tonnes, with Vietnam being responsible for 75% of total fish supplies (Globefish, 2014). Striped catfish gained its role as one of the most important aquatic species in this Asian country, as its production dramatically increased from 22,500 tonnes in 1997 to 1.2 million tonnes in 2007 (Dung et al., 2008). The Vietnamese striped catfish industry has achieved a bigger role in the world market, as it provides about 90% of total exports (WWF, 2012).

Pangasius World exports and fraud

The story of exports of striped catfish has been remarkably successful around the world. The top destinations for this fish, as of 2014, are the United States and the European Union, which represent 22% and 18%, respectively, of the total export market (Pangasius-Vietnam, 2015). In the US (and in other regions of the world), imports of the fish have boomed due to the high acceptability and its relative cheap cost. Striped catfish is imported as a food product into the US in the form of frozen fillets, and the imports have been growing in recent years (Figure 1.2). The importance of its importation is translated in the statistic that this foodfish commodity is ranked sixth in the top 10 seafoods of the American diet (NFI, 2016). However, careful attention needs to be paid to how this trend continues given the tougher USDA mandatory inspections, which will probably increase fillet prices.

Striped catfish has been subject to food mislabeling or substitution. Given its fast growth, high yields, and cheaper cost, the fish has been used before as replacement for more expensive white-flesh fishes, such as cod, grouper or flounder (Carvalho et al., 2015; Helyar et al., 2014). In the US, this fish species was marketed as catfish several years ago, which brought about confusion with the domestically cultured catfish (Ictaluridae family) until the FDA ruled that only members of this family could be granted the term "catfish" for labeling purposes (FDA, 2002). Cheap labor and inexpensive animal feed have been two of the key factors reducing the production costs, and resulting in the lack of transparency resulting from the fish product substitution activities. However, the trend of using lower quality feed has been avoided in recent years in the striped catfish industry, greatly due to food safety and consumer demands (FAO, 2015).

In addition to the exports of striped catfish as frozen fillets, this fish species is also an important part of the ornamental trade in the US, and marketed as iridescent shark. Production for aquarium trade purposes occurs in Florida, where it is sold at an early juvenile size. In spite of the fish exhibiting aquaculture potential, its production as a food fish has not started in the US, probably due to the colder winter temperatures being a limiting factor, and also due to competition from the domestic channel catfish industry.

Challenges and limitations to optimizing striped catfish health

Despite the explosive growth of the striped catfish industry, there are several constraints that affect the culturing of this species, including deterioration of water quality and higher mortalities due to microbial diseases. *Edwardsiella ictaluri* is a major pathogen of Pangasius species. It is a Gram-negative bacillus that is ubiquitous in aquatic environments. This bacterium was first associated with fish farming practices, when Hawke isolated it from channel catfish (1979). Symptoms of disease in striped catfish were first described as multifocal white lesion visible in the liver, spleen and kidney (Ferguson et al., 2001). This disease is now described as "bacillary necrosis" of Pangasius. One external sign that is a hallmark of infection associated with this pathogen is the appearance of a hole-in-the-head lesion (Waltman et al., 1986). In 2002, the rod-shaped pathogen was isolated from catfish farmed in the Mekong Delta region that exhibited the previously described internal lesions (Crumlish et al., 2002). The FAO and the few literature papers available indicate that *E. ictaluri* is a major pathogen to the Pangasius industry, but there are no reports on the specific economic burden that the disease poses to that industry. But the story of the bacterium and channel catfish and how it inflicts annual losses of $40-50

million to that industry in the US, definitely highlights its importance as a deleterious pathogen (Yeh et al., 2005).

Historically, antibiotics have been used to treat *E. ictaluri* infections of catfish, taking advantage of the pathogen's innate susceptibility to these compounds (Stickney, 1994; Stock and Wiedemann, 2001). However, a recent study found that 73.4% of *E. ictaluri* isolates from Vietnam showed resistance to at least 3 antibiotics *in vitro* (Dung *et al*., 2008). The finding of naturally resistant strains in the environment is probably a result of improper use of such compounds, and explains why their use is not recommended (FAO, 2015; Phuong and Oanh, 2010). Moreover, the issue of potential transmission of antibiotic resistant genes, as with other animal products, is also of concern from both a human food safety standpoint.

Bacterial disease treatment in this Pangasius fish is complicated, and the cost-ineffectiveness of antibiotics calls for a shift in focus in prevention rather than a cure. Prophylaxis in fish is a rather complex endeavor, particularly since fish are poikilothermic, and their basic metabolism is under the control of the quality of water. The latter comprises multiple variables that could predispose fish to disease, therefore proper maintenance of water quality must not be overlooked. Before one can proceed to investigating and validating alternative methods to control disease in striped catfish, it is important to point out how there is limited work determining the normal physiology of this piscine species (the importance of different physiological blood and immunology analytes will be discussed in section 2.4). A good understanding of what a healthy fish would entail physiologically, could prove to be key to ensuring disease preventions.

2.2 Tilapia

Habitat and fish biology

Tilapia is the common name given to three economically important genera of fish in the Cichlidae family, including *Oreochromis*, *Sarotherodon* and *Tilapia*. Anatomically, tilapia has a compact and tough body, and has scales covering the skin unlike all Siluriformes species. Tilapia adults are generally smaller than striped catfish, given that the former may reach up to 60 cm and 4.3 kg in length and weight, respectively (Luna, 2017). Tilapia species are capable of living in fresh, brackish and saltwater conditions.

These fish species are native to Africa, and the Middle East but have been introduced to practically every region of the world for culturing. Most of their production takes place in regions with tropical and subtropical climates in developing countries, yet farming also occurs in temperate regions by use of indoor systems. Among many of the tilapia species cultured globally, Nile tilapia (*Oreochromis niloticus*; Figure 1.3) is preferred due to their rapid growth rates, slow sexual maturation, resistance to disease agents, tolerance to poor water quality, and inexpensive feeding habits as omnivorous grazers (Tsadik and Bart, 2007). These preference are evidenced by Nile tilapia accounting for >80% of the total tilapia production (El-Sayed, 2006).

The history of tilapia culture is possibly one of the oldest ones in the history of man. Aquaculture practices of this fish go back to ancient Egypt around 4000 BC, where tilapia was reared and captured in ponds (Linseele et al., 2014). In Christian mythology, the farming of tilapia is also well known since the apostle Peter caught tilapia from the Sea of Galilee to feed to the multitudes. Moving forward to the 1900's, farming of Nile tilapia started becoming more widespread in 1960 (FAO, 2017a). Since then, China has become the largest producer of tilapia, providing more than 50% of the total production, followed by Indonesia, Egypt, Thailand, and

Bangladesh (Yacout et al., 2016). The farming of tilapia has resulted in yields increasing >500% in the past 2 decades, and total production being at its peak with 5.3 million tonnes by 2016 (Figure 1.4).

Tilapia continues to be of increasing importance because of globalization. It holds a prestigious place by being one of the most farmed fish species around the world, with total production being second only to carp species. Over the past decades, consumption has transcended the more traditional Asian and African markets to making its way into the European and the Americas. In the US, tilapia is the fourth most widely consumed seafood, exemplifying its role and acceptance in the American diet (NFI, 2016).

Tilapia in the US

The demand for tilapia for food consumption is high in the US. The farming of tilapia primarily occurs in the US via the use of indoor recirculating aquaculture systems (RAS, discussed in greater detail later), with Blue Ridge Aquaculture being the largest producer of tilapia in the US. The domestic production is approximately equivalent to 10 thousand metric tons of tilapia. Although there has been an increase in the domestic output of tilapia in the past decade, this quantity is insufficient to meet the nation's demand. Therefore, over the years this shortage of tilapia in the US market has created an excellent opportunity for other tilapia producing countries to export tilapia into the US. The rate of US tilapia imports has increased dramatically since the early 2000's (Fig 1.5). Imports account for over 95% of the total demand of tilapia. The countries that contribute to most of the US tilapia supplies are China, Indonesia, Costa Rica, Taiwan, Honduras, and Ecuador. Tilapia is imported into the US in the main forms of frozen whole tilapia, frozen fillets, or fresh fillets.

Problems of the tilapia aquaculture sector

The tilapia industry shares some similarities to the striped catfish industry, in that fish health issues affect outputs, resulting in significant economic losses. The common trend that underlies these problems is that of increasing output to meet the world demands of tilapia. Since aquaculture of this species is more global and has been around for longer, compared to that of striped catfish, the use of technology has been implemented for both intensifying production while also aiming for greater farming sustainability. However, intensive farming is often accompanied by induction of animal stress and disease outbreaks. In this kind of farming, environmental conditions (e.g. inadequate levels of oxygen, pH, alkalinity, nitrogen species, and temperature) and poor husbandry practices (e.g. malnutrition, inadequate stocking densities, and frequent netting) can exert much stress on the animals being reared, compromising their immune response and making them more vulnerable to diseases.

The main bacterial pathogens that affect the tilapia industry include *Aeromonas hydrophila* and *Streptococcus iniae*. These are often opportunistic pathogens that would not affect healthy fish, but become problematic when certain conditions subject the fish to stress. In the case of *A. hydrophila*, this is a bacterium that is part of the normal fish microbiota, and can cause septicemia in the animal. Therefore stress is an integral part frequently preceding disease outbreaks in fish farming. Increasing densities of fish, a practice associated with intensification, can also facilitate the horizontal transmission of disease agents. This is the case with diseases such as streptococcosis, which is a major health complication in tilapia (Plumb and Hanson, 2010). The disease is most often caused by *S. iniae* and *S. agalactiae*, which invade and affect the brain and the eye of the host. This disease is of concern because of the economic loss that it exerts on the tilapia industry which amounts to over $250 million USD annually, in addition to

being a potential for zoonosis (Amal and Zamri-Saad, 2011). Such as with many bacterial diseases in aquaculture, both of these diseases are commonly treated with antibiotics, yet their ineffectiveness calls for the development of more efficacious strategies.

2.3 Solving the loopholes in fish disease management

Even though antibiotic application is the gold standard when dealing with bacterial diseases, their use is a double-edge sword. Antibiotics can be effective in reducing the numbers of bacteria as long as the recommended guidelines of application are followed. Yet, if application is shorter than expected, the outbreak may happen again. On the opposite end, if the drugs are applied for an extensive period of time, there is the risk of the "bugs" developing antimicrobial resistance. Therefore this often becomes a very expensive option that does not solve the issues of disease, especially if detecting disease on later stages.

Vaccination has been another option considered to address the problems in striped catfish and tilapia, however this can be a more labor-intensive option with unreliable success rates. It is also worth noting that vaccines can be more expensive than antibiotics in the short term, which would not be attractive for farmers. Furthermore, the success of vaccination can be complicated by mutations in the target pathogen. In addition, the regulatory and approval process of vaccines is a time-lengthy one by nature. Yet, it is worth noting that the field of fish vaccinology is still a young one that needs much work.

All of these concerns have promoted the idea of both increasing biosecurity, and exploring alternative and novel compounds that can have a functional benefit to the health of the fish, while also ensuring environmentally friendly farming practices. Consumer views are critical, given that people nowadays demand that animal farmers abstain from the use of

antibiotics, and turn to the use of more natural alternatives (Ortega et al., 2014). In the search for such alternative in the present era, the impact of manipulating the fish gut microbiota in the animal's health has been examined.

2.4 Nutritional value striped catfish and tilapia

Although not the focus of this work, it is worth mentioning the benefits associated with eating fish. Fillet products from white-flesh fish, such as striped catfish and tilapia, are appetizing to the consumers due to their excellent quality, which has a firm texture, limited number of intramuscular bones, a delicate flavor and absence of a strong fishy odor. The health benefits of fish consumption are popularly associated with the intake of omega-3 highly unsaturated fatty acids (HUFA). This is grounded on the knowledge that these lipids are linked to a decrease in the risk of various cardiovascular diseases. Striped catfish and tilapia contains low to moderate levels of omega-3 HUFA, but have a high content of proteins (Asdari et al., 2011; Young, 2009).

2.5 Fish blood

The blood is a very dynamic system and an important indicator of the health status of all vertebrates. Just like any other vertebrate, the blood of bony fish is made up of cells, ions, proteins, sugars and fat components that are suspended in fluid. These integral parts of the blood are generally classified as hemotologic and biochemical analytes.

Teleost blood morphology and function

The different types of cells present in the circulating blood of fish vary by species, but generally include the following: erythrocytes, reticulocytes, thrombocytes, lymphocytes neutrophils, heterophils, monocytes, eosinophils, and basophils (Fänge, 1992; Hrubec and Smith, 2010). White blood cells (WBC), or leukocytes, are among the most variable analytes among all the hematologic components, and their presence and numbers depend on multiple factors, including fish species. Tilapia has lymphocytes, neutrophils, monocytes and eosinophils (Hrubec et al, 2000). Channel catfish is reported to have lymphocytes, neutrophils, monocytes, basophils and heterophils (Tavares-Dias and de Moraes, 2007; Cannon et al., 1980). Rainbow trout has lymphocytes, neutrophils, monocytes, and eosinophils (Lone et al., 2012).

Teleost erythrocytes, or red blood cells (RBCs), are nucleated and have an oval shape, and represent the most numerous blood cells in fish. They contain hemoglobin and carry oxygen similar to all vertebrates. Their cell morphology is similar in appearance and ultrastructurally to other vertebrates, except mammals, which have lost their nucleus (Campbell, 2015). Additionally, these are found in fewer numbers relative to RBCs present in mammals. Fish RBCs are also more metabolically active compared to their mammalian counterparts, due to the poikilothermic nature of the former (Witeska, 2013). Reticulocytes are a form of immature RBC, and consist of smaller and more rounded cells than mature erythrocytes, and their cytoplasm stains slightly basophilic.

Thrombocytes are the cells responsible for all coagulation processes. This type of cell has the same morphological characteristics in all bony fish, with a condensed nucleus and a clear or lightly stained cytoplasm. The shape these cells display range from a spindle-form to a more round shape upon activation, when observed in a blood smear. Thrombocytes are similar in

function to platelets of higher vertebrates, and are activated by the same mechanisms as the latter. Additionally, thrombocytes can cross-react with mammalian platelet markers (Hrubec and Smith, 2010). Despite these similarities, it is worth mentioning that thrombocytes and platelets do not arise from the same cell lineages (Daimon et al., 1979). This suggests that the latter probably came to existence via convergent evolution. One function that sets thrombocytes apart from platelets nonetheless, is that of greater immune functionality, given their potential for phagocytic activity (Nagasawa et al., 2014).

Fish lymphocytes are the most abundant type of WBC in peripheral blood circulation in healthy fish (Campbell, 2015). This is not the case in mammals, where these cells predominate in tissue, not in the blood (Fänge, 1992). They are small and round cells that are part of the innate and adaptive immune responses. These cells have abundant N:C ratio, with a light blue cytoplasm and a more abundant nucleus, which stains a more basophilic to purple color (Hrubec and Smith, 2010). Based on size, these cells have been classified as either small or large lymphocytes, with the former representing antigen naïve cells (Campbell, 2015). Yet, functionally, these cells can be further divided by function into B, T, and Natural killer (NK)-like cells (also termed non-specific cytotoxic cells). B cells express B cell receptors (BCR), which are composed of membrane bound immunoglobulins, and serve as an antigen receptor. Similarly, T cells also have transmembrane proteins, for antigen recognition, called T cell receptors (TCR). These types of cells are predominantly involved in adaptive responses just like their mammalian counterparts, of the same name; whereas, NK-like lymphocytes are only involved in nonspecific responses, just like NK cells in mammals (Nakanishi et al., 2015).

Neutrophils are one of the largest cells in blood circulation. The term heterophil, a granulocyte observed in avian species, has been often used instead of neutrophil, yet differences

in cytochemistry and function between the two remain to be characterized (Hrubec and Smith, 2010). Neutrophils consist of a pale blue/gray cytoplasm, with fine to no visible granules. The nucleus of these cells can vary from oval to multilobed, depending on the fish species (Fänge, 1992). These cells are professional phagocytic cells whose primary role is in the inflammatory response, and their function of killing involves the production of reactive oxygen species during phagocytosis, just like in mammals.

Eosinophils and basophils occur in very small numbers in fish, and often absent from blood circulation (Hrubec and Smith, 2010). Eosinophils are medium sized granulocytes, which can be differentiated from neutrophils by the occurrence of abundant coarse or rod-shaped granules that stain eosinophilic (or red) that can obscure the nucleus (Campbell, 2015). Basophils on the other hand are distinguished by the presence of round basophilic (or blue) granules, which can often obscure the nucleus as well (Fänge, 1992). The functions of these types of cells have not been clearly defined in fish (Campbell, 2015; Hrubec and Smith, 2010).

Monocytes are one of the largest cells in blood circulation. Just like in mammals, these cells are mostly found in the blood, but can migrate into tissue to differentiate into macrophages or dendritic cells (Bassity et al., 2012; Ellis, 1977). They are big round, agranulated, mononuclear cells that have a cytoplasm that stains basophilic, and a round to kidney-shaped nucleus (Fänge, 1992). Additionally, monocytes can often contain vacuoles in the cytoplasm (Hrubec and Smith, 2010). Monocytes, macrophages and dendritic cells are key components of the natural immune response via phagocytosis, yet they also serve as a paramount bridge for the creation of memory via antigen presentation.

Fish hematology

Some of the frequently assessed hematologic analytes include packed cell volume (PCV), mean corpuscular volume, erythrocyte counts, hemoglobin levels, and examining cell morphology. The majority of the studies available to date use these tools to assess the changes caused by biological or chemical agents on fish hematology. All this information is helpful, yet it could be difficult to interpret given our lack of understanding of the relationship of normal fish physiology and hematology per fish species. This proves to be more difficult given hematologic reference intervals (RIs) are only available for a handful of fish. Reference intervals are ranges that are defined from individuals that are seemingly healthy, and generally encompass the central 95% of the subjects (Sikaris, 2014). Therefore, this information could be used to monitor analytes in a small sample of blood comparatively to guide treatments options. However, the low volume of information regarding these values, and how to interpret blood tests in fish, can be expected if the number of different fish species is considered. Despite the advances in fish medicine, RIs are not appreciated and are rarely used for correlating to pathology cases.

Packed cell volume is the percentage of RBCs present in the circulating blood. The values for this blood analyte typically range 20-45% in fish (Hrubec and Smith, 2010). Generally, fish that are on the more active end have higher PCVs than the less active ones. The values of PCV are among the most sensitive to physiological changes, and tend to increase upon increases of RBC numbers, or decrease during episodes of anemia (Fänge, 1992; Kumar et al., 2016). These fluctuations are directly affected by changes in RBC counts. The numbers of RBCs vary and depend on species of fish, but typically range from $1 – 3 \times 10^6$ cells/μL, with sedentary and active fish being on the lower and higher end, respectively (Campbell, 2015).

Counting of leukocytes and differentials (leukogram) are other examples of hematologic analyses done yet their occurrence is more infrequent given the greater expertise these require. This is because identification of white blood cells in fish has to be done manually, as opposed to the automatic cell counters available for mammals, and that can be difficult and require training. The leukogram tends to vary by fish species, but lymphocytes (small lymphocytes predominantly) are the most abundant WBC, followed by monocytes and neutrophils, then eosinophils and rarely basophils, if present (Campbell, 2015).

Fish blood chemistry

Assessment of blood chemistry has been considered the gold standard in the fish clinical setting, due to automation, which gives it an advantage over the more complicated and less standardized fish hematology. Although there are numerous studies focusing on changes in plasma chemistry after exposure to a toxin, or a biological agent, this kind of analytes suffer from the same problem as their hematologic counterpart: the absence of RIs for diagnostic purposes. And worth noting is the fact that plasma components are highly sensitive to multiple factors including fish handling, anesthesia, blood sampling, and diet (McDonald and Milligan, 1992). Therefore all these little things must be taken into account before looking at the effects of other agents. The plasma is made up of proteins, electrolytes, salts, glucose, lipids, and other metabolism by-products.

The major proteins in the blood plasma are albumin and the globulins, but other transport and binding proteins, in addition to blood clotting factors are present as well (McDonald and Milligan, 1992). Of those mentioned, albumin and globulins are the ones of interest in most studies, because of their larger role in regulation of blood vessel osmotic pressure, and in the

immune response, respectively. The majority of the information available about these fish proteins is descriptive (e.g. molecular weight), and there is limited data about their relative abundance in the plasma per fish species. Total plasma protein in fish generally ranges 2-8 g/dL (McDonald and Milligan, 1992). Shifts in plasma protein levels often occur either after changes in hydration or stress (McDonald and Milligan, 1992).

There are enzymes of tissue origin that are also present in the blood plasma. Alkaline phosphatase (ALP) and aspartate aminotransferase (AST) are examples of these non-plasma specific enzymes that are often measured for clinical cases. These enzymes are normally present in liver, heart, gill, kidney, and muscle. Greater titers of these enzymes in the plasma could be indicative of tissue damage, sexual maturation, or stress (Shahsavani et al., 2010). Although there are studies linking stress to activities of these enzymes, there is no sound understanding on how to interpret their values. Therefore, more studies to decipher how these enzymes relate to metabolism are necessary before these values can be of used for diagnostic purposes.

There are some metabolism by-products that can also be found in the plasma. Creatinine, an example of this, is the result of creatine metabolism. This metabolite is normally excreted by the kidneys in the piscine. The levels of creatinine are normally low in fish, ranging 0.11 – 0.88 mg dL^{-1}, and these analytes appear to be unaffected by stress (McDonald and Milligan, 1992). Bilirubin, another metabolite found in the plasma, is the product of heme metabolism in the liver. Just like with creatinine, the purpose of levels of this pigment in the blood is not understood. Thus, the clinical significance of the two analytes remains to be described.

The levels of glucose can be used as an indicator of stress. Glucose, along with cortisol (a hormone), is linked to stress responses as a result of fish handling, transport, disease, and poor water quality (McDonald and Milligan, 1992). Active fish tend to have higher levels of glucose

than the more sluggish ones (McDonald and Milligan, 1992). The concentration of this analyte can also be affected by nutritional status, size and age (McDonald and Milligan, 1992).

Electrolytes make up some of the most studied analytes in the piscine plasma (Hrubec et al, 1996). The role of these chemicals underlies the fish's ability to osmoregulate. Changes in electrolyte level can be due to stress, disease or lesions of the gills that increase the permeability of ions (McDonald and Milligan, 1992). Sodium and chloride are the major ions present in the plasma. There are other electrolytes present in the plasma such as calcium, phosphorous, potassium and magnesium, but their contribution to fish pathology is not well understood.

Hematology and plasma chemistry of Striped catfish

There are scant studies focusing on blood analytes on striped catfish. To date, there are only two studies using hematological parameters to understand the pathology of certain chemical and bacterial agents (Hedayati and Tarkhani, 2014; Sirimanapong et al., 2014). Moreover, there are no studies describing the plasma chemistry of this fish species. The scarcity of studies, and more importantly the absence of RIs, impedes the use of these tools to evaluate the health status of this catfish species.

2.6 Fish immunology

Bony fish, just like higher vertebrates, have an innate and adaptive immunity to defend themselves from disease insults. However, the innate portion of immunity contributes to the largest proportion of overall immunity in finfish, a higher proportion of utilization when compared their mammalian counterparts (Ellis 2001). This is due to the poikilothermic nature of fish, given that the adaptive immune responses can take up to several weeks and anamnestic

responses do not occur. The major immune organs in fish include the anterior kidney (AK), the spleen and the thymus. These organs give rise to the classic cells involved in the non-specific response (mononuclear phagocytes, granulocytes, and NK-like cells) and cells involved in the adaptive response (T and B lymphocytes). It is important to note that although there is much improvement in our knowledge of fish immunology, much of it is still poorly characterized, and our understanding suffers from the lack of available molecular probes for ease of investigation. Much of the immune responses have been conserved through evolution, so the immune responses in fish are comparable to those from higher vertebrates (Table 1.1). However, immune-related differences unique to teleosts exist, and a few of these will be discussed next.

Piscine immunoglobulins

Immunoglobulins (Igs) are a central part of the humoral response of both the innate and acquired immunity, and they are produced by B-cells. These cells express Igs either as membrane bound molecule, termed B-cell receptor (BCR), and use it for antigenic recognition, or as an antibody in the secreted form. Most of the knowledge available to date about Igs roots from our understanding of antibodies of mammalian origin. In these vertebrates, antibody isotypes are classified as IgM, IgG, IgA, IgD, and IgE. In teleost fish, nonetheless the isotypes described to date include an IgM-like antibody, IgD and, IgT/IgZ.

The IgM-like molecule was the first Ig identified in fish, and it represents the most commonly studied antibody class in the piscine. In the membrane of B cells, it is expressed in a monomeric form, or as a tetrameric antibody in the secreted form. This Ig molecule can be found in the blood circulation, gut, gills, and bile (Morrison and Nowak, 2002) In most fish species, IgM-like isotype in the plasma is expressed as a tetramer, and its molecular weight ranges from 600-800 kDa (Smith et al, 1993). However, it can be also be found as a monomer as

in the case of the giant grouper (Clem, 1971), or as a dimer in salmonids (Magnadottir, 1998). The importance of this Ig isotype lies in its role in complement activation for lysing pathogens, in addition to pathogen marking for facilitating phagocytosis (Mashoof and Criscitiello, 2016). Typically, levels of the IgM-like antibody in the serum range from 0.8-9 mg/mL (Ye et al., 2013). Despite being the most abundant and most studied fish Ig, RIs of the IgM-like isotype in fish are rare.

For a while, the IgM-like isotype was considered the only Ig isotype present in teleosts until the discovery of IgD. The presence of the IgD isotype in fish demonstrates its importance from an evolutionary standpoint by being the only immunoglobulin present across all vertebrate classes (Das et al., 2012). The IgD isotype has recently been described in tilapia (Wang et al., 2016), rainbow trout (Ramirez-Gomez et al., 2012), carp (Savan et al., 2005a), atlantic salmon (Hordvik et al., 1999), and zebrafish (Zimmerman et al., 2011). The IgD isotype can be found both in the transmembrane or secreted forms, where it exists as a monomer. This isotype is found in the blood, anterior and posterior kidneys, spleen and gills (Bengten and Wilson, 2015). Its function, nonetheless, remains unknown.

The most recent addition to the known repertoire of teleosts antibody classes is that of IgZ or IgT. This Ig isotype does not exist in any other vertebrate but teleost fish. The IgZ or IgT molecules receive different nomenclatures due to their discovery in zebrafish (Danilova *et al*., 2005) and trout (Hansen *et al*., 2005), yet these represent the same isotype as their gene sequences are similar (Zhang *et al*., 2011). In recent years, gene orthologs for IgT have been found also in Atlantic salmon (Tadiso *et al*., 2011), common carp (Savan *et al*., 2005a), and fugu (Savan *et al*., 2005b). However the antibody has either been reported to be absent in some fish, e.g. channel catfish (Bengtén *et al*, 2006), or has not been investigated in other fish species both

due to incomplete fish genomes and lack-there-of molecular probes. Structurally, IgT is found as a tetramer in mucosal membranes, whereas it has a monomeric form in the blood plasma (Zhang *et al*., 2010). The only functional characterization of this isotype has been shown in rainbow trout, where higher titers of IgT were found in the gut mucus relative to IgT levels in the serum (Zhang *et al*., 2010). This work demonstrated evidence of the phylogenetically oldest antibody class with dedicated mucosal immunity functionality, and comparable to IgA in mammals.

Mucosal immunity

The mucosal membranes are major barriers in all vertebrates that prevent and protect the host from the entry of pathogens, and are therefore considered an active site of immune responses. In teleost fish, the skin, the gill and gut constitute the major mucosal surfaces, and are at the forefront as the first line of defense. In these, mucus produced by goblet cells covers the intestinal walls and complicates an easy entrance for intruders. Additionally, the mucus acts as a medium where bioactive molecules (e.g. antimicrobials, lysozyme, antiproteases) are suspended to complicate the penetration of pathogens.

Among all the mucosal sites, the gut is an important immune site (Figure 1.6), and will be the one relevant to this work. Fish are different than mammals in that they lack organized lymph structures, such as gut associated lymphoid tissue (GALT), but rather the gut has small aggregates of intraepithelial lymphocytes (IEL) in the lamina propria (LP), which is also rich in other immune cells of the adaptive and innate immune responses (Salinas *et al*., 2011). Functionally, nonetheless, the fish gut mucosal immunity is comparable to that observed in mammals. In both animal models, intestinal epithelial cells (enterocytes) have an important role in antigen uptake, processing and then priming of T-cells for development of memory (translated

to tolerance or pathogen clearance). As mentioned previously, IgM and IgT can also be found in the gut mucosa (Zhang *et al*., 2010). An important and abundant component of the gut is that of commensal bacteria, which are ultimately in control of the development of its protective functionalities.

2.7 Gut microbiota and probiotics in aquaculture

When addressing the mucosal function of the GIT in vertebrates, it is difficult to avoid mentioning the gut microbiota, which is crucial to the overall functions of the intestine. This microbial component, acting as an additional organ, constantly has cross-talk with the host cells, resulting in the development of a functional GIT, and additional benefits for the animal. The occurrence of this intimate association of gut-microbes also occurs in the piscine.

Gut microbiota in teleosts

The fish digestive tract is home to a diverse and dynamic community of microbes (commensal, mutualistic and pathogenic). Normally, the composition of the resident gut microbiota ranges 10^7-10^8 CFU per gram (Austin, 2002). In freshwater fish, members of the genera *Acinetobacter, Aeromonas, Flavobacterium, Lactococcus, Pseudomonas, Bacteroides, Clostridium* and *Fusobacterium* are the dominant components of the gut microbiota (Perez *et al*., 2010). But, the microbial composition of the gut is influenced heavily by species, host genetics, nutrition, age, and environmental factors (Austin, 2002). These symbiotic organisms are able to thrive within the gut via active signaling.

These bacterial communities appear to have a complex role within their host, and are influential in the development of the intestinal epithelium, fish nutrition, homeostasis, immune

responses and likelihood of disease. The importance of the gut microbiota in fish has been shown in a study where absence of the gut microbiota in larvae zebrafish promoted phenotypes of an immature digestive tract (e.g. low density of microvilli in enterocytes), but bacterial inoculation at later life stages was able to reestablish a functional gut (Bates et al., 2006).

The gut microbiota strengthens the first line of defense (i.e. the gut mucosa). It is well known that an established microbial consortium in the gut of vertebrates is capable not only of aiding with digestion functions, but also with protection of the host (Gomez et al., 2008). Although the exact mechanism of action can be convoluted, it is generally accepted that the endogenous microbes protect the host from invaders via competition for nutrients and attachment sites, in addition to production of antimicrobials. Additionally, these bacteria during the early life stage of the fish educate the immune system so that it can distinguish resident, or good, bacteria from foreign entities via the use of pattern recognition receptors (PRRs). These receptors are typically expressed on the surface of both enterocytes and immune cells (Yiu et al., 2017). A type of PRR known to recognize bacteria includes the toll-like receptor (TLR), which identify surface antigens of pathogens, and can direct the appropriate immune responses. A healthy and undisturbed gut microbiota is sufficient enough to keep invaders out, but when breaches occur, humoral (e.g. complement, lysozyme, antibody mediated opsonization and agglutination) and cellular (e.g. phagocytosis) immunity start taking action.to contain the infection.

Nowadays, it is accepted that the microbiota is a player with an unquestionable role in enhancing the integrity of the gut mucosa. Therefore, growing attention has been oriented towards manipulation of the intestinal microbiota for health promoting benefits in the host. The use of probiotics for this purpose in fish and shellfish is an excellent example.

Probiotics and aquaculture

The word probiotic is derived from the Latin "pro" and Greek "bios" roots meaning "for life". There have been multiple definitions to the word probiotic, but the most widely accepted one was proposed by Fuller (1989), describing it as a live microbial dietary supplement that exerts a beneficial effect by improving microbial balance of the body. Then there was an important modification to include that the beneficial effect occurs when the live microbes are administered in the adequate amount (FAO/WHO, 2001). Some of the properties that categorize a microbe as one with probiotic potential include the organism's abilities to colonize the gut, improve digestion, antagonize pathogenic microbes and stimulate the host's immune response. The true intent of supplementing these microbes is to modify a disturbed microbiota (by stress, antibiotics, or other extraneous factors), to return it to its healthier and balanced composition.

There are many microbial organisms that have been investigated for their probiotic potential. As far as sources of the microbes, there is not really a consensus as to which is considered the standard, or of greater benefit, and microorganisms of both endogenous and exogenous origin have been explored as probiotics. Some examples of probiotics include members of the bacterial genera *Lactobacillus*, *Bifidobacterium* and *Bacillus* species, and the *Saccharomyces* yeasts (Montalban-Arques et al., 2015). The application of these is important as a functional food for humans, in agricultural practices, and has also gained momentum in aquaculture in recent years.

The application of probiotics is an emerging practice for protecting farmed fish, while also helping to minimize the use of antibiotics. Probiotics can be administered via the feed or water. However, feed supplementation is preferred to ensure a successful delivery into, and colonization of, the GIT. Most of the studies available to date assess the effect of direct-fed

microbes, and typically show positive health results, such as improved growth and immunity on the host (Guo et al., 2016; Gupta et al., 2014; Newaj-Fyzul et al., 2007; Zokaeifar et al., 2012). It is worth mentioning that some probiotic strains have been used as water additives and have demonstrated beneficial effects in the host (Gupta et al., 2016; Zhou et al., 2010a), yet this delivery method will not be addressed in this work.

Selecting a probiotic

Before considering the positive impact in the host, there are multiple parameters to consider when choosing a probiotic candidate. The first one to consider is that it must not be pathogenic to the aquatic host, or to humans (especially if talking about a foodfish). Then, another relevant characteristic involves the microbe's ability to resist the high temperatures during the feed making process (e.g. extrusion or pelleting). Other key attributes include the microbe's innate ability to resist gastric and bile acids of the GIT. For the purpose of resisting harsh conditions, bacterial strains capable of endospore formation (such as *Bacillus* species) have been of great consideration (Olmos and Paniagua-Michel, 2014). Only after surviving these adverse conditions of the stomach, will the probiotic candidate be able to adhere and colonize the gut to create the symbiotic relationship with the host.

Colonization of the GIT is another vital criterion when choosing a probiotic. After resistance to the conditions of the upper digestive tract, the probiotic has to adhere to the mucous or epithelial cells of the mucosal surface in the intestine (Tassell et al., 2011). One study found that feeding *B. subtilis* and *B. licheniformis* to rainbow trout for three weeks resulted in over 35% of the culturable microbes of the gut mucosa (Merrifield et al., 2010a). Another study found that feed supplementation with *Kocuria* SM1 and *Rhodococcus* SM2 in rainbow trout for 14 days

resulted in high degree of gut colonization (90-100% total culturable bacterial population; Sharifuzzaman et al., 2014). It is important to mention that probiotics, do not permanently colonize the gut, and their presence continues until they are no longer administered orally (Newaj-Fyzul et al., 2007; Robertson et al., 2000). This is logical provided the dynamics of the gut microbiota of fish. However there are circumstances where the microbiota can suffer drastic reduction in number and diversity (e.g. medicated antibiotic application in the feed). One study showed that application of probiotics after antibiotics was able to repopulate the gut bacterial communities, and to bring back the phenotypes associated with a stable microbiota (Merrifield et al., 2010b). Thus, and just like in mammals, it appears that the use of probiotics after treatment with antimicrobials must not be overlooked.

By definition, a probiotic has to confer a health benefit to the host, which would translate either to a positive in growth or disease protection. To date, most of the screening for probiotic candidates has focused on the search of a strain capable of being active against a pathogen. This search has taken form of *in-vitro* studies (Sahoo et al., 2015; Vine et al., 2004a). Meanwhile other studies have proceeded to validate their findings with an *in-vivo* work by doing challenge studies (Guo et al., 2016; Ran et al., 2012; Vaseeharan and Ramasamy, 2003). Although these proceedings can have meaningful findings, there is one drawback to this approach in which only one possible mode of action is being looked at. In other words, this excludes other modes of action of probiotics (e.g. competition for binding sites, stimulation of immunity, etc).

Modus operandi

A good understanding of how the probiotics work in the host of interest is important for the probiotic supplementation efforts. The general mechanisms of action (Figure 1.7) include

stimulation of immune responses, stability of the gut microbiota (discussed above), competition for binding sites and for available nutrients, and production of antimicrobials (Montalban et al., 2015). It is important to consider that not all probiotics have the same function, neither would a strain have the same effect in different aquatic species. Additionally, other factors such as rearing conditions, health status, and interactions with native microbiota can also alter the outcomes of probiotic supplementation.

- Production of antimicrobial compounds

Several microorganisms have been documented for their inhibitory capacities against relevant microbial pathogens in aquaculture. These inhibitory properties typically involve bacteriostatic and bactericidal effects against unwanted bacteria. Lactic acid bacteria (LAB) have been a promising group of probiotic candidates due to their antimicrobial capacities. For example, *Lactococcus lactis* strain RQ516 has been shown to inhibit the growth of *A. hydrophila in-vitro* (Zhou et al., 2010b). Another study demonstrated that six *Lactobacillus* isolates from shellfish had antimicrobial activity against *Edwardsiella tarda*, *S. iniae*, and *Vibrio parahaemolyticus* (Kang et al., 2016). Other studies have gone a bit further and tried to characterize these inhibitory compounds from *L. lactis*. For instance, one study demonstrated that the marine isolate *L. lactis* TW34 was able to produce nizin Z, a bacteriocin, and this acted against *L. garvieae,* the responsible agent of lactococcosis (Sequeiros et al., 2015). Therefore expanding on the inhibitory potential of organisms of the LAB group.

Other organisms, such as members of the genus *Bacillus,* have been scrutinized as probiotics, due to their large antibacterial arsenal. In a study, different isolates of *Bacillus subtilis* from both the soil or the intestine of channel catfish exhibited inhibitory activity against multiple

A. hydrophila, *E. ictaluri*, *E. tarda, Flavobacterium columnare, S. iniae* and *Yersinia ruckeri* (Ran *et al*., 2012). Lalloo *et al* (2007) reported that three isolates of *B*. subtilis, *B. cereus* and *B. licheniformis* inhibited the growth and reduced the numbers of *A. hydrophila in-vitro*. The inhibitory results observed, *in-vitro*, against these pathogenic bacteria are probably due to the naturally produced antibiotics by members of the genus *Bacillus* (Hong *et al*., 2005). Another research area that has gained attention in the world of probiosis, is the ability of the *Bacillus* spp to sabotage quorum sensing, the mechanism of communication among bacteria for coordinated gene expression, of Gram-negative bacteria. Previous work found the ability of *Bacillus* sp. QS-1 to interrupt quorum sensing in *A. hydrophila*, leading to a reduction in biofilm formation, hemolytic and protease activities (Chu et al., 2014). Although all of this is promising, careful attention has to be given to the fact that *in-vitro* findings do not translate in their entirety to *in-vivo* work because of the more intricate environment in the latter.

Although antibacterial effects of probiotics are a major focus for probiotics in finfish aquaculture, antifungal and antiviral effects are still, but less commonly, investigated. Control of viral disease is more problematic than bacterial outbreaks due to the lack of antiviral therapy options available. In one study, orange spotted grouper (*Epinephelus coioides*) that were fed with *B. subtilis* E20 for 28 days, and that were then later challenged with grouper iridovirus, showed more than 50% higher survival rate relative to the non-probiotic treatment control (Liu et al., 2012). A similar study in olive flounder (*Paralichthys olivaceus*) found that upon diet supplementation of the commercial probiotic Lactobacil ® and later infected with lymphocystis disease virus resulted in lower mortalities (30%) compared to the control group that were not fed probiotics (80% mortality; Harikrishnan *et al*., 2010). The exact mechanism of antiviral activity of probiotics is unknown, but it is speculated that probiotic candidates produce antiviral

substances (Kamei *et al*., 1988). The available literature is limited also for antifungal effects of probiotics in finfish. One study showed that use of *L. plantarum* FNCC 226 prevented the growth of *Saprolegnia parasitica* in striped catfish (Atira *et al*., 2012).

- Competition for nutrients

The existence and further proliferation of microbes in a physical space is dependent upon the availability of nutrients within that environment. Given the complexity of the microbial community and the limited amount of nutrients in the GIT, competition for these sources of energy is an inevitable occurrence. Therefore, microorganisms have evolved mechanisms by which to sequester the available nutrients. Iron for example, is a metal that many organisms need for growth. Meanwhile, siderophores are low molecular weight molecules that chelate iron from ferric-iron complexes and make it readily available for bacterial growth. The use of siderophores is an important tool to sequester iron in a low-iron environment, and thus depriving competitors from it. Siderophore producing, and harmless, bacteria offer potential for probiosis to outcompete pathogens by further limiting available iron (Weinberg, 2004). *Pseudomonas fluorescens* AH2 has been shown to inhibit the growth of *V. anguillarum* in co-culture experiments, while also reducing the mortality of rainbow trout challenged with *V. anguillarum* from 47% to 25% when applied as a probiotic feed supplement (Gram *et al*., 1999). Another study reported the ability of siderophores produced by *B. cereus* to inhibited *A. hydrophila* by speeding up the rate of iron uptake *in-vitro*, while also facilitating greater use of organic carbon in the medium (Lalloo *et al*., 2010).

- Competition for binding sites

The ability of pathogens to adhere to the digestive mucosa is the first, and a crucial, step to become established in the fish intestine, and later initiate the development of disease. To mitigate this, a probiotic that could prevent pathogen-tissue interactions is desirable. This also helps to further explain the importance of one of the first criteria in choosing a probiotic candidate, which lies in its innate capacity to bind and colonize the gut, and consequently minimizing available sites for adhesion, a phenomenon known as "competitive exclusion". One study demonstrated that the addition of an intestinal isolate of clownfish to intestinal mucus, already colonized by *V. alginolyticus,* was able to displace the pathogen, reducing pathogen attachment (Vine et al., 2004b). Although this study also showed that prior colonization with the probiotic enhanced pathogen attachment, the major takeaway was how the probiotic showed promise as a treatment to reduce the numbers of *V. alginolyticus*. Another study found that feeding *B. subtilis* to ornamental fish from the genera *Poecilia* and *Xiphophorus* conferred high survival and low infectivity after *A. hydrophila* challenge (Ghosh *et al*., 2008).

- Modulation of the immune response

The body of knowledge about the effects of probiotics in the immunity of fish, specifically of tilapia, is vast. Most of these studies generally focus on innate and adaptive responses, and try to distinguish between localized and systemic responses (Table 1.2). Although the effects of altering the gut microbiota on immunity can be hard to comprehend, this only explains the great extent by which the indigenous gut bacteria (and probiotics) alter the host immune responses.

Supplementation of probiotics affects the systemic immune responses in finfish. Even though, the majority of the studies cover this occurrence – the exact process on how this happens is not fully understood in the piscine. In tilapia, probiotic administration has been reported to improve serum lysozyme, alternative complement, myeloperoxidase activity, phagocytic activity and respiratory burst of immune tissue leukocytes, and antibody levels (Aly *et al*., 2008a; Chiu e*t al*., 2014; Ferguson *et al*., 2010; Iwashita *et al*., 2015; Liu *et al*., 2017; Pirarat *et al*, 2006; Ramos *et al*., 2017; Standen *et al*., 2013; Telli *et al*., 2014; Wang *et al*., 2008; Zhou *et al*., 2010a). Other studies have demonstrated that peripheral hematologic profiles can be altered via the supplementation of probiotics (Aly *et al*., 2008b; Hassaan *et al*., 2014; Jatobá *et al*., 2011; Reda and Selim, 2015; Telli *et al*., 2014; Ng *et al*., 2014).

Although the gut is the site where probiotics establish and execute their functions, few studies have focused on the mucosal immunity in tilapia. This is primarily due to the lack of molecular probes to assess this kind of immunity in fish, while also the gut mucosal immunity remains uncharacterized in tilapia. One study, however, found that the levels of IELs increased after supplementation of a probiotic candidate, *Pedicoccus acidilactici,* in Nile tilapia for six weeks (Standen *et al*., 2013). The study also found that this microorganism appeared to have an effect in raising the number of goblet cells, which are responsible for mucin production, a major component of mucus. Similar effects were also recorded in another study that found elevated levels of IELs and goblet cells after dietary supplementation with *B. amyloliquefaciens* after sixty days (Reda and Selim, 2015). A different study in Nile tilapia found that supplementation of *L. rhamnosus* GG increased the abundance of IELs and, more specifically granulocytes in the intestine (Pirarat *et al*., 2011).

Within the intestine, a few studies have shown that cytokines such interleukin (IL)-1ß, tumor necrosis factor (TNF)-α, transforming growth factor (TGF)-ß are upregulated upon probiotic supplementation in tilapia (He *et al*, 2013; Liu *et al*., 2013; Standen *et al*., 2013). Other studies in sea bass and rainbow trout showed that expression of probiotics increased TCR-related (Picchietti *et al*., 2009) and IgT (Pérez-Sánchez *et al*., 2011) transcripts, respectively. However, these components of the immune system have not been investigated due to their incomplete characterization in tilapia. Cytokine gene expression after dietary administration of probiotics has also been reported in a few studies as well. The levels of IL-1 and TNF-α were increased in *L. rhamnosus* GG supplemented tilapia (Pirarat *et al*., 2011). These cytokines, which relate to pro-inflammatory conditions, are in agreement with the increased levels of phagocytosis from the same study. A different work also reported that the gene expression of IL-1β and transferrin were up-regulated in both the spleen and the anterior kidney (AK) following supplementation with *L. acidophilus* (Villamil *et al*., 2017).

2.8 Role of water quality in fish health

Unequivocally, water quality is the single most important factor affecting fish welfare in aquaculture systems. Water quality affects the health status of the fish and, vice-versa, fish health can reflect the quality of the system where the animal is cultured. There are multiple physico-chemical parameters that are of crucial importance for water conditions, and every fish species has a defined list of optimal range for each. Those parameters include, but are not limited to, dissolved oxygen (DO), carbon dioxide (CO_2), temperature, nitrogen species, pH, alkalinity, hardness, and salinity. They are all important in fish farming, but it is worth mentioning that as

operations intensify, fish waste management becomes a problem. However, this is where a biological process known as nitrification comes into play.

Nitrification and fish production

Ammonia (NH_3) is a nitrogen species that accumulates in the water due to fish metabolic wastes (urine and feces) and uneaten fish food. It is an important compound in fish production activities due to its toxicity when accumulated in large concentrations. This compound is detoxified naturally in a two-step biological process, known as nitrification. The first step of the cycle consists of the bacterial oxidation of NH_3, into the less toxic nitrite (NO_2) compound. Most biology textbooks give credit of the first step of the reaction to *Nitrosomonas* spp, but other non-culturable species are probably important in the process (Bothe et al., 2000). After NO_2 levels increase, this nitrogen species is further oxidized to, the even less harmful, nitrate (NO_3) by function of bacteria of the genus *Nitrobacter,* but probably other nonculturable species are involved as well. Accumulation of NH_3 or NO_2 is not desired as they are both toxic to aquatic species (Manthe et al., 1983; Svobodova et al., 2005). The final product of nitrification, NO_3, is less toxic compared to its precursors, and it can accumulate in the aquaculture system allowing the growth of algae, until these compounds are flushed out via water exchanges, or utilized by plants, or it is converted to nitrogen gas (N_2) by heterotrophic bacteria via denitrification.

Good water quality practices in fish farming operations include monitoring levels of nitrogen species. Most commercial kits only measure total ammonia nitrogen (TAN), which is a sum of the molecules NH_3 (unionized form) and NH_4+ (ionized form) in water. Out of the two, NH_3 is more toxic, and the fraction of this unionized form can be determined if the temperature

and pH of the system are known. Typically if temperature and pH are high, the levels of NH_3 will be high. Toxic levels of NH_3 in fish range from 0.2-3mg/L (Boyd and Tucker, 1998).

Just as in the case of ammonia, NO_2 toxicity is dependent on other water quality parameters as well, particularly, the pH. In acidic conditions, NO_2 binds to hydrogen ions to form HNO_2, nitrous acid, which is of greater toxicity. The toxicity of NO_2 varies per aquatic species, but levels above 0.1 mg/L are of health concern in aquaculture important species (Stickney, 1994). The mechanism by which NO_2 causes disease is by entrance into the gills, and then being absorbed into the bloodstream, combining with hemoglobin to form methemoglobin, and leading to "brown blood disease". This disease is characterized by the inability of red blood cells to transport sufficient amounts of oxygen to organs and tissues, resulting in suffocation of the fish. NO_2 toxicity, however, is more concerning to freshwater species, because in the saltwater scenario chloride ions out-compete and block the entrance of nitrite to the gills. Hence, salt can be added to freshwater systems to protect the fish from NO_2 poisoning.

Dissolved oxygen and carbon dioxide levels

Fish utilize oxygen to break down organic material (feed), to then convert that to energy, and biomass. While respiring, fish give off carbon dioxide. For freshwater species, it is recommended that the concentration of DO be kept above 5mg/L (Svobodova et al., 1993). This value represents the minimum requirement of oxygen in fish for healthy growth and reproduction. Another important aspect about DO concentration is that it helps to reduce the levels of CO_2.

After carbon dioxide is released through the fish gills, it is found in two forms, as molecular gas (CO_2) and as carbonic acid (H_2CO_3). Carbon dioxide has an important role in

regulating the buffering capacity of the water. High levels of this parameter may become problematic, either by lowering the pH of the water, or when levels of DO are low, raising the amount of CO_2 in the blood of the fish, and leading to acidosis. However, carbon dioxide is not a problem when the DO concentration is above saturation levels. Thus, constant aeration and monitoring the DO levels of the rearing medium is vital during culturing of fish.

Taken all into account, poor water quality conditions can cause a substantial amount of stress to the fish and render them vulnerable to diseases, resulting in significant financial losses and the possibility of losing the whole population. Therefore, attempts to reduce the likelihood of diseases should employ the old business proverb of "lower the risks to increase profits". In efforts to reduce these losses in intensive operations, the use of a recirculating aquaculture system (RAS) has gained attention in recent years.

Recirculating aquaculture system technology

The RAS units entail complex systems to minimize intense human labor, pollution and disease associated with traditional fish farming operations. It represents an alternative and an innovative method to culturing fish outdoors where fish are farmed in tanks systems indoors in a more controlled environment; both allowing for higher intensification in production and for the possibility of culturing marine and freshwater species. This technology system works by constant filtration and recycling of the water being used to culture fish. Therefore re-using over 90% of the water, and creating minimal effluents.

The system entails multiple components that control five key aspects: clarification, aeration, circulation, nitrification and degassing. First, fish are grown in the main tank, supplied with food to encourage growth and survival. The tank water, containing remains of feed and fish

by-products, circulates to mechanical filter that will trap large solids and clarify the water. Filtered water then travels to a bioreactor that will detoxify metabolic waste products from fish, ammonia (and the subsequent nitrogen product of nitrification). After this step, purified water will recirculate back to the main tank (Figure 1.8). Additional parts of RAS include a water pump to circulate the water in the system, and sometimes ultraviolet filtration or ozonation to eliminate microorganisms from filtered water before it recirculates back into the tank.

Nitrification in RAS is vastly facilitated via the use of biofiltration. Unlike, open water systems, RAS employs biofilter media that helps in creating larger surface area for nitrifying bacteria to proliferate and bio-remediate ammonia and nitrite. However, the biofilter will only start working after it has been colonized with the appropriate ammonia-oxidizing bacteria, which can take up to several weeks.

Current status on the use for RAS for sustainable fish farming

Recirculating culture systems certainly offer several benefits over traditional open systems. They are considered more environmentally friendly in management of fish wastes, and have greater control of biosecurity of the fish crop (Piedrahita, 2003; Summerfelt et al., 2009). Certainly, the use of this technology is more common in urban sites, where there is increased interest in producing food near highly populated areas, but also where flow-through production systems (e.g. ponds and raceways) are limited because of the absence of nearby water natural sources. Virtually, any aquatic organism should be able to be raised under these controlled conditions, and space can be better managed for the culture of aquatic species, compared to in-land production activities. The use of RAS does provide that advantage to circumvent increasing land prices and water shortages that will probably continue to challenge aquaculture activities in

the future. However, some disadvantages for the use of these systems include the substantial technical knowledge for proper operation and maintenance, but also the high initial economic input they require.

The use of RAS units is currently advantageous for research purposes, but one with great potential to become popular in the world in the years to come. As previously mentioned, Blue Ridge Aquaculture in VA, USA takes advantage of this technology and manages to produce over 4 million lbs annually of tilapia. As the global population continues towards a trend of greater urbanization, and natural sources of water become more limited, there will be a greater need especially for foodfish. Although a high monetary investment for RAS is required initially, there is the potential economic advantage of a steady and controlled production year-round. Furthermore, the idea of safer, more environmentally friendly practices and locally produced fish may be appealing to a world population that appears to be following that trend.

2.9 Conclusion of literature review

The literature provides adequate evidence to justify that ameliorating the health of foodfish can help in increasing their survival. A lot of normal physiology information does not exist for important cultured fish species, such as striped catfish. This data can prove to be nothing but beneficial in diagnosis during diseases cases of fish. Additionally, the use of probiotic as an alternative for antibiotics shows promise, not only in prophylaxis but showing a degree of protection during infection. Additional work is needed for this, nonetheless, especially as neither do all probiotic have the same mechanisms of action nor do they have the same benefits in different piscine hosts. Provided the complexity and large role the microbiome has on

immunity, it is imperative to understand how alteration of the indigenous microbiota affect the immune responses of the host.

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Figure 2.1 Striped catfish (*Pangasius hypophthalmus*)

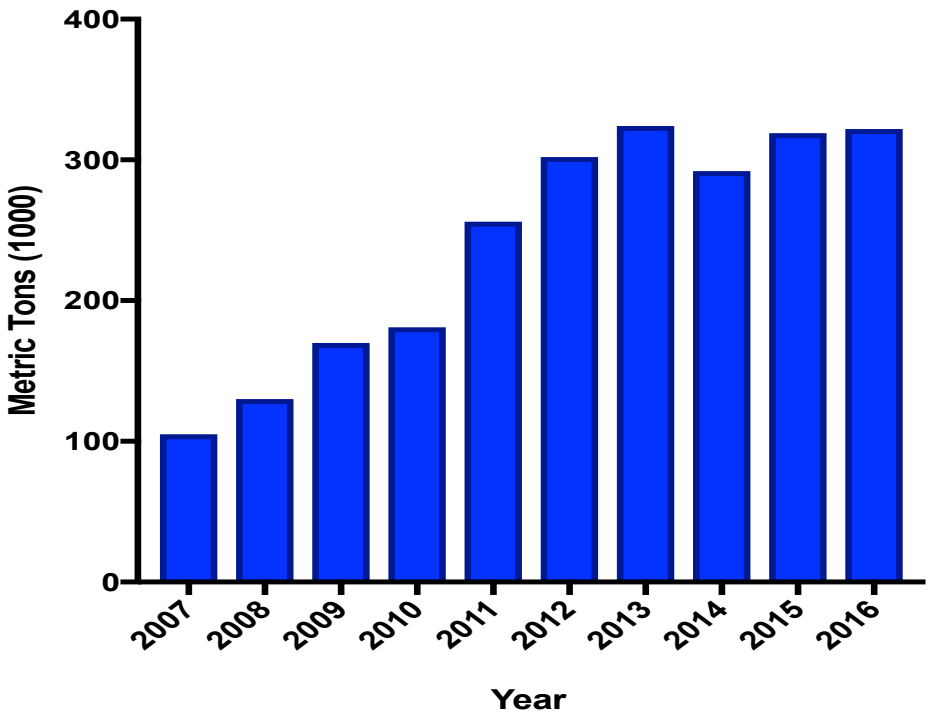

Figure 2.2 US Pangasius imports. Source: NFI, Urner Barry 2017

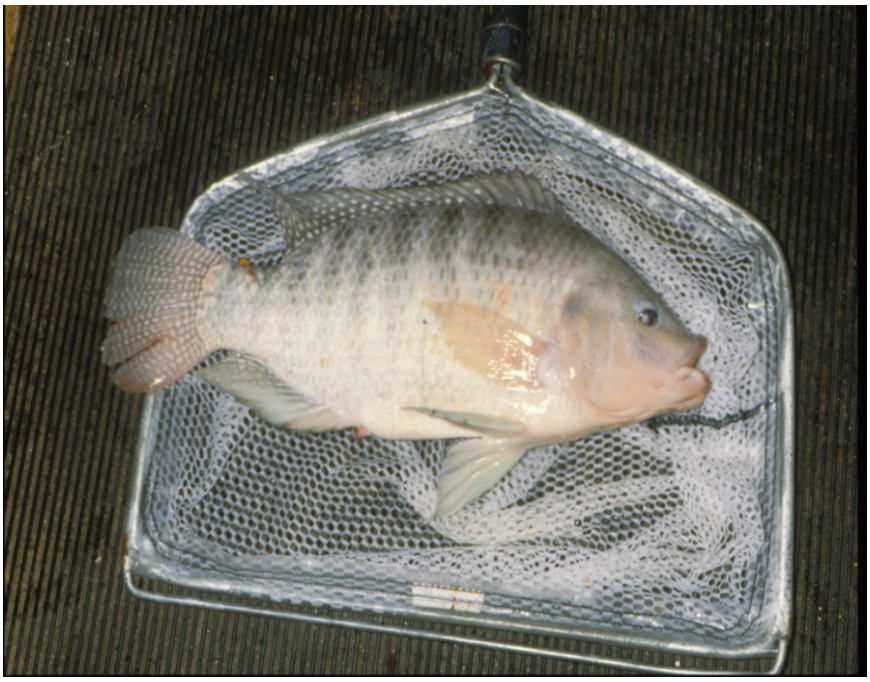

Figure 2.3 Nile tilapia (*Oreochromis niloticus*). Photo courtesy of SA Smith.

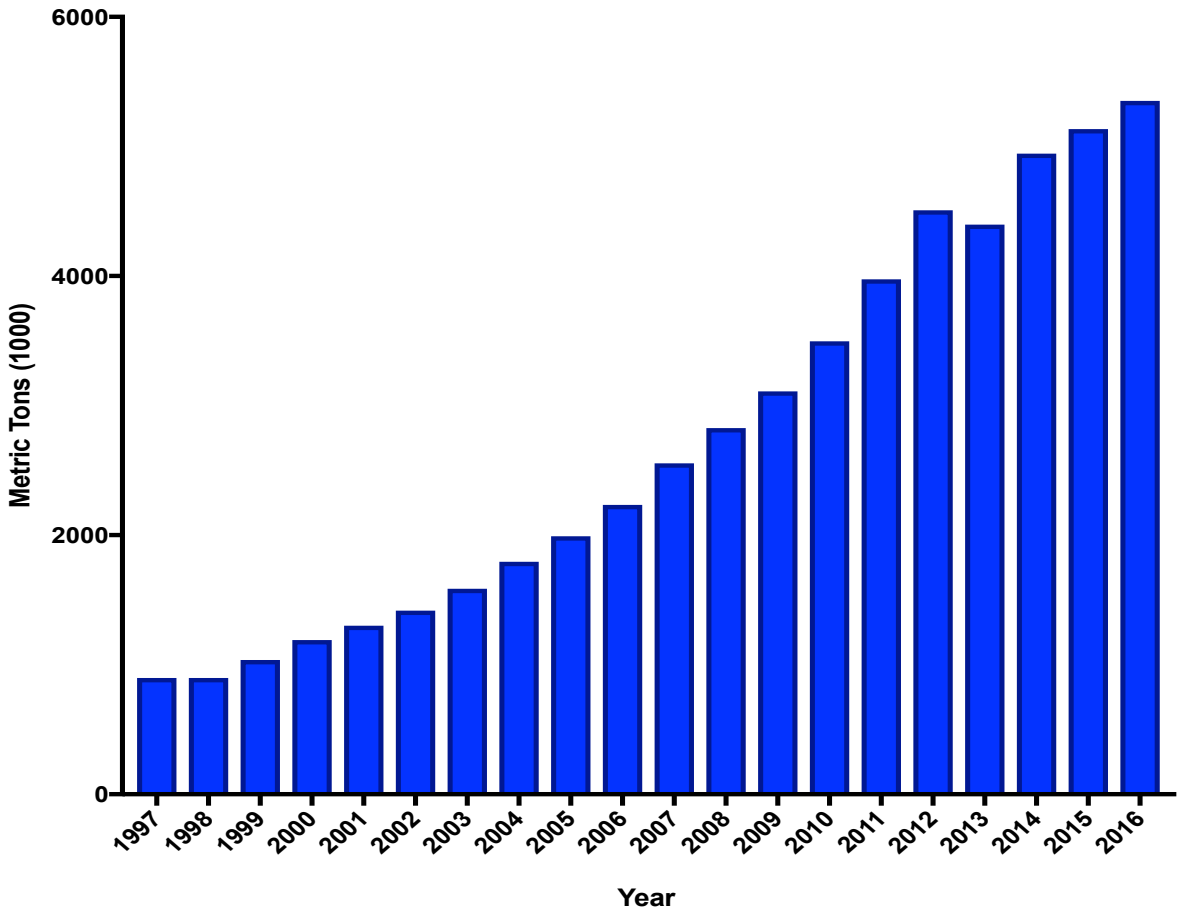

Figure 2.4 Global production of tilapia, years 1997-2016. Source FAO 2013-2017

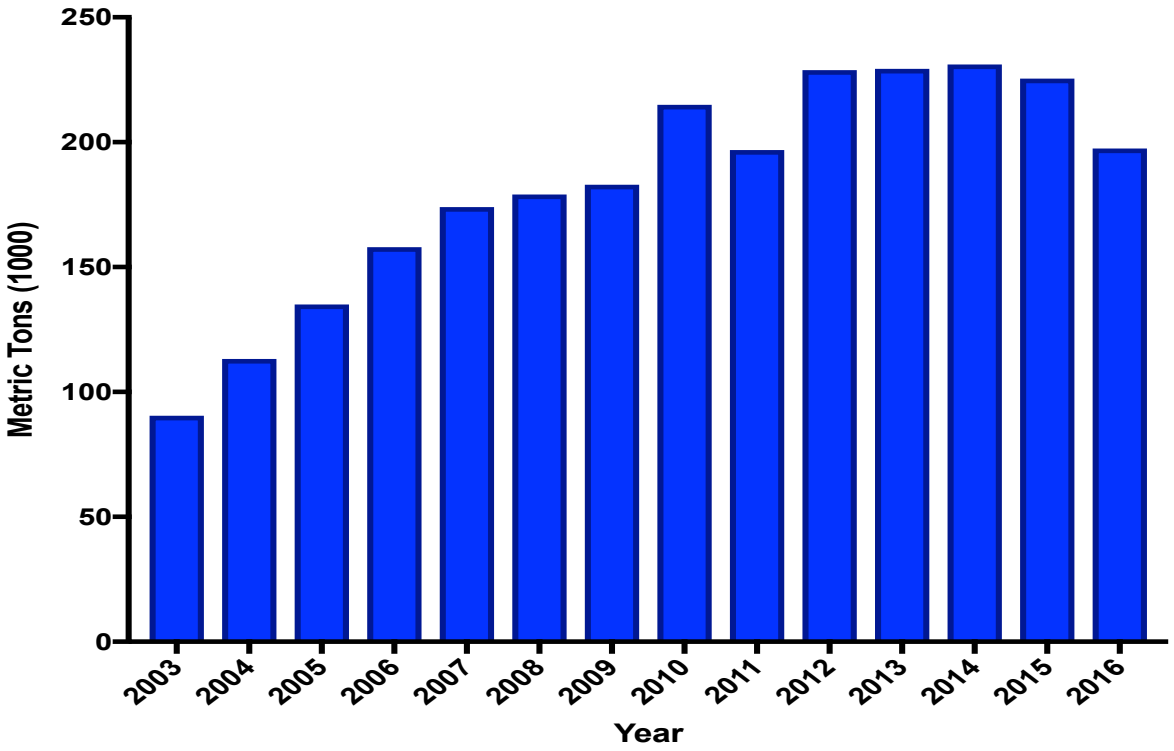

Figure 2.5 US imports of tilapia 2003-2016. Source: USDA, 2017

Table 2.1 Comparison of the teleost and mammalian immune responses

	Bony fish	**Mammals**
Innate immunity	More diversity	Limited diversity
Adaptive immunity	Poor (sluggish)	Strong
Lymphoid organs	Anterior kidney, spleen and thymus	Bone marrow, spleen, thymus, lymph nodes
Immunoglobulins	IgM-like, IgD, IgT/IgZ	IgM, IgG, IgA, IgD and IgE
MHC I and II; CD4 and CD8	Present	Present
Mucosa associated lymphoid tissue	Present	Present
Dependence on temperature	High	Low

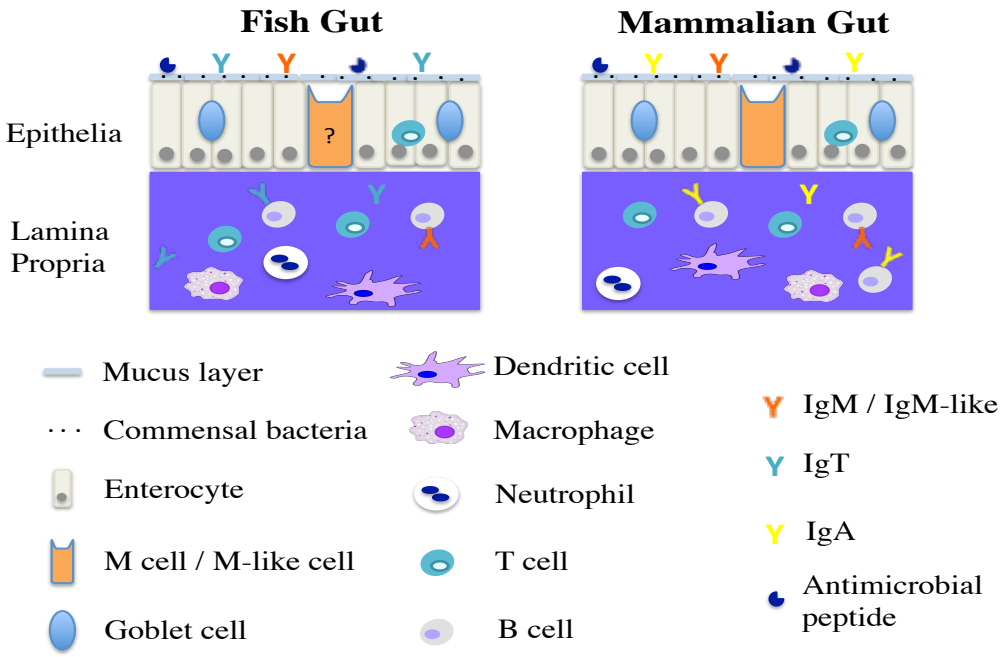

Figure 2.6 Schematic comparing fish and mammalian gut mucosal surfaces. Similarities between the types of immune cells (dendritic cells, neutrophis, T and B cells), as well as in the structure (goblet cells, mucus layers, enterocytes) are displayed. A prevalent humoral difference (mucosal Ig isotype present) is also depicted. (Source: modified figure from Gomez et al, 2013).

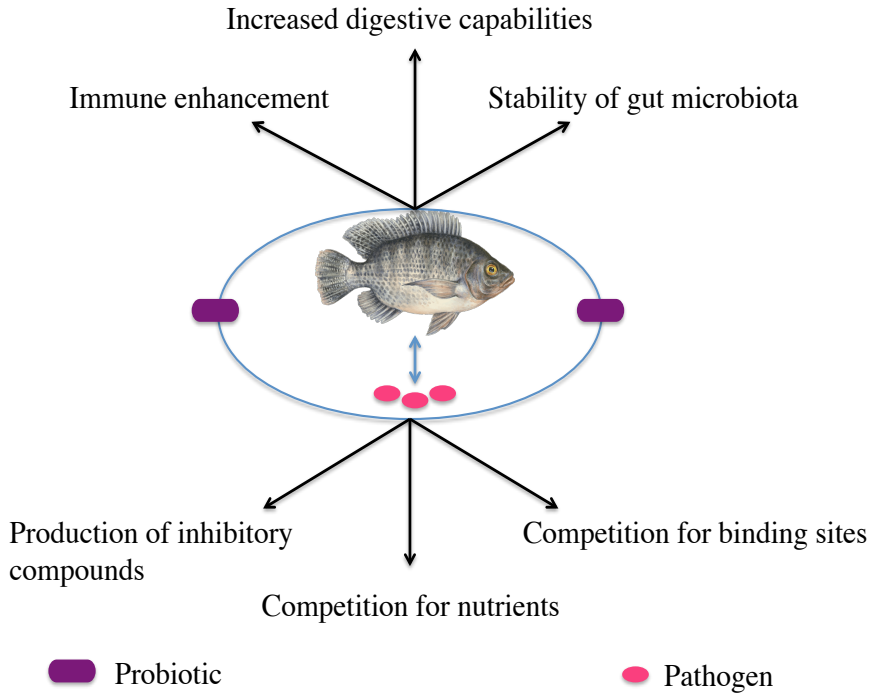

Figure 2.7 Modes of action of probiotics in fish. Source: adapted from Montalban-Arques et al., 2015

Table 2.2 Studies that report immunological modulation after probiotic supplementation in Nile tilapia (*Oreochromis niloticus*)

Probiotic organism	Dose and trial duration	Immune parameters altered	Reference
Bacillus amyloliquefaciens and *Lactobacillus* spp	10^8 CFU g^{-1}, 99 days	↑ lysozyme activity + SOD + TIg; no change in hematology	Ridha and Azad, 2012
B. amyloliquefaciens	10^4, 10^6 CFU g^{-1}, 60 days	↑ intestinal villi + IEL + RBC + WBC	Reda and Selim, 2015
B. amyloliquefaciens	10^4, 10^6 CFU g^{-1}, 60 days	↑ intestinal villi + IEL + RBC + WBC	Reda and Selim, 2015
B. amyloliquefaciens R8	10^9 CFU g^{-1}, 2 months	↑ SOD + PA + RB + serum LA + survival rates	Saputra *et al*., 2016
B. cereus var. toyoi and *B. subtilis* C-3102	0.25%+0.25% and 0.5% w/w, 127 days	↑ hemoglobin, hematocrit and neutrophils	Garcia-Marengoni *et al*., 2015
B. coagulans and *Rhodopseudomonas palustris*	10^7 CFU ml^{-1}, 40 days	↑ SOD + catalase + MPO + RB; no change in LC	Zhou *et al*., 2010a
B. licheniformis	0.24, 0.48, 0.96 x 10^6 CFU g^{-1}, 12 weeks	↑ RBC + WBC + hematocrit + hemoglobin	Hassaan *et al*. 2014
B. licheniformis	2 x 10^{10} CFU g^{-1}, 10 weeks	↑ classical complement + LA; unchanged SOD	Han *et al*., 2015
B. pumilus	$10^{6,}$, 10^{12} CFU g^{-1}, 8 months	↑ NBT + WBC + lymphocytes + neutrophils + monocytes	Aly *et al*., 2008b

Bacillus sp, *Pediococcus* sp., *Enterococcus* sp., *Lactobacillus* sp	10^6 CFU g^{-1}, 8 weeks	↑ villi height + plasma alternative complement	Ramos *et al*., 2017
B. subtilis and *L. acidophilus*	10^7 CFU g^{-1}, 56 days	↑ RB + serum lysozyme; no change in hematocrit	Aly *et al*., 2008a
B. subtilis	5 x 10^6 CFU g^{-1}, 84 days	↑ LA + PA; ↓ hematocrit + RBC	Telli *et al*., 2014
B. subtilis	1.5 and 3 x 10^9 g^{-1}, 4 weeks	↑ RB + WBC; ↓ mortalities upon challenge with *Aeromonas hydrophila* and *Streptococcus iniae*	Iwashita *et al*., 2015
B. subtilis HAINUP40	10^8 CFU g^{-1}, 8 weeks	↑ total antioxidant activity + SOD after 8 weeks; ↑ LC and RB only after 2 weeks	Liu *et al*., 2017
Enterococcus faecium	10^7 CFU ml^{-1}, 40 days	↑ SOD + classical complement + MPO; unchanged LA	Wang *et al*., 2008
L. acidophilus	10^6 CFU g^{-1}, 15 days	↑ IL-1ß + transferrin transcripts	Villamil *et al*., 2014
L. plantarum	10^8 CFU g^{-1}, 12 weeks	↑ WBC + thrombocytes	Jatobá *et al*., 2011

L. plantarum	0.5, 1, 2% w/w, 40 days	↑ T helper cell cytokines in liver + WBC + TIg + PA + LA	Hamdan *et al*., 2016
L. rhamnosus GG	10^{10} CFU g^{-1}, 30 days	↑ alternative complement + villi height + IEL + phagocytosis + bactericidal activity + proinflammatory cytokines in anterior kidney	Pirarat *et al*., 2011
Lactococcus lactis	10^{7} CFU ml^{-1}, 40 days	↑ SOD + MPO + LC + RB	Zhou *et al*., 2010b
Pediococcus acidilactici	10^{7} CFU g^{-1}, 32 days	↑ WBC + LA; unchanged gut WBC	Ferguson *et al*., 2010
Pediococcous acidilactici	2.81 x 10^{6} CFU g^{-1}, 6 weeks	↑ IEL + TNF-α intestinal transcripts + monocytes + neutrophils	Standen *et al*., 2013
Pseudomonas fluorescens	1.8x10^{8} CFU g^{-1}, 45 days	↑ serum globulin + RBC + lymphocytes, monocytes; ↓ mortalities	Eissa and Abou-ElGheit, 2014
Psychrobacter namhaensis SO89	10^{7} CFU g^{-1}, 50 days	↑ RBC, WBC + IgM + alternative complement + PA + LA + T helper cell cytokines in the liver	Makled *et al*., 2017

↑ denotes increasing values, and ↓ indicates decreasing levels

IEL, intraepithelial lymphocytes
LA, lysozyme activity
LC, lysozyme content
MPO, myeloperoxidase
RB, respiratory burst
RBC, red blood cell
SOD, superoxide dismutase
TIg, total immunoglobulin

NBT, nitroblue tetrazolium

WBC, white blood cell

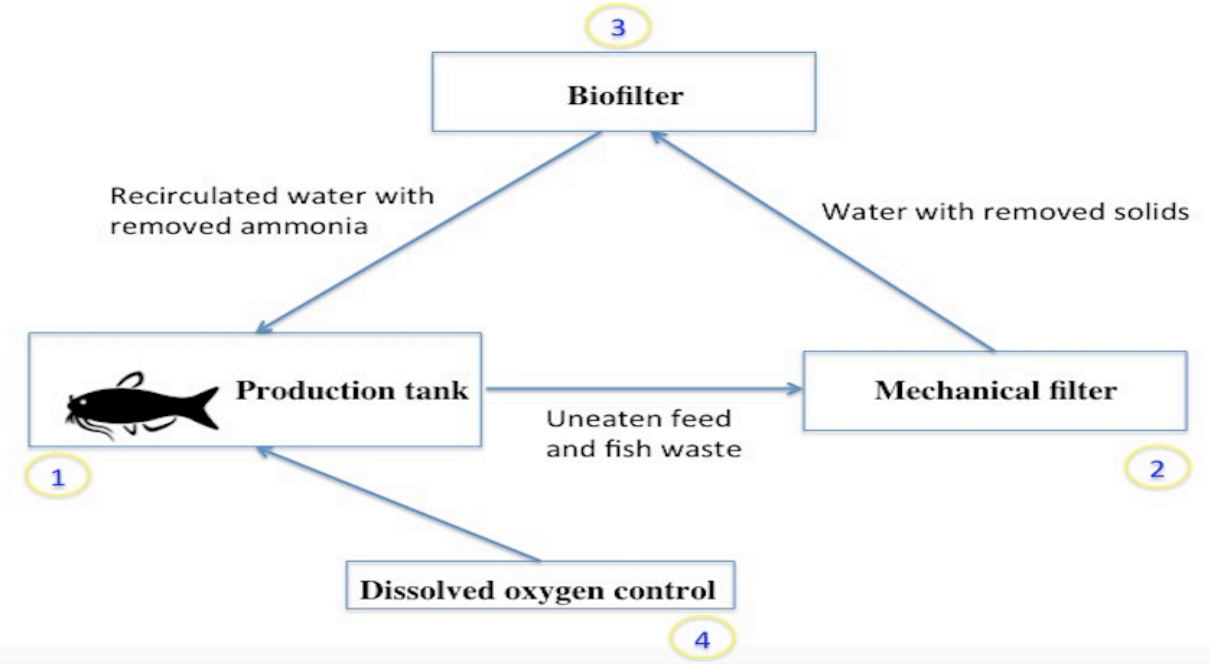

Figure 2.8 Simplified diagram of recirculating aquaculture systems. 1- Water with fish waste and feed remains flows out from tanks into chamber with mechanical filtration. 2- Mechanically filtered water move into biofilter to reduce levels of ammonia. 3- Filtered water recirculates back into the grow-out tank. 4- Air is constantly being added to the fish

CHAPTER III

GENERAL METHODOLOGY

The following protocols were carried out for the completion of the three objectives for this dissertation work. Specific materials and methods relevant to each objective are discussed in the corresponding chapters. All animal protocols were approved and carried out by following the standards of the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech. The IACUC protocol 15-021 was associated with Objectives 1 and 2, and protocol 16-047 was linked to Objective 3.

3.1 Recirculating systems and management of water quality

Striped catfish that were used for Objectives 1 and 2 were reared in recirculating aquaculture system (RAS) units (Figure 2.1) in the Food Science and Technology Building of Virginia Tech. Municipal water that was treated with carbon filtration for removal of chloramines was used for both studies. Each RAS unit had an approximated capacity of 1,100 L. Each unit consisted of a 800L rearing fiberglass tank, and a 300L fiberglass sump. A custom mechanical filter was also in place for preventing accumulation of uneaten feed and fecal wastes. Biological filtration (nitrification) was provided by the presence of submerged KMT filter media (Anoxkaldness, Lund, Sweden) in the sump for the removal of ammonia and nitrite. An ultraviolet unit helped disinfect the water before recirculating back into the main culture tank. Within the rearing tank there was an aerator that constantly added air and removed carbon dioxide. Fresh water was added every two days to the sump to account for water lost to evaporation and splash, and 20% water exchanges were done twice a month. An automated 16h light : 8h dark photoperiod was maintained though the studies.

The fish for Objective 3 were reared in a RAS unit (Figure 2.2) in the Human and Agricultural Biosciences Building 1 of Virginia Tech. The RAS unit, of approximately 2,600 L in capacity, had six 300L circular tanks and an 800 L fiberglass sump. Other components of the RAS unit involved a sand filter (Hayward Industries, Elizabeth, NJ, USA) for mechanical filtration, biofiltration media in the sump, a bubble bead filter (Aquaculture Systems Technology, New Orleans, LA, USA) for nitrification and water clarification, and an ultraviolet filter for water sanitation. Water in the recirculating system was from a municipal source with prior carbon filtration to remove chloramines. Each of the tanks had an air stone to add oxygen and strip out carbon dioxide. Backwashes were performed once daily for removing the nitrogenous species and preventing build up of organic matter in filters.

Water quality parameters were monitored daily throughout the studies. These parameters involved alkalinity, dissolved oxygen (DO) and temperature. The alkalinity was measured via the titration method using sulfuric acid (HACH, Loveland, CO, USA), and ranges between 100-150 mg L^{-1} were considered acceptable for both fish species. Adjustments to alkalinity were done by addition of sodium bicarbonate. Temperature was maintained by use of a thermostat-linked coil heat exchanger and kept set either at 27°C for striped catfish, or at 30°C for tilapia. The levels of DO were kept above 5.5 mg L^{-1} saturation. The pH was measured every other day, but levels were kept approximately at 7.5.

All nitrogenous species were also evaluated in the studies. These were monitored by use of an automated spectrophometer (DR 2800, HACH). Total ammonia nitrogen and nitrite were monitored three times a week, whereas nitrate was done once weekly. Acceptable levels for these freshwater species were <0.1 mg L^{-1}, <0.1 mg L^{-1}, <50 mg L^{-1} for ammonia, nitrite and nitrate, respectively.

3.2 Experimental fish and handling

Two batches of fingerlings were obtained for the three studies. For Objectives 1 and 2, striped catfish were obtained from Sos Tropical Inc (Riverview, FL, USA); whereas Nile tilapia for Objective 3 were sourced from Spring Genetics (Miami, FL, USA). Upon receipt, temperature, DO and ammonia were taken, and gradual water changes were carried out, with water from rearing tanks. After 30 minutes fish were transferred to the rearing tanks and allowed to acclimate. Fish were fed commercial feed (Rangen, Inc, Buhl, ID, USA) suitable for their size, and were allowed to acclimate for a minimum of 2 months.

Sedation and euthanasia were important parts for this work. For the three animal studies, fish had to be sedated for intraperitoneal (IP) injections (Figure 2.3), and collection of blood from caudal vessels (Figure 2.4). Sedation was achieved by use of sodium bicarbonate-buffered tricaine methanesulfonate (MS-222, 100mg/mL; Western Chemical Inc., Ferndale, WA, USA) until fish lost equilibrium (stage III anesthesia). After completion of procedures following sedation, fish were placed in a container of fresh water with proper aeration to allow for visible recovery and then returned to the corresponding tank. Euthanasia preceded tissue collection procedures of Objective 3. This was accomplished by utilization of sodium bicarbonate-buffered MS-222 (200mg mL^{-1}) until fish lost operculum movement (stage IV anesthesia). This was followed by cervical dislocation of the animal by disconnection of the spinal cord from the brain.

3.3 Feed preparation and animal feeding

Feed for Objectives 1 and 2 was ground from a commercial feed, and then pelleted in a pellet mill, resulting in ~1 cm pellets. Fresh produced pellets were allowed to dry in a well-ventilated room for 24 hours at room temperature. A proximate analysis was obtained from this

prepared diet indicating a profile of 45.5% carbohydrates, 28.2% protein, 8% ash, and 5.98% fat. A commercial 3mm floating feed (Rangen, Inc, Buhl, ID, USA) was utilized as the basal diet, and in the making of the probiotic diets. *Bacillus* spores were suspended in distilled water, which was then top-coated onto the feed using a low-pressure air sprayer. The basal diet without the probiotic strains served as the control. All diets were kept at 4°C during the course of the probiotic trial. Probiotic viability was checked in the feed by use of the standard plate count method.

The fish species for the three objectives were weighed at day zero, and at the end of the study. They were fed a rate of diet relative to their biomass per day for a 16 h period using 24 h automated belt feeders (Pentair AES, Apopka, FL, USA). Daily feeding rates were adjusted based on feeding behavior (responsiveness), mortalities, or feed conversion rates (FCRs).

3.4 Hematology

Blood was collected from the caudal vessels of sedated fish by use of a 21 and a 23-gauge needle attached to a 1mL syringe for tilapia and catfish respectively. A sample of blood was placed in 0.6 mL heparinized tubes and mixed gently by inversion. Tubes were then centrifuged for 5 minutes to separate plasma, which was transferred to new tubes and stored at -20 °C until further processing.

White blood cell differentials were also done by first making a blood smear. A drop of fresh blood (no anticoagulant) was placed towards the end of a slide. The blood was spread by carefully pushing a second slide towards the opposite end of the first slide to make a thin smear of blood. Smears were allowed to air dry before staining. Dried blood smears were then stained by use of Romanowsky dyes (Wright-Giemsa or May Grunwald-Giemsa for Objectives 2 and 3

respectively), and observed under a microscope at 1000x for counting of the peripheral WBC populations.

3.5 Fish necropsy procedures

Tilapia subjects were dissected by use of appropriate aseptic techniques and sterile instruments. The abdomen of the fish was punctured with the tip of a sterile knife, and a cut was made along the ventral body surface of the animal to provide access to the coelomic cavity. The entire viscera was carefully removed, and tissue samples were collected and stored as needed for the respective analytical assays.

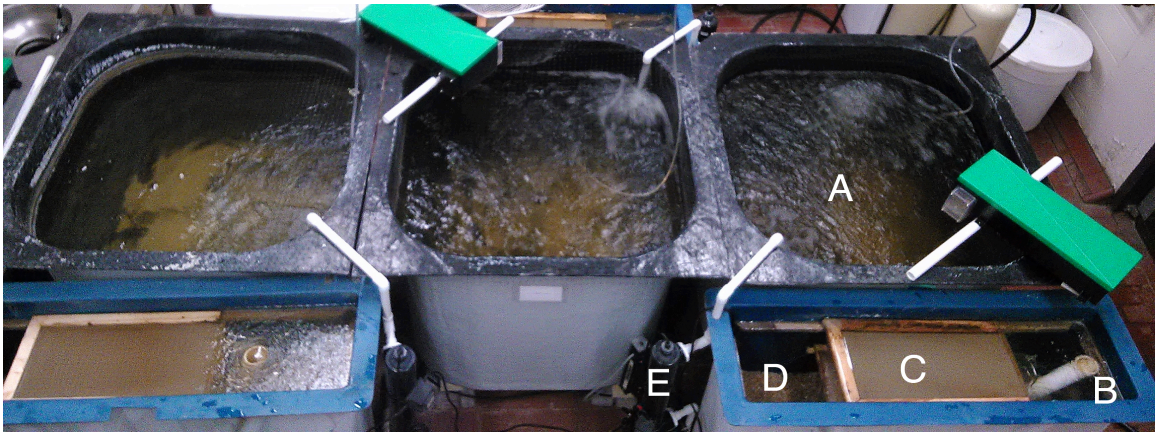

Figure 3.1 Recirculating aquaculture systems located in the Food Science and Technology building at Virginia Tech. Water circulates from the rearing tank (A) to the sump (B) where it is filtered mechanically (C) and biologically (D), and passes via a UV filter unit before returning to the main tank.

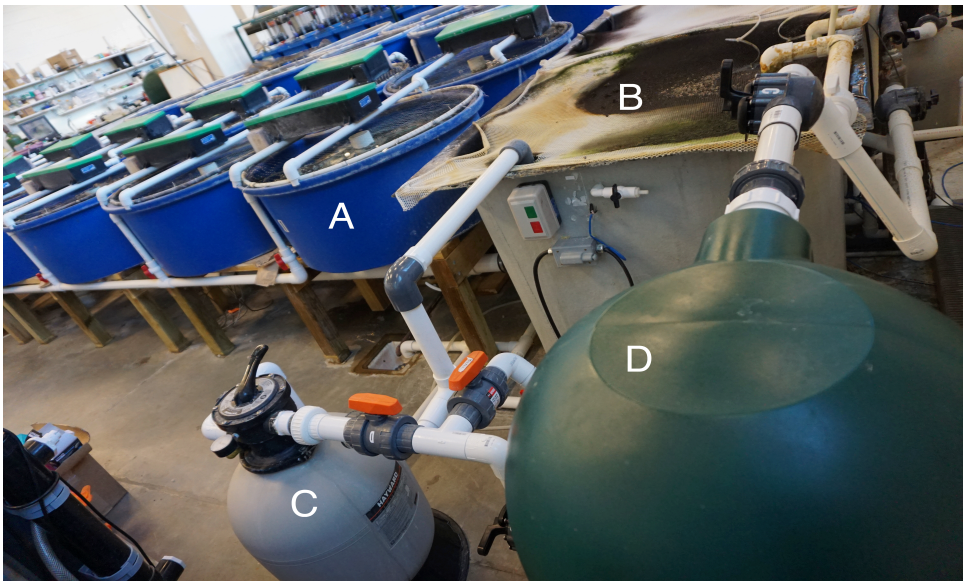

Figure 3.2 Recirculating aquaculture systems located in the Human and Agricultural Biosciences building 1 at Virginia Tech. Water flows from the rearing tank (A) to the sump (B), from which the water is then channeled to a sand filter (C) and a bubble bead filter for mechanical and biological filtration, respectively, and the water then returns to the rearing tanks.

Figure 3.3 Intraperitoneal injection of fish.

Figure 3.4 Blood collection from caudal vessels of fish.

Chapter IV

ISOLATION, PURIFICATION AND DETERMINATION OF TOTAL PLASMA DERIVED IgM-LIKE IMMUNOGLOBULIN FOR REARED *PANGASIUS HYPOPHTHALMUS* IN RECIRCULATING AQUACULTURE SYSTEMS

Abstract*

A good understanding of the normal immune responses in important aquaculture species is vital for evaluating the health status of the species of interest. However, basic information such as titers of immunoglobulin (Ig) is limited for fish, which could be attributed to the lack of available specific immunological probes to assess such immunity analytes. This study aimed to isolate, purify and characterize the IgM-like antibody from striped catfish (*Pangasius hypophthalmus*); and then to determine the reference intervals for this macromolecule of the immune system in the catfish species. An SDS-PAGE analysis of the purified striped catfish immunoglobulin revealed a heavy and a light chain with molecular weights of 70 and 25 kDa, respectively, and thus an estimated size of at least 760 kDa for the tetrameric form of this antibody isotype. A polyclonal rabbit anti-striped catfish antibody was produced with reactivity exhibited only against fish of the genus *Pangasius*, while also showing none against three non-*Pangasius* species. The plasma levels of the IgM-like molecule in striped catfish were determined via a sandwich enzyme-linked immunosorbent assay (ELISA). The reference interval for this analyte had a range of 1.2 – 7.6 mg mL^{-1}, and a median value of 3.3 mg mL^{-1}. The findings of this study can help in monitoring changes in the health status of striped catfish

* Manuscript to be submitted to Fish and Shellfish Immunology journal

undergoing different types of stress agents (e.g. vaccination, biological, chemical) in recirculating conditions. Furthermore, the methodology presented here could help in efforts to reliably quantify the levels of total immunoglobulin in other species of fish.

1. Introduction

Immunoglobulins (Igs) are a paramount and a conserved component of humoral immunity, across all vertebrate taxa. These compounds confer protection to the host via several immune mechanisms including neutralization, opsonization and agglutination of foreign agents, overlapping functionalities of both the innate and adaptive immune responses. In teleosts, a tetrameric Ig, termed IgM-like, similar to mammalian IgM is the predominant Ig isotype found in blood circulation or as a membrane bound molecule [1]. Moreover, other Ig classes such as IgD and IgZ/T have been added to the repertoire of piscine antibodies, given their presence in a few fish species [2-3]. Although the research on these shows some promise in advancing our understanding of the fish immune response, the role of these in the immune response has not fully been established. The IgM-like molecule, on the other hand, is the one whose function is most widely understood. The tetrameric IgM-like class is commonly found as a 600-800 kDa macromolecule [4]. Each monomer component of the tetrameric form is made up of two heavy (H) and two light (L) chains.

The striped catfish (*Pangasius hypophthalmus*), of the Order Siluriformes, is an economically important freshwater species in southeast Asia. Its production exceeds 1.6 million metric tonnes, and it represents a common whitefish commodity around the world [5]. Despite its evident aquaculture importance, there have been a limited number of studies focusing on humoral immune responses in this fish species. The absence of this basic immunology knowledge has exacerbated the economic losses occurring in the striped catfish industry due to bacterial diseases. An additional component that makes diagnostic decisions difficult is that reference intervals (RIs) of the total IgM-like antibody concentration have not been determined in striped catfish.

In an effort to understand the humoral immune responses in striped catfish, the purpose of this work was to isolate, purify, characterize the plasma Ig, and generate antibodies against the purified Ig from this fish species. This polyclonal antibody was employed as a tool to determine total Ig in the plasma of striped catfish, and thus helping define RI for this immune analyte. Furthermore, the cross-reactivity of the polyclonal antibody was tested against plasma from other fish species.

2. Materials and Methods

2.1 Experimental animals

Striped catfish were acquired from a commercial source (Sos Tropical Inc., Riverview, FL, USA). Four 9-month old fish with weights ranging 280-380 g were used for this study. Fish were stocked in a single recirculating aquaculture system (RAS) at the Food Science and Technology aquaculture facility at Virginia Tech, Blacksburg, VA. The 1,100 L RAS was equipped with a mechanical filter for solids removal, bio-filter media for nitrification, an ultraviolet unit for disinfection, and an aerator to add oxygen and strip carbon dioxide out of the water. Municipal water that underwent carbon filtration was utilized to emulate a freshwater environment and to maintain the experimental animals. Catfish were kept under controlled conditions of dissolved oxygen (>5 mg L^{-1}), temperature (25-27°C), alkalinity (90-130 mg L^{-1}), pH (~7), total ammonia nitrogen (<1 mg L^{-1}), nitrite (<0.1 mg L^{-1}) and nitrate (<50 mg L^{-1}). To ensure acclimatization to these conditions, the fish were reared in the RAS units 2 months prior to immunization.

Striped catfish were fed an in-house produced and pelleted diet (45.5% carbohydrates, 28.2% protein, 5.98% fat) throughout the trial. This feed was given at an initial rate of 3% body

weight day $^{-1}$, but the rate was reduced to 1.5% body weight day $^{-1}$ by the end of the trial. Feeding of the animals was achieved by use of an automated 24 h belt feeder (Pentair AES, Apopka, FL, USA), with feed being continuously delivered over a period of 12 h.

2.2 Immunization of fish

Before immunizations, fish were sedated individually in sodium bicarbonate-buffered tricaine methanesulfonate (MS-222, 100mg/mL; Western Chemical Inc., Ferndale, WA, USA). Prior to each intraperitoneal (IP) injection, fish were individually weighed. Each fish received an IP injection on day 0 with 195 μg goat anti-mouse IgG ($F[ab']_2$ fragment of goat IgG; Cappel Laboratories, West Chester, PA, USA). Each immunized fish was placed in a container with freshwater and proper aeration, to allow for visible recovery, before being returned to the rearing tank. On days 14 and 28, the fish were given a booster with a dose of 195 and 100 μg, respectively. Intraperitoneal administrations of immunogen were carried out without the use of an adjuvant.

2.3 Fish blood collection

Non-lethal blood samples were collected on days 35, 42 and 49. A 23-gauge needle attached to a 1 mL syringe was utilized for blood collection from the caudal vessels. The blood was collected in 0.6 mL pediatric tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ, USA). Plasma separation was achieved by centrifugation at 14,000 x *g* for 3 minutes, and then plasma samples were pooled and stored at -20 °C.

2.4 Purification of Pangasius Ig

Striped catfish-derived antibodies were separated from plasma by affinity chromatography as previously described [4]. Briefly, two milliliters of goat anti-mouse IgG agarose beads (Sigma Aldrich Co., St Louis, MO, USA) were packed by gravity into a 10 x 0.5 cm glass column (Bio-Rad Laboratories, Richmond, CA, USA). The packed column was washed with 150 mL of phosphate buffered saline (PBS, pH 7.4) at a slow flow rate of 30 mL/h, and stripped with 10 mL elution buffer (0.1 M glycine, pH 11.0). Two milliliters of plasma of the goat-IgG-immunized striped catfish were diluted with an equal volume of PBS and filtered through a 0.45 μm pore filter. The filtrate was applied to the column, allowing the sample to pass through the beads. The column was then washed with 150 mL PBS. The bound fish immunoglobulins were eluted by application of 5 mL elution buffer to the column, and neutralized by addition of 0.1 M Tris buffer (pH 7.2). The neutralized eluate was extensively dialyzed against several changes of PBS overnight at 4 °C, concentrated in 10,000 molecular weight centrifugal filters (Merck Millipore, Burlington, MA, USA) and stored at -20 °C.

2.5 Molecular weight determination of striped catfish IgM-like

The purity and molecular weight of the isolated striped catfish antibody was determined by use of the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) as previously described [6]. The catfish Ig sample was mixed 1:1 with Laemmli buffer (Sigma Aldrich Co) and heated to 95 °C in a water bath for 5 mins. Samples were allowed to cool to room temperature, before being loaded to a 10% polyacrylamide precast gel (Bio-Rad Laboratories). A pre-stained molecular weight marker (Bio-Rad Laboratories) was used as reference. The samples were electrophoresed at a constant voltage of 200 V for 30 mins. The gel

was then stained in a 10% acetic acid – 40% methanol solution containing 0.02% Coomassie Blue R-250 (Bio-Rad Laboratories) for 10 mins and later destained with 10% acetic acid – 40% methanol for 20 mins. Prior to production of rabbit anti-Pangasius serum, the concentration of the fish Ig sample was determined via BCA protein assay following the manufacturer instructions (Thermo Fischer Scientific, Waltham, MA, USA).

2.6 Preparation of rabbit anti-Pangasius polyclonal antibody

Antiserum to the affinity-purified striped catfish Ig was produced via intramuscular immunizations in two New Zealand white rabbits in the commercial laboratories of Thermo Fisher Sci. The initial immunization consisted in 300 μg of affinity-purified striped catfish Ig in 0.5 mL PBS emulsified with an equal volume of Freund's complete adjuvant per rabbit on day 0. Each rabbit received three boosters of 100 μg affinity-purified striped catfish Ig in 0.5 mL PBS emulsified with an equal volume of Freund's incomplete adjuvant on days 14, 42 and 56. Blood collection via cardiac puncture of sedated rabbits occurred on days 28, 56, 70 and 72. Serum was separated via centrifugation and stored at -20 °C until further processing.

Immunoglobulins were separated from day 56-rabbit serum by protein A-affinity chromatography (Thermo Fisher Sci) using a 5 mL gravity fed column. The column was equilibrated by addition of 5 mL PBS (pH 7.4). Rabbit serum was diluted 1:1 in PBS, then filtered through a 0.45 μm pore filter, and the filtrate was applied to the column. This was followed by a wash with 15 mL PBS to flush unbound material out of the column. Rabbit IgG was eluted by application of 5 mL 0.1 M glycine through the column. Collected eluate was neutralized by addition of 1 M Tris, pH 8.8. Samples were concentrated via use of 10 kDa centrifugal filters (Merck Millipore) and then stored at -20 °C until use. Purity of the sample was

confirmed via SDS-PAGE (Appendix A) as previously described. Rabbit anti-Pangasius IgG was then biotinylated using a commercial conjugation kit (Thermo Fisher Sci) for immunoassays.

2.7 Cross-reactivity and specificity of anti-Pangasius IgG

Reactivity of anti-Pangasius IgG to plasma from striped catfish, giant pangasius (*Pangasius sanitwongsei*), Nile tilapia (*Oreochromis niloticus*), rainbow trout (*Oncorhynchus mykiss*), and brown bullhead (*Ameiurus nebulosus*) was evaluated via use of Ouchterlony double-diffusion gels. A 10 mL solution of 1% agarose was prepared by dissolving agarose (Sigma Aldrich Co) in distilled water in a boiling water bath. The liquid agarose was then poured on a 3.5" by 2" square of plastic on a glass plate that was placed on a horizontal surface. The agarose was allowed to solidify for 20 mins. A template was placed above the gel and wells were made with the help of a gel puncher, amounting to a total of 7 wells with one center well surrounded by six wells in a circle. The central well was dedicated to the rabbit anti-Pangasius IgG, whereas the other wells were either used for the test sera, or for a negative control (PBS). The reaction volume for each well was 10 μL. Gels were stained and destained using the same solutions as for the SDS-PAGE described previously.

The specificity of the rabbit anti-Pangasius IgG was assessed via Western blot. Striped catfish affinity-purified IgM-like antibody that was electrophoresed in a 10% SDS-PAGE gel under reducing conditions, was used for the electro-transfer. A Trans-Blot SD semi-dry transfer unit (Bio-Rad) run at 15V for 60 mins was utilized to transfer the content of the 10% SDS-PAGE gel to a 0.2 μm nitrocellulose membrane (Bio-Rad). After the transfer, the nitrocellulose membrane was blocked for 1 h at room temperature in PBS containing 5% skim milk powder (Nestlé S.A., Glenndale, CA, USA). The membrane was washed in PBS containing 0.05%

Tween 20 (PBST) three times, with 5 mins intervals in between washes. The membrane was then incubated in diluted biotinylated rabbit anti-Pangasius IgG to 1:1000 in PBST for 1 h at room temperature. This was followed by three additional washes with PBST. Then a 1:3500 dilution of streptavidin-horseradish peroxidase (HRP, Thermo Fischer Sci) in PBST was applied to the membrane for 1 h at room temperature. The membrane was washed three times in PBST, and the blot was developed by addition of 3, 3', 5, 5'-Tetramethylbenzidine (TMB, Sigma-Aldrich Co) for 20 mins. The color development was stopped by the addition of distilled water.

2.8 Quantification of total Ig in striped catfish plasma via ELISA

A sandwich enzyme-linked immunosorbent assay (ELISA) was utilized for determining the total Ig concentration in plasma of striped catfish. The plasma samples of 69 one year old, healthy striped catfish (141.7 ± 41.2 g), from a different study, were used on this assay. Each sample was run in triplicate. Briefly, a ninety-six well plate was coated overnight at 4 °C with 50 μL per well of unbiotinylated rabbit anti-Pangasius IgG diluted 1:1000 in 0.05 M sodium carbonate-bicarbonate buffer (pH 9.6). Following coating, each well was rinsed 3 times with PBST. After washing, each well was blocked for 1 h at 37 °C with 200 μL 5% skim milk powder in PBS, and then washed an additional three times with PBST. Each well was loaded with 50 μL of two-fold serial dilutions of either purified striped catfish Ig (standard) or striped catfish plasma (unknown) or PBST only (negative control). Standard concentrations of the purified striped catfish Ig ranged from 13.0 – 0.0127 μg mL^{-1} and were prepared in PBST. The dilutions of the unknown plasma samples were also prepared in PBST. In preliminary runs, it was determined that the dilution of plasma most suitable for IgM-like concentrations was 1:8000 (data not shown). Plates were coated with these diluted samples, and incubated for 90 mins at

room temperature. After the incubation period, the plates were washed three times with PBST. Biotinylated anti-Pangasius IgG diluted to 1:1000 in PBST was added at a volume of 100 μL to each well, and incubated for 1 h at room temperature. The plates were washed in PBST as described previously, and then loaded with 100 μL of the streptavidin-horseradish peroxidase probe diluted to 1:3500 in PBST and incubated for 1 h at room temperature. After 3 more washes with PBST, the substrate TMB (Sigma-Aldrich Co) was added at a volume of 100 μL per well. The color was allowed to develop for 6 mins at room temperature, and the reaction was stopped by addition of 50 μL 1 M H_2SO_4 per well. The absorbance was read at 450 nm in an automated microplate reader (Molecular Devices LLC, Sunnyville, CA, USA). The absorbance values were plotted against the concentration of purified striped catfish Ig, and the best-fit curve was generated in Microsoft Excel. Using the generated equation from the standard curve, the absorbance for each unknown sample was transformed to Ig concentration.

2.9 Data analysis

Reference intervals of IgM-like antibody concentration were established by following the guidelines outlined by the American Society for Veterinary Clinical Pathology [7]. The normal distribution of the raw data was evaluated according to Andersson-Darling utilizing histograms and Q-Q plots, with $p < 0.05$ suggesting normality. Data were screened for outliers using Tukey's interquantile range method. Given the sample size $40 \leq n < 120$, reference intervals were determined by the robust method with Box-Cox transformation since distribution was not Gaussian. The 90% confidence intervals were obtained by the use of a parametric bootstrap method. Data were processed using Microsoft Excel (Microsoft Office: Mac 2011) and statistical

analysis was completed by the use of the Reference Value Advisor freeware set of macroinstructions for Excel [8].

3. Results

3.1 Purification of striped catfish immunoglobulins

The eluate sample, after goat IgG affinity chromatography, only showed two bands following SDS-PAGE under reducing conditions (Fig. 4.1). By using the molecular marker as a reference point, it was approximated that the sizes of these two bands were 70 and 25 kDa, which would correspond to the H and L chains of the IgM-like isotype, respectively. Taken together, it could be estimated that the size of the IgM-like antibody would be of 760 kDa for the tetrameric form in this catfish species (Table 4.1).

3.2 Specificity of polyclonal rabbit anti-pangasius antibody

A Western blot analysis of the rabbit anti-Pangasius IgG against the affinity purified striped catfish IgM-like antibody revealed reactive bands at 70 and 25 kDa, thus reacting selectively against both the heavy (H) and light (L) chains of the immunoglobulin from this catfish species (Fig. 4.2).

3.3 Cross-reactivity of polyclonal rabbit anti-Pangasius antibody

An Ouchterlony double immunodiffusion test revealed that the rabbit anti-Pangasius IgG only formed a precipitate line to the striped catfish and to the plasma of giant pangasius (Fig. 4.3), but not to the plasma of rainbow trout or tilapia. A Western blot run also showed that the polyclonal rabbit IgG reacted not only against the H and L chains of the Ig present in the plasma

of both striped catfish and giant pangasius (Fig. 4.2). The rabbit IgG did not react to the brown bullhead plasma in either assay.

3.4 Concentration of immunoglobulins in normal striped catfish plasma

The estimated concentration of IgM-like molecules in the plasma of striped catfish had a median concentration value of 3.3 mg mL^{-1}. The reference interval for this immune analyte is summarized in Table 4.3.

4. Discussion

Production of antibodies is a way by which the host's immune system responds to an ongoing condition compromising its health. In Osteichthyes, an IgM-like isotype is the primordial antibody class produced during a pathogenic infection, and thus is the most studied isotype; yet little is known about its concentration in most fish species. This range of values corresponds to the expected range for a given population of the species of interest; while also being narrow enough to help detect changes due to diseases.

In the current study, all the tools necessary to establish the RI of such humoral immunity analyte in striped catfish were developed by following a previously described method to generate a polyclonal anti-fish probe [4]. Based on findings, purified striped catfish immunoglobulins can be considered an IgM-like compound given the two observed bands after analysis via an SDS-PAGE under reducing conditions (Fig. 4.1). The H chain of the denatured and reduced plasma Ig of striped catfish was visually estimated to have the molecular weight of 70 kDa, whereas the light chain had an approximate molecular weight of 25 kDa. Comparatively, these H and L components have been determined to have the sizes of 72 kDa and 23 kDa in channel catfish [9];

74.8 kDa and 27 kDa in African catfish [10]; 75 kDa and 28 kDa in silver catfish [11]; 83 kDa and 27 kDa in Asian sea bass [12]; 72 kDa and 27 kDa in Atlantic salmon [13]; 70 kDa and 27 kDa in Nile tilapia [14]; and 77 kDa and two light chains of 20.5 and 21.5 kDa in blue tilapia [4]. The secreted form of the IgM-like antibody, in plasma, is generally found as a tetrameric macromolecule, composed of fours subunits, each consisting of two H and two L chains [15]. Under this assumption, the molecular weight of the IgM-like antibody in striped catfish can be estimated to be at least of 760 kDa, which is comparable to the same antibody isotypes from other fish species (Table 4.2).

By use of the rabbit anti-Pangasius IgG through a Western blot and Ouchterlony double immunodiffusion gel, our study was able to reveal several interesting characteristics of the striped catfish specific IgM-like. First, the Western blot demonstrated that the rabbit anti-Pangasius IgG reacted against both the H and L chains components of the IgM-like antibody in the affinity purified sample of striped catfish (Fig. 4.2). The same Western blot analysis also showed that same specificity of the rabbit IgG for IgM-like present in the whole plasma of either striped catfish or giant pangasius. This result suggests that the genetic sequences of both the fragment antigen-binding (Fab) and fragment crystallizable (Fc) portions of the natural IgM-like antibody are possibly conserved among species of the genus *Pangasius*. This appears to be the case as the rabbit anti-Pangasius did not react against the whole plasma of brown bullhead, which is another catfish species but different at the family level.

The findings of specificity and cross-reactivity from the Western analysis were consistent with those of the Ouchterlony gel (Fig 4.3). This method showed that the rabbit anti-Pangasius generated a precipitate line to plasma only of members of the genus *Pangasius,* but not to the plasma of rainbow trout, Nile tilapia, or brown bullhead. Moreover, it was observed that when

plasma samples of the two *Pangasius* spp were placed in adjacent wells (4.3B), two precipitate lines formed a zone of equivalency, which is indicative of identical antigens. Upon closer examination, double bands of precipitation were observed with the striped catfish plasma. A previous study found similar occurrences using a polyclonal anti-tilapia antibody against three species of *Oreochromis*, and suggested the possibility of multiple forms for the IgM-like isotype [4]. Therefore, multiple forms for this macromolecule remain to be defined in this catfish species. Immunological assays such as double immunodiffusion are employed for studying phylogenetic relationship between organisms, and the results from the Ouchterlony of our study demonstrated that striped catfish and giant pangasius are closely related species, and seemingly not related to brown bullhead, Nile tilapia and rainbow trout.

Historically, a number of assays have been utilized to determine total Ig in the plasma of fish, namely single radial immunodiffusion and ELISAs. While the former has been used for quantifying IgM-like antibody in some fish species [16-18], it is time inefficient and is not a sensitive assay, in addition to also requiring large volumes of reagents. On the contrary, the ELISA assay is a more high-throughput method and is a far more sensitive option. Thus, a sandwich ELISA was developed for quantifying the total IgM-like levels of sixty-nine individual striped catfish. The calculated median value for the concentration of this immunity analyte was 3.3 mg mL^{-1}. This is similar to values reported from other freshwater species such as 3.3 mg mL^{-1} in rainbow trout [17]; but varies from values of Ig in marine species, such as 5.4 mg mL^{-1} in Asian sea bass [12], 7.37 mg ml^{-1} in Atlantic menhaden [19], and 14.3 mg mL^{-1} in Antarctic dragonfish [20]. These differences could be attributed to multiple factors including culturing methods, environmental conditions, age, sex, among others. Possibly even the design of the ELISA can yield variability in the results, given that in our study a sandwich ELISA yielded less

variability compared to an indirect ELISA, which suffered from all dilutions not being helpful to estimate the concentration of fish IgM-like molecule in the plasma accurately (Appendix B). The sandwich ELISA technology allowed for determining the normal levels of IgM-like (1.2-7.6 mg mL^{-1}) in the plasma of striped catfish grown in RAS conditions.

The wide range of the reference interval observed in this work could be attributed to both the fish and possible rearing conditions. A study in Pacific Herring found that levels of IgM-like antibody in the peripheral blood were positively correlated with fish length [21]. Since the plasma samples utilized in this study came from a group of striped catfish of varying whole body weights, this could possibly explain the variation in the Ig concentration. Similar to the immunized catfish used in this study, the plasma came from striped catfish raised in RAS units. It is possible that these rearing conditions affect the plasma Ig levels of this catfish species.

In conclusion, this work has helped further validating the use of a rabbit anti-fish polyclonal antibody technology for specificity of fish at the genus level. Additionally, a reliable sandwich ELISA method was optimized for quantification of fish plasma Ig. Since the IgM-like antibody contributes to both innate and adaptive immune responses in fish, levels of this could convey invaluable information in regards to the health status of the animal. Future studies should be tailored towards determining the effects of pathogenic and environmental agents on the levels of IgM-like antibody in striped catfish.

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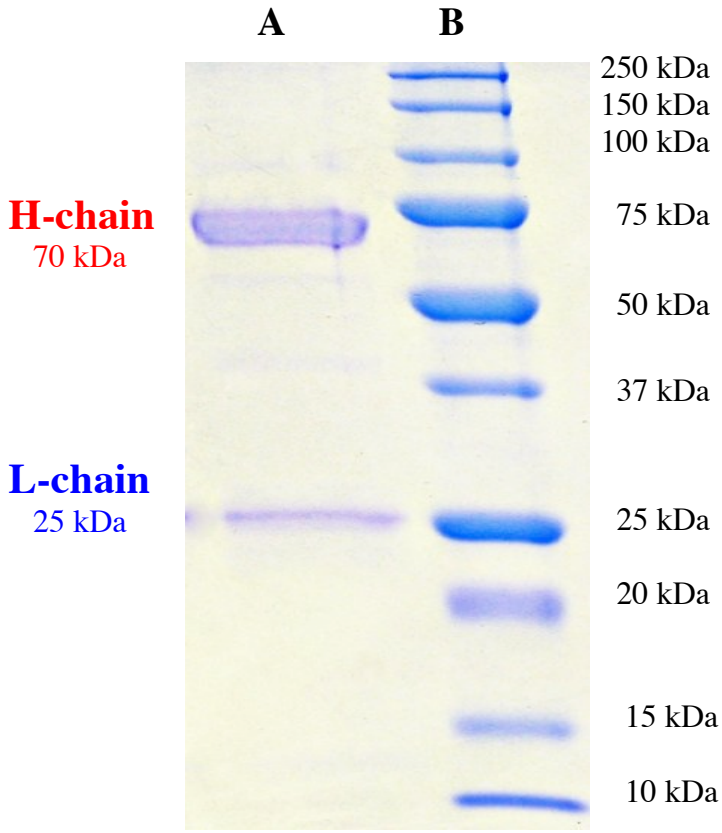

Figure 4.1 SDS-PAGE analysis of affinity purified striped catfish IgM-like immunoglobulin. Purified samples were subjected to electrophoresis in a 10% sodium dodecyl sulfate polyacrylamide gel under reducing conditions. Lanes "A" and "B" denote the affinity purified striped catfish immunoglobulin and a molecular marker, respectively. The purified IgM-like antibody from striped catfish was separated into 70 kDa heavy chain (H) and 25 kDa light chain (L) subunits.

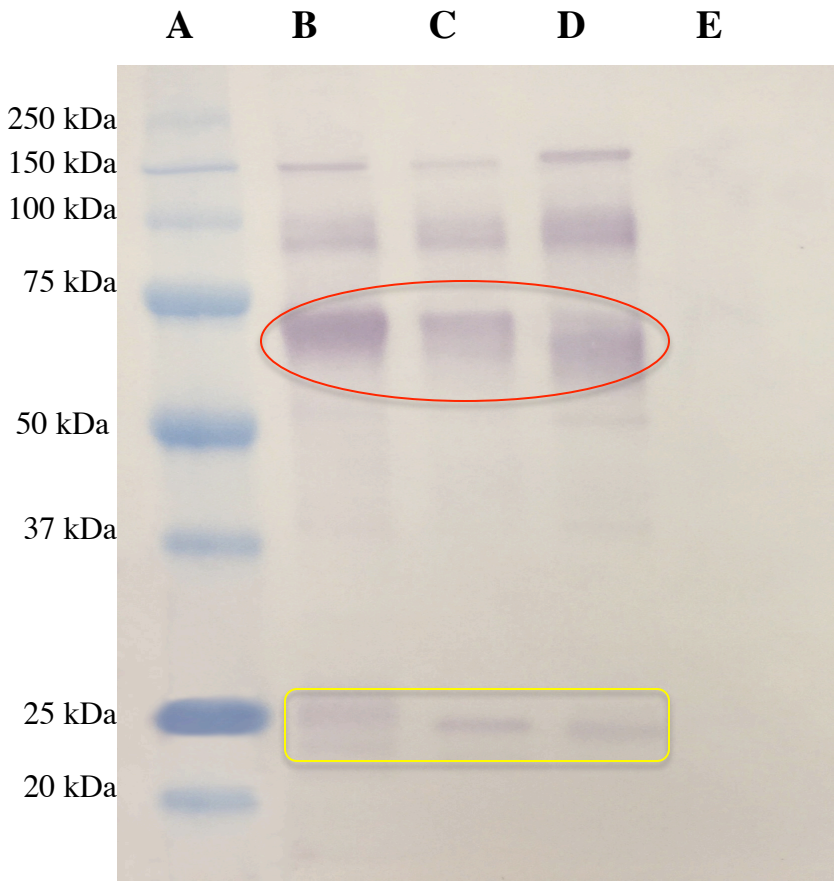

Figure 4.2 Western blot analysis of rabbit polyclonal anti-*Pangasius hypophthalmus* antibody against samples of various fish species. Red circle and yellow rectangle indicate the IgM-like antibody's heavy chain and light chain, respectively. Lane: A, the electro-transferred pre-stained molecular marker; B, affinity purified striped catfish IgM-like antibody; C, striped catfish whole plasma; D, giant pangasius whole plasma; E, brown bullhead whole plasma. Rabbit polyclonal antibody showed reactivity against the assumed heavy and light chains of the IgM-like antibody in the striped catfish samples, and also in the giant pangasius plasma.

Table 4.1 Comparison of IgM-like immunoglobulin of *Pangasius hypophthalmus* to that found in other fish species

Fish species	**Common name**	**IgM-like (kDa)**	**H-chain (kDa)**	**L-chain (kDa)**	**References**
Pangasius hypophthalmus	Striped catfish	760 (predicted)	70	25	This study
Ictalurus punctatus	Channel catfish	760 (predicted)	72	23	Phillips and Ourth, 1986
Clarias gariepinus	African catfish	816 (predicted)	74.8	27.2	Rathore et al., 2006)
Rhamdia quelen	Silver catfish	824 (predicted)	75	28	Kreutz et al., 2016
Oreochromis niloticus	Nile tilapia	776 (predicted)	70	27	Kuendee et al,. 2015
Oreochromis aureus	Blue tilapia	780 and 788 (predicted)	77	21.5 and 20.5	Smith et al., 1993
Salmo salar	Atlantic salmon	792 (predicted)	72	27	Havarstein et al., 1988
Lates calcarifer	Asian sea bass	880 (predicted)	83	27	Choudhury and Prasad, 2011

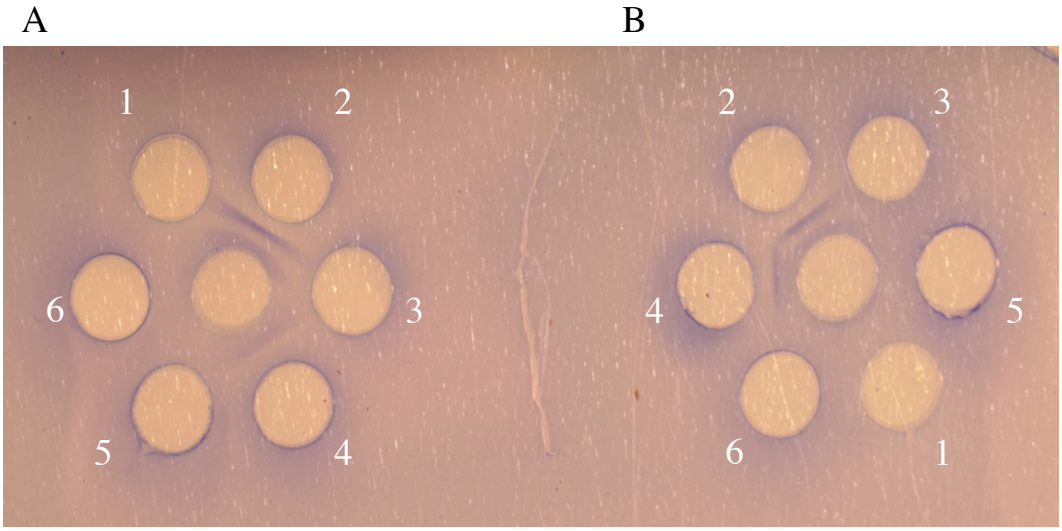

Figure 4.3 Ouchterlony double immunodiffusion showing reactivity of rabbit anti-*Pangasius hypophthalmus* IgG with plasma of multiple fish species. Contents of the wells in "A" and "B" were as follows: 1, PBS; 2, striped catfish; 3, Nile tilapia plasma; 4, giant pangasius; 5, rainbow trout; 6, brown bullhead; center well, rabbit polyclonal anti-*P. hypophthalmus* antibody. Anti-*P. hypophthalmus* IgG reacted only against the plasma of species of the genus *Pangasius*. Additionally, a zone of equivalence can be observed in "B", suggesting identity of antigens.

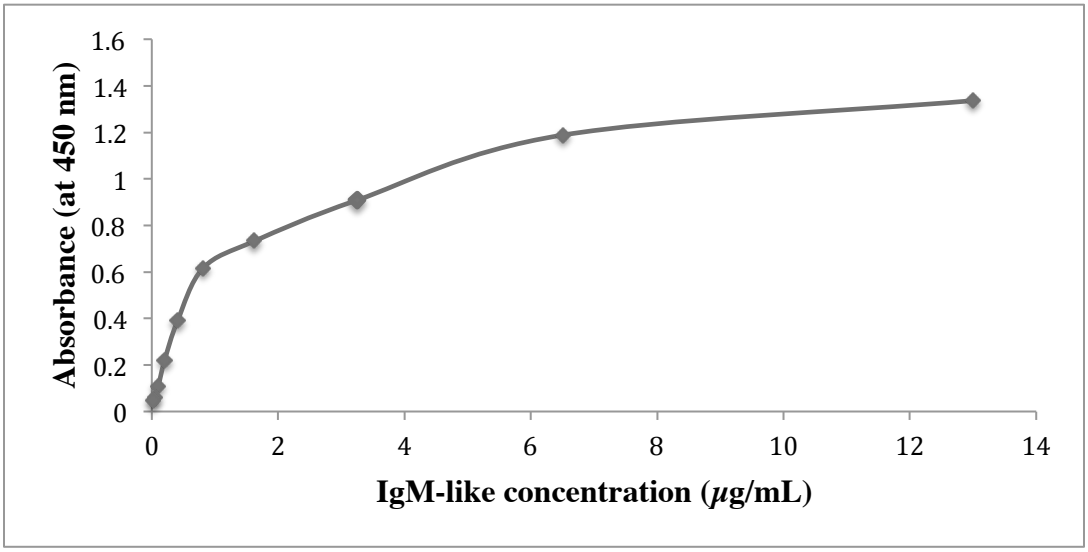

Figure 4.4 Standard curve for sandwich enzyme-linked immunosorbent assay of absorbance against affinity purified striped catfish immunoglobulins. $R^2 = 0.96$

Table 4.2 Plasma IgM-like immunoglobulin reference values for juvenile striped catfish (*Pangasius hypophthalmus*) (n=69) grown in a recirculating aquaculture system

Analyte	Min	Max	Median	Reference Interval	90% CI		D‡	Method
					Lower limit	Upper limit		
[1]IgM-like, mg mL^{-1}	0.9	7.7	3.3	1.2–7.6	1.0–1.4	6.5–8.7	n	RT

[1]Immunoglobulin M(IgM); ‡ distribution of the data – non-Gausian (n); RT, robust method post Box-Cox transformation

CHAPTER V

HEMATOLOGIC AND PLASMA CHEMISTRY REFERENCE INTERVALS FOR CULTURED STRIPED CATFISH (*PANGASIUS HYPOPHTHALMUS*) IN RECIRCULATING AQUACULTURE SYSTEMS

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Abstract

Background: Striped catfish (*Pangasius hypophthalmus*) are a valuable aquaculture fish species that is produced primarily in Southeast Asia. In the US, this fish is primarily produced as an ornamental species. Striped catfish has high productivity and great demand in numerous countries around the world, yet little is known about its normal physiology. **Objectives:** The objective of this study was to establish hematological and blood chemistry reference intervals for healthy juvenile striped catfish. **Methods:** Blood samples were collected from 70 striped catfish raised in recirculating aquaculture systems. Whole blood and plasma samples were analyzed for multiple hematological and chemistry analytes using standard techniques. **Results:** The reference intervals for hematology were as follows: PCV 23.5–35.9%, MCV 106.3–156.6 fL, RBC count 1.79-2.75 x 10^6 cells/μL, thrombocytes 26,318–73,333 cells/μL, total WBC count 36,294–94,286,cells/μL, total lymphocytes 18,997–59,998 cells/μL, small lymphocytes 13,763–51,490 cells/μL, large lymphocytes 715–21,200 cells/μL, granulocytes 4,504–18,291 cells/μL, monocytes 0–7,549 cells/μL. Plasma chemistry reference intervals were the following: alkaline phosphatase 32.7–74.6 U/L, aspartate aminotransferase 20.3–1,235.8 U/L, sodium 135.2–147.7 mmol/L, potassium 3.3–5.0 mmol/L, chloride 120.1–133.6 mmol/L, calcium 2.7–3.6 mmol/L, magnesium 0.9–1.3 mmol/L, phosphorous 1.4–2.7 mmol/L, glucose 4.6–7.6 mmol/L, cholesterol 2.8–5.3 mmol/L, total protein 30–42 g/L, albumin 7-11 g/L, globulin 22–32 g/L, albumin:globulin ratio 0.27–0.37, creatinine 0–8 μmol/L, and osmolality 251.8–327.9 mOsm/kg. **Conclusions:** Reference intervals reported here can help veterinarians and fish health specialists monitor the health status of striped catfish under recirculating aquaculture conditions for research, exhibit and production purposes.

Introduction

Striped catfish (*Pangasius hypophthalmus*) are a ray-finned fish belonging to the order Siluriformes. This catfish, native to the Mekong River in Vietnam, represents one of the most important aquaculture species in southeast Asia. Its production is primarily localized to this region of the world. Vietnam is the major producer, though aquaculture production of striped catfish is developing in other Asian countries, including Bangladesh, China, India, Indonesia, Malaysia, Myanmar and Thailand.[1] To date, there have been no efforts to produce striped catfish as a food commodity in the western hemisphere, yet production of this fish as an ornamental species for the aquarium trade does occur in the United States.

With food fish production exceeding 1.5 million tons per year, this fish represents roughly 40% of the annual global production of all catfish species. Export values in 2014 were $1.8 billion USD.[2] These positive trends in production of striped catfish are due to the species' high adaptability to a wide range of water quality parameters. Additionally, there is growing interest to move production of fish indoors into recirculating aquaculture systems (RAS) due to greater control of water quality parameters, increased biosecurity, and reduced water usage and effluent discharge. Moreover, design and implementation of such technology will allow production of this warmwater fish in temperate climates near major fish markets, including the US. The use of this technology is potentially the future of food fish production, and is currently used in the ornamental, exhibit, and research settings.

Current intensive production of striped catfish, however, is adversely affected by infectious diseases. Two of the main problems in the striped catfish industry are bacillary necrosis of Pangasius and motile *Aeromonas* septicaemia, caused by *Edwardsiella ictaluri* and

Aeromonas hydrophila, respectively. Research efforts to assess and mitigate these issues do exist, yet there is limited work focusing on normal physiology of this catfish species.

Hematology and blood chemistry values can provide substantial diagnostic information of physiological state once reference intervals are established. Reference intervals are commonly utilized in human and veterinary medicine, but they are currently underutilized for fish. In fact, baseline blood values for fish have been described in only a few species, relative to the extent of the class Osteichthyes . As culturing of this aquaculture species continues to increase, it is important to develop methods to evaluate the health status of this fish during production. Hence, the objective of this work was to establish hematologic and chemistry reference intervals for healthy striped catfish. Future work will investigate the influence of different stressors including disease, therapeutic agents, nutrition, or environmental factors in this fish species.

Materials and Methods

Striped catfish fingerlings were obtained from a commercial source (Sos Tropical Inc., Riverview, FL, USA). The fish for this study were sexually immature and approximately 1 year old, and were not classified by sex. Fish were stocked in a single RAS unit at the Food Science and Technology aquaculture facility at Virginia Tech, Blacksburg, VA. The 1,100 L RAS was outfitted with a mechanical filter for solids removal, a bio-filter for nitrification, an ultraviolet unit for disinfection, and an aerator to add oxygen and strip carbon dioxide out of the water.

Municipal water that contained chloramine was carbon filtered prior to use in RAS units. Water quality was monitored on a daily basis for dissolved oxygen and temperature, and three times a week for ammonia, nitrite, nitrate, alkalinity and pH using methods adapted from

APHA.[3] Levels of unionized ammonia were calculated by the method presented by Emmerson et al.[4] Striped catfish were fed a pelleted diet (45.5% carbohydrates, 28.2% protein, 5.98% fat) at a rate of 3% body weight per day, with feed being delivered continuously over a period of 12 h using an automated 24 h belt feeder (Pentair AES, Apopka, FL, USA).

Striped catfish were conditioned to the RAS for a period of four months at which time they were bled. Blood was collected from seventy fish for hematologic and blood chemistry analysis within a seven-hour period. To reduce stress that could affect the blood chemistry values, careful netting and handling were implemented. Each fish was sedated one a time in sodium bicarbonate–buffered tricaine methanesulfonate (MS-222, 100 mg/mL; Western Chemical Inc., Ferndale, WA, USA) for 1 minute until loss of equilibrium was observed. Following sedation, weights and lengths were determined for individual fish. Blood was collected by puncture of the caudal tail vessels, using a 23-gauge needle attached to a 1mL tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ, USA). Blood smears were immediately made with fresh blood upon sample collection without samples coming into contact with anticoagulant, and allowed to air dry. Collected blood was then transferred in equal amounts to two different 0.6mL pediatric plasma separation tubes containing lithium heparin (Becton Dickinson, Franklin Lakes, NJ, USA), one was used for plasma chemistry analysis, while the other was for hematological examination. After respective blood collection, each fish was placed in a recovery container with clean, aerated freshwater for about 5 minutes. Recovered fish were then returned to a separate RAS unit to ensure that the same fish would not be bled a second time.

Hematological and plasma chemistry analysis were conducted as previously described.[5] Blood samples for hematological evaluation were placed on ice until further processing (<1 h).

Meanwhile, a sub-sample of blood from each fish was placed into a hematocrit capillary tube, centrifuged (Autocrit Ultra 3; Becton Dickinson, Franklin Lakes, NJ, USA) at 10,000*g* for 3 minutes at room temperature, and the packed cell volume (PCV) was determined using the built-in reader. Plasma protein concentration was determined with a hand-held dual salinity/specific gravity refractometer (Shenzhen Guanshenpeng Ind Co, China), using the separated plasma from the capillary tubes. Specific gravity was transformed to plasma protein content using the method of Moore and Van Slyke.[6] A Neubauer hemocytometer with Natt-Herrick's solution (Vetlab Supply, Palmetto Bay, FL, USA) was used to determine the total red blood cell (RBC) and total white blood cell (WBC)-plus-thrombocyte counts.[7] Within 15 minutes of blood smears being air-dried, they were then stained with Wright's-Giemsa dye (Thermo Fisher Scientific, Waltham, MA, USA). Stained slides were used to determine WBC + thrombocyte counts, differential WBC counts, and assess cellular morphology. Leukocyte and thrombocytes were identified and counted on smears until 100 WBC and a variable count of thrombocytes were achieved. The obtained percentage for each leukocyte type and thrombocytes were multiplied by the total WBC-plus-thrombocyte number to estimate final cell counts. The final thrombocyte number was subtracted from the leukocyte-plus-thrombocyte final number to give the total leukocyte count.

Blood samples for chemistry analysis were centrifuged at 14,000*g* for 3 minutes. Plasma was collected and frozen at -20C until later examination within one month. An automated clinical chemistry system (Olympus AU-400, Olympus America Inc, Melleville, NY, USA) was utilized to analyze plasma for total protein, albumin, creatinine, glucose, cholesterol, sodium, chloride, potassium, calcium, magnesium, and phosphorous concentrations, alkaline phosphatase (ALP) and aspartate aminotransferase (AST) activities. Globulin levels were estimated from the difference between total protein and albumin values. Osmolality was determined using a freezing

point osmometer (Multi-Osmette, Model #2430; Precision Systems, Inc, Natick, MA, USA).

Plasma chemistry and hematological reference intervals were determined using guidelines described by the American Society for Veterinary Clinical Pathology (ASVCP).[8] Three samples for chemistry analysis and two samples for hematology were discarded prior to analysis due to hemolysis and blood clotting respectively. Normal distribution of data were assessed according to Andersson-Darling using histograms and Q-Q plots, with $p < 0.05$ indicating normality. Data were screened for outliers using Tukey's interquantile range method. For each analyte, given the sample size $40 \leq n < 120$, reference intervals were determined by the robust methods when the distribution for the analyte was Gaussian, or by the robust method with Box-Cox transformation when distribution was not Gaussian, or by a nonparametric transformation if distribution was not Gaussian after Box-Cox transformation. The 90% confidence intervals were obtained by the use of a parametric bootstrap method. Data were processed using Microsoft Excel (Microsoft Office: Mac 2011) and statistical analysis was completed by the use of the Reference Value Advisor freeware set of macroinstructions for excel.[9]

Results

During the entirety of the study, there was no morbidity or mortality in the experimental population of striped catfish. The mean weight of fish used for this study was 141.7 ± 41.2 g, while the mean length for the catfish used in this study was 21.9 ± 2.1 cm. Status of water quality during the entire trial is presented in Table 5.1. Reference intervals, median, mean and standard deviations for plasma chemistry are tabulated (Table 5.2).

Reference intervals for hematological analytes are summarized in Table 5.3. As in the case of other fish species, there were three major types of circulating blood cells: RBCs, WBCs,

and thrombocytes. The morphology of the blood cells was comparable to that seen in other finfish species (Figure 5.1). The RBCs (approximately 11.2 x 7.4 μm) were nucleated and with an oval shape. The nuclei of the RBCs stained purple, and the cytoplasm showed a reddish color. Reticulocytes, which had a light-blue tone to the cytoplasm, were rarely observed in the blood of this fish species. Thrombocytes (approximately 8.5 x 4.3 μm) were small round to oval shaped cells with an oval-shaped purple nucleus (Figure 5.1B, D, E and F). The cytoplasm of thrombocytes was distinctively clear.

There were four types of WBCs that were identified and counted: small lymphocyte (SL), large lymphocyte (LL), granulocytes, and monocytes. The nucleus of all WBCs stained purple. Lymphocytes had high nucleus:cytoplasm ratio. Among the lymphocytes observed, small lymphocytes (approximately 4.6 x 4.0 μm) were the smallest cells observed and the most abundant type of white blood cells (Figure 5.1C, D and E). These SLs consisted of a round and condensed purple nucleus, and a thin blue cytoplasm. Large lymphocytes (approximately 7.0 x 6.6 μm) had a blue but more abundant cytoplasm compared to SLs, and a larger round nucleus (Figure 5.1A, and B). Both SLs and LLs often had cytoplasmic pseudopodia. Monocytes (approximately 11.7 x 10.9 μm) were the largest type of white blood cell observed, with an abundant cytoplasm that stained blue (Figure 5.1B, C and F) and often showed clear vacuoles (Figure 5.1C). The nucleus of monocytes was round to kidney shape. Granulocytes (approximately 10.4 x 9.6 μm) represented the second most abundant type of white blood cells in circulating blood of striped catfish (Figure 5.1A, C, and D). The size of these cells was variable, ranging from the second to largest in size to approximately the size of a LL. The nucleus from these cells varied in shape, being round, indented, or segmented. Nonsegmented nuclei predominated in the granulocytes of striped catfish. The nucleus was frequently pushed to a side

of the cell by the present granules. Under this Romanowsky staining, the granules present in these cells stained a light red. The cytoplasm was pale grey and the granules imparted a light pink to dark pink cast to the cell based on the numbers and density of granules present in the cytoplasm. In some cells the granules were clustered, leaving a rim of grey cytoplasm, while in other cells the granules were more diffuse. Two cell types sharing characteristics of neutrophils (Figure 5.1E and F) were observed infrequently in a few individuals. Basophils, as in many fish species, were not observed in striped catfish.

Discussion

Current diagnostic clinical pathology tools are limited in aquatic veterinary medicine due to scant reference intervals for the majority of fish species.[10] This proves to be a paramount limitation for the culturing of healthy aquaculture species. Discrepancies in sampling techniques, methodology, age and strain of fish selected may produce variable results. Moreover, extraneous factors can affect the blood values of fish, including water quality and environmental conditions (e.g. temperature, season), stress induced by fish handling, diet and culturing conditions (e.g. oxygen levels, alkalinity). However, this work followed guidelines utilized in other work,[5, 11, 12] which minimized variability and yielded reproducible results.

Although reference intervals have been reported for a few important aquaculture finfish species, this is the first study defining the basic hematological and plasma chemistry reference intervals in striped catfish. Two previous studies examined changes in blood analytes from a bacterial and a chemical stressor, but only mean values were reported.[13, 14] Mean values for blood cannot be used clinically as they provide no information regarding a specific individual or distribution of values within the population. Therefore, the previous studies in *Pangasius* are

only useful in shedding light on how a fish might respond to a specific stressor. The power of reference intervals lies in that they encompass the central 95% of a healthy population and, therefore, can be used for interpreting disease on an individual basis.

Packed cell volume is the most frequently reported blood analyte in fish since it is a direct indicator of animal health status.[10] The values for this analyte generally range between 20 - 45% in fish[10], with more active fish species having a higher PCV than less active ones.[15] This range was in agreement with our findings. Like PCV, the RBC counts in fish are also lower than those of mammals.[5] Normal values of RBC in finfish generally range from $1x10^6 – 5x10^6$ cells/μL with sedentary fish being in the lower end, and pelagic fish being on the higher end of the spectrum. Furthermore, they are comparable with other fish from the order Siluriformes.[16, 17] Mean corpuscular value (MCV) normally ranges from 150 to 350 fL in fish.[10] Active fish present higher oxygen demands, smaller RBCs, and lower MCV volume. Therefore, the low MCV volume in the striped catfish in this study can be attributed to this species' high migratory habits in their normal riverine habitat, where they travel upstream for spawning purposes, and downstream for feeding.[1]

The reference intervals established for some of the types of WBC's have a wide range but this is a normal occurrence in fish. Reference intervals are expected to become tighter under the laboratory setting due to the more controlled conditions and more optimal quality of water, yet this was not the case. Manual counting of cells could be the major contributor of the variability observed in the reference intervals of the different types of WBC's. Fish blood is generally richer in leukocytes counts per μL, compared to mammals.[18] In fish, there is a greater number of lymphocytes in peripheral blood circulation, compared to mammals where lymphocytes mostly remain in tissue. However, stress conditions have been reported to decrease the levels for these

cells, causing lymphopenia, in fish.[19, 20] The pattern of abundance of cell types of WBCs found in this study with lymphocyte counts being the greatest, however, is consistent with that observed in other non-related freshwater fish species.[5, 11, 12]

Definitive identification of granulocytes in fish is problematic. Historically, granulocytes in fish have been categorized as neutrophils, basophils, eosinophils and heterophils.[5,11,12,13,14,21] The ultrastructure of basophils, neutrophils and eosinophils has been defined in fish. With light microscopy, staining is variable, and makes classification into cell type difficult. In this study, however, granulocytes could not be definitively separated into the different categories. The granulocytes observed in striped catfish seemed to share characteristics more similar to those of eosinophils and contained reddish granules, yet an abundance of eosinophils in the piscine appears to be rare.[10, 15] The granulocytes in this study were previously described as neutrophils in striped catfish.[14] In our striped catfish, a cell type sharing the morphological characteristics of a typical neutrophil was present, however, this cell type was observed in approximately 20% of individuals (Figure 5.1E). Another cell, similar to the neutrophil described in channel catfish neutrophil[22] with purple or blueish granules was observed; but this cell type was encountered even less frequently (Figure 5.1F). These two types of cells were not observed in all individuals, and the numbers were too infrequent to include in the differential counts. The granulocytes could also be heterophils, as this cell type has been reported in other fish species. However, a distinction at the functional level between heterophils and neutrophils has not been established in piscine literature, and there is no distinction of these cells on the ultrastructural and cytochemical characteristics level.[23] Future work should be tailored towards stimulation of either of these granulocytes to define distinction criteria for these cells in striped catfish.

The reference intervals for blood chemistry analytes were generally consistent with those previously reported for other freshwater teleosts. Among the few exceptions for consistency, the values for albumin in this catfish (7-11 g/L) did not predominantly fall within the generally normal values seen in fish of 10-24 g/L.[24] However, reported reference intervals for albumin:globulin ratios (0.27–0.37) are consistent with previous findings describing these ratios to be lower than that of mammals. Albumin and globulins are the major blood proteins in fish, with globulin levels being greater than those for albumin. These serum proteins have a large role in innate immunity, thus higher concentrations, in particular of globulins, in serum may be reflective of enhanced non-specific responses in fish. Overall, the reference values for plasma protein (as determined spectrophotometrically) were consistent with those from other freshwater fishes.

Total plasma protein had higher values when determined by refractometry. The two methods – refractometry, and spectrophotometry – yielded different reference intervals of 12.6-17.3 g/dL and 30-42 g/dL, respectively. The use of the refractometer is often preferred because of its greater practicality compared to the more labor intensive and expensive spectrophotometer method. Total plasma protein in fishes typically range 2-8 g/dL when determined by use of the refractometer,[24] and the values in this work did not fall within such range. Protein is a major contributing factor of plasma refractive index, but nonprotein components such as glucose, cholesterol and chloride can interfere with measurements in the refractometer.[25] Due to these discrepancies, there should be awareness of the elevated values when using refractometry for estimating total plasma protein in striped catfish.

The activities for the enzymes ALP and AST values are other frequently assessed analytes in the clinical setting. These are circulating isozymes in the blood, which are also

normally present in tissue organs like the liver and the heart. Increased levels in circulation can be indicative of tissue or organ damage. The reference interval for AST was wide compared to the one for ALP. However, it is difficult to interpret their values for different fish species due to lack of information on the metabolic contribution of these enzymes.[12] The levels of creatinine are generally low in fish, ranging 0.11 – 0.90 mg/dL;[24] the reference values for this analyte were within that range in this work.

Electrolytes are reliable indicators of a fish's ability to osmoregulate, and constitute some of the best-understood blood analytes in fish.[12] Sodium and chloride are the major ions in the blood of all fish, with sodium levels being slightly higher than chloride values.[24] The reference intervals established here for those ions are comparable to those reported for other freshwater finfish species. Values for potassium in freshwater fish normally range 2-4 mmol/L.[24] Reference intervals for that analyte in this study (3.3-5.0 mmol/L), however, were on the higher end, and were also greater than values previously reported for channel catfish.[16, 26] Possible explanations for the increase in this value include increased activity, and use of anesthetics.[27] The observed values of 251.8-327.9 mOsm/kg for osmolality for the striped catfish are comparable to those reported in other freshwater fish species.

The slightly elevated reference intervals established here for calcium (2.7-3.6 mmol/L) are not entirely consistent with the normal range reported for freshwater fish of 2.0-2.9 mmol/L.[24] Increase in the levels of this divalent ion could be attributed to acute stress,[28] or increased blood protein concentration.[24] As for the other divalent ion, magnesium, the levels determined in this study were lower than that of calcium. Such findings are consistent with those seen in other freshwater fish. Levels of these two ions could have been influenced also by the moderately hard water used during the study (125 mg/L total hardness), which could explain the

higher levels of calcium in the blood. The values for these ions in striped catfish, however, are low when compared to marine species. This makes sense given the relatively low hardness of freshwater environments, resulting in lower movement of these ions across the gills.

Glucose is frequently used as an indirect indicator of stress responses. Fluctuations in the blood levels for this analyte appear to be linked to cortisol and thyroid hormone variations.[24, 29] The values for this analyte were generally twice as high in our study compared to other freshwater fish, which could be attributed to a number of factors including diet, age, and stress related to rearing fish conditions. It is also possible that MS-222 contributed to the increased levels in this analyte, as seen in another study in zebrafish.[30] The dose of 100 mg/L is commonly used to quickly induce a stage 3 of anesthesia in fish. Yet, it is important to mention that anesthetic drugs generally cause hyperglycemia.[31] Our findings suggest that MS-222 may not be appropriate for glucose metabolism studies, but this claim requires further validation.

Fish inhabit a highly dynamic environment, where their physiology is constantly adapting to their environment. Factors affecting their normal physiology include water quality parameters, environmental contaminants, pathogens, and predators. The number of studies defining normal references values levels for fish is limited. As with mammals, and with other animals of domestic and commercial importance, reference intervals should be determined for different populations and strains of the striped catfish cultured under specific conditions. Additionally, the fish were reared in a RAS unit, which is different from their production in southeast Asia, where the food fish strain is cultured in ponds and cages in rivers. The use of RAS is increasing in acceptance in the world for research and production purposes due to greater control of rearing conditions, and conservation of water resources. Therefore, the work from this research can prove to be a beneficial tool for future intensive production of striped catfish in RAS.

The exact numbers of value and trade of striped catfish-specific ornamental industry do not exist, yet the American ornamental fish industry is an economically important one since it is worth over $1 billion USD.[32] The strain of striped catfish used was the one dedicated for ornamental purposes in the US. Since striped catfish is part of this aquarium trade, the work presented here can be beneficial to the maintenance of this fish in optimal health to maximize profits for the ornamental industry. The results observed in this study, however, are an important first step for assessing the baseline physiology for this economically important aquacultured freshwater fish.

As aquaculture practices continue to expand and intensify, nonlethal and inexpensive diagnostic tools to monitor the welfare and health status of the fish are necessitated. Use of standardized hematological and plasma chemistry reference intervals can help maintain a healthy population of striped catfish and help assess outbreaks of disease, and/or detection of disease during the early stages. Furthermore, knowledge and utilization of reference values of normal blood physiology of striped catfish can help current producers enhance animal health and wellbeing and aid in propagating the culture of this fish species worldwide.

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Table 5.1 Water quality values for juvenile striped catfish (*Pangasius hypophthalmus*) raised in a recirculating aquaculture system (1,100 L)

Parameter	**[1]Range**	**Mean**	**Standard Deviation**
Temperature (C)	26.2-27.9	27.8	0.2
Alkalinity as $CaCO_3$ (mg/L)	95-160	121.5	16.1
pH	7.7-7.9	7.8	0.06
NH_3-N, unionized (mg/L)	0.001-0.010	0.005	0.002
NO_2-N (mg/L)	0-0.02	0.008	0.005
NO_3-N (mg/L)	8.5-35	22.7	9.1
Dissolved Oxygen (mg/L)	6.9-7.7	7.4	0.1

[1]Minimum, maximum values

Table 5.2 Plasma chemistry reference values for juvenile striped catfish (*Pangasius hypophthalmus*) (n=67) raised in a recirculating aquaculture system (1,100 L)

Analyte	Min	Max	Median	Reference Interval	90% CI		D‡	Method
					Lower limit	Upper limit		
[1]ALP, U/L	0	74	53.6	32.7–74.6	26.0–39.3	68.3–81.1	g	R
[2]AST, U/L	12	2422	135	20.3–1235.8	15.9–35.2	736.9–2269.1	N	RT
Sodium, mmol/L	130	147	141.4	135.2–147.7	133.9–136.4	146.1–149.1	g	R
Potassium, mmol/L	3.2	5.7	4.1	3.3–5.0	3.1–3.5	4.8–5.1	g	R
Chloride, mmol/L	117	135	124.4	120.1–133.6	118.8–121.5	132.5–134.6	n	RT
Calcium, mmol/L	2.7	3.9	3.1	2.7–3.6	2.6–2.8	3.5–3.7	n	NP
Magnesium, mmol/L	0.9	1.4	1.1	0.9–1.3	0.9–1.0	1.3–1.4	g	R
Phosphorous, mmol/L	1.3	2.9	2.0	1.4–2.7	1.2–1.5	2.5–2.8	g	R
Glucose, mmol/L	4.3	8.6	6.1	4.6–7.6	4.3–4.9	7.3–7.9	g	R
Cholesterol, mmol/L	2.8	6.7	3.8	2.8–5.3	2.6–3.0	5.0–5.6	n	NP
Total Protein, spectrophotometry g/L	29	44	36	30–42	29–31	41–43	g	R
Albumin, g/L	7	11	9	7–11	6.8–7.2	10–11	g	R
Globulin, g/L	22	33	27	22–32	22–23	31–33	g	R
Albumin:Globulin ratio	0.27	0.39	0.32	0.27–0.37	0.26–0.28	0.37–0.38	g	R
Creatinine, μmol/L	0	7.9	3.6	0–8	0–0	7.2–8.8	g	R
Osmolality, mOsm/kg	243	355	289.8	251.8–327.9	242.2–259.2	319.8–335.1	g	R

‡ Distribution of the data – Gausian (g) or non-Gausian (n); R, robust method; RT, robust method post Box-Cox transformation; NP, nonparametric method of transformation; [1]ALP, alkaline phosphatase; [2]AST, aspartate aminotransferase

Table 5.3 Hematologic reference values for juvenile striped catfish (*Pangasius hypophthalmus*) (n=68) raised in a recirculating aquaculture system (1,100 L)

Analyte	Min	Max	Median	Reference Interval	90% CI		D‡	Method
					Lower limit	Upper limit		
PCV, %	22	36	29.8	23.8–35.9	22.5–25.0	35.0–37.0	g	R
[1]MCV, fL	12	2422	131.5	106.3–156.6	102.7–110.5	152.1–161.3	g	R
RBC, x $10^6/\mu$L	1.65	2.71	2.27	1.79–2.75	1.71–1.88	2.67–2.83	g	R
Total WBC, cells/μL	34,574	98,802	65,290	36,294–94,286	31,614–41,301	89,095–98,578	g	R
Total Lymphocytes, cells/μL	18,357	65,120	39,498	18,997–59,998	16,046–22,159	56,259–64,117	g	R
Small Lymphocytes, cells/μL	11,077	58,383	32,627	13,763–51,490	10,660–17,282	47,910–54,670	g	R
Large Lymphocytes, cells/μL	727	22,593	5,868	715–21,200	430–1,176	17,666–24,632	n	RT
Granulocytes, cells/μL	4,987	19,293	11,397	4,504–18,291	3,351–5,673	17,233–19,461	g	R
Monocytes, cells/μL	0	9,074	1,600	0–7,549	0–14	6,320–8,969	n	NP
Thrombocytes, cells/μL	24,937	83,358	49,826	26,318–73,333	22,374–31,155	69,566–77,275	g	R
Plasma protein concentration, g/dL refractometry	11.7	17.1	15.0	12.6–17.3	12.2–13.0	16.8–17.7	g	R

‡ Distribution of the data – Gausian (g) or non-Gausian (n); R, robust method; RT, robust method post Box-Cox transformation; NP, nonparametric method of transformation; [1]MCV, mean corpuscular value

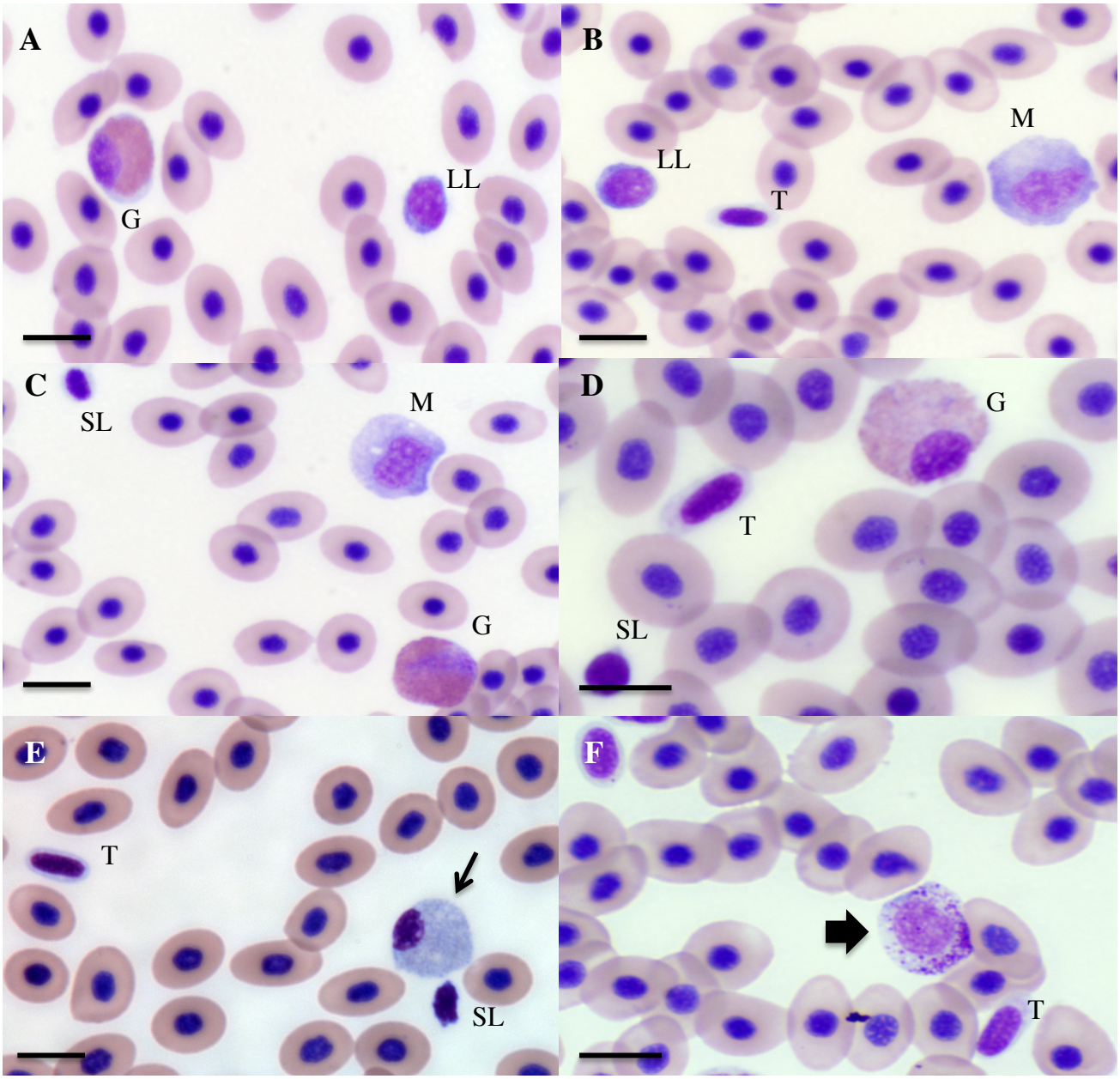

Figure 5.1 Representative blood cells of the striped catfish (*Pangasius hypophthalmus*) reared in recirculating aquaculture systems (**A**-**F**). (**A**) A granulocyte (G) showing a nucleus pushed to the side and a lower clear edge of the cytoplasm, and a large lymphocyte (LL). (**B**) A large lymphocyte (LL), a thrombocyte (T), and a monocyte (M). (**C**) A small lymphocyte (SL), a monocyte showing vacuoles (M), and a granulocyte (G) with granules that are possibly eosinophilic. (**D**). A small lymphocyte (SL), a thrombocyte (T), and a granulocyte (G). (**E**) A

thrombocyte (T), a cell (small arrow) resembling the characteristics of a neutrophil, but with no granules. (**F**) Two thrombocytes (T), and a cell (large arrow) with granules that are possibly neutrophilic. Wright's Giemsa, bar = 10μm

CHAPTER VI

MODULATION OF IMMUNITY IN NILE TILAPIA BY DIETARY SUPPLEMENTATION OF *BACILLUS SUBTILIS* STRAINS

Abstract

[†]Dietary supplementation of probiotics is an alternative, with growing scientific validation, to the misuse of antibiotics for prophylaxis in aquaculture. Strains of *Bacillus subtilis* are regarded as attractive probiotic candidates to the fish farming industry; however, the literature is limited in the number of studies assessing the use of these in tilapia. The objective of this study was to examine the effect of strains NZ86 and O14VRQ of *B. subtilis* on various parameters of the innate immunity in Nile tilapia (*Oreochromis niloticus*) in a 51-day feeding trial. Supplementation of tilapia with either strain resulted in significant increases ($p < 0.05$) in plasma lysozyme concentration at varying degrees throughout the trial. Meanwhile, alternative complement activity was significantly elevated ($p < 0.05$) only after feeding of the NZ86 strain after 14 and 51 days. Conversely, supplementation of O14VRQ resulted in a significant increase ($p < 0.05$) in the percent of neutrophils in the peripheral blood of tilapia by day 28. At the end of the trial, there was a trend towards increased phagocytic and respiratory burst activities observed in immune organ derived leukocytes. Consistent with this data, there were slightly elevated levels of mRNA of the cytokines TNF-α and IL-1β in the spleen and anterior kidney. Feeding with either probiotics appeared to have a greater effect on the gene expression of both pro-inflammatory cytokines in the intestine, yet only O14VRQ was significantly different than the control. Moreover, the occurrence of these results could be more tightly associated with

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supplementation of the probiotic strains, given that *Bacillus* bacteria were observed to populate the intestines of the treatment groups. These results suggest the potential roles of these *B. subtilis* probiotic candidates to stimulate immune responses both locally and systemically in tilapia.

1. Introduction

Aquaculture is the fastest growing food production sector, with more than 327 finfish species farmed around the globe [1]. Tilapia is among the top economically important fish, characterized by its robustness, rapid growth, and great adaptability to a wide range of rearing conditions [2]. As tilapia aquaculture has intensified and become more global, problems such as poor water quality and disease outbreaks continue to be a few of the major issues compromising sustainability [3-4]. Historically the use of antibiotics has been the standard control method against bacterial infections, yet their administration is restricted in many countries because of fear of promoting bacterial resistance up the food chain [5]. In recent years, and because of the controversies surrounding antibiotic use, probiotics have been considered as safer alternatives to antibiotics in disease prevention.

Probiotics are live microorganisms that can survive, proliferate and colonize the animal gut, when administered in the appropriate doses, to promote a healthy balance in the gut microbiota. Greater microbial stability in the intestine often translates to beneficial outcomes such as pathogen exclusion of invading microbes, improved digestive capabilities, enhanced growth performance, and increased animal survival [6].

The use of probiotics is not rare to aquaculture, and *Bacillus* species include one of the most scrutinized bacteria in fish. These bacteria are ubiquitous in nature, inhabiting soil, water and air, and with the remarkable characteristic of a tough endospore for enduring adverse environmental conditions [7]. *Bacillus subtilis* is a commonly investigated species with probiotic potential, demonstrated by its ability to produce several antimicrobial compounds, and its status as a safe organism in animals [8]. Although various studies have been carried out for nutritional supplementation of probiotics in fish and shellfish, work on *B. subtilis* in tilapia is limited [9-11].

In preliminary studies from our lab (unpublished work), two environmental isolates of *B. subtilis* strains NZ86 and O14VRQ were shown to enhance survival after bacterial challenge, in catfish and shrimp, respectively. However, assessment of immunity was excluded from these studies, making it difficult to understand the mode of action of these probiotic strains. Therefore, the present study was designed to investigate multiple functional parameters of systemic innate and adaptive immunity to shed light on the mechanism of action of these probiotic candidates in Nile tilapia (*Oreochromis niloticus*) in a 51-day trial. Additionally, an assessment of intestinal immune gene expression was completed to uncover local immune effects upon probiotic supplementation.

2. Materials and Methods

2.1 Bacterial preparation and experimental diets

Spores of two proprietary probiotic *Bacillus subtilis* strains O14VRQ and NZ86 were used independently as additives to a basal diet. Both probiotic strains consisted of dry powder concentrates containing each *B. subtilis* strain (provided for testing by Novozymes Biologicals). The basal diet consisted of a commercial 3mm floating feed (Rangen, Inc, Buhl, ID, USA) with a content of 36% protein and 6% lipid. Spores of *B. subtilis* strains NZ86 and O14VRQ were each suspended in distilled water, and were top-coated using low-pressure air spraying onto the commercial feed. Briefly, batches of feed were placed in 19 L buckets and mixed constantly with a spiral-arm mixer fixed to a handheld drill at approximately 500 rpm. Spores were mixed with filtered municipal water at a ratio of 10:1 (volume of solution per gram of spores) to reach a final concentration of 10^7 spores per gram of feed. The opening of the mixer was covered with a lid and the spore suspension was applied continuously to the feed at a rate of 10.5 cubic feet per

minute at a pressure of 50 PSI with an air sprayer (Ingersoll Rand 210G, Ingersoll Rand Inc., Davidson, NC, USA) through a small orifice cut into the lid of the bucket. Feed was allowed to tumble for 10 minutes after spraying and later air-dried. The basal diet without the probiotic strains served as the control. All diets were stored at 4°C during the course of the experimental trial.

2.2 Bacillus *quantification in probiotic diets*

Samples of the probiotic diets were analyzed to quantify each of the *B. subtilis* strains present per gram of feed. Briefly, 5-10 g per probiotic diet was ground using a coffer grinder for 1 min. One gram of the resulting ground feed was transferred to 99 mL of PBS, and then agitated for 30 mins using a wrist action shaker. The solution was then incubated in an 80 °C water bath for 10 mins. Each solution was cooled for 30 mins, vortexed, then serially diluted in PBS and plated in plate count agar medium and grown overnight at 35 °C. Bacterial colonies were quantified based on the normal morphology of bacilli.

2.3 Fish

Nile tilapia (*Oreochromis niloticus*) were obtained from Spring Genetics (Miami, FL, USA) and were maintained in a recirculating aquaculture system (RAS) at the Human and Agricultural Biosciences Building aquaculture laboratory (Blacksburg, VA, USA). The RAS unit was equipped with aeration, a UV filter for disinfection, a sand filter for mechanical filtration, and a bead filter for improved water clarification and nitrification. Sixty non-sexed fish (161 ± 3.9 g) were divided equally into three groups and placed in three 300-L tanks under the same RAS unit, with each tank corresponding to each *B. subtilis* strain and the control diet. The length

of the study was of 51 days. At day 0, fish (n = 12 per treatment) were randomly tagged intramuscularly with t-bar anchor tags by the dorsal fin of the animals. This procedure was to facilitate the monitoring of individual immune performances over time. Tilapia were fed at rates ranging 2.6–4.3% body weight per day, with feed being delivered continuously over a period of 18 h using an automated 24 h belt feeder (Pentair AES, Apopka, FL, USA). Water quality was monitored on a daily basis for alkalinity, dissolved oxygen and temperature, and three times a week for ammonia, nitrite, nitrate, and pH using methods adapted from APHA [12]. The experimental trial was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech.

2.4 Blood sampling

Collection of blood was carried out in an n=12 per group on days 14, 28 and an n=9 per group on day 51. Individual fish were carefully netted and anesthetized in sodium bicarbonate buffered MS-222, and blood was collected by puncture of the caudal vessels using a 21-gauge needle attached to a 1 mL tuberculin syringe (Becton Dickinson, Franklin Lakes, NJ, USA) without anticoagulant. Blood smears, prepared only on days 14 and 28, were immediately made with fresh whole blood samples and allowed to air dry. Remaining blood was placed in 0.6 mL plasma separation tubes containing heparin-lithium (Becton Dickinson, Franklin Lakes, NJ, USA) and these were centrifuged at 14,000 x g for plasma separation. Plasma was collected and stored at -20°C until lysozyme and alternative complement analyses were conducted.

2.5 Tissue sampling and isolation of leukocytes

Anterior kidney (AK) and spleen were collected from an n=9 per group on day 51. Fish were first euthanized by an overdose of MS-222 followed by cervical dislocation. This was followed by aseptic removal of the immune organs. A small fraction of each organ (~50mg) was stored in RNAlater (Sigma) for RNA extraction. The rest of each organ was placed in small petri dishes containing 0.4 ml L15+ medium (Sigma, supplemented with 10 U ml^{-1} heparin, 2 mM L-glutamine, 100 μg mL^{-1} gentamicin, and 15 mM HEPES). Tissues were homogenized through a 100 μm cell strainer using the plunger of a 3 mL syringe. The resulting 4mL cell suspension was layered over 6mL Ficoll-Paque (GE Healthcare, Chicago, IL, USA) for density gradient separation and centrifuged at 600 x g for 25 minutes at 4°C. Leukocytes were collected from the interface layer and washed twice in L15+ medium (without gentamicin) at 200 g for 10 minutes at 4°C. Cells were counted by use of trypan blue exclusion in a hemocytometer, and adjusted with L15+ medium to a concentration of 6 x 10^6 live cells mL^{-1} prior to flow cytometry procedures.

2.6 Sampling of intestines

Intestine samples were collected for both RNA extraction and quantification of probiotics in the gut. Prior to collection, the contents (fecal and uneaten feed) of the intestines were carefully squeezed out of the intestine. For consistent results, about 50 mg of the section of the intestine that makes a loop around the posterior end of the liver was collected in 1 mL RNAlater and stored at -20 °C. The remainder of the intestine was flash frozen in dry ice/ethanol and stored at -20 °C for bacterial quantification.

2.7 *Bacilli quantification in intestines*

Frozen gut samples were sent to the facilities of Novozymes (Salem, VA, USA) for determining germination of bacilli in the intestines of tilapia. Approximately 3.5 g of the gastrointestinal tract of tilapia were homogenized in 3.5 mL PBS. The total DNA of the samples was recovered by use of 0.1mm glass beating beads to quantify both vegetative cells and spores, whereas DNA from vegetative cells was extracted using 1.4 mm ceramic beating beads. Both methods employed the same kit, and were carried out following the manufacturer's instructions for the Powerlyzer-Powersoil DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA). Following DNA extraction, quantitative real time PCR (RT-qPCR) for 16S rRNA was run on the resulting samples. A set of forward and reverse primers and probes specific to each of the *Bacillus* strains were utilized (Appendix C). For the RT-qPCR, samples were heated to 95 °C to denature the DNA double helix, and annealing occurred at 60 °C over 41 cycles using Thermo Fisher LightCycler 960 (Thermo Fisher Sci, Waltham, MA, USA)

2.8 Hematology

White blood cell differentials were determined using the May Grünwald and Giemsa stain method [13] and with guidance of WBC morphology in tilapia [14]. Smears prepared from fish blood were used to assess the abundance profile of peripheral blood leukocytes. Within 15 minutes of blood smears being air-dried, they were immersed in 100% methanol for 30 seconds for later cytological analysis. Methanol-fixed smears were stained with May Grünwald dye (Sigma, St Louis, MO, USA) for 10 minutes, followed by dipping in Giemsa stain (Sigma) for 15 minutes and finally rinsing for 2 minutes in distilled water. Leukocytes were identified and counted on smears until a count of 100 white blood cells (WBC) was achieved.

2.8 Plasma lysozyme concentration

The content of plasma lysozyme was determined using the EnzChek lysozyme assay kit (Thermo Fisher Sci). 25 μL of plasma sample were diluted with 25 μL of 1X reaction buffer (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.5) and incubated with 50 μL fluorescein isothiocynate (FITC) labeled *Micrococcus lysodeikticus* (50 μg/mL) in a 96-well plate at 37°C for 30 minutes. Fluorescence intensity was determined with a fluorescence microplate reader (BioTek Instruments, Winooski, VT, USA) at excitation/emission wavelengths of 485/535 nm. Lysozyme content (U/mL plasma) of each sample was calculated by comparing the reading to the standard curve prepared with lysozyme from chicken egg white (0 to 250 U).

2.9 Plasma alternative complement activity

The activity of the alternative complement pathway (ACH50) was measured by use of the method of hemolysis of rabbit red blood cells (RRBC; Innovative Research, Novi, MI, USA), as previously described [15]. Plasma from tilapia from days 14, 28 and 51 were used for estimation of alternative complement activity.

2.10 Flow cytometry assay of phagocytosis

Phagocytosis of AK cells and splenocytes was assessed via flow cytometry [16]. WBCs to be used for phagocytosis were prepared as mentioned above from AK and spleen. A stock solution of FITC-labeled *Escherichia coli* (Thermo Fisher Sci) was reconstituted in phosphate buffered saline solution (PBS, supplemented with 5 mM sodium azide) resulting in a concentration of 3 x 10^9 CFU mL^{-1}. Phagocytic activity for each sample was carried in duplicate. Shortly, 368 μL (2.2 x 10^6 cells) of AK and spleen WBC suspension, from the previously

adjusted stock solution of 6 x 10^6 live cells mL^{-1} with L15+ medium (without gentamicin), were placed in 5mL round-bottom tubes (Thermal Scientific Inc, Odessa, TX, USA). Subsequently, cells were infected with a multiplicity of infection (MOI) of 10 (2.2 x 10^7 CFU). Cells were then incubated for 1 hour at room temperature. Phagocytosis was stopped by transferring samples to microcentrifuge tubes preloaded with 500 μL ice-cold PBS. Free FITC was removed by centrifugation at 100 g for 10 minutes at 4°C, and a washing step with 500 μL PBS at 1200 rpm for 5 minutes at 4°C. Cells were resuspended in 50μL ice-cold PBSE (PBS pH 7.3, 1% BSA, 0.1% sodium azide, 25 mM EDTA), and propidium iodine (PI) was added to a concentration of 1 μg mL^{-1} to stain dead cells. Controls of FITC treated and untreated cells were included. Samples were analyzed with a Mark II Imager flow cytometer (Millipore, Seattle, WA, USA). Five thousand cells were recorded per sample. The anterior kidney leukocyte (AKL) and spleen leukocyte (SL) samples were analyzed for size (forward scatter, FSC), granularity (sideward scatter, SSC), for green/FITC fluorescence (detected with 530/30 nm bandpass filter; FL1), and red fluorescence (PI, detected with 585/40 nm band pass filter; FL2). Data was collected in the form of fluorescence histograms with relative fluorescence on a logarithmic scale. Data was also collected in the form of live cells ingesting bacteria. Dead cells (PI positive cells) were excluded by gating, and only FITC positive cells were included in subsequent analyses. Phagocytic activity was defined as the median fluorescence intensity of the cells.

2.11 Flow cytometry assay of respiratory burst

Respiratory burst of tilapia AKL and SL was measured via flow cytometry [17], and it was carried out in duplicate per sample. WBC's used for this assay were prepared as described above. Phorbol 12-myristate 13- acetate (PMA, Sigma) was utilized to activate leukocytes, via

signal transduction, in conjunction with dihydrorhodamine 123 (DHR, Sigma), which is oxidized to the green fluorescent rhodamine 123 (RHO). Briefly, 500 μL (3 x 10^6 cells) of the AK and spleen leukocyte suspension, from the stock solution of 6 x 10^6 live cells mL^{-1} with L15+ medium (without gentamicin), were placed in 5mL round-bottom tubes (Thermal Scientific Inc). Samples were incubated at room temperature for 10 minutes. This was followed by addition of 1 μg mL^{-1} PMA and samples were then incubated at room temperature for another 10 minutes. Consequently, DHR was added to samples (to a concentration of 5 μM), and incubated at room temperature for 15 additional minutes. The reaction was stopped by putting the samples on ice, and adding 300 μL ice cold PBS (supplemented with heparin 10 U mL^{-1}). Prior to flow cytometry analysis, PI was added to samples to a 1μg mL^{-1} for dead cell exclusion. Controls of DHR treated and untreated cells were included for analysis. Respiratory burst analysis was carried by use of a BD FACSAria I flow cytometer (BD Sciences, San Jose, CA, USA). Ten thousand cells were recorded per sample. The settings of the instrument were the same as described for phagocytic activity. The AKL and SL samples were analyzed for size (forward scatter, FSC), granularity (sideward scatter, SSC), for green/FITC fluorescence (detected with 530/30 nm bandpass filter; FL1), and red fluorescence (PI, detected with 585/40 nm band pass filter; FL2). Data was collected in the form of fluorescence histograms with relative fluorescence on a logarithmic scale. Dead cells (PI positive cells) were excluded by gating, and only FITC positive cells were included in subsequent analyses. Respiratory burst was reported as the median green fluorescence intensity of the cells.

2.12 RNA extraction, cDNA synthesis and RT-qPCR

Approximately 50 mg (per organ) of anterior kidney, spleen and mid intestine were removed at necropsy from tilapia (n=9) on day 51 and placed in 1 mL RNAlater (Sigma) and stored at -20°C. Samples were removed from RNAlater, surgically cut into smaller pieces and placed in RLT buffer (Qiagen, Germantown, MD, USA) for RNA extraction. Tissues in RLT buffer were homogenized using an Omni TH homogenizer (Omni International, Kennesaw, GA, USA) at medium speed for 1 minute. The resulting suspension was further broken down, by passing the lysate through a 23G needle attached to a 3 mL syringe. Total RNA was extracted from the different tissues using the RNeasy minikit (Qiagen), following the manufacturer's instructions. During the extraction process, samples were DNase (NEB, Ipswich, MA, USA) treated to eliminate genomic DNA on the columns. Total RNA concentrations were measured by use of a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Only RNA with a 260/280 ratio above 1.9 was used for cDNA synthesis. RNA samples were stored at -80 °C. Complementary DNA was synthesized from mRNA (1 μg) using the high capacity reverse transcription kit (Thermo Fisher Scientific) and following the manufacturer instructions. Samples were diluted to 5 ng/ μL with nuclease free water (GE Healthcare, Chicago, IL, USA) and stored at -20°C.

The NCBI primer-BLAST tool was used to design primers and probes of interleukin-1beta (IL-1β), tumor necrosis factor alpha (TNF-α) and beta–actin (actβ), with sequences spanning exon-exon regions (Table 6.5). Gene expression analysis was carried quantitative real time PCR (RT-qPCR) in a total sample volume of 10 μL, containing 300 nM both forward and reverse primers (Integrated DNA Technologies, Coralville, IA), 150 nM 6-carboxyfluorescein (FAM) probe (Integrated DNA Technologies), and 2x Primetime Gene Expression Master Mix

and 12.5 ng of cDNA. All samples were run on a CFX Connect Real Time System (Bio-Rad Laboratories, Hercules, CA, USA). Samples were heated to 95 °C for 3 min once, and then 38 cycles of 95 °C for 15 sec and 55 °C for 1 min. The resulting quantitative cycle (Cq) values were automatically determined by the thermocycler instrument. No template control (NTC) samples were included for each primer pair and probe combination to rule out DNA contamination. The expression of the immune genes was normalized using the reference gene for actβ in the non-treatment control.

2.14 Statistical analysis

Experimental data are expressed as means ± standard error of the mean (SEM). Data of control and probiotic treatment groups were analyzed and compared by a one-way analysis of variance (ANOVA), followed by Tukey's honest significant difference (HSD) post hoc test.

3. Results

3.1 Bacillus subtilis quantification in the tilapia intestine

The RT-qPCR analyses to quantify the presence of total *B. subtilis* strains NZ86 and O14VRQ in the whole tilapia intestine, before and after supplementation with either of the bacilli strains, are shown in Table 6.1.

3.2 Hematology

Influence of probiotic diets on the hematological profile of Nile tilapia are summarized in Table 6.2. The types of white blood cells observed included lymphocytes, neutrophils, monocytes and eosinophils (Figure 6.1). Differences were observed on day 28, under the

O14VRQ diet, with a significant decrease on lymphocytes ($p<0.05$) favoring the levels of neutrophils ($p<0.05$) in circulating blood.

3.3 *Plasma lysozyme concentration*

After fish were fed diets containing spores of B. *subtilis* (10^8 CFU/g feed) of either strain NZ86 or O14VRQ, lysozyme content was significantly increased ($p<0.05$) at varying degrees on days 14, 28 and 51 (Figure 6.2A).

3.4 Plasma complement activity

Plasma alternative complement activity (AH50) was increased after supplementation of spores of *B. subtilis* NZ86 and O14VRQ in every time point, but differences were significantly higher ($p< 0.05$) only for the NZ86 treatment group on days 14 and 51 (Figure 6.2B) relative to the control diet.

3.5 Phagocytic activity

Phagocytic activities of AKL after stimulation with FITC-labeled *E.coli*, were higher after supplementation of Nile tilapia with spores of *B subtilis* NZ86 and O14VRQ after 51 days, but the effects were not statistically significant (Table 6.3). The levels of phagocytic activity were higher in the WBC isolated from the spleen. A representative flow cytometry image of a white blood cell internalizing FITC-labeled *E. coli* is shown in Figure 6.3.

3.6 Respiratory burst

Stimulation with PMA resulted in potent respiratory burst in Nile tilapia leukocytes. However, differences were only observed in between treatment probiotic diets in SL as shown in Table 6.4. Median fluorescent intensity (MFI) and percentages are RHO-positive cells are tabulated in Table 6.4. Representative histograms and dot plots of respiratory burst from PMA stimulated SL and AKL are included in Figure 6.4. Only a fraction of both SL and AKL had strong fluorescent intensity (Fig. 6.4a and 6.4b). Leukocytes from the spleen with both low granularity, and small size exhibited the greatest respiratory burst (Figure 6.4a). Among the AKL, both larger cells with medium granularity and small cells with low granularity had the strongest oxidative activity (Figure 6.4b).

3.7 Immune gene expression

The mRNA expression of the pro-inflammatory cytokines IL-1β and TNFα in the AK and spleen is displayed on Figure 6.5. Expression of the same pro-inflammatory cytokines in the mid intestine of tilapia is shown in Figure 6.6. Significant levels ($p<0.05$) of pro-inflammatory cytokines were only observed in the intestine after supplementation with *B. subtilis* O14VRQ.

4. Discussion

Supplementation of probiotics in aquaculture is appealing in the context of both improved fish health, and reduction in the use of antibiotics for control of bacterial diseases. Furthermore, application of members of the genus *Bacillus* has an advantage for ease of dissemination given its cost-effectiveness of production and delivery, and ability for long-term storage [7]. Other studies have demonstrated the ability of *B. subtilis*, and specific strains of species of interest, to

enhance immunity and improve disease protection in many finfish and shellfish species, such as rainbow trout (*Oncorhynchus mykiss*), rohu (*Labeo rohita*), Nile tilapia, and white shrimp (*Litopenaeus vannamei*) [9, 18-20]. Preliminary work from our group found that application of *B. subtilis* strains NZ86 and O14VRQ was associated with increased disease resistance in *Pangasius hypophthalmus* and white shrimp (unpublished work). Thus this study aimed to demonstrate the immune responses as a plausible mode of action underlying the greater effects of supplementation with these probiotic candidates.

In this study, it was discovered that feeding of tilapia with *B. subtilis* at a final count 10^7 CFU g^{-1} feed was translated into medium levels of either strains of *B. subtilis* strains NZ86 or O14VRQ inhabiting the gut of the respective tilapia group (Table 6.1). This reflects the occurrence of a crucial step since detection of these probiotic populations reveals information about their ability to survive and propagate as part of the tilapia gut microbiota. Moreover, it allows making a stronger connection with the local and systemic immunostimulatory effects observed in this study.

The present work showed that the functionality of the tilapia gut was influenced immunologically following administration of the *B. subtilis* strains. Such supplementation resulted in elevated levels of the pro-inflammatory cytokines IL-1β and TNFα, but the heightened effects were only significant ($p < 0.05$) with diet O14VRQ. These cytokines are biological markers that contribute to host responses during the event of invasion or colonization of the gut [21]. Since dietary supplementation of the tilapia with strain O14VRQ resulted in lower transient probiotic counts (Table 6.1), it appears the tilapia gut is more sensitive at the gene expression level to this probiotic strain of *B. subtilis*. Dietary supplementation with probiotics can lead to increased immune gene expression level in the gut as evidenced in another

study with the use *Pediococcus acidilactici* in Nile tilapia [22]. The abundance of these two cytokines would lead to recruitment of more lymphoid cells to the site of colonization/invasion. Additional microscopy work for this study partially demonstrated this given an increase in the intraepithelial lymphocytes (IEL) after O14VRQ supplementation (Appendix D). The implication of all these would be that the *B. subtilis* strain O14VRQ appears to result in a higher state of immune-readiness at the gut level, relative to strain NZ86, which could be beneficial against pathogenic insults.

A similar effect as a result of the two pro-inflammatory cytokines related to the gut, occurred systemically. Since IL-1β and TNFα orchestrate the recruitment of macrophages and neutrophils to the inflammation site, this could explain the increased percentages of monocytes and neutrophils only in the O14VRQ treatment group after 28 days. Other studies using dietary supplemented *B. subtilis* in tilapia and major carp have shown an increase total WBC in blood [23-24]. Overall, this may be an indicator that the O14VRQ strain can stimulate the gut associated lymphoid tissue (GALT) of the host, leading to an effect in the hematopoietic organs [6]. Inconsistent with this, nonetheless, was that mRNA levels for these cytokines in the AK and spleen showed much variability, most likely due to the actß not being an appropriate reference gene for these two tissues in Nile tilapia. Thus, future studies should consider the use of multiple reference genes for more appropriate and sound normalization of gene expression across different tissues.

Lysozyme, a crucial part of the first line of defense, is an enzyme widely distributed across all vertebrates. In fish, this bacteriolytic enzyme has wider activity than the mammalian lysozyme, demonstrated in the greater lytic ability of the peptidoglycan component of the cell wall in both Gram-positive and Gram-negative bacteria [25]. The enzyme can be found in the

mucus, the lymphoid tissue, the plasma and other fluid components of bony fish [26]. The plasma lysozyme concentration is often investigated since its activity can be enhanced following dietary supplementation of *Bacillus* spp. In this study, feeding the fish with *B. subtilis* NZ86 and O14VRQ had a differential effect on plasma lysozyme content on three different time-periods. Similar to other studies [9,19], direct feeding only with strain O14VRQ had an effect in enhancing lysozyme content compared to the control diet on day 14 ($p=0.03$). On day 28, it was the other strain, NZ86, which influenced positively the plasma lysozyme content ($p=0.003$), suggesting that this probiotic requires a longer time to exhibit an effect in systemic immunity. Plasma lysozyme content was also increased after 51 days of supplementation with *B. subtilis* NZ86 and O14VRQ ($p<0.05$). The result from this time-period indicates the ability of both candidates for long-term effects in immunity, which is similar to the findings of Sun et al. after feeding grouper (*Epinephelus coioides*) with two different bacilli for 60 days [27]. Our results show that feeding tilapia with *B. subtilis* NZ86 or O14VRQ may or may not improve lysozyme content in the short-term, but feeding with either of the candidates does have a remarkable increase after long-term feeding.

Complement is a multi-protein component system that plays a key role in the innate immune response. Just like in higher vertebrates, the complement system in teleosts can be initiated by three different pathways: the classical, alternative and lectin. The alternative complement is particularly unique in bony fish, since it is more active at lower temperatures and has a broader recognition of foreign particles, compared to mammals [15]. In the present study, only supplementation of spores of *B. subtilis* NZ86 had a significant improvement ($p<0.05$) in AH50 on days 14 and 51. Even though the essential role of complement in clearing of pathogens is unquestionable, its continued activation can still lead to adverse effects in the host [27]. Thus,

it could be advantageous that AH50 levels were not as enhanced under the NZ86 diet regimen at day 28.

Phagocytosis is a function of cellular immunity that is of key importance in teleosts due to its low responsiveness to temperature changes [26]. Just like in higher vertebrates, the leukocytes capable of phagocytic activity in fish include neutrophils, monocytes, macrophages, dendritic cells, but unlike mammals, B-cells also exhibit potent phagocytic capability [28-29]. Leukocytes undergoing phagocytosis start a complex cascade of pro-inflammatory responses including respiratory burst, cytokines, chemokines, leukotrienes, degranulation and release of lysozymes [30]. Supplementation with *Bacillus* strains can stimulate phagocytic activity in fish [6,19,27]. In the present study, using flow cytometry, a trend towards greater phagocytic activity was observed after supplementation of tilapia with strain NZ86 in both AKL and SL, also with O14VRQ but only in the AKL, yet these findings were not significant ($p>0.05$). Additionally, the phagocytic activity in SL appeared to be greater than in AKL, which makes sense from the perspective of the spleen being a blood-filtering organ and predominantly constituted by macrophages and B-lymphocytes, which are potent phagocytes in teleosts. Since this immune function was assessed at a late time period following probiotic supplementation, it is possible that an enhanced effect could have happened much earlier and that the activities just decreased over time [31]. Future work should investigate the effect of feeding these bacilli strains on phagocytosis at multiple time points.

Respiratory burst is a measure of the generation of reactive oxygen species (ROS) by phagocytes, except lymphocytes, which lack the full NADPH oxidative complex machinery that drives this process [32-33]. Production of ROS, such as superoxide anion (O_2^-) or hydrogen peroxide (H_2O_2), contributes to the killing of internalized pathogens after their fusion with the

lysosome. There was increased activity for this innate immune function in the AKL following supplementation for 51 days with either strain NZ86 or O14VRQ, yet findings were not significant ($p>0.05$). The SL exhibited lower activity compared to the AKL, and respiratory burst granted by O14VRQ was only significantly higher compared to NZ86. The difference in respiratory activity in both hematopoietic organs can be attributed to the scarcity of the NADPH-positive leukocytes in the spleen. Since respiratory burst is a process associated with phagocytosis, it makes sense that the results are comparable.

All tilapia treatments grew well, and showed a similar growth performance throughout the 51-day dietary regimen. None of the treatments improved survival (all > 95%), and the FCRs were not statistically different (Appendix E). A possible explanation of this could be the recirculating conditions for this study at least meeting the requirements for the optimal growth of tilapia [34]. Therefore, despite the apparent elevated local and systemic immune responses in tilapia granted differentially upon dietary supplementation with either *Bacillus subtilis* diet, it is important to keep in mind that the growth performance was neither benefited nor impaired as a result of such probiotic diets.

In conclusion, the evidence from this study shows promise for the use of NZ86 or O14VRQ strains of *B. subtilis* as probiotics in aquaculture. This study demonstrated that supplementation of NZ86 resulted in more frequent systemic immune responses than localized effects. Strain O14VRQ, in the other hand, led to increases of the innate immunity both locally and systemically. Future studies of these strains should include disease challenge work with enteric pathogens (e.g. *Edwardsiella tarda* in tilapia) to have a better understanding of the elevated immune responses after supplementation with either *Bacillus subtilis* strain.

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Table 6.1 Bacterial quantification of total *Bacillus subtilis* strains in the intestine of Nile tilapia (*Oreochromis niloticus*) following RT-qPCR analysis. Values are mean ± SD

Treatments	**Day 0, Concentration (CFU g^{-1})**	**Day 51, Concentration (CFU g^{-1})**
Control	ND	ND
NZ86	ND	$1.07 \pm 0.2 \times 10^4$
O14VRQ	ND	$1.23 \pm 0.3 \times 10^3$

ND, not detected

Table 6.2 Peripheral blood leukocyte profile for Nile tilapia (*Oreochromis niloticus*) under different *Bacillus subtilis* probiotic diets. *n*=12 per group

Cell types	Day 14				
	Control	**NZ86**	**O14VRQ**	**p value‡**	**Pooled error§**
Lymphocytes, %	82.8	86.0	80.8	0.21	7.1
Neutrophils, %	8.58[a,b]	5.75[a]	12.4[b]	0.012	5.1
Monocytes, %	8.33	7.67	6.75	0.54	3.5
Eosinophils, %	0.25	0.50	0.17	0.27	0.5
	Day 28				
	Control	**NZ86**	**O14VRQ**	**p value**	**Pooled error**
Lymphocytes, %	88.9[a]	92.5[a]	81.7[b]	0.0001	4.8
Neutrophils, %	6.92[a]	4.33[a]	12.1[b]	0.0001	3.2
Monocytes, %	3.92[a,b]	2.92[a]	5.50[b]	0.01	2.1
Eosinophils, %	0.25	0.25	0.75	0.09	0.6

Values expressed as means

‡ p values shown pertain to one way ANOVA

§ Pooled error is the square root of within group variance (MSE)

[abc]Superscript letters in the same row indicate significant differences between groups ($P < 0.05$) after Tukey's HSD test.

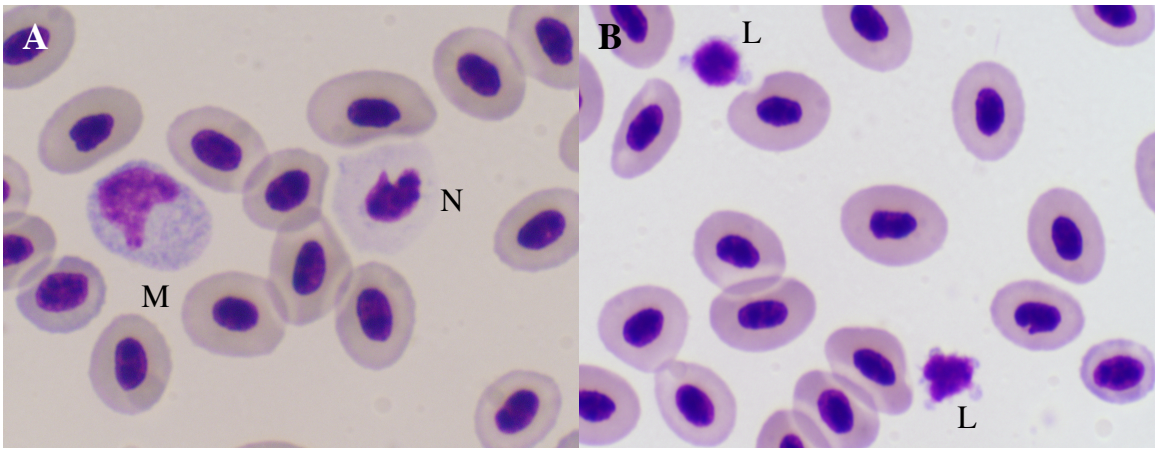


Figure 6.1 Micrograph of a blood smear showing the predominant types of peripheral blood cells found in Nile tilapia (*Oreochromis niloticus*), raised in recirculating aquaculture systems (**A-B**). May-Grünwald Giemsa stained. (**A**) A monocyte (M) and a neutrophil (N) with a few granules. (**B**) Two lymphocytes (L).

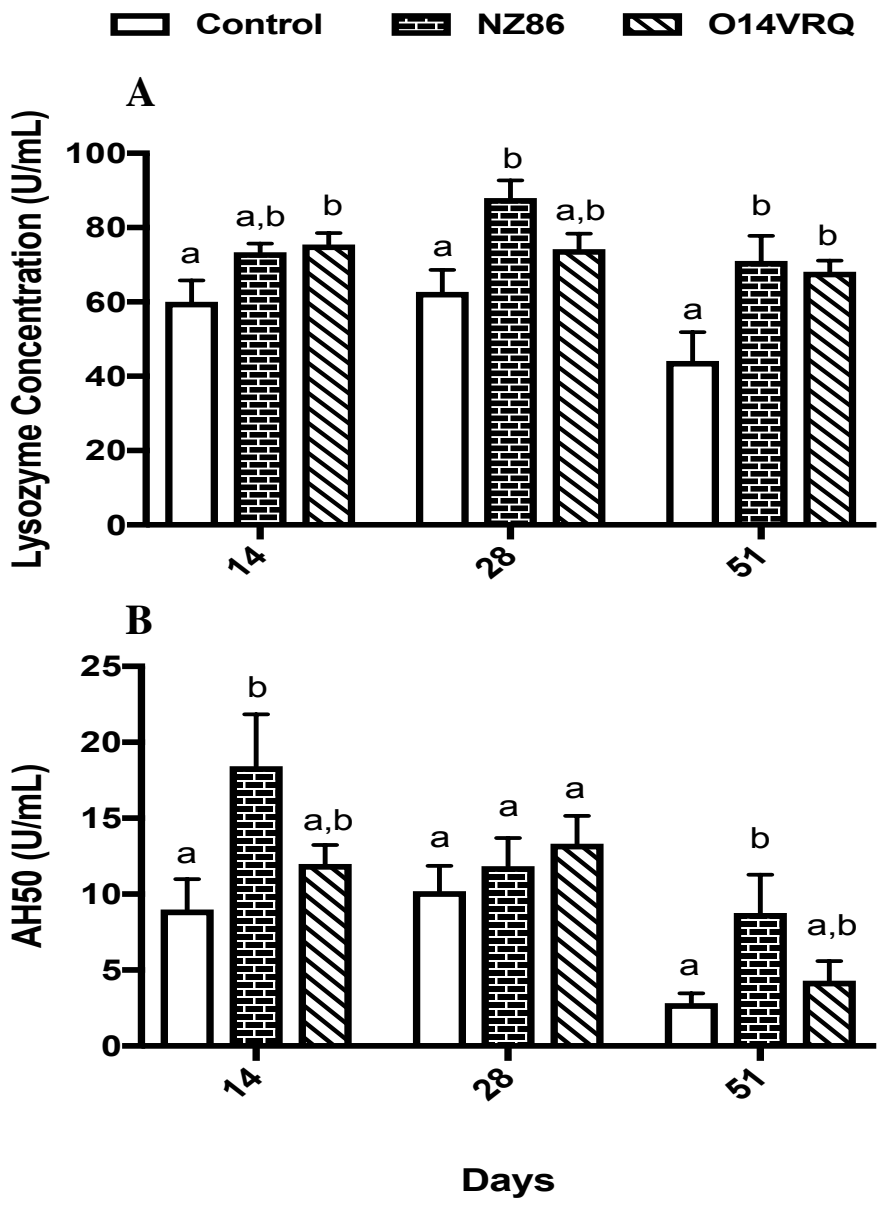


Figure 6.2 Plasma lysozyme concentration (U mL^{-1}) (A) and plasma alternative complement activity (AH50 U mL^{-1}) (B) after supplementation of Nile tilapia (*Oreochromis niloticus*) with different *Bacillus subtilis* probiotic diets. At Days 14 and 28, *n*=12 per group. At day 51, *n*=9 per group. Values graphed are means ± SEM. [abc]Different superscript letters denote significant differences between treatment groups per time period ($p < 0.05$) after one way ANOVA, followed by Tukey's HSD test.

Table 6.3 Proportions of FITC positive cells and median fluorescence intensity (MFI) of anterior kidney and spleen leukocytes (AKL and SL, respectively), after stimulation with FITC-labeled *E. coli*, from Nile tilapia (*Oreochromis niloticus*) following probiotic supplementation. *n*=9.

	Phagocytosis-Positive Cells (%)		**MFI of Phagocytosis-Positive Cells (10^3)**	
Treatments	AKL	SL	AKL	SL
Control	95.2	92.1	38.1	65.6
NZ86	93.7	93.3	74.1	73.5
O14VRQ	93.8	89.4	44.7	64.9
§Pooled Error	7.47	10.3	39.7	14.5
‡p value	0.893	0.722	0.145	0.907

Values expressed as means

‡p values shown pertain to one way ANOVA

§Pooled error is the square root of within group variance (MSE)

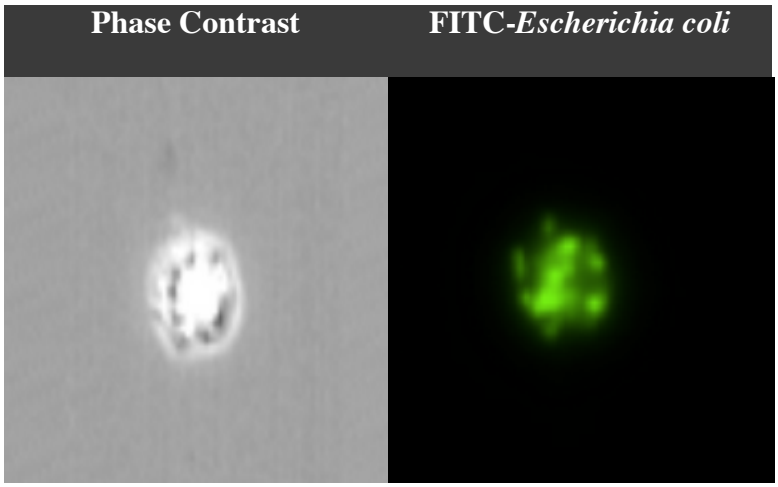


Figure 6.3. Representative image of flow cytometry analysis of phagocytic activity of FITC-labeled *E. coli* by tissue immune cells of Nile tilapia (*Oreochromis niloticus*) following probiotic supplementation.

Table 6.4 Proportion of rhodamine (RHO) positive cells and median fluorescence intensity (MFI) of PMA stimulated anterior kidney and spleen leukocytes (AKL and SL, respectively) from Nile tilapia (*Oreochromis niloticus*) following probiotic supplementation. *n*=9.

	RHO-Positive Cells (%)		**MFI of RHO-Positive Cells**	
Treatments	AKL	SL	AKL	SL
Control	90.9	27.2[a,b]	380	46.4[a,b]
NZ86	90.7	23.8[a]	387	32.5[a]
O14VRQ	94.0	48.5[b]	475	69.4[b]
§Pooled Error	4.52	19.2	172	18.9
‡p value	0.239	0.0243	0.441	0.00280

Values expressed as means

[ab]Superscript letters in the same row indicate significant differences between groups ($P < 0.05$) after Tukey's HSD test.

‡p values shown pertain to one way ANOVA

§Pooled error is the square root of within group variance (MSE)

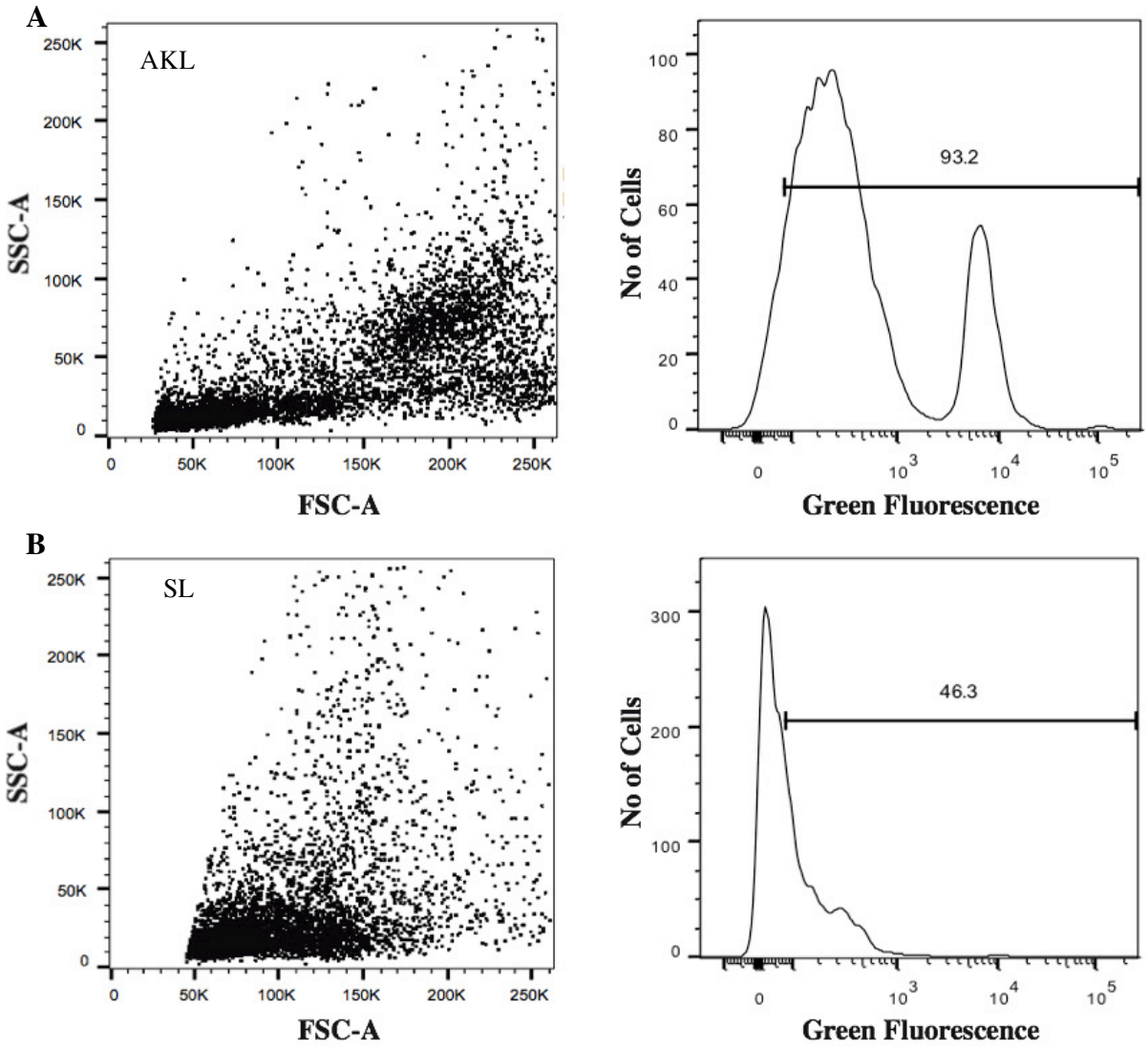


Figure 6.4 Flow cytometry analysis of respiratory burst from anterior kidney (**A**) and spleen (**B**) leukocytes (AKL and SL respectively) of Nile tilapia fed different probiotic diets. Representative size/granularity (FSC/SSC) dot plots show rhodamine-positive cells (green) in tilapia. The horizontal bars on fluorescence histograms are indicative of percentage of RHO-positive cells.

Table 6.5 Primers and probes specific for *Oreochromis niloticus* genes for RT-qPCR analyses

Gene target	**Forward primer (5' – 3')**	**Reverse primer (5' – 3')**	**Probe (5'FAM/ZEN/3'IBFQ)**	**Amplicon size (bp)**	**Access number**
TNF-a	ATAGCTTCTCAGACC ACGGC	GCAAACACGCCAA AGAAGGT	CACGCTGTGGACGG AAACCAAC	145	AY428948.1
IL-1β	GGCATCAAAGGCAC AAACCTC	GGTGTCGCGTTTGT AGAAGAG	CAAGCCAACCCTCC ACCTGGAG	150	KF747686.1
actβ	CTCCATCGTCCACCG CAAA	CTGCGCCTGAGTTG TGTATG	CAAACGCCCAACAA CTTCAGCTCT	131	XM_0034431 27.4

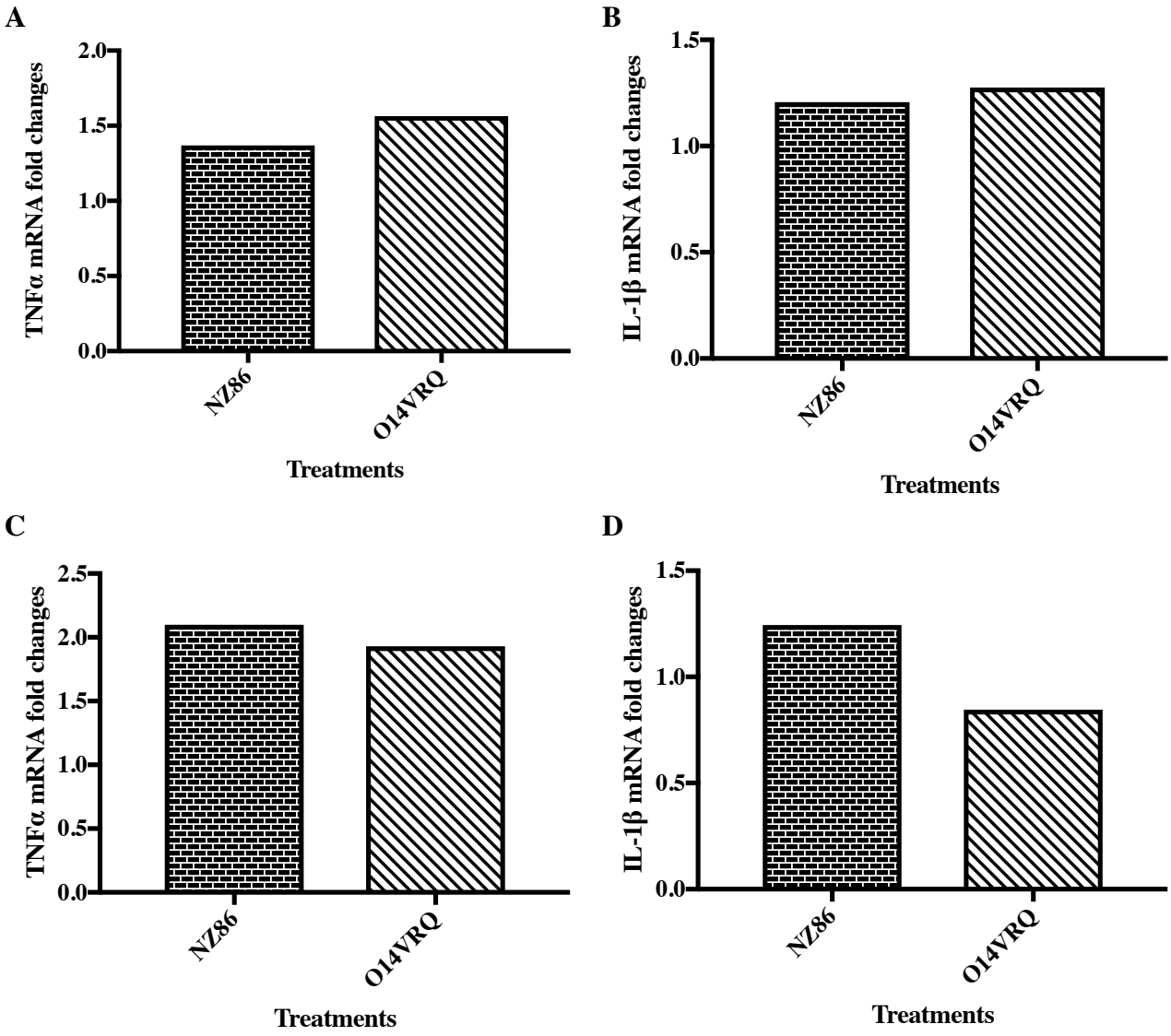


Figure 6.5 Expression of pro-inflammatory cytokine mRNA from the AK and spleen of Nile tilapia (*Oreochromis niloticus*) after probiotic supplementation for 51 days. TNFα in the AK (A), IL-1β in the AK (B), TNFα in the spleen (C), and IL-1β in the spleen (D). Values graphed are means. n=9

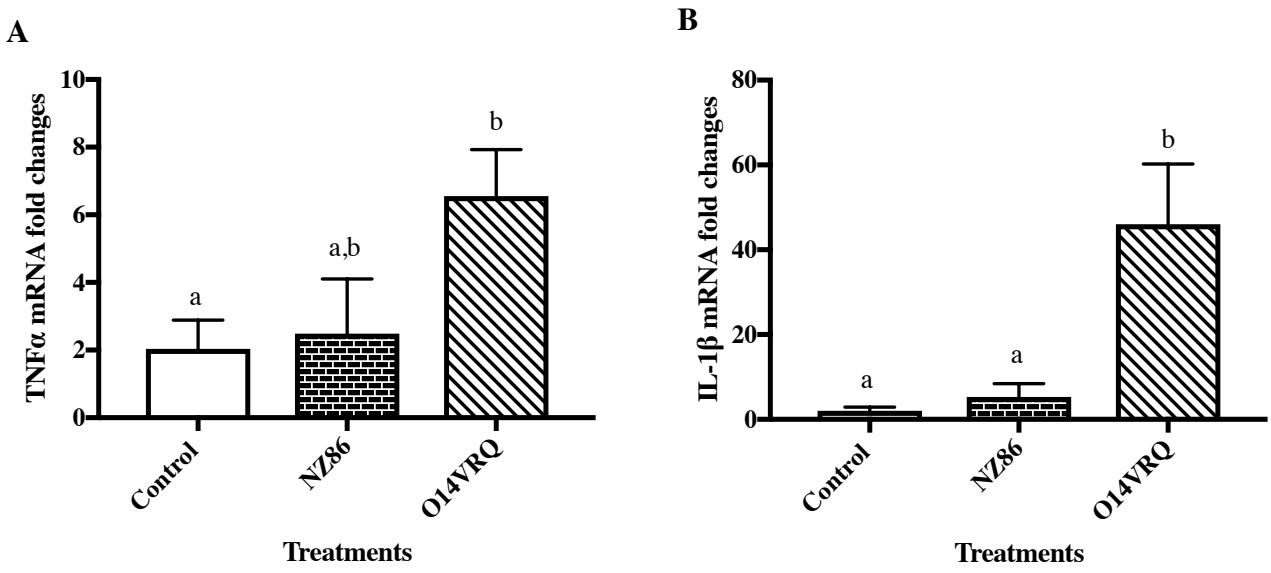


Figure 6.6 Expression of pro-inflammatory cytokine mRNA from the mid intestine of Nile tilapia (*Oreochromis niloticus*) after probiotic supplementation for 51 days. TNFα in the intestine (A), and IL-1β in the intestine (B). Values graphed are means ± SEM. Statistical analyses were carried out with one-way ANOVA followed by Tukey's HSD test for multiple comparisons. Different letters indicate significant differences ($p < 0.05$). n=8

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

7.1 Conclusions

A fish with poor health translates to decreased productivity, less income, less food for human consumption and greater food insecurity. To address this problem, this dissertation focused on generating and evaluating information that could be vital for addressing the fish health issues that concern farming of the commercially important striped catfish, and Nile tilapia.

In the first study, we isolated an affinity purified IgM-like antibody from striped catfish. Characterization of this macromolecule via SDS-PAGE allowed us to determine that it is made up of a heavy and light chain components of approximately 70 kDa and 25 kDa, respectively. Taking these into consideration, and the general consensus that the IgM-like isotype is tetrameric in form, we estimated that size of the whole molecule is at least of 760 kDa.

By using a previously described methodology, a rabbit polyclonal anti-*Pangasius* antibody was developed. This antibody was shown to have specificity not beyond the genus level, but to be efficacious enough for immunoassays. This work revealed a range for the plasma IgM-like antibody concentration, which can serve as a working reference range for diagnostic cases, immunization and other research trials involving this humoral component of the immune system of this catfish species. It must be taken into consideration, however, that these values are only applicable to the same or similar recirculating conditions of our study.

In the second study, hematologic and plasma chemistry reference intervals were established for striped catfish grown in RAS conditions. The values for the analytes investigated in this work were comparable to those reported in other freshwater species. One problem that was encountered in this study related to our difficulty in distinguishing between the types of

granulocytes in peripheral blood circulation. Under light microscopy, the most abundant type of granular cells were morphologically similar to eosinophils when using Romanowsky stains; yet this was confusing as this type of WBC is typically found in low numbers in most fish species. Since the literature describing leukocytes in striped catfish is scant, in addition to nomenclature and identification of WBCs in the piscine being complicated, we were not able to classify them beyond that category. The helpfulness of the reference intervals established here could be greatly appreciated to address welfare issues compromising the health of striped catfish grown in similar RAS conditions.

In the third study, we demonstrated that the NZ86 and O14VRQ strains of *B. subtilis* have various immunostimulatory effects at the local and systemic level upon their establishment in the tilapia intestine. Localized immunity effects were linked to greater expression of pro-inflammatory cytokines resulting in a state of heightened immunity in the intestine. Several systemic innate immunologic effects, including lysozyme content, alternative complement activity, phagocytic activity, respiratory burst, and peripheral blood WBC profiles, were found to be elevated during probiotic supplementation. Therefore this study adds evidence to modulation of the immune system being a possible mechanism of action of the two probiotic candidates in the piscine.

Use of piscine models can present some problems, especially for statistical analysis, due to fish to fish variation when it comes to immune responses. Sometimes statistical significance could be missed as a result of using an inadequate sample size, and therefore complicating making links of these findings to address larger population problems in fish. Although statistical significance was possibly missed due to variation, the positive trends

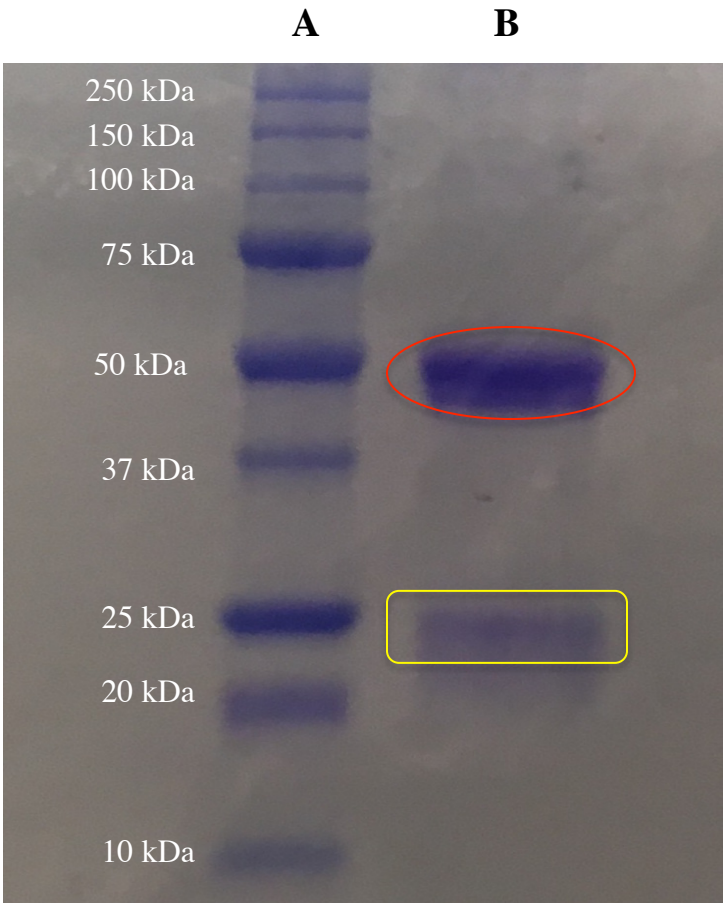
could be indicative of biological relevance, and therefore be meaningful or practical for fish farmers.

7.2 Future Directions

While many important foodfish species remain with limited characterization, especially when it comes to their physiology, this says a lot about the numerous research opportunities that are possible. The findings of the immunologic, hematologic and plasma chemistry reference values is intended to serve as a stepping stone for any health and welfare issues involving striped catfish. It would be interesting to see how varying one rearing conditions (e.g. ammonia levels) would affect any of the analytes explored here. While this work was carried out in RAS conditions, it is intended to also motivate similar research in other culturing conditions for comparison purposes.

The area of probiotic supplementation is one that will continue to gain more attention in aquaculture research and in the industry. This work is laying groundwork for future studies involving the use of these two strains in fish. Since trends for some immunity analytes, such as phagocytic activity and respiratory burst, were observed, future work should consider increasing sample size to achieve statistical significance (Appendix F). It would be rather helpful to also the same immune responses in tilapia following exposure with a relevant fish pathogen (e.g. *Streptococcus* spp, *Edwardsiella tarda*). While determining the mode of action of probiotic candidates is important, in time, it would also be helpful to pinpoint the effective dose of a specific probiotic strain. Another meaningful pieces of information to assess would include how the composition of the gut microbiota is affected, and the prevalence of the observed effects upon terminating the dietary regimen.

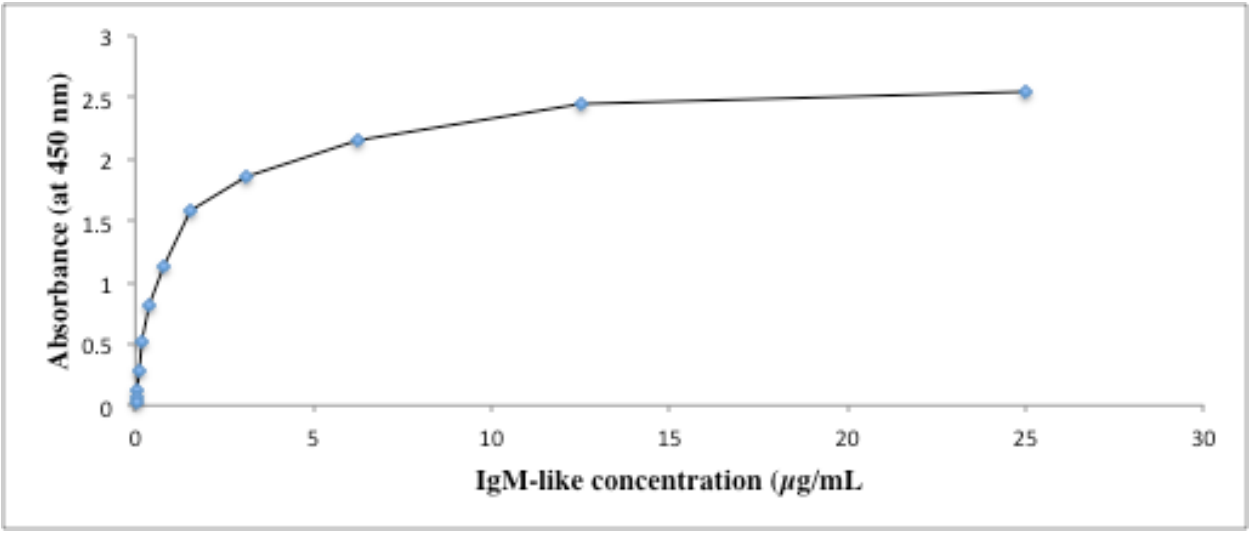
Appendix A. Molecular weight determination of rabbit anti-*Pangasius hypophthalmus* IgG purified via protein A column chromatography



Following the purification of the rabbit anti-*P. hypophthalmus* IgG via protein A affinity chromatography (Chapter IV), its purity was verified via SDS-PAGE. This technique showed that only two bands at ~50 kDa (heavy chain) and at ~25 kDa (light chain) were present in the sample as depicted by the red circle and yellow square, respectively.

Appendix B. Quantification of IgM-like molecule in *Pangasius hypophthalmus* via indirect enzyme-linked immunosorbent assay (ELISA).

Indirect ELISA assay involved was different from the sandwich ELISA (described in Chapter IV) in that assay was in the following manner: Plates were coated with striped catfish plasma, followed by addition of rabbit anti-Pangasius IgG, then a sheep anti-rabbit IgG conjugated with horseradish peroxidase, and finally the TMB substrate. The standard curve produced for this assay is shown below ($R^2 = 0.96$)



When trying to find out the appropriate dilution for the appropriate estimation of total IgM-like antibody in the plasma, non-specific binding of the primary and secondary antibodies was likely to be occurring since all dilutions of the test sample were yielding drastically different values for the concentration of that immunoglobulin in the plasma, as seen in table below.

Dilution of plasma	**Estimated total concentration (mg/mL)**
1:50	1.10
1:100	2.50
1:200	6.00
1:500	10.4
1:1000	22.3

Appendix C. Custom primers and probes specific for strains NZ86 and O14VRQ of *Bacillus subtilis* for RT-qPCR analysis.

Strain of *B. subtilis*	**Forward primer (5' – 3')**	**Reverse primer (5' – 3')**	**Probe (5'-6FAM/3'-BHQ1)**
NZ86	CTGTTCTCATGAACTGGGGC	GCTAACTCTGCAGGTACCCC	AAGGTCGAAGTTGAGGCAAA
O14VRQ	CCCTTGGGTAATGCTATGTAAAGG	GATGGCTTTCCACAGCGATGG	CAACCCCACTAGACCCTCAATAAAGC

Appendix D. Intestinal morphology of Nile tilapia (*Oreochromis niloticus*) fed different probiotic diets. The different parameters of the gut villi were measured via light microscopy, whereas the analyses of the microvilli parameters were done via transmission electron microscopy. Values are mean ± SEM. n = 9

Morphological Parameter	**Treatment**		
	Control	**NZ86**	**O14VRQ**
Villi length (μm)	991.7 ± 22.74	1028 ± 74.80	1067 ± 37.57
Villi perimeter (μm)	2383 ± 42.68	2528 ± 150.1	2693 ± 101.2
Goblet cell counts / 100 μm	2.40 ± 0.182	2.69 ± 2.40	2.58 ± 0.157
IEL counts*	47.3 ± 2.41	44.1 ± 2.65	51.8 ± 3.05
Microvilli length (μm)	1.74 ± 0.0499	1.54 ± 0.0781	1.63 ± 0.0583
Microvilli density (number / 25 μm^2)	1191 ± 25.84	1196 ± 25.17	1161 ± 69.03

*IEL, intraepithelial lymphocytes

Appendix E. Growth performance and feed utilization in Nile tilapia (*Oreochromis niloticus*) fed different diets. Values are presented as mean ± SEM. n = 9

	Control	**NZ86**	**O14VRQ**
Gained weight (g)	377±6.21	351±16.6	377±8.73
Feed intake (g)	463	435	433
Final Length (cm) ◊	24.9±0.156	24.3±0.343	23.6±0.261
K, condition factor ß	3.50±0.0640[a]	3.57±0.120[a]	4.07±0.103[b]
VSI *	6.80±0.292	6.66±0.277	7.20±0.442
SGR ‡	2.37±0.0654	2.28±0.0921	2.42±0.0769
FCR §	1.23	1.24	1.15
Survival rate (%)	100	95.0	100

◊ Length = distance from the tip of the snout to the end of the caudal peduncle

ß K factor = 100 (Weight / length^3)

*VSI, viscero-somatic index

‡SGR, cumulative specific growth rate = [ln (final weight) – ln (initial weight)] / 51 days

§FCR, cumulative feed conversion rate = feed consumed / body weight gain

Superscript letters denote significant differences ($p < 0.05$)

Appendix F. Power analysis for future work involving flow cytometry analysis of phagocytic activity and respiratory burst in Nile tilapia, following probiotic supplementation.

Assay	Effect Size	Sample Size
Phagocytic activity (AK)	0.6	13
Phagocytic activity (spleen)	0.6	32
Respiratory burst (AK)	0.6	28
Respiratory burst (spleen)	0.7	15

The power analysis was carried out using the G*Power software, and with the objective that data could achieve statistical differences via One-way ANOVA. Sample size was calculated considering power and alpha levels at 0.95 and 0.05, respectively.