Enhancing Seafood Quality and Safety by Reducing Reliance on Antibiotics: Applying a Novel Antibody in Tilapia

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Academic Abstract

Disease outbreaks have overwhelmed the aquaculture industry as a whole and have been catastrophic for many single operations. To minimize disease outbreaks, efforts are underway to enhance animal health and disease resistance to pathogens without the use of antibiotics. The overall purpose of this study was to explore a potential prophylactic, a novel antibody diet, for the bacterial pathogen, *Aeromonas hydrophila*, in tilapia.

The tilapia were on an anti-interleukin-10 antibody diet as a neutralization of interleukin-10 (IL-10), an anti-inflammatory cytokine. The anti-inflammatory function of IL-10 has been shown to allow persistence of gastrointestinal pathogens. Tilapia were fed the novel diet and were challenged via bath immersion or oral gavage with *A. hydrophila*. Four trials of challenge studies were conducted. Clinical signs of the disease and survival were monitored post-challenge of the bacteria. Out of the 4 trials, one bath immersion trial showed significantly lower survival in the group fed the novel antibody diet ($p=0.044$) compared to the control fed group, after challenged with *A. hydrophila*. The other trials tested showed no significant differences in survival between diets. Among the survival percentages collected as a whole, it cannot be determined from *in vivo* results whether this anti-IL-10 diet is effective in preventing mortality from *A. hydrophila* in tilapia. Therefore, an *in vitro* study using an enzyme-linked immunosorbent assay (ELISA) was used to determine the neutralization capability of anti-IL-10 on IL-10 using tilapia splenocytes. Interferon-γ, a pro-inflammatory cytokine, was quantified in order to find a trend in expression of IL-10 *in vitro* in various tilapia cell treatments. The protocol for the ELISA study is under development being that the use of this antibody is novel and has never before been done in fish.

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General Audience Abstract

Aquaculture is the fastest growing animal-production sector for food in the world. Health and safety issues are prevalent among aquatic animals during massive growth and production. Disease outbreaks within aquaculture facilities can cause losses worth billions of dollars. Antibiotics are currently in use in aquaculture as a therapeutant for treating disease. However, over time the use of antibiotics has brought up a new set of issues; antibiotic resistant bacteria/genes and transfer of these to the environment and to humans via consumption.

A novel antibody feed, containing an antibody to interleukin-10, with potential as a preventative disease measure was used to study disease development after exposure with a bacterial pathogen. Thus, tilapia were exposed to the bacteria, *Aeromonas hydrophila*, and signs of disease and survival were monitored to see if the novel antibody feed would prevent disease onset. Further research is necessary on the antibody before confirming effectiveness on disease prevention.

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Dedication

I'd like to dedicate this work to my parents, Barbara and Ken Garry. Thank you for giving me the strong foundation I needed to succeed and always instilling confidence in me. You are always my number one cheerleaders in anything I do. Without your love and encouragement, I don't know where I'd be. I love you both.

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List of Abbreviations

Anti-IL-10, antibody to IL-10

BSL-2, Biosafety Level-2 laboratory

C-2hAh, control diet and 2h *Aeromonas hydrophila* growth culture

C-4hAh, control diet and 4h *Aeromonas hydrophila* growth culture

C-5.5hAh, control diet and 5.5h *Aeromonas hydrophila* growth culture

C-Ah, control antibody diet and *Aeromonas hydrophila* challenge

C-C, control antibody diet and control challenge

Con A, Concanavalin A

DO, dissolved oxygen

ELISA, enzyme-linked immunosorbent assay

FCR, feed conversion ratio

FDA, Food and Drug Administration

IFN-γ, interferon gamma

IL-10, interleukin-10

IL-10-2hAh, anti-IL-10 diet and 2h *Aeromonas hydrophila* growth culture

IL-10-4hAh, anti-IL-10 diet and 4h *Aeromonas hydrophila* growth culture

IL-10-5.5hAh, anti-IL-10 diet and 5.5h *Aeromonas hydrophila* growth culture

IL-10-Ah, anti-IL-10 diet and *Aeromonas hydrophila* challenge

IL-10-C, anti-IL-10 diet and control challenge

MAS, Motile *Aeromonas* Septicemia

OD, optical density

rpm, revolutions per minute

TSA, trypticase soy agar

TSB, trypticase soy broth

Chapter 1: Introduction

Despite their tolerance to a wide range of environmental conditions, tilapia are frequently exposed to stressful situations which can lead to disease outbreaks. Disease often strikes a host while accompanied by an external stressor such as pollutants, overcrowding, etc. (Austin, Austin, 1999). A stressful environment will cause fish to be more susceptible to disease infection, especially bacteria that comes secondary to stress such as *Aeromonas hydrophila* (Harikrishnan, Balasundaram, 2005). *Aeromonas hydrophila* is considered one of the most common bacterial diseases infecting farmed and feral tilapia (El-Sayed, 2006), making this pathogen a target for control. This pathogen has been shown to cause economic loss in fish farms around the world from the United States to the Philippines (Hossain, et al., 2014; Yambot, 1998).

Currently, *A. hydrophila* is treated with antibiotics in the aquaculture industry (FAO, 2005b). However, over time the use of antibiotics has brought up a new set of issues; antibiotic resistant bacteria. *Aeromonas hydrophila,* a bacterial threat to aquaculture, has reportedly acquired resistance to several antibiotics. According to a study done by Odeyemi, Asmat, & Usup in 2012, various isolates of *A. hydrophila* have shown resistance to multiple antibiotic drugs. For this reason, efforts are underway to replace antibiotics in the aquaculture industry, while maintaining a safe environment for the animals.

This research focused on a potential prophylactic treatment against the bacterial fish pathogen, *A. hydrophila*, using a novel antibody to avian interleukin-10 (anti-IL-10) via diet. The anti-IL-10 has had neutralization of interleukin-10 (IL-10) capabilities *in vivo* in chickens, showing prevention of reduced body weight post-pathogen exposure (Arendt, et al., 2016; Sand, 2016). As an anti-inflammatory cytokine, IL-10's function is critical to balance the pro-inflammatory response of a host. However, the research outlined by Cyktor, Turner (2011),

suggests that in the absence of IL-10, the host has an improved ability to regulate pathogenic infection.

The long term goal of this study was to create a prophylactic for tilapia against bacterial pathogens, using *A. hydrophila* as a model to alleviate the reliance the aquaculture industry has on antibiotic use. This work used *Oreochromis aureus* as a host model and *A. hydrophila* as a bacterial model due to their prevalence in the aquaculture industry. The prophylactic anti-IL-10 diet could potentially be used in aquatic host species beyond tilapia and for protection against other pathogens.

One specific objective of this research was to investigate the effect anti-IL-10 diet had on survival post-*A. hydrophila* infection of tilapia fingerlings using two routes of exposure; bath immersion and oral gavage. Utilizing the antibody diet *in vivo* gave insight on clinical signs of disease and survival patterns comparing two routes of bacterial infection. This study was the first of its kind to implement oral gavage as a route of infection for *A. hydrophila* in tilapia. Oral gavage was necessary to compare with bath immersion being that the pathogen was introduced to the tilapia at the same site of anti-IL-10 digestion.

Another objective of this research was to explore the neutralization capabilities of anti-IL-10 to IL-10 *in vitro* by measuring cytokine activity derived from tilapia splenocytes. By applying the anti-IL-10 to various cell treatments, an enzyme-linked immunosorbent assay (ELISA) was used to quantify interferon gamma (IFN-γ), which has been shown to be inhibited in the presence of IL-10. This was used as the mechanism for exploring expression of IL-10 in the presence of anti-IL-10. The *in vitro* study will give more insight of the function of anti-IL-10 in tilapia.

Chapter 2: Literature Review

A Global Need for Aquaculture

Aquaculture is the production: breeding, rearing, and harvesting of aquatic animals and plants. It is also referred to as fish or shellfish farming (NOAA Fisheries). The global need for aquaculture stems from a growing human population, need for nutritious food, sustainability, and growing demand for seafood. The global human population is expected to exceed 9 billion by 2050, a 2.3-billion-person spike from 2009 to 2050 (FAO, 2009). This drastic rise in population will elicit a drastic rise in food supply. According to the National Oceanic and Atmospheric Administration Fisheries, the demand for seafood is growing rapidly and the wild capture fisheries can no longer sustain the necessary supply (NOAA Fisheries). The global food fish consumption amounted to 47.1 million tons and 64.5 million tons from aquaculture and wild capture respectively in 2006. These numbers are projected to be 93.6 million and 58.1 million tons in 2030 (World Bank, 2013).

Not only is food security an issue in this never-ending increase in human population, but the issue of nutrition security is also alarming (FAO, 2009). Furthermore, the challenge is to sustainably feed the world a nutritious and wholesome diet to maintain good health. One essential nutrient in a wholesome diet is protein, which can be absorbed from foods such as meats, eggs, milk, and fish or fishery products. According to the Food and Agriculture Organization (FAO), global seafood consumption per capita has been increasing and will continue to do so as consumers realize the nutritional benefits of fish. Fish have many health benefits besides protein. Fish are a valuable commodity for omega 3 fatty acids and many micronutrients (FAO, 2017a). The consumption of many types of fish are heathier options than some of the other animal proteins when it comes to total and saturated fats, while maintaining the

amount of protein consumed. Fish, a low-saturated fat and high protein-food, is represented as a generally healthy food for the population (Yaktine, 2007).

With the realizations of the health benefits of fish and other seafood products, there has been an increase in global fish consumption. Seventeen percent of the global population's animal protein intake stemmed from fish consumption in 2013 (FAO, 2016). A rise in fish for food demand by humans globally, presses for an equivalent rise in fish supply. This supply is collectively coming from fisheries and aquaculture, however wild capture production has leveled out in the past decade while aquaculture production has been increasing (FAO, 2016). Of the millions of tons of food supplied by aquaculture and fisheries in 2006, 47% of it came from aquaculture alone. This was an astronomical increase from 3.9% in 1970 (Peeler, Taylor, 2011). In 2012, the total wild capture production amounted to 91.3 million tons, while total aquaculture production was about 66.6 million tons. Utilization of these products for human consumption amounted to 136.2 million tons (FAO, 2014). Then in 2014, the production of food-fish for human consumption from aquaculture surpassed the production from wild capture for the first time in history (FAO, 2016). The reason for the increase in aquaculture being that wild capture is no longer a sustainable option for our boundless human population growth.

The FAO has outlined the general situation of world fish stocks. Twenty-five percent of the marine fish stocks in the world fall into the "overexploited, depleted, or recovering from depletion" categories. In other words, (overexploited) these fisheries are being exploited above a long-term sustainable level, (depleted) wild catches are well below past records, or the fisheries are recovering from depletion with catches increasing (FAO, 2011). With that being said, there is obvious danger of the progression of overexploitation of fishing from wild caught fisheries. Even though commercial fisheries will remain a vital industry for our coastal communities we need to

increase seafood production using alternative means to meet the growing global demand. Thus, the aquaculture industry is fulfilling the need for a sustainable resource for supplying the world animal protein without completely depleting or further exploiting natural fishing stocks.

Not only is aquaculture a more sustainable source of food than its wild capture counterpart, it demonstrates exceptional feed conversion ratio (FCR). Feed conversion ratio is the amount of feed required of livestock or aquaculture production to gain one pound of body mass (Bourne, 2014). Fish are the most efficient animal in converting protein when compared to other meats such as chicken, pork, and beef. For comparison, salmon is about seven times more efficient at converting protein than cattle (Global Aquaculture Alliance, 2018). The average FCR of fishmeal for global fed aquaculture is expected to decrease even further over the next decade (World Bank, 2013). Thus, aquaculture can maximize cost and time efficiency by producing valuable protein.

Tilapia Industry

Tilapia farming is the most widespread type of aquaculture, globally (FAO, 2014). Tilapia express many key characteristics that make them appealing for growth in aquaculture. Due to their ability to grow quickly and resilience, tilapia are widely grown around the globe (El-Sayed, 2006). There are many tilapia production practices including water and land-based systems. Ponds, floating cages, tanks and raceways, and recirculating systems are all techniques used for tilapia production across the world (FAO, 2005a). Cages in open water such as lakes and reservoirs have become popular for tilapia production in many Asian countries as well as Mexico and Columbia (Gupta, Acosta, 2004). Indoor recirculating systems for tilapia production require less water and land and there is more control over environmental conditions which is especially important in the U.S. with changing climates (Watanabe, et al., 2002). Focusing on

domestic tilapia production, the world's largest indoor fishery is in Virginia, called Blue Ridge Aquaculture. According to the company's website, Blue Ridge Aquaculture raises approximately four million pounds of tilapia each year, shipping live tilapia to cities across the United States (Blue Ridge Aquaculture, 2017).

Not only are tilapia's resilience a key factor in their massive role in the aquaculture industry, but so is the fact that they are a widely consumed food fish by humans. In the United States in 2010, tilapia was the fourth most consumed seafood; just behind shrimp, canned tuna, and salmon (NOAA Fisheries, 2010) and the industry is only expected to increase from there. Between 2010 and 2030, tilapia is in the top 2 of aquaculture species production and is expected to nearly double to about 7.3 million tons, globally (World Bank, 2013).

Farm raised tilapia, dubbed as the "aquatic chicken," are low-priced and the market demand is increasing (Perschbacher, 2017). On a large scale, according to the United States Department of Agriculture, "The United States annually imports nearly $1 billion worth of tilapia while producing another 30 million pounds ourselves… worldwide, farm-raised tilapia is nearly an $8 billion yearly industry (USDA, ARS, 2017)." The U.S. is the largest market for tilapia consumption, with the main suppliers being countries in Asia and Central America (FAO, 2016). China is the main supplier (FAO, 2017b).

The ever-growing supply and demand for not only aquaculture but tilapia specifically is due in part to its durability as a species as well as nutritional benefits when consumed by humans. A warm water fish, tilapia are known to withstand temperatures as low as 7-10ºC, very low levels of dissolved oxygen, and a wide range of pH (El-Sayed, 2006). The diverse environmental conditions the fish can bear make it simple to farm for the most part. Also, their non-fishy taste and firm texture are desirable features of tilapia to consumers. Even with various

regions of the world having different partialities for foods, tilapia reaches a wide variety of human consumers (Suresh, Bhujel, 2012).

Overall, tilapia is an important commodity to the economic welfare of fish farmers globally, as well as important to sustain the demand from consumers. This has led to the growth of the tilapia industry. However, despite the species' resilience to harsh environments, tilapias are still susceptible to contracting infectious diseases, possibly due to immunosuppression and often due to poor water quality (El-Sayed, 2006).

Water Quality

Maintaining an aquaculture system with good water quality is essential for the health and well-being of the aquatic organisms living in the system. However, the water quality in a system not only affects the fish in a system, but the fish affect the water quality. Water quality parameters such as dissolved oxygen, temperature and ammonia are vital in controlling because of the effect they can have on fish health (Buttner, et al., 1993). Other parameters such as pH and alkalinity are important for monitoring due to their effect on the nitrification process. Nitrification is a biological filtration process in which biofilm forming bacteria such as *Nitrosomonas* and *Nitrobacter* oxidize toxic ammonia and nitrite into less toxic nitrate (Water Environment Federation, 2015). Generally, methods for measuring important water quality parameters are well established. Standard methods of measurement in the United States are typically done with manufactured test kits, which involve adding water to vials and adding chemicals. Then, colors of the samples are measured to quantify the water quality parameter of interest such as ammonia, alkalinity, nitrite, and nitrate (Noga, 2010). Commercially available probes can also be used for testing DO, temperature, and salinity.

The water quality in a system will regulate the development and health of an aquatic organism. Depending on the species, water quality parameters will vary and what is deemed as "good" quality for one species may be detrimental to the health of another (Stickney, 2009). Specifically, tilapia are well-adapted fish to varying water conditions. Optimal temperature of tilapia is in the range of 25-30ºC, with 30ºC preferred (El-Sayed, 2006), dissolved oxygen (DO) above 5.0 mg/L and ammonia below 1.0 mg/L (Riche, Garling, 2003). The resilience of tilapia is part of why it is such a popular farmed fish, however poor water quality can still lead to a stressful environment and environmental quality is an important aspect in preventing stress in the animal.

Stress, is the first step towards disease susceptibility (El-Sayed, 2006) and one of three components; host, stressor, and a disease causing situation (Austin, Austin, 1999). It would be desirable to maintain good water quality within aquaculture facilities, but with high fish stocking densities and possible biosecurity issues, this may become difficult and diseases can be introduced. In the event disease is suspected in an aquaculture facility, water quality measurements should be taken immediately to resolve any conditions that may be compromising the fish (Noga, 2010).

Disease in Aquaculture

The rapid development and expansion of the aquaculture industry has not been without issues. Maintaining the health of the animals is not only appropriate welfare but is also crucial to the livelihood of farmers and the other fish in the farm. Economic losses due to disease in the production of finfish is difficult to assess, however it has been approximated by the FAO that $6 billion is lost annually (World Bank, 2014). Most disease outbreaks happen outside of the United

States, where most of global aquaculture takes place, but ultimately disease in aquaculture is ubiquitous (World Bank, 2014).

Emerging diseases are a major limitation in the aquaculture industry. Some pathogens are commonly found in a specific species, however, a system of polyculture can lead hazardous to other species (Murray, Peeler, 2005). There are a variety of routes of infection that disease can emerge in an aquaculture facility. A pathogen can spread through transportation of live animals into a new stock or facility, transporting of fish products for food fish feed, evolution of virulence of a pathogen, etc. (Murray, Peeler, 2005). In addition to these scenarios of infection introduction, a compromised environment will allow the infection of opportunistic pathogens to invade a host as well. According to Derome, et al. (2016), opportunistic pathogens are present in the microbiota of healthy fish and take advantage of their host in unfavorable conditions.

Bacteria, fungi, viruses, and parasites are common pathogens negatively affecting the aquaculture industry. While these are all of great concern, bacteria's ability to independently survive in water makes it a pathogen detrimental to the industry (Pridgeon, Klesius, 2012). With both Gram-negative and Gram-positive bacteria expressing infection in fish, increased attention has been placed on bacterial diseases in the industry in recent years (El-Sayed, 2006). Some common bacteria affecting the tilapia industry as well as other fish include *Aeromonas*, *Streptococcus, Mycobacterium*, and many others (FAO, 2005a). All of these pathogens have caused outbreaks leading to economic loss and money would have been better spent in prevention rather than trying to treat and eradicate the bacteria in an aquaculture facility (Deng, et al., 2017; Francis-Floyd, 2011).

For example, an outbreak of the bacterial pathogen, *Aeromonas hydrophila* in commercially raised catfish in Alabama in 2009 cost the industry $12 million (Hossain, et al.,

2014). After further etiological studies of the pathogen, it was believed to have an Asian origin. A study by Hossain, et al. (2014), suspected that the strain causing the outbreak in catfish in southeastern U.S. was carried by imported fish from Asia. An epidemic of *A. hydrophila* in China and the importation of live fish or seafood thereafter, along with genotypic analysis, solidified the Asian origin of the pathogen.

The global aquaculture industry is vulnerable to diseases; domestically and internationally. Thus, the future of aquaculture and its sustainability relies heavily on prevention of disease outbreaks. If the industry focuses on prophylactic disease measures as opposed to therapeutic, this may be the means of decreased disease dissemination.

For the purpose of the research outlined in this thesis, *A. hydrophila* was used as the model bacterial pathogen in testing a potential prophylactic. The use of *A. hydrophila* as opposed to other bacterial fish pathogens mentioned above is due to its low zoonotic potential in order to ensure human handler safety (Lowry, Smith, 2007; Swann, White, 1991).

Aeromonas hydrophila: Opportunistic Pathogen

Aeromonas hydrophila is an opportunistic bacterial pathogen, meaning it exploits the host when living conditions are unfavorable and the fish becomes stressed. The pathogen affects various freshwater species such as tilapia, carp, catfish, salmon, etc. (Hossain, et al., 2014). The organism is naturally found in the microbiota of healthy fish. Thus, in environmentally adverse conditions that may cause stress in the animal, *A. hydrophila* takes advantage of its host (Derome, et al., 2016).

In the past few decades, *A. hydrophila* has been the source of various outbreaks in aquaculture facilities across the globe. A study in China investigated the pathogenic source causing morbidity in fish and crab across 8 farms. Of 95 bacterial isolates taken from 6 fish

farms and 2 crab farms, 35 were identified as *A. hydrophila* by biochemical methods and 29 (of the 35) were identified as *A. hydrophila* by PCR analysis. The *A. hydrophila* isolates originated from 5 of the fish farms (Nielsen, et al., 2001). The identification results of the isolates taken from moribund fish showed predominance in *A. hydrophila.* This study was carried out in an environment with no capabilities of controlling water quality. Thus, further confirming that neglected water quality is reservoir for the opportunistic pathogen, *A. hydrophila.*

Another example of an outbreak of *A. hydrophila* in Nile tilapia lead to severe economic loss in the Philippines (Yambot, 1998). Mortalities presented systemic infections, with various organs affected. Mortality rates were up to 100% in some cages. This outbreak occurred during colder months and rainy season, when conditions for tilapia may not have been optimal. Conclusively demonstrating *A. hydrophila* as an opportunistic pathogen that necessitates alternative control.

Aeromonas hydrophila has also been detected in the United States for decades. In North Carolina in 1973, 37,500 fish mortalities were observed over about 2 weeks from the pathogen in a lake (Miller, Chapman, 1976). According to a study done by Hazen, et al. (1978), the pathogen had been isolated from water samples taken from 135 of the 147 natural aquatic habitats across 30 states. With prevalent presence of this opportunistic pathogen in water systems, aquatic species are at risk due to common environmental stresses that can aide in the infection of this pathogen.

Motile *Aeromonas* Septicemia

The genus *Aeromonas* represents Gram-negative, motile, facultative anaerobic bacteria. Caused by the bacteria *A. hydrophila, A. caviae, and A. sobrai,* Motile *Aeromonas* Septicemia (MAS) (Savan, Sakai) is a disease prevalent in fish and other aquatic organisms in ponds and

recirculating systems (Camus, 1998; Hanson, 2014). An outbreak of MAS in Alabama and Mississippi in 2009 caused severe mortality of catfish, an industry worth $361 million in sales in 2015 (Abdelhamed, et al., 2017; Zhang, et al., 2016).

According to Camus (1998), clinical signs of the disease may include external hemorrhaging, skin lesions, exophthalmia (eye popping), severe ulcerations, and abdominal distension. Behavioral changes caused by *Aeromonas* infections include lack of appetite, lethargic swimming, or swimming in a corkscrew pattern. The bacteria are transmitted horizontally and can be shed from open lesions on a fish or from fecal shedding. Probable routes for infection are oral and dermal (Hanson, 2014). Being one of the most common bacterial diseases in tilapia, the infection of *Aeromonas* in farmed raised tilapia may lead to heavy mortality (El-Sayed, 2006). Diagnosing MAS in fish is as simple as observing clinical signs of disease as outlined above, as well as aseptically sampling the kidney onto nutrient rich agar plates (Hanson, 2014).

Experimental Induction of *Aeromonas hydrophila*

Many researchers have investigated methods of experimentally inducing pathogens into an aquaculture system to study things such as the effects of immune response, a novel prophylactic treatment, etc. Various methods have been explored such as waterborne challenges, intraperitoneal injection, intramuscular injection, and oral gavage, which are outlined in Table 2.1. In a study by Zhang, et al. (2016), the exposure of *A. hydrophila* was done by waterborne challenge, deemed as a more natural route of infection than other common methods. In the results of this study, it was found that even minor skin abrasion or external injuries to the fish can be detrimental to the life of the fish. Twenty percent mortality was shown in fish exposed to *A. hydrophila* via waterborne challenge when artificial external injuries were a factor (i.e. skin

scrapes). This is an important finding, that in high stocking densities of fish in an aquaculture facility, the collision of fish with one another can inflict skin abrasions, which leads to fish health vulnerability.

Although less natural of infection route, intraperitoneal injection (IP) is a method commonly used due to its uniformity and reproducibility. The pathological changes were studied in tilapia hybrids IP injected with *A. hydrophila* (Rey, et al., 2009). Severe intussusception was shown in the gastrointestinal tract of the tilapia, which is seen as wrinkling or folding. Although injected internally, the fish sustained superficial hemorrhages, as well as typical clinical signs of lethargy and decreased appetite.

Oral administration or oral gavage is another method that can be used to experimentally induce bacterial disease in fish. This technique may use a syringe with a tubing or feeding needle while inserting the needle down the esophagus of an anesthetized fish (Collymore, et al., 2013). *Aeromonas hydrophila* has been orally administered in common carp in order to study the effects of the pathogen on the intestinal mucosal membrane (Schroers, et al., 2009) and in channel catfish to study portals of entry of the pathogen (Ventura, Grizzle, 1987). The oral gavage method has never been used for administration of *A. hydrophila* in tilapia and by introducing the bacteria directly to the gastrointestinal tract, it is present in the same place digestion of a potential prophylactic is taking place. The motive of this alternate route of infection is to explore the differences between infection portal of the bacteria while feeding a potential prophylactic diet.

Table 2.1. A representative sample of studies showing the experimental induction of *Aeromonas hydrophila* in teleost fish species

Paper	Animal (Host) Species	Exposure Method	Relevant Findings
Zhang, et al. (2016)	Channel catfish (*Ictalurus punctatus*)	Waterborne	• Healthy fish challenged at concentration $2.0x10^7$ CFU/ml had 0% mortality after 48h • External surface wounds predisposed catfish to *A. hydrophila* via waterborne route – 90% mortality occurred post infection in fish with clipped fins and 20% mortality in fish with skin scrapes
Kong, et al. (2017)	Grass carp (*Ctenopharyngodon idella*)	Intraperitoneal injection	• Intestinal mucosal barrier permeability increased post-challenge at concentration $2.0x10^7$ CFU/ml • Up-regulation of inflammation-related gene expression post-pathogen challenge • Intestinal IL-10 expression significantly increased post-challenge
Sarder, et al. (2001)	Nile tilapia (*Oreochromis nioloticus)*	Intraperitoneal Injection	• Mortality onset post-injection was 12h with $5.0x10^6$ CFU/ml • All dead fish presented typical symptoms of *A. hydrophila* – reddish anus, swollen abdomen, and swollen injection site
Rey, et al. (2009)	Tilapia hybrids	Intraperitoneal Injection	• Expressed intussusception of intestinal walls and congested liver • Injection of *A. hydrophila* suggested target of gastrointestinal tract
Saraceni, et al. (2016)	Zebrafish Larvae *(Danio rerio)*	Waterborne	• Healthy larvae exposed to the pathogen (10^8 CFU/ml) expressed 33% mortality while larvae with clipped tail fin expressed 77% mortality
Schroers, et al. (2009)	Common carp	Oral Gavage	• The intestinal epithelium mucus was not protected against *Aeromonas hydrophila*, damaging the intestinal barrier

Potential for *Aeromonas hydrophila* in Humans

Recently, *A. hydrophila* has been viewed not only as a pathogen to effect aquatic species, but also a danger to human public health (Igbinosa, et al., 2012). In 1984, for the first time, the Food and Drug Administration (FDA) announced *A. hydrophila* as a new foodborne pathogen (Isonhood, Drake, 2002). Although considered as a zoonotic disease, this pathogen is not likely to harm healthy exposed individuals but there have been cases. Open sores in infected waters or handling infected fish with sharp fins are certainly possible routes of infection (Swann, White, 1991). A review of the pathogen importance in food safety by Daskalov (2006), outlined the prevalence of *A. hydrophila* in the environment; a toddler who consumed contaminated water and various adults on different occasions that consumed contaminated fish or meat were infected with *A. hydrophila*.

Although limited, there have been cases of *A. hydrophila* detection in food. In a market study in Mexico City, *A. hydrophila* was detected from various samples of frozen tilapia, along with other *Aeromonas* species (Castro-Escarpulli, et al., 2003). With that being said, not only is the pathogen a danger to the health of aquatic species, but potentially to consumers.

Regulations of Antibiotic Use in Aquaculture

The FDA has outlined the approved aquaculture drugs for use (FDA, 2017). There are 9 classes of drugs approved for use in the United States in the aquaculture industry, including antibiotics, antimicrobials, antiparasitic compounds, and sedatives (FDA, 2017). One of specific importance due to its potential negative effects in the aquaculture industry is the use of antibiotics. With more than 100 bacterial species known to cause disease in aquaculture, antibiotics have been the main antibacterial therapeutant (Lunestad, Samuelsen, 2008).

Regulations of antibiotic use and other drugs in aquaculture have been set by the FDA (FDA, 2017) and must be followed by aquaculture production within the United States, as well as the aquaculture supply being imported into the country. Considering less than one percent of global aquaculture for human consumption takes place in the United States (Fry, et al., 2014), thorough inspections of imported seafood is crucial. According to the World Health Organization (WHO), few countries responsible for massive aquaculture production regulate antibiotic usage (WHO, 2006). For example, China is the largest producer of aquaculture globally (FAO, 2014), making the country the largest producer and user of antibiotics as well (Liu, et al., 2017). A review of antibiotics in Chinese aquaculture compiled a list of 20 antibiotics that were reportedly used from 1996-2013 (Liu, et al., 2017). Some of these antibiotics were designed for human use, but were implemented in aquaculture disease treatment or prevention. The use of these human antibiotics can be the means to spreading bacterial resistance in not only animal medicine, but human medicine as well.

Regulating antibiotic usage in aquaculture is essential due to the implications antibiotic use brings the industry. Antibiotics have been shown to leave residues in fish tissues for human consumption as well as cause antibiotic resistant bacteria and multi-drug resistant bacteria (Heuer, et al., 2009). With that being said, the transfer of antimicrobial agents into the environment stemming from aquaculture, could be harmful to consumers (Love, et al., 2011).

Most antibiotics are administered via food, however, sick fish often experience loss of appetite and may not be receiving the treatment (Secombes, 2012). This allows antibiotics to enter the water, and spread among plants and other unintended organisms (Lunestad, Samuelsen, 2008). For the safety of seafood consumers, the FDA has set withdrawal times that are labeled on the drug for fish intended for human consumption. Withdrawal times vary from drug to drug but

have been established by the FDA to eliminate the transfer of drug residues to human through food consumption (Department of Health and Human Services, 2011). However, about 90% of the seafood in the United States is imported (NOAA Fisheries), and although the FDA has regulations for seafood inspections, only a select amount is inspected for products that have violated regulations in the past (FDA, 2008). Unfortunately, there are still cases of drug residue detection even when used within the regulation of the FDA. For example, a market study conducted in the U.S. with fish samples from various countries found antibiotic residues above the detection limits (Done, Halden, 2015). This study detected antibiotics that are currently approved for use in the U.S.; the most detected being oxytetracycline which is the most commonly used antibiotic in aquaculture (Sapkota, et al., 2008). According to a study done by Sapkota, et al. (2008), on the potential impact of human health from antibiotics used in aquaculture, antibiotic residues consumed in low-levels are unlikely to cause negative health effects but increased levels have not been well studied so it is possible that consumers are at risk.

A larger issue of antibiotic use that is less controllable is the development of antibiotic resistance (Heuer, et al., 2009). A review by Defoirdt, et al. (2011), compiled a list of 12 antibiotics that have shown multiple drug resistance isolated from various aquaculture farms globally. Expansion of antibiotic resistance, while antibiotics are heavily utilized to treat infected aquatic organisms, raises an issue where alternative therapeutants or prophylactics are necessary.

Antibiotic Resistance: *Aeromonas hydrophila*

According to a review done by Harikrishnan, Balasundaram (2005), *A. hydrophila* has been shown to be sensitive to the following antibiotics: chloramphenicol, nitrofurantonin, oxolinic acid, and trimethoprime-sulfamethoxazol. The therapeutic use of antibiotics for *A. hydrophila* can and has led to bacterial resistance. In the same review, *A. hydrophila*

demonstrated resistance to antibiotics such as ampicillin, cephalothin, flumeguine, gentamycin, nitrofurantoin, oxytetracycline, penicillin, streptomycin, tetracycline, trimethoprime, and trimethoprime-sulfamethoxazol. Among these antibiotics is one of the few U.S. FDA approved drugs for aquaculture; oxytetracycline (FDA, 2017).

The multi-drug resistance of this pathogen was also outlined by Wooley, et al. (2004). An isolate of *A. hydrophila* was cultured from an ulcerated koi and an antibiogram was determined using the disc diffusion method. *Aeromonas hydrophila* (isolate 5046) was shown resistant to 2 of the 8 tested antibiotics including ampicillin and sulfasoxazole (Wooley, et al., 2004). In another study done with tilapia from a pond in Thailand, 53 out of 55 strains of *A. hydrophila* tested demonstrated resistance to ampicillin, while 100% of strains showed sensitive to florefenicol (Tipmongkolsilp, et al., 2012). The studies demonstrating multi-drug resistance or sensitivity, make other prophylactic measures of aquatic diseases not only desirable, but also necessary.

The prevalence of antibiotic resistant bacteria and antibiotic resistant genes in the aquatic environment is increasing. Aquaculture is already a contributor to the spread of these bacteria and genes because of the use of antibiotics. A study done from retail aquaculture products in China found antibiotic resistance genes present in tilapia, crucian carp, catfish, and shrimp (Ye, et al., 2013). This also brings about the issue of spreading these genes to humans after consumption of aquaculture products. Since the development of antibiotic resistance is said to be unavoidable with the use of antibiotics (Sharma, et al., 2016), development of alternatives to treating and preventing disease is necessary to prevent aquaculture from contributing any further to the rising issue.

Teleost Immune System: General

With the opportunity for disease and economic loss on commercial fish farms, it is imperative to study the immunological defenses of fish. Extensive immunological research in fish is limited; much of what we know about the immune system is from mice or humans (Lieschke, 2009). However, we do know that in teleosts, the immune system is divided into the innate (nonspecific) and adaptive (specific) systems (Magnadóttir, 2006). When a fish is exposed to a pathogen or other foreign molecule, the initial response from the host is by the innate immune system (Thompson, 2017). Nonspecific defense mechanisms include surface barriers, humoral factors, cellular factors, eosinophils, basophils, mast cells, and inflammation (Secombes, 2012). The surface barriers mentioned of the nonspecific defense mechanism are simply the external features of the fish such as scales, skin, mucus, etc. (Thompson, 2017). The innate immune system does not have complex mechanisms like the adaptive system does, but is efficient in immediately responding to common or shared features of microorganisms (Jørgensen, 2014).

The innate system is an essential activation mechanism for the response of the adaptive system (Magnadóttir, 2006). The development of the adaptive immune response in the early stages of life can take up to 12 weeks in fish, emphasizing the importance of the innate immune system prior to its development (Magnadóttir, 2006). Adaptive immunity is the response from a host that is specific to the antigen presented and that has been adapted to respond since the first encounter with that specific antigen (Mutoloki, 2014). Adaptive immunity may be a delayed response and is necessary for enduring immunity overtime after repeated exposure to foreign molecules or pathogens in a host (Secombes, Wang, 2012). It is prompted when the innate system is unsuccessful at clearing foreign molecules or pathogens (Dixon, Becker, 2011). Much

like other vertebrates, as a part of specific defense mechanisms, fish have T- and B- lymphocytes as a cellular response and immunoglobulins as a humoral response (Thompson, 2017). In addition, the specific immune response factors include lymphoid organs, antibody responses, memory, etc. (Secombes, 2012). The combination of these immune responses and two immune systems are fundamental in combatting foreign substances, and especially disease in fish.

Teleost Innate Immune System: Cytokines

Specific fundamental proteins, called cytokines, have a primary role of signaling the immune system and its responses (Magnadóttir, 2006; McInnes, 2017). Working as a mediator between innate and adaptive immunity, cytokines are recruited by the innate system to the site of a foreign infection. Fish cytokines have been studied extensively in the last decade and have been shown to express similar features to other vertebrates (Secombes, 2016). In responding to and clearing an immune response, both pro-inflammatory and anti-inflammatory cytokines are necessary to maintain proper health of the fish. With that being said, the production of cytokines influences the production of other cytokines. Being that cytokines have different functional characteristics and properties, they are separated into classes such as interferons, interleukins, chemokines, colony-stimulating factors, tumor necrosis factors, and others (Secombes, et al., 1996).

Interleukin-10 (IL-10) is a cytokine of particular interest to this research. Interleukin-10 is an anti-inflammatory cytokine responsible for counter-balancing the actions of pro-inflammatory responses in a host to prevent inflammation damage to host tissue. Interleukin-10 has been identified in various species of teleosts, including rainbow trout, sea bass, common carp, and Atlantic cod (Zou, Secombes, 2016).

Interferon gamma (IFN-γ) is a cytokine associated with innate and adaptive immunity (López-Muñoz, et al., 2011). It is part of the type II IFN receptor family (Zou, Secombes, 2011). IFN-γ has a defined role in the immune system of fish and has now been sequenced in various fish species from rainbow trout to Atlantic salmon (Wang, Secombes, 2013). According to Zou, Secombes (2016), cells that are treated with IFN-γ, activate pro-inflammatory response genes. Thus, when an immune response is elicited in a host, IL-10 and IFN-γ are expressed antagonistically of each other. Also, IL-10 has a well-known capability of inhibiting production of IFN-γ (Commins, et al., 2008; Hillyer, Woodward, 2003; Pestka, et al., 2004). The contrasting relationship of IL-10 and IFN-γ can be used to study the capacity of either immune parameter to perform under specific circumstances.

Fish Spleen Function

In regards to the immune system, teleost fish lack bone marrow and lymph nodes. Thus, if a pathogen should invade to the blood circulation, the kidney and spleen are responsible for clearance (Press, Evensen, 1999). According to Secombes, Wang (2012), the spleen is a major secondary lymphoid organ in fish and generally functions similarly to mammals. The spleen is home to cells such as macrophages and lymphocytes (Press, Evensen, 1999). With the production of IL-10 by macrophages among other cells, detection of IL-10 in fish spleen has been documented on many occasions. In the characterization of IL-10 in goldfish, quantitative expression showed IL-10 mRNA levels the highest in the spleen tissue (Grayfer, et al., 2011). In another study of Atlantic cod stimulated with formalin killed-*Vibrio anguillarum,* increased expression of IL-10 was shown in the spleen (Seppola, et al., 2008). These studies are indicative of the immunoregulatory aspects of IL-10 in teleost spleen cells. Therefore, splenocytes can be used as a means to characterize the expression of IL-10 under specific circumstances.

Understanding the role of IL-10 and splenocytes will be essential in clearing bacterial pathogenic infections with novel developing methods.

Manipulation of Interleukin-10

As previously stated, IL-10 is an anti-inflammatory cytokine, intended to balance an elicited pro-inflammatory response. Without the immunosuppressive properties of IL-10 *in vivo*, a pathogenic infection has the potential to lead to death by inflammation, (Cyktor, Turner, 2011) demonstrating IL-10's importance in the immune system. However, IL-10 can be manipulated by pathogen invasion. Cyktor, Turner (2011) reviewed the behavior of pathogens during invasion in the absence or neutralization of IL-10. There is evidence that pathogens have the ability to persist by finding a balance between pro-inflammatory and anti-inflammatory responses during infection, through a variety of pathways. In an instance where a pathogenic invasion can find a balance in an immune response, the IL-10 production is able to establish pathogen persistence in the host (Cyktor, Turner, 2011).

Information on the expression of cytokines in fish is limited. However, we can infer that they act similarly to those in mammals, although there are some differences between species (Secombes, 2012). Likewise, we can compare the bioactivity of IL-10 between different fish species. In a study done on catla, major carp, IL-10 expression was compared between catla challenged with *A. hydrophila* and IL-10 expressed in a control challenge. Twenty-four hours post-challenge with the bacteria showed a significant increase in the expression of IL-10 in the gill, kidney, and liver (Swain, 2012). Another study done by Kong, et al. (2017), suggests that IL-10 mRNA levels significantly increased in the intestine in grass carp experimentally infected with *A. hydrophila*, 24 and 72 hours post-infection. The results from these studies demonstrate the fact that IL-10 expression is increased with bacterial infection.

Anti-IL-10

The use of a novel antibody to avian IL-10, "anti-IL-10" was developed to test the neutralization of IL-10 and its effect when exposed to a pathogen in broiler chicks (Arendt, et al., 2016; Sand, 2016). Anti-IL-10 was made by injecting hens with chicken IL-10-bovine gamma globulin with an 8 amino acid peptide emulsified with Freund's Complete Adjuvant (Sand, 2016). Twenty-one days post-injection, the eggs were collected, yolks separated from albumin, and the yolks were lyophilized. Using a dried egg yolk powder, the anti-IL-10 was fed to chicks challenged with mixed *Eimeria spp.,* which causes coccidiosis, a parasitic disease that invades the intestinal tract and causes tissue damage. The study demonstrated that feeding anti-IL-10 prevented a reduction in body weight in challenged chicks when compared to the control fed chicks (Sand, 2016). It was concluded that anti-IL-10 served as a possible protectant from decreased nutrient absorption post *Eimeria* challenge.

The anti-IL-10 egg yolk powder was fed to broiler chicks in another study by Arendt, et al. (2016), which studied the intestinal luminal levels of IL-10. The study demonstrated that IL-10 was indeed secreted into the intestinal lumen and thereby a pathogenic infection increases the amount of IL-10 secreted. However, the anti-IL-10 had no effect on the level of IL-10 in the lumen. These unexpected findings could have been due to the sampling time interfering with the ability to detect luminal levels (Arendt, et al., 2016). Further studies utilizing anti-IL-10 are necessary in order to fully understand its effect on IL-10 and efficiency in clearing a pathogenic infection (Sand, 2016). The implementation of this novel antibody to IL-10 in tilapia has never been done before.

Conclusions

The economic burden of disease outbreaks, the safety of the animals and human handlers, and rising global demand for tilapia are all indications of the need for prophylactic disease measures. The majority of bacterial diseases are currently treated with antibiotics; however, any further development of antibiotic resistance bacteria is undesirable in the production of food fish.

This research outlines the implementation of the avian anti-IL-10 into the diet of tilapia fingerlings that were disease challenged with bacteria, *A. hydrophila*. Clinical signs of disease and survival percentages were recorded over time after exposure. The challenge studies are an in progress development of a model of this specific pathogen with two routes of infection, bath immersion and oral gavage. These studies are the first to expose *A. hydrophila* to tilapia via oral gavage. Therefore, not only was the use of anti-IL-10 in the diet novel, method development in fish was also novel. *In vitro* studies were considered for studying neutralizing function of anti-IL-10 when in the presence of IL-10. This was done by Enzyme Linked Immunosorbent Assay (ELISA). Tilapia splenocyte cytokine IFN-γ was measured as an indicator of the expression of IL-10 *in vitro.*

Chapter 3: Experimental Exposure of *Aeromonas hydrophila* in Tilapia Fingerlings on a Novel Antibody Diet

Introduction

The up-regulation of interleukin-10 (IL-10), an anti-inflammatory cytokine, has been determined by recent studies as the reason for lack of immune detection by gastrointestinal pathogen invasion (Cyktor, Turner, 2011). This study focuses on the use of anti-IL-10 egg yolk antibody in tilapia fingerling diet as a neutralization of IL-10, when challenged with the pathogen, *Aeromonas hydrophila*. To understand the effectiveness of the anti-IL-10 diet in tilapia fingerlings, the diet was administered for a minimum of 3 days prior to an experimental exposure of *A. hydrophila* by bath immersion and oral gavage. Thus, the objective of this study was to experimentally introduce *A. hydrophila* into tilapia fingerlings subjected to an anti-IL-10 diet and monitor clinical signs of disease and survival using bath immersion and oral gavage. It was hypothesized that the anti-IL-10 diet would neutralize IL-10 *in vivo* and allow the tilapia fingerlings to clear infection of *A. hydrophila.*

This study focused on method development of exposure trials of *A. hydrophila* via bath immersion and oral gavage. Common challenge studies of *A. hydrophila* in tilapia are done via intraperitoneal injection (IP) (AlYahya, et al., 2018; Ardó, et al., 2008; Rey, et al., 2009), which may be more effective for infection, but is not as natural. The stressor implemented in the bath immersion studies was hypoxia, paired with the exposure of *A. hydrophila*. Hypoxia is a common stressor in reared tilapia when raised in high stocking densities and during transportation of the animals. Inducing acute hypoxia has been shown to increase cortisol levels in tilapia, thus, demonstrating an effect on the immune system (M. dos Santos, et al., 2016).

Materials and Methods

All lab and animal protocols were approved by Virginia Polytechnic Institute and State University's Institute for Biosafety Committee (IBC) and Institutional Animal Care and Use Committee (IACUC) . Tilapia fingerlings (*Oreochromis aureus)* were purchased from Tilapia Depot (St. Augustine, FL) and delivered at <1.5 inches in length. The fish were acclimated to the facility for approximately 72h prior to experimentation. Once the bacterial pathogen was experimentally infected via either bath immersion or oral gavage, fish were monitored for signs of disease and mortality. An aseptic swab of the posterior kidney was sent to the Virginia-Maryland College of Veterinary Medicine for confirmation of bacterial presence in fish that were euthanized due to morbidity or in the case of mortality.

Feed Preparation

Antibody to avian IL-10 and a control antibody was received from Dr. Mark Cook, University of Wisconsin-Madison, Animal Sciences Department. The anti-IL-10 was prepared as outlined in Sand (2016). In summary, hens were injected with a chicken IL-10 peptide conjugated to bovine gamma globulin. Twenty-one days post-injection the eggs were collected, yolks separated, and lyophilized. The control diet used in this study was a control egg yolk antibody and the anti-IL-10 diet was the anti-IL-10 peptide antibody.

Each egg yolk powder (1.0g) was added to 250 mL of DI water and homogenized until uniform using a VirTis VirTishear homogenizer (Gardiner, NY). The suspension was then transferred to a sprayer and sprayed onto 2 mm finfish starter feed (Zeigler Bros., Inc.). The feed was placed in a covered 5 gal bucket while mixing with a power mixer and spraying simultaneously through the lid to allow a uniform coating. The feed was dried at room temperature for 48h and stored at 4ºC for the remainder of the trial. Three kilograms of feed was

prepared for each diet, applied at a concentration of 1g egg yolk powder/kg feed. This application rate was modified as outlined by Sand (2016) to compensate for antibody loss in tank water prior to feed consumption by the fish. The concentration of antibody in the egg yolk powder was confirmed at 0.4 mg/g egg yolk powder (Sand, 2016). Adherence of antibody to feed was confirmed by optical density (OD) prior to use for experimental trials, shown in Figure A.1. The control egg yolk antibody and anti-IL-10 peptide antibody have equal nutritional assets. The presence of an anti-IL-10 peptide was the only difference between the diets. Three days prior to disease challenge, fish were fed twice daily until satiation.

Water Quality

Water quality measurements were taken and recorded from each tank daily, for the extent of each trial. The measurements taken daily included temperature, dissolved oxygen (DO), pH, alkalinity, ammonia, and nitrite. Hach® equipment such as spectrophotometer, Permachem® reagents, and DO meter were used (Loveland, CO) and Hach® protocols were followed. Nitrate was analyzed once a week. Water quality data was collected, data points were averaged by treatment, and recorded as mean ± standard error in Tables A.1-A.4 by experimental trial.

Aeromonas hydrophila Growth Curve Development and Starter Culture Preparation

A clinical isolate of *A. hydrophila* was obtained from Dr. Stephen Smith from the Virginia-Maryland College of Veterinary Medicine, that was isolated from a tilapia outbreak in 1996. A single pass through a healthy tilapia via intraperitoneal (IP) injection was performed to re-isolate the bacteria from the posterior kidney. The culture was isolated by swabbing the posterior kidney of the moribund fish. The isolate was confirmed at the Virginia-Maryland College of Veterinary Medicine by Matrix-Assisted Laser Desorption/Ionization (MALDI). Once the identity of the pathogen was confirmed *A. hydrophila*, a growth curve of the pathogen was

developed to further understand its growth patterns. A 100ml flask of Trypticase Soy Broth (TSB) was inoculated and grown at 30ºC overnight (14h) while shaking at 250 rpm. After overnight incubation, 1ml was transferred to four, 100ml flasks of sterile TSB as replicates to develop a growth curve of the bacteria. Three flasks served as replicate samples on the growth curve and the fourth flask was used for making glycerol stock cultures for later use in challenge experimentation. The inoculum from 3 samples were plated onto Trypticase Soy Agar (TSA) and OD was measured in a spectrophotometer at wavelength 600 nm at time points 2h, 3h, 4h, 5h, 6h, 7h, 8h, and 24h of growth at 30ºC while shaking.

Glycerol stock cultures were prepared once the inoculum reached OD=0.5. One ml stocks of the bacteria were made with glycerol and stored at -80ºC until used for challenge trials. The concentration of *A. hydrophila* was determined by plate count method. The growth curve with OD measurements developed is shown in Figure A.2.

Bath Immersion Challenge

Tilapia ranging in size of 1-3g were divided into eight, 110L recirculating tanks in a Biosafety Level-2 Laboratory (BSL-2), with 15 fish per tank. Two tanks were used for each treatment as replicates. Treatment groups included: control antibody diet x control challenge (fish were exposed to sterile TSB) (C-C), anti-IL-10 antibody diet x control challenge (IL-10-C), control antibody diet x *A. hydrophila* challenge (C-Ah), and anti-IL-10 antibody diet x *A. hydrophila* challenge (IL-10-Ah).

In preparation of the culture used for bacterial challenge, four (1ml) frozen stock cultures were taken out of the -80ºC freezer to thaw at room temperature for 15 minutes. Four sterile samples of 100ml TSB were inoculated using a 1ml culture at separate time points. Each sample was incubated at 30ºC while shaking (250 rpm) for approximately 5.5h in order to reach a target

concentration of $1.0x10^9$CFU/ml and OD=1.35. Target concentrations were determined through the prediction model shown in Figure A.3. After 5.5h of incubation, the sample was taken out of the incubator, used for the challenge and was immediately plated on TSA in duplicate. Plates incubated for 24h at 30ºC and were counted.

Trial 1: One tank at a time, (n=15) fish were transferred to 800ml of tank water in a 1L beaker. With a DO probe suspended into the water, saran wrap was used to cover the top of the beaker as a seal to restrict oxygen, inducing a hypoxic state. A visual of this method is shown in Figure A.4a. For 15 minutes, the fish were exposed to a low oxygen environment; bottoming out at approximately 0.45mg/L DO. Immediately following the stressor, the fish were netted and transferred to 1L of fresh tank water in a 2L bucket with aeration, shown in Figure A.4b. For C-C, 1 ml of sterile TSB was added to the exposure bucket. For the IL-10-Ah and C-Ah, cultured TSB was incubated at 30ºC for 5.5h while shaking (250 rpm). The bacteria was grown to a target concentration of $1.0x10^9$ CFUs/ml determined by OD. After the addition of 1ml of the inoculum into the 999ml exposure bucket, the fish were exposed to a target concentration of approximately $1.0x10^6$CFU/ml with aeration for 60 minutes. After 60 minutes of exposure, fish were netted out of exposure tank and returned to respective tanks for monitoring of clinical signs of disease and mortality.

Trial 2: The method of *A. hydrophila* inoculum growth as well as the exposure to hypoxic stress (15 minutes) was the same as in Trial 1. The concentration (CFU/ml) of pathogen the fish were exposed to increased. After 5.5h of incubation of *A. hydrophila* in TSB at 30ºC shaking (250 rpm), 50ml of inoculum was transferred to the (950ml) fish exposure bucket for 60 minutes with aeration. The target concentration of exposure was approximately $5.0x10^7$CFU/ml. The C-C and IL-10-C treatments were exposed to 50ml of sterile TSB. Post-exposure, fish were

netted and returned to respective tanks where they were monitored for clinical signs of disease and mortality until the conclusion of the trial.

Oral Gavage Challenge

Trial 1: At the time of experimentation, tilapia were 4.5-5.5g. The tank layout and treatment groups were the same as the bath immersion trials. A 1mL frozen stock culture of *A. hydrophila* was thawed at room temperature for 15 minutes. Once the culture was thawed, 100mL of sterile TSB was inoculated. The *A. hydrophila* inoculum incubated for 5.5h at 30ºC while shaking (250 rpm). After 5.5h of incubation, the target concentration of pathogen was approximately $1.0x10^9$ CFU/ml, determined by OD. Four separate cultures of *A. hydrophila* in 100ml TSB were used for the 4 challenge tanks. A 10-fold dilution was done with sterile TSB from the inoculum prior to gavage.

Beginning with the C-C and IL-10-C treatments, fish (n=15) were removed from their tanks and placed in a 1.5L holding bucket, containing water from the respective tank, with a lid to prevent fish from escaping. One by one a fish from the holding tank was lightly sedated using 50 mg/L Tricaine-S/L (Western Chemical Inc., Ferndale, WA) of water buffered with 50 mg/L sodium bicarbonate. Each fish was orally gavaged with a volume dependent on their body mass. A standard of 0.02ml *A. hydrophila* culture per gram of fish was orally gavaged. A table of approximate fish weights and volume gavaged was used during the experiment to immediately determine volumes to gavage. The target concentration of *A. hydrophila* in 0.10ml gavage was $1.0x10^7$ CFU/ml.

The inoculum was administered to each fish by oral gavage using a 0.5ml glass syringe with 18Gx2” plastic animal feeding needle (Cadence Science, Cranston, RI). This technique is shown in Figure A.5. The needle was placed into the anesthetized fish’s mouth, just enough to

surpass the gills and pass between the two pharyngeal plates into the stomach. Once the fish was gavaged, the needle was carefully removed and the fish was placed into a recovery bucket, containing 1.5L of its respective tank water. Feeding needles were replaced between tanks (every 15 fish). Sterile TSB was used for gavaging the control challenge treatment tanks. After recovery from anesthesia, the fish (n=15) were returned to their respective recirculating tanks. All fish were then monitored for clinical signs of disease such as lack of appetite, skin lesions, abdominal distention etc. and mortality until the conclusion of the study.

Trial 2: The purpose of oral gavage Trial 2 was to determine the pathogenicity of *A. hydrophila* at different points of the bacterial growth curve while still implementing the anti-IL-10 diet. Treatments for this trial included C-C, IL-10-C, 2h *A. hydrophila* growth culture (2hAh), 4h (4hAh), and 5.5h (5.5hAh) on both control and anti-IL-10 diets. Tilapia fingerlings were kept in recirculating tanks with n=8 per treatment. Frozen stock cultures were used for this experiment and were thawed at room temperature for 15 minutes, prior to inoculating TSB for experimentation. The inoculum was grown for 2h at 30ºC with 250 rpm shaking, was removed from the incubator, plated on TSA, used for 2hAh treatment, and was placed back in the incubator for 4hAh and 5.5hAh treatments.

Statistical Analysis

Statistical analysis was performed in JMP Pro (version 13.0) statistical software. The effect of treatment group on average survival percentage for each tank was compared using a one-way ANOVA followed by a post hoc test. One-way ANOVA was also used for water quality measurements followed by a post hoc test. Student's t-tests were used to compare the means of CFU/ml or CFU/g in *Aeromonas hydrophila* treatment groups. Results were recorded by mean ± standard error. Differences with $p<0.05$ were considered significant.

Results

Bath Immersion Challenge

Trial 1: The exposure concentrations per treatment were determined by plate count method. The C-Ah treatment was exposed to $1.71x10^6 \pm 7.00x10^4$ CFU/ml (mean ± standard error) and the IL-10-Ah treatment was exposed to $1.25 \times 10^6 \pm 2.00x10^5$ CFU/ml. These values are average values of n=2 replicate plate counts. There were no significant differences of CFU/ml concentrations between treatments ($p>0.05$). Fish were monitored hourly for the first 6h post-infection and then daily for 13 days. There was 100% survival across all treatments. The first 9 days following the challenge, fish in all treatments experienced a mild decrease of appetite. By the tenth day post-challenge there were no observed clinical signs of disease from any treatments; all fish were responding to feed. At the conclusion of the study, one fish from each of the *A. hydrophila* tanks was humanely euthanized and an aseptic swab was taken of the posterior kidney (n=4). No growth of bacteria was detected in any of the samples.

Water quality data was compiled and is outlined in Table A.1. Overall, there were significant differences ($p<0.05$) in temperature and DO between treatments. The IL-10-Ah on average had significantly lower temperature.

Trial 2: A more concentrated sample of *A. hydrophila* was used for Trial 2 bath immersion. By plate count method, it was determined that the C-Ah treatment was exposed to $7.88x10^7 \pm 4.00x10^6$ CFU/ml and the IL-10-Ah treatment was exposed to $8.47x10^7 \pm 3.60x10^6$ CFU/ml. These exposure concentrations were not significantly different ($p>0.05$). Mortalities and moribund fish were observed within the first 50h post-challenge. The survival results reported include mortalities as well as moribund fish that were euthanized. The controls challenged with sterile TSB had 100% survival on both diets. The C-Ah treatment had 66.7 ±

6.67% (mean ± standard error) survival 24h post-infection, while the IL-10-Ah had 40.0 ± 6.67% survival, as shown in Figure 3.1. At 24h post-infection, IL-10-C and C-C had significantly higher survival than the IL-10-Ah (p=0.0029) and C-Ah (p=0.025). However, there were no significant differences between diets of *A. hydrophila* challenged groups (p=0.053) at 24h. This trial displayed clinical signs of disease and mortality in fish past the 24h post-infection mark. At 50h post-infection, the C-Ah showed 63.3 ± 10.0% while the IL-10-Ah showed 33.3 ± 0% survival. The IL-10-C and C-C groups' survival was significantly higher than the IL-10-Ah (p=0.0025) and C-Ah (p=0.022). The IL-10-Ah group had significantly less survival than the C-Ah group (p=0.044). Moribund tilapia and all mortalities had swabs taken of the posterior kidney for isolation of *A. hydrophila*. All swabs were positive for the bacterium.

Clinical signs of disease in "moribund" fish included white and red external skin lesions, decreased appetite, and loss of equilibrium; fish swimming in corkscrew pattern or slowly tilting back and forth from side to side. Fish presenting these symptoms were immediately and humanely euthanized. Fifty hours post-challenge, after the mortalities had occurred, the surviving fish, despite lethargy, appeared healthy externally. Average water quality for this trial is outlined in Table A.2 by treatment. There were significant differences ($p<0.05$) in temperature and DO.

Oral Gavage Challenge

Trial 1: The average exposure concentrations for *A. hydrophila* challenged treatments were $1.29x10^6 \pm 7.00x10^4$ CFU/g fish and $2.25x10^6 \pm 3.50x10^5$ CFU/g fish for C-Ah and IL-10-Ah, respectively. A Student's t-test between *A. hydrophila* exposed treatments, showed the IL-10-Ah group was exposed to a significantly higher concentration of bacteria (p=0.047) than C-Ah which led to lower survival, with a pooled standard error of $2.02x10^5$. Despite the difference

in *A. hydrophila* concentration, there were no significant differences in survival percentage 24h post-infection between any of the treatment groups ($p=0.54$). There was 100% survival in fish gavaged with sterile TSB for both diets. The survival for C-Ah was 96.7 ± 3.34% and 86.7 ± 13.3% for IL-10-Ah. Results are shown in Figure 3.2. Twenty-four hours post-infection, despite the mortalities recorded in *A. hydrophila* challenged treatments, the remaining fish appeared healthy and fed normally. Swabs of the posterior kidney from euthanized fish appearing healthy from each treatment group were confirmed to have no growth of bacteria. Water quality by treatment for this trial is outlined in Table A.3. Significant differences were seen in temperature, ammonia, and DO ($p<0.05$).

Trial 2: The concentration of *A. hydrophila* exposed to fish in Trial 2 is outlined in Table 3.1. Fish were monitored hourly for the first 6h post-challenge. As seen with timing of mortalities in the previous challenge trials, all mortalities occurred within 24h of challenge. The survival of fish in C-2hAh was 87.5% and 75.0% for IL-10-2hAh, 87.5% from C-4hAh and 75.0% for IL-10-4hAh, 75.0% from C-5.5hAh and 100% from IL-10-5.5hAh, as shown in Figure 3.3. From the survival percentages recorded, there were no trends in survival by treatment group. As this was an exploratory challenge trial, there were no replicates of treatment groups, thus significant difference analysis could not be run.

Swabs taken from the posterior kidney in Trial 2 were from both moribund fish or mortalities. Swabs were all confirmed with *A. hydrophila* growth. The fish remaining post-challenge appeared healthy. Water quality measurements are outlined in Table A.4. The only significant differences between treatments were seen in alkalinity.

Discussion

The purpose of this study was to test the effect of an anti-IL-10 diet on survival of tilapia fingerlings after challenged with *A. hydrophila* via bath immersion and oral gavage. Implementing acute hypoxia in the bath immersion challenge introduced a stress component with the bacterial infection. *Aeromonas hydrophila* is said to be an opportunistic pathogen, and when coupled with stress in tilapia, it causes disease (AlYahya, et al., 2018; Cipriano, 2001; Schroers, et al., 2009; Swann, White, 1991). The introduction of stress is one of the three factors needed to elicit disease; which also include a susceptible host and a disease causing agent according to Austin, Austin (1999).

Challenge studies with *A. hydrophila* in tilapia are often via IP injection (AlYahya, et al., 2018; Ardó, et al., 2008; Rey, et al., 2009). However, the utilization of bath immersion and oral gavage in the present study was to mimic a more natural route of infection of the pathogen contaminating tank water versus IP injection. There have been several bath immersion or oral gavage *A. hydrophila* challenges in catfish and common carp (Schroers, et al., 2009; Zhang, et al., 2016). In the catfish study, healthy fish challenged via bath immersion at $2.0x10^7$ CFU/ml had 100% survival after 48h and mortality was only demonstrated in fish with skin scrapes or fin clips prior to challenge (Zhang, et al., 2016). Similar findings were seen in this study in bath immersion Trial 1, with 100% survival in healthy fish challenged with $1.48x10^6$ CFU/ml ± $1.58x10^5$ and pathogen clearance from the kidney. This was credited to minimal concentration of exposure despite the added hypoxic stress. The occurrence of decreased appetite in all treatment groups is due to well-known appetite suppression from stress (Bernier, 2006).

The second bath immersion trial exposure concentration was $8.17x10^7 \pm 3.21x10^6$ CFU/ml and caused mortalities in both diets of the *A. hydrophila* challenged treatments. After

50h post-infection, the C-Ah showed 63.3 ± 10.0% while the IL-10-Ah showed 33.3 ± 0% survival. With significantly lower survival shown in the IL-10-Ah treatment, further testing is needed to ensure the cause of mortalities was due to the presence of anti-IL-10. Positive swabs for the pathogen from mortalities indicate this concentration of bacteria was enough to infect the tilapia via bath immersion. Thus, it cannot be determined based off of these survival results how the anti-IL-10 is functioning internally.

The oral gavage route of infection in Trial 1 did not show any significant differences in survival percentage between diets and Trial 2 did not show trends of survival. However, these trials demonstrated an effective route of infection for experimentally inducing *A. hydrophila* in tilapia. Oral gavage can be used as an experimental infection model for this enteric pathogen in tilapia as it has been in common carp (Schroers, et al., 2009).

Water quality plays a substantial contribution to the health of fish. In the experimental trials in this study, there were significant differences ($p<0.05$) for some water quality parameters between treatments. A few trials had significant differences in temperature, DO, as well as ammonia and alkalinity. In regards to temperature, from the trials that showed differences, the temperature range was 26.2 ± 0.13 ºC to 28.2 ± 0.10 ºC. These temperatures are still within the optimal range of 25.0ºC-30.0ºC for tilapia growth (El-Sayed, 2006). Therefore, it is likely that these differences in temperature played a minimal role on the survival rate, if at all.

Dissolved oxygen preferred for tilapia health is above 5.0mg/L (Buttner, et al., 1993; Riche, Garling, 2003). Despite significant differences between treatments, the lowest recorded mean for DO was 6.88mg/L (bath immersion Trial 1), this is above the optimal range. According to Ross (2000), tilapia have efficient respiratory action at dissolved oxygen above 3.0mg/L.

Again, despite significant differences between treatments, the DO data that was collected in these studies on a daily basis remained above the optimal range for tilapia.

When considering differences in ammonia levels, there is unionized ammonia (NH_3) and ionized ammonia (NH_4). The forms of ammonia are dependent on pH and temperature of the water. Smaller fish are more susceptible to the negative effects of unionized ammonia and tilapia experience decreased growth and performance at ammonia 1.0 mg/L (Riche, Garling, 2003). With that being said, the ammonia levels were significantly higher in the C-Ah and IL-10-Ah treatments of oral gavage Trial 1, reaching as high as 0.578 mg/L. This potentially could have acted as an unintentional added stress on the fish exposed to the pathogen, affecting the survival rates of these treatments.

Overall, further research must be conducted to confirm the functionality of the anti-IL-10 diet *in vivo*. These experimental trials did not provide sufficient evidence to conclude if an anti-IL-10 diet would benefit the survival of tilapia subjected to bacterial pathogen, *A. hydrophila*. Therefore, future research can be conducted *in vitro* to understand the functionality of anti-IL-10 and its capability of neutralizing IL-10. By isolating tilapia cells that produce cytokines of interest, IL-10 and interferon-γ, cells can be treated with the novel antibody *in vitro* and an enzyme-linked immunosorbent assay (ELISA) can be used to quantify the cytokine of interest.

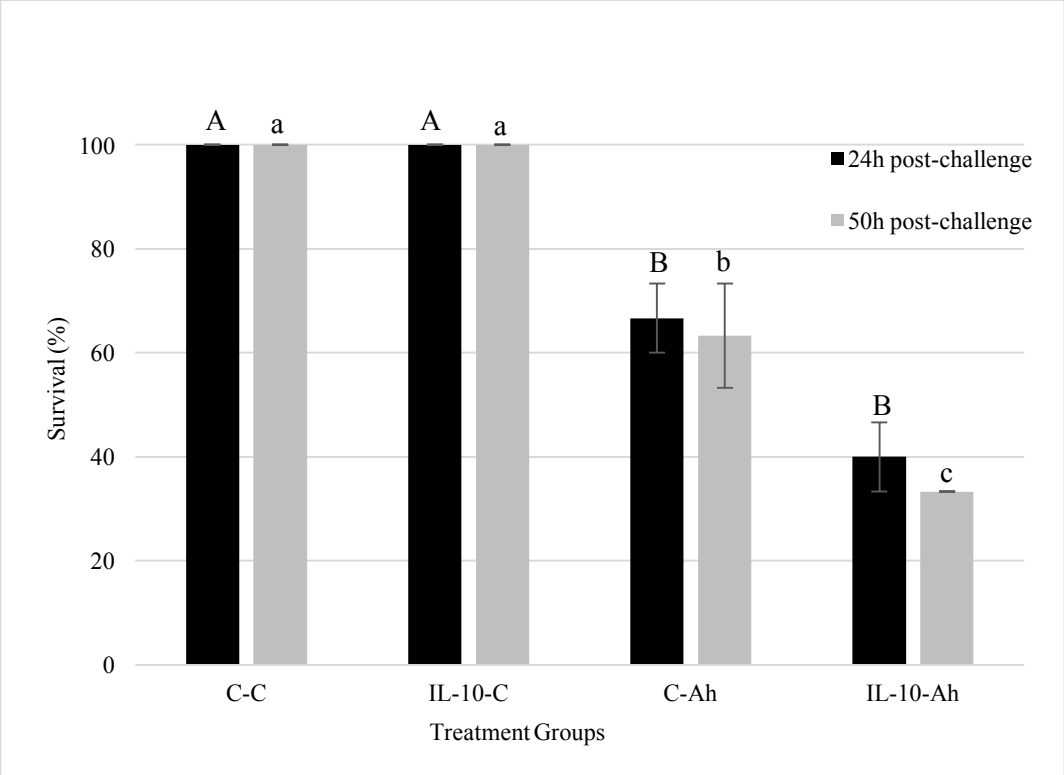

Figure 3.1. Mean percent survival 24h and 50h post-challenge with *A. hydrophila* via bath immersion with standard error bars. C-Ah was challenged with $7.88x10^7$ CFU/ml and IL-10-Ah, $8.47x10^7$ CFU/ml. Significant differences are indicated by different letters. There were significant differences at 24h in control challenged groups vs. *Aeromonas hydrophila* groups ($p<0.05$). The IL-10-Ah group at 50h showed significantly less survival then the C-Ah group ($p=0.044$).

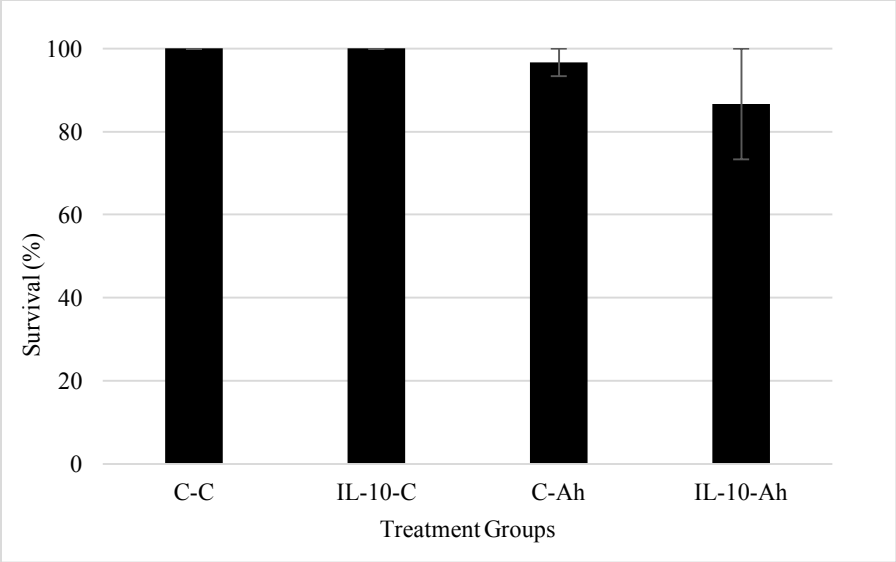

Figure 3.2. Mean percent survival 24h post-challenge with *Aeromonas hydrophila* via oral gavage Trial 1 with standard error bars. C-Ah was challenged with $1.29x10^6$ CFU/g fish and IL-10-Ah, $2.25x10^6$ CFU/g fish. A significantly higher concentration of bacteria led to a lower survival percentage in IL-10-Ah group.

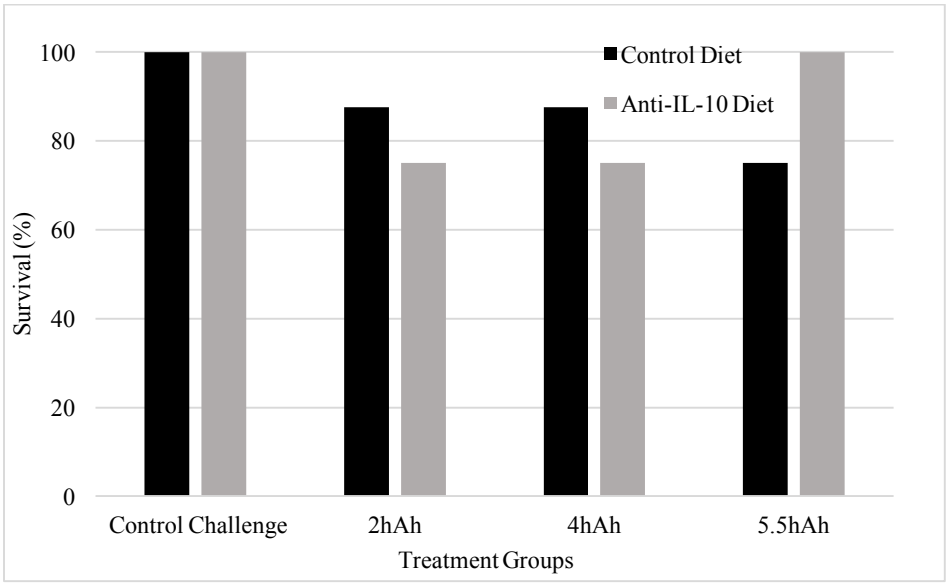

Figure 3.3. Percent survival of fish orally gavaged *Aeromonas hydrophila* 24h post-challenge in Trial 2. The control challenge was sterile TSB, 2hAh was $2.78x10^6$ CFU/g, 4hAh was $1.68x10^7$ CFU/g and the 5.5hAh was $3.72x10^7$ CFU/g.

Treatment	CFU/g fish
C-C & IL-10-C	N/A: sterile TSB
C-2hAh & IL-10-2hAh	$2.78x10^6$
C-4hAh & IL-10-4hAh	$1.68x10^7$
C-5.5hAh & IL-10-5.5hAh	$3.72x10^7$

Table 3.1. Treatment vs. the CFU/g that was administered into fish for oral gavage Trial 2.

Chapter 4: Assessment of the Bioactivity of Interleukin-10 Neutralizing Antibody *in vitro* in Tilapia Splenocytes

Introduction

Interleukin-10 (IL-10) is an anti-inflammatory cytokine; thus, the function of this protein is to downregulate an inflammatory immune response (Commins, et al., 2008). On the contrary, an inflammatory immune response elicits pro-inflammatory cytokine responses. One of the pro-inflammatory cytokines of interest to this study is interferon-γ (IFN-γ). The activity of IFN-γ is inhibited in the presence of IL-10 (Commins, et al., 2008; Hillyer, Woodward, 2003; Pestka, et al., 2004). The counteractivity of these pro- and anti-inflammatory cytokines was used as the basis to quantify the IFN-γ in an enzyme-linked immunosorbent assay (ELISA) while hypothesizing a specific trend of IL-10 activity within various tilapia cell treatments *in vitro*. The objective of this study was to develop a cell culturing and ELISA method based off of *in vitro* chicken research (Arendt, et al., 2016) to explore the bioactivity of avian anti-IL-10 antibody *in vitro* in cultured tilapia splenocytes by quantifying IFN-γ.

Being that this novel avian antibody has never been used before in any species of fish, method development was crucial for the progress of this study. Four points of interest were investigated in the progression of the ELISA method development. These four categories included: (i) the initial cell concentration, (ii) use of a standard that was reconstituted minutes before use versus frozen aliquots, (iii) the use of a phosphate coating buffer versus a carbonate coating buffer, and (iv) utilizing different volumes of samples or standard to investigate the volume's effect on the absorbance intensity.

In order to compare the IFN-γ concentrations, tilapia spleen cells were treated with and without the anti-IL-10, the treatments used were mimicked from the study done by Arendt, et al.

(2016) with modifications. Tilapia spleen cells were targeted for use in this study due to the spleen's known expression of IL-10 (Grayfer, et al., 2011). Interleukin-10 has also been shown to increase in spleen tissue after the immune system of its host was stimulated (Seppola, et al., 2008). Thus, creating a spleen cell suspension, and extracting the buffy coat containing white blood cells that produce IL-10, was the mechanism for extracting the cell cytokines for use in the ELISA.

The four main treatments of this *in vitro* study included (1) tilapia spleen cell supernatants (containing cytokine material), (2) cell supernatants with added mitogen, Concanavalin A (Con A), (3) cell supernatants with Con A and chicken IL-10, and (4) cell supernatants with Con A, IL-10, and anti-IL-10. It was expected that the treatment including the anti-IL-10 would yield the highest concentration of IFNγ, due to the neutralization of IL-10 and presence of IFN-γ in the absence of IL-10. It was also expected that the concentration of IFN-γ would be significantly lower in the treatment containing cell supernatants, Con A, and IL-10 due to the presence of anti-inflammatory IL-10 as well as the treatment containing only cell supernatants due to the lack of the mitogen, Con A. In the treatment with cell supernatants containing Con A, there should be some amount of IFN-γ detected due to the presence of Con A simulating cell mitosis and therefore increasing the cytokine concentration coming from the cells (Yin, et al., 1999). These expectations are based off of the results found by Arendt, et al. (2016).

Materials and Methods

Multiple variables for change were considered in the method development of this ELISA. One variable taken into consideration in the development of this assay was (i) the initial cell concentration which was expected to make a difference in the ultimate concentration of IFN-γ in the different treatment groups. Concentrations $4x10^6$ cells/ml and $1x10^7$cells/ml were tested. In

developing the standard curve for the commercial IFN-γ chicken matched antibody pair kit (Invitrogen, Frederick, MD), (ii) using a standard chicken IFN-γ that was reconstituted minutes before the assay was run or the use of frozen stock stored at -20ºC was another variable considered. The use of a (iii) phosphate coating buffer (pH=7.4) versus a carbonate coating buffer (pH=9.4) for coating the ELISA plate was explored, as well as (iv) the volume of the samples and standard used in the ELISA; 100µl versus 150µl to compare the intensity of absorbance readings of the ELISA were also considered as variables in protocol development.

Based on the preliminary results of the variables tested above, the best combination of them can be used to conduct the ELISA for determining the bioactivity of anti-IL-10 and its capability to neutralize IL-10 *in vitro* using tilapia splenocytes.

Cell Culturing: Splenocytes

Tilapia weighing in range from 40-80g were used for this study. The fish were hand fed a 3mm commercial feed once daily until apparent satiation. Fish were humanely euthanized using buffered Tricaine-S (Western Chemical Inc., Ferndale, WA) and aseptically dissected to remove the spleen. Three spleens were collected and pooled together for cell culturing to assure enough viable cells per sample would be present for the assay. Spleens were collected in sterile petri dishes containing 4ml of 'complete media' which contained RPMI-1640 media (Sigma-Aldrich, R7509), 10% Fetal Bovine Serum (Sigma-Aldrich, F4135), and 100U penicillin – 100ug/ml streptomycin (Sigma-Aldrich, P4333). Spleen samples were maintained on ice.

In a biosafety cabinet, spleens were macerated through a sterile mesh cell strainer using the rubber portion of a sterile syringe. The complete media from the dish was used to rinse the spleen tissues through the strainer. This was done until the tissues were completely macerated. With the cell suspension in the petri dish, using a pipette, the cell suspension was mixed by

pipetting up and down about 10 times while rinsing the plate to collect residual cells. The cell suspension was gently overlaid on 30/70% room temperature Histopaque-1077 (Sigma-Aldrich 10771). It was then centrifuged at 600xg for 20min at room temperature. After centrifugation, about 2ml of the buffy coat layer containing lymphocytes, were removed and transferred to a new tube. To this tube, the suspension of lymphocytes was diluted with complete media until the mixture was 5ml, and was vortexed for 10s. This mixture was then centrifuged at 300xg for 4mins at room temperature. The supernatant was collected and discarded, 5ml of complete media was added back, vortexed, and centrifuged again. Two ml of complete media were added back to the pellet, vortexed, and the viable cell count was done using a hemocytometer and trypan blue. Spleen cell extraction procedure was a modification of chicken spleen cell extraction outlined by Ren, et al. (2015).

(i) Point of Interest: Initial Cell Concentration

The cells were diluted with complete media to a cell concentration of either $4x10^6$ or $1x10^7$ cells/ml and were added to a 96-well tissue culture plate (Fisher Scientific: Costar, Pittsburgh, PA) in triplicate for each of the 4 treatments. Treatment wells included (1) cells only: 100µl cell suspension and 120µl complete media, (2) cells and Con A (Type IV, Sigma-Aldrich C2010): 100µl cell suspension, 40µl of 20µg/ml ConA, and 80µl of complete media, (3) cells, ConA, and recombinant chicken IL-10: 100µl cell suspension, 40µl of 20µg/ml ConA, 40µl of 0.2ng/ml IL-10, and 40µl of complete media, (4) cells, ConA, chicken IL-10, and anti-IL-10: 100µl cell suspension, 40µl of 20µg/ml ConA, 40µl of 0.2ng/ml IL-10, and 40µl of 0.85µg/ml of anti-IL-10. Treatments incubated for 24h in a 5% CO_2 chamber at 30ºC. These procedures were modified from Arendt, et al. (2016) and Hillyer, Woodward (2003). The initial cell concentration

of splenocytes was expected to have an impact on the concentration of IFN-γ; the higher the cell concentration the higher the cytokine production from those cells.

Post-incubation, cultures were transferred to microcentrifuge tubes for centrifugation at 4,000 rpm, 28ºC for 20 min. Cell supernatants were removed and used for application in sandwich ELISA.

ELISA

A commercial IFN-γ chicken matched antibody pair kit (Invitrogen, Frederick, MD) was used for the ELISA to quantify IFN-γ to express neutralization capabilities of anti-IL-10 on chicken IL-10 in tilapia cell supernatants. A Buffer Kit for Antibody Pairs (Thermo Fisher CNB0011) was used per manufacturer's instructions in this assay. Ninety-six well flat bottom ELISA plates (EIA/RIA Costar, Pittsburgh, PA) were coated with capture, anti-chicken IFN-γ, and incubated overnight at 4ºC. The modifications of (iii) using a phosphate coating buffer (pH=7.4) and carbonate coating buffer (pH=9.4) were compared as a point of interest (BioSource Cytoset Buffer Set, CNB0011). The plate was washed once with washing buffer, blocked with 300µl assay buffer for 1h at room temperature. After blocking, cell supernatants post-centrifugation were transferred from the tissue culture plate to the ELISA plate (either 100µl or 150µl), and chicken IFN-γ diluted in assay buffer was used for development of the standard curve and incubated at room temperature for 1h. The use of (iv) 100µl or 150µl of sample and standard was also explored as a point of interest in this step to measure intensity differences in absorbance readings at different well depths. After one washing, 50µl of detection antibody, anti-chicken-IFN-γ-biotin, was added to each well and incubated for 1h at room temperature with shaking. The plate was washed 3 times, and 100µl of streptavidin-HRP solution was added for 45min at room temperature with shaking. The plate was washed 3 more times, and 100µl of

TMB stabilized chromagen was added to each well, and incubated with shaking for 30min. One-hundred µl of stop solution was added to each well and the plate absorbance was read using a SpectraMax 340PC Microplate Reader at wavelength 450 nm.

Statistical Analysis

Absorbance data readings were analyzed using one-way ANOVA in jmp statistical software (Version jmp Pro 13) followed by Tukey's post-hoc test with a defined p-value < 0.05 as significant. Interferon-γ concentrations were calculated based off of the equations output from the standard curves.

Results and Discussion

The methods for quantifying IFN-γ in the presence of anti-IL-10 in tilapia splenocytes *in vitro* are currently under development. However, preliminary testing of multiple variables has shed light on the appropriate protocol to be used for this assay.

(i) Point of Interest: Initial Cell Concentration

The effect of different starting cell concentrations, $4x10^6$ versus $1x10^7$ cells/ml was not concluded from this research. It would be expected that the higher the cell concentration, the higher the cytokine expression, however further testing is necessary to draw this conclusion.

(ii) Point of Interest: Standard Reconstitution

In preparation of the chicken IFN-γ standard curve, it was determined that the storage temperature of reconstituted standard in assay buffer was critical to the stability of the cytokine. In instances when the standard was reconstituted and used immediately, a standard curve with a mean (n=5) $R^2=0.99$ was developed. In ELISA experiments where a -20ºC stored aliquot of standard was used (stored less than 7 days before use), there was no detection of a standard curve, indicating the degradation of IFN-γ at -20ºC. Studies have shown differences in necessary

storage conditions across various cytokines to prevent degradation over storage (Lipiäinen, et al., 2015).

(iii) Point of Interest: Coating ELISA with Phosphate Buffer or Carbonate Buffer

The difference in pH of coating buffer may have an impact on the overall intensity of the absorbance reading according to a comparison of standard curve using a phosphate buffer and a carbonate buffer shown in Figure 4.1. The lower pH (phosphate buffer) was shown to express a higher intensity absorbance reading.

(iv) Point of Interest: Differences in Well Depth and Initial Cell Concentration

The use of 100µl versus 150µl of sample or standard in the well would be expected to make an impact on the ultimate intensity of the absorbance reading of IFN-γ. The results to this variable are still being tested.

Preliminary Results: IFN- γ Quantification

Trial 1: Although method development is currently underway, absorbance results from completed ELISAs can be analyzed as a preliminary data set of IFN-γ concentrations in each of the treatment groups. For example, a pooled sample of 3 spleens was used for the assay which contained $1x10^7$cells/ml for use in the ELISA. Compared to a standard curve made from newly reconstituted standard, and using a well depth volume of 100µl, and coated with a phosphate coating buffer, there were no significant differences in calculated IFN-γ. The concentrations of IFN-γ were calculated using the following equation developed from the standard curve, y=1261.5x-113.72 and are outlined in Figure 4.2. There were no statistical differences between treatment groups (p=0.6088).

It is possible that the particularly low concentrations of IFN-γ could be reflective of the lower sample volume used. Being that the absorbance levels were so low in comparison to the standard curve, it is difficult to refer to this as reliable data set.

Trial 2: Another data set collected using the following parameters was developed: 2 separate pooled spleen samples were used, the ELISA plate was coated with carbonate coating buffer, 150µl was used for well depth, and cell concentration was $1x10^7$ cells/ml. The IFN-γ concentrations were calculated based on the equation, y=2528.1x-209.2 developed from the standard curve. Again, it was seen that there were no significant differences between treatment groups based on these method parameters (p=0.6308). The results are shown in Figure 4.3.

The higher overall absorbance values and thus calculated IFN-γ could be credited to the higher volume, or well depth, used in this trial. With methods for this assay not yet defined, it is difficult to conclude whether or not anti-IL-10 has neutralization capabilities of IL-10 *in vitro* using tilapia splenocytes. There must be a defined protocol for completing this assay before concluding whether or not the IFN-γ concentrations within these treatment groups are significantly different.

Conclusions

Preliminary data collected that was outlined in this chapter, showed that storage of the standard chicken IFN-γ was imperative to its effectiveness. This particular standard degraded in less than one week at -20ºC. Coating the ELISA plate with a phosphate buffer at a closer to neutral pH than the carbonate buffer appears to elicit a higher intensity absorbance value. Method development of initial cell concentration will elicit further research. The expectation of IFN-γ concentrations from tilapia splenocyte treatments in this work is outlined in the results of chicken splenocyte IFN-γ in Figure A.6.

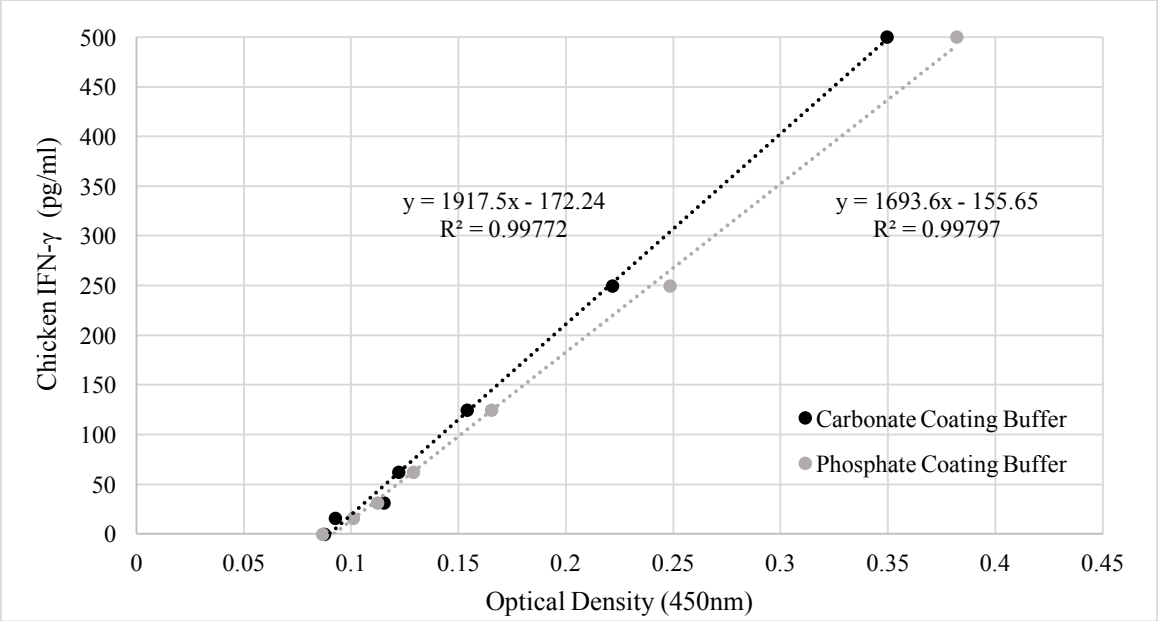

Figure 4.1. Comparison of standard curve development between the use of different coating buffers with the phosphate buffer at pH=7.4 and the carbonate buffer at pH=9.4.

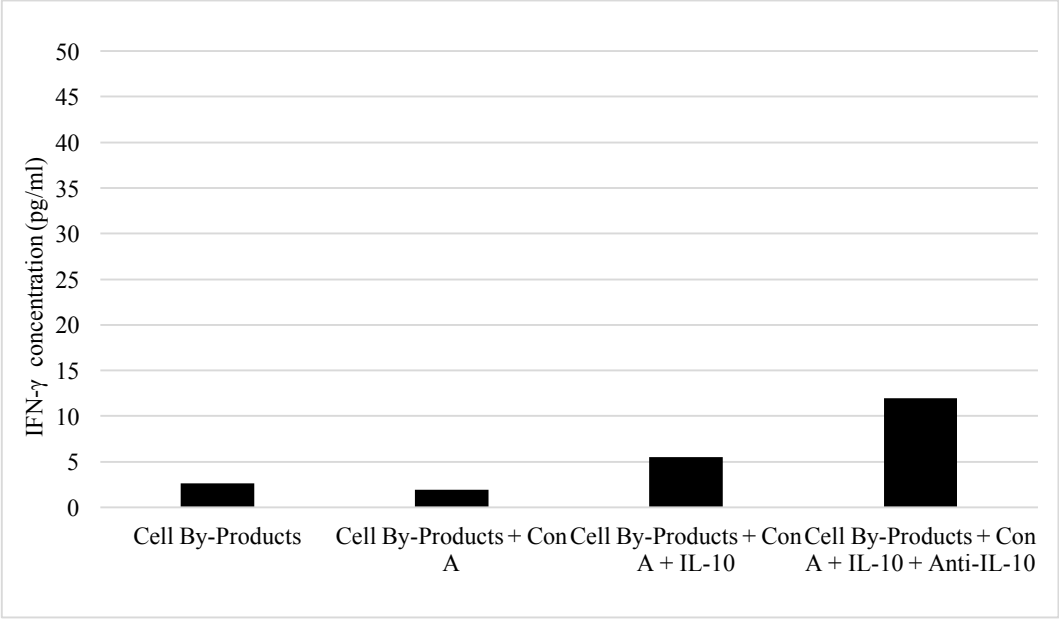

Figure 4.2. Preliminary data set comparing calculated IFN-γ between treatments. This experiment was done using a 100µl well depth, coated with phosphate coating buffer, and cell concentration of $1x10^7$cells/ml. There were no significant differences between groups (p=0.6088).

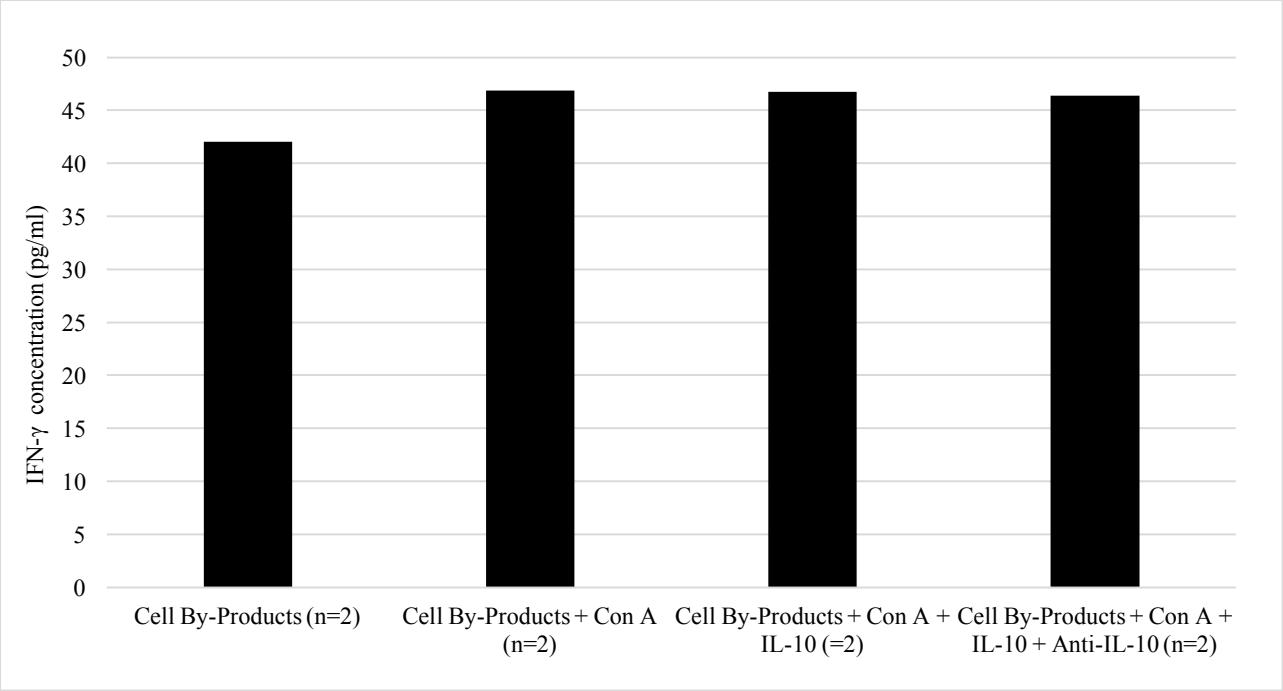

Figure 4.3. Comparison of preliminary results of treatment groups with the following ELISA method variables: the ELISA plate was coated with carbonate coating buffer, 150µl was used for well depth, and initial cell concentration was $1x10^7$ cells/ml. There were no significant differences between treatment groups (p=0.6308).

Chapter 5: Conclusions and Future Research

The long term goal of this study was to develop a prophylactic for *A. hydrophila* in tilapia using an antibody to IL-10. By testing different routes of entry, bath immersion and oral gavage, clinical signs of disease and mortalities were observed in both challenge routes of the pathogen in all treatments challenged with *A. hydrophila*. Within this research, a challenge model for oral gavage using *A. hydrophila* was developed, which had never before been done in tilapia. Based on the mortalities observed throughout the bacterial challenge trials between control diet and anti-IL-10 diet, further research on the functionality of the anti-IL-10 *in vivo* and *in vitro* is necessary to determine anti-IL-10 as effective for prevention of disease in tilapia.

Another objective of this study was to quantify IFN-γ as a mechanism to investigate IL-10 expression when using the anti-IL-10 *in vitro*. Since this anti-IL-10 is novel and has never been utilized in tilapia, or any fish species, protocol development was crucial in finding the most reliable results. Once this ELISA protocol is fully developed, the IFN-γ can be quantified in the tilapia splenocyte treatments, indicating the effectiveness of anti-IL-10 in neutralizing IL-10.

The expression of IL-10 can be quite different among fish species. As of 2013, "IL-10 genes were discovered in common carp, rainbow trout, silver carp, zebrafish, sea bass, cod, goldfish, and grass carp" (Wang, Secombes, 2013). Thus, this research was based on the bioactivity of IL-10 in fish species other than tilapia, with the assumption that the expression is similar in tilapia. The expression patterns of IL-10 have been shown to be different among species of fish (Inoue, 2005). With more extensive research done on the bioactivity and expression of IL-10 in tilapia, a more appropriate method to target the actions of the cytokine can be used.

Further research should also be done repeating the challenge studies that were done in this research with different pathogens. *Aeromonas hydrophila* was used due to its prevalence in the industry and relative safety for human handlers, but a pathogen that invades the gastrointestinal tract of the fish specifically, would be interesting in the use of oral gavage challenge. *Streptococcus iniae,* for example is a more invasive bacterial pathogen and can colonize the gastrointestinal tract (Agnew, Barnes, 2007). Thus, the pathogenesis of the pathogen may play a role in the effectiveness of anti-IL-10.

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Appendix

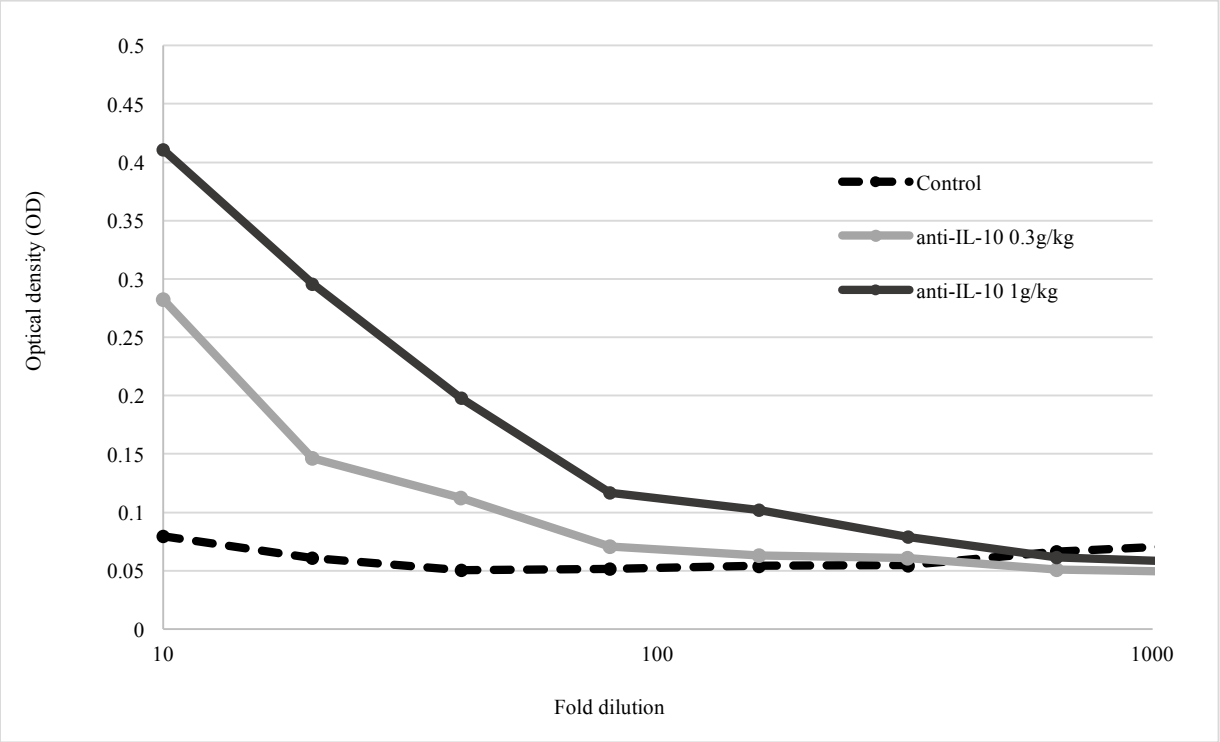


Figure A.1. Confirmation of antibody adherence to the feed post-spray using two concentrations of coating techniques.

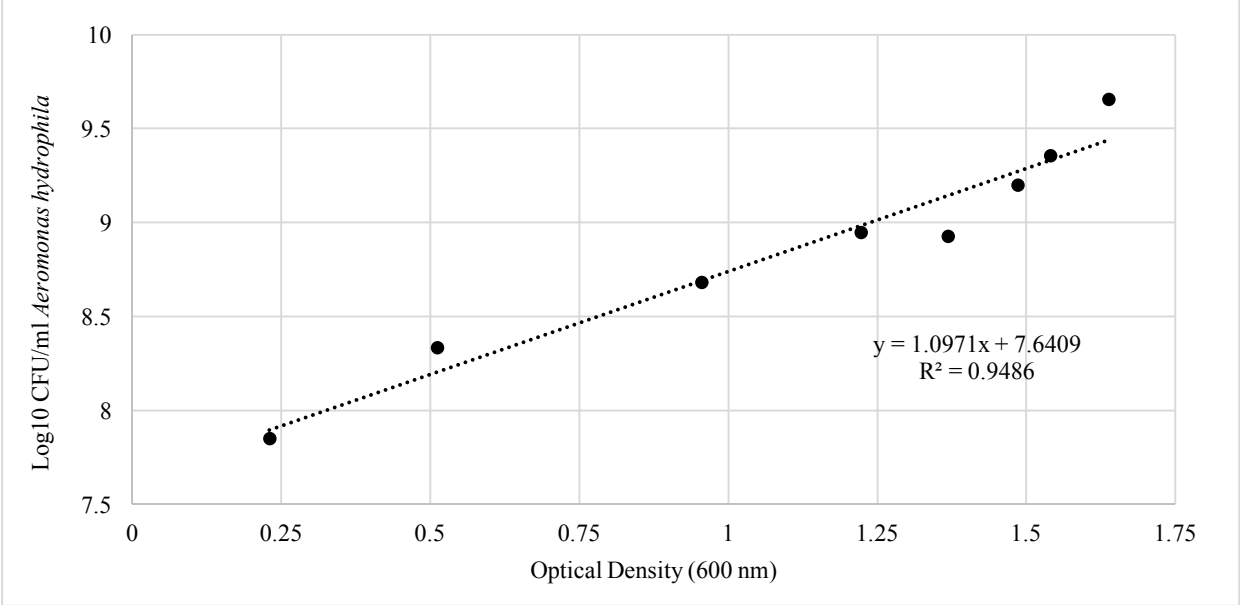


Figure A.2. Prediction model for concentration of *Aeromonas hydrophila* (log_{10}CFU/ml) as a function of optical density (600 nm) using the equation y = 1.0971x + 7.6409.

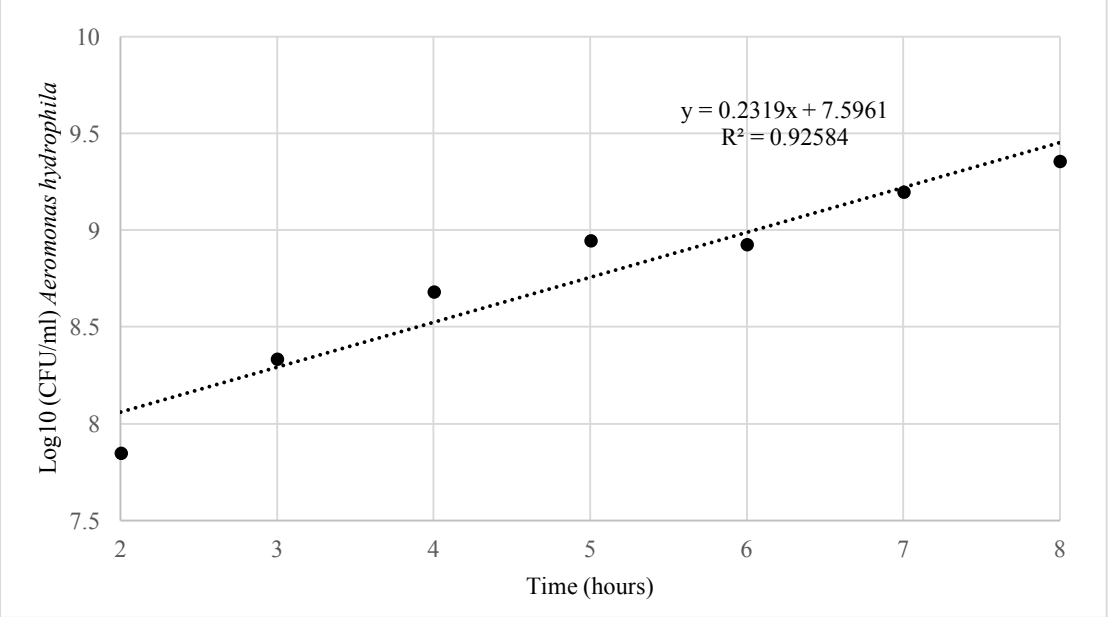
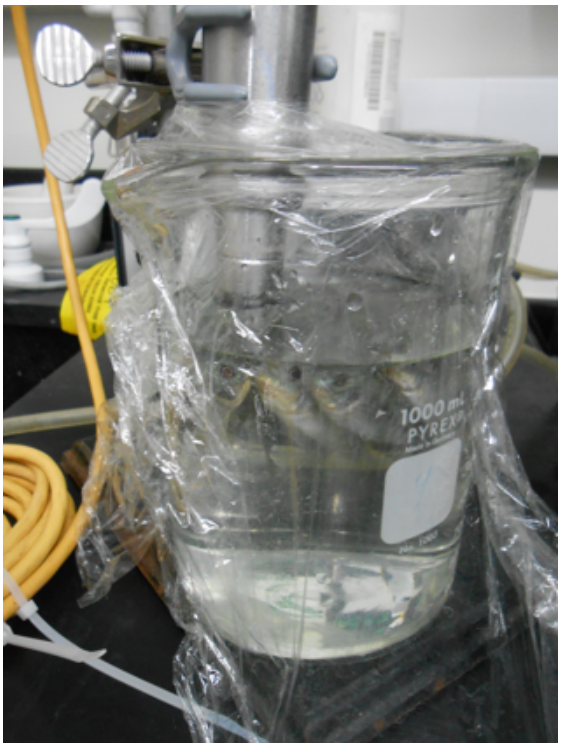


Figure A.3. Concentration of *Aeromonas hydrophila* as a function of time $y = -8E+06x^2 + 4E+08x - 9E+08$.

a)



b)

Figure A.4. (a) Tilapia fingerlings exposed to hypoxic stress for bath immersion trials. Probe of dissolved oxygen meter is submerged in water with saran wrap covering the beaker. (b) Exposure to pathogen in bucket with aeration for bath immersion trials.

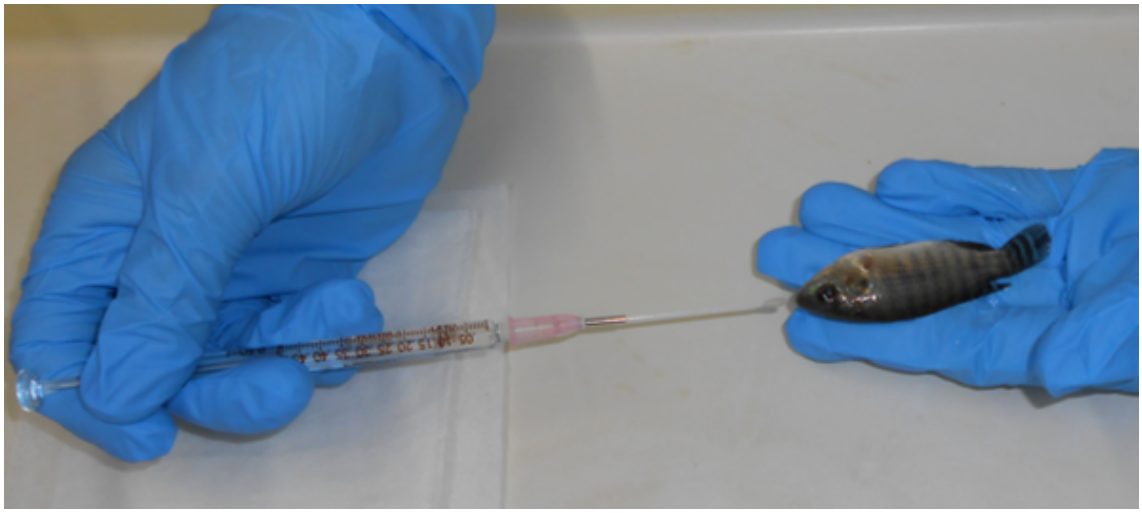


Figure A.5. Oral gavage technique for tilapia fingerlings using with 18Gx2" plastic animal feeding needle and 0.5ml glass syringe.

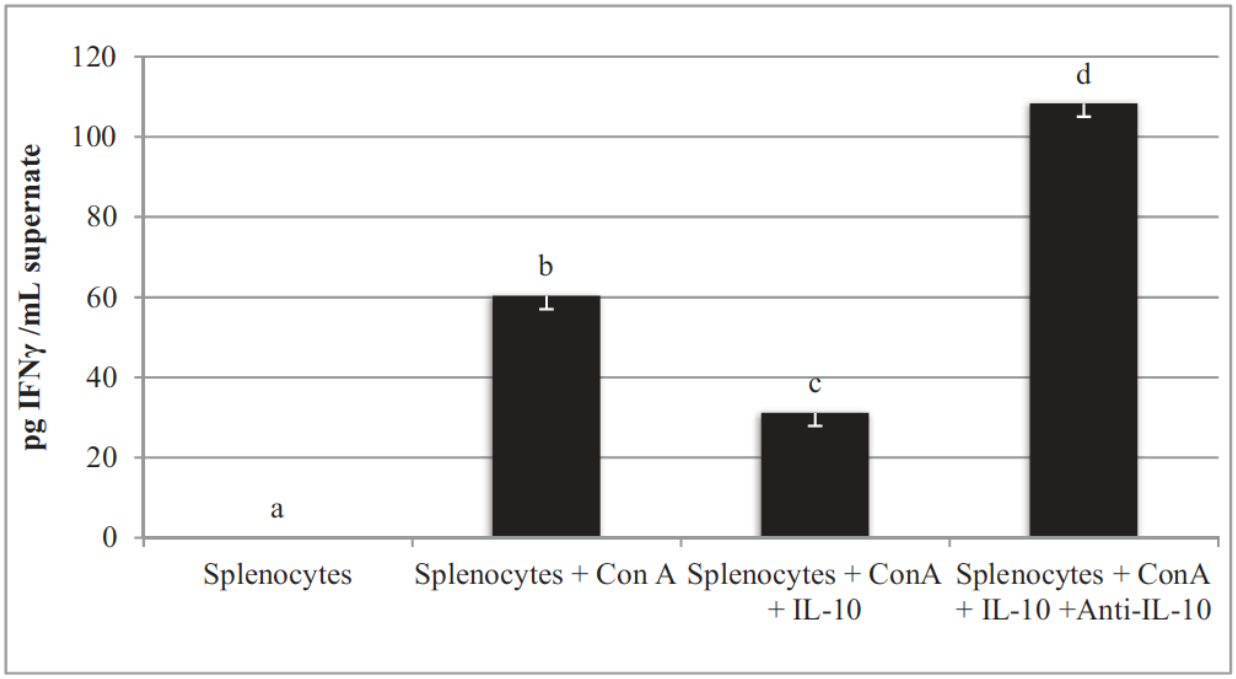


Figure A.6. The neutralizing effect of anti-IL-10 result done by Arendt, et al. (2016) from chicken splenocytes *in vitro*. The results of IFN-γ presence in these splenocyte treatments are the expectation of pending research with tilapia splenocytes.

Treatment (Diet x Challenge)	Temperature (ºC) (n=44)	pH (n=42)	Alkalinity (n=36)	Ammonia (mg/L) (n=40)	Nitrite (mg/L) (n=40)	Nitrate (mg/L) (n=8)	DO (mg/L) (n=42)
C-C	28.05±0.21 A	7.13±0.034	143.36±4.06	0.303±0.028	0.033±0.004	3.16±0.77	7.03±0.039 A
IL-10-C	27.92±0.21 A	7.15±0.034	146.31±4.06	0.280±0.028	0.035±0.004	3.00±0.77	7.05±0.039 A
C-Ah	28.01±0.21 A	7.06±0.034	135.89±4.05	0.305±0.028	0.034±0.004	5.05±0.77	6.93±0.039 AB
IL-10-Ah	27.01±0.21 B	7.07±0.034	138.47±4.05	0.345±0.028	0.035±0.004	3.90±0.77	6.88±0.039 B

Table A.1. Mean ± standard error of water quality taken during bath immersion Trial 1. Different letters indicate significant differences ($p<0.05$) base on Tukey test. N= data points taken over 22 day trial.

Treatment (Diet x Challenge)	Temperature (ºC) (n=18)	pH (n=16)	Alkalinity (n=16)	Ammonia (mg/L) (n=16)	Nitrite (mg/L) (n=16)	Nitrate (mg/L) (n=16)	DO (mg/L) (n=18)
C-C	27.59±0.13 A	7.36±0.04	155.38±4.55	0.42±0.23	0.034±0.016	3.5±1.0	7.11±0.047 AB
IL-10-C	26.76±0.13 BC	7.41±0.04	160.25±4.55	0.92±0.23	0.057±0.016	2.6±1.0	7.26±0.047 A
C-Ah	26.95±0.13 B	7.35±0.04	143,38±4.55	0.95±0.23	0.079±0.016	3.5±1.0	7.07±0.047 B
IL-10-Ah	26.2±0.13 C	7.39±0.04	147.13±4.55	1.04±0.23	0.076±0.016	4.6±1.0	7.19±0.047 AB

Table A.2. Mean ± standard error of water quality measurements by treatment in the bath immersion Trial 2 (9 days). Means with different letters are significantly different ($p<0.05$) based on the Tukey test. N= data points taken over the 9 day study.

Treatment (Diet x Challenge)	Temperature (ºC) (n=42)	pH (n=38)	Alkalinity (n=22)	Ammonia (mg/L) (n=44)	Nitrite (mg/L) (n=34)	Nitrate (mg/L) (n=6)	DO (mg/L) (n=42)
C-C	28.2±0.10 A	7.24±0.036	146.91±2.99	0.248±0.085 C	0.029±0.0078	2.93±0.69	6.98±0.027 B
IL-10-C	27.8±0.10 AB	7.22±0.036	151.36±2.99	0.264±0.085 BC	0.0339±0.0078	3.40±0.69	7.12±0.027 A
C-Ah	27.45±0.10 BC	7.28±0.036	140.46±2.99	0.576±0.085 AB	0.0322±0.0078	3.20±0.69	7.07±0.027 AB
IL-10-Ah	27.2±0.10 C	7.29±0.036	145.55±2.99	0.578±0.085 A	0.0444±0.0078	3.28±0.69	7.03±0.027 AB

Table A.3. Mean ± standard error of water quality measurements taken during oral gavage Trial 1 (30 days). Different letter indicate significant differences ($p<0.05$), based on the Tukey test. N= data points taken over the 30 day study.

Treatment (Diet x Challenge)	Temperature (ºC) (n=4)	pH (n=3)	Alkalinity (n=3)	Ammonia (mg/L) (n=4)	Nitrite (mg/L) (n=4)	DO (mg/L) (n=4)
C-C	26.85±0.47	7.65±0.05	124±3.0 BC	0.11±0.005	0.012±0.005	7.18±0.13
IL-10-C	25.98±0.32	7.57±0.01	159.67±2.4 AB	0.24±0.038	0.016±0.008	7.34±0.095
C-2hAh	27.10±0.15	7.77±0.15	102±13.0 C	0.16±0.064	0.006±0.002	7.25±0.043
IL-10-2hAh	26.80±0.07	7.84±0.14	105.33±11.41 C	0.19±0.063	0.014±0.005	7.26±0.047
C-4hAh	26.20±0.20	7.60±0.003	171.67±8.29 A	0.34±0.062	0.027±0.013	7.28±0.085
IL-10-4hAh	27.15±0.10	7.79±0.18	98±12.66 C	0.245±0.10	0.012±0.005	7.30±0.028
C-5.5hAh	26.53±0.11	7.44±0.07	95.67±5.61 C	0.99±0.73	0.107±0.074	7.18±0.091
IL-10-5.5hAh	25.63±0.82	7.54±0.01	158.67±1.45 AB	0.15±0.041	0.011±0.005	7.20±0.120

Table A.4. Mean ± standard error water quality measurements of oral gavage Trial 2 (4 days) by treatment. Nitrate measurements were not taken for this study due to the short time length. Different letters indicate significant differences, ($p<0.05$) based on Tukey test. N= data points taken over the 4 day study.

Date	Was there color development on the standard curve?	R^2 developed from standard curve	Well depth volume	Initial cell concentration (cells/ml)	Standard reconstitution; fresh or frozen (-20ºC)?	Incubated at 30ºC, 5%CO2?	Coating buffer used A- Phosphate buffer B- Carbonate buffer	Other Comments
3/9/18	no	N/A	100ul	N/A	fresh	yes	A	A tissue culture plate was used instead of ELISA plate.
3/14/18	no	N/A	100ul	N/A	frozen	N/A	A	A tissue culture plate was used instead of ELISA plate.
3/15/18	yes (same plate as below - 3/15/18)	0.99797	100ul	N/A	fresh	N/A	A	This plate only included standard curves (no cell samples).
3/15/18	yes (same plate as above - 3/15/18)	0.99772	100ul	N/A	fresh	N/A	B	This plate only included standard curves (no cell samples).
3/21/18	no	N/A	100ul	$4x10^6$	frozen	yes	A	
3/24/18	yes	0.99699	100ul	N/A	fresh	N/A	A	This plate only included standard curve (no cell samples).
3/28/18	Used same plate as 3/24/18 to run samples	N/A	100ul	$4x10^6$	N/A	No; it was incubated at 34C w/out CO_2	A	The 3/28/18 samples that were run were done on an ELISA plate that was reused from a prior standard curve run done on 3/24/18.
3/28/18	no	N/A	100ul	$4x10^6$	frozen	yes	A	
3/31/18	no	N/A	100ul	N/A	frozen	N/A	A	
4/1/18	yes	0.99967	100ul	$1x10^7$	fresh	yes	A	Tissue culture wells were pooled and then split into (3), 100ul samples for ELISA replicates.
		sample	100ul	$1x10^7$	fresh	No; it was incubated at 34C w/out CO_2	A	
		sample	100ul	$1x10^7$	fresh	Both incubation conditions diluted in Assay Buffer	A	
4/1/18	yes	0.99789	100ul	N/A	fresh	N/A	A	This plate only included standard curve (no cell samples).
4/6/18	Color was very faint to the eye	0.94877	200ul	$1x10^7$	frozen	yes	B	
4/8/18	no	N/A	200ul	$1x10^7$	frozen	yes	A	Plate reading not taken - no absorbance data was collected.
4/14/18	yes	0.95869	150ul	$1x10^7$	fresh	yes	B	There were no replicates at ELISA level.
4/28/18	yes	0.99151	100ul	N/A	fresh	yes	B	This 4/28/18 run was all done in 1 ELISA plate with 3 different standard curves done in duplicate and 2 different pooled spleen samples added in varying well depths -100ul or 150ul.
	yes	0.98407	150ul	N/A	fresh	yes	B	
	yes	0.96812	100ul	N/A	fresh	yes	B	The standard curve developed in this curve was diluted in RPMI complete media (as opposed to assay buffer).
		samples	100ul	$4x10^6$	N/A	yes	B	
		samples	150ul	$4x10^6$	N/A	yes	B	There were no replicates at ELISA level.

Table A.5. Compiled ELISA data outlining several points of interest that are discussed in Chapter 4; initial cell concentration, standard reconstitution, coating buffer used, and well depths.