Disease Prevention in Channel Catfish (*Ictalurus punctatus*) Through the Use of an Attenuated *Aeromonas hydrophila* Vaccine or the Probiotic Effects of *Bacillus velezensis* AP193.

by

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Abstract

Since 2009, Motile *Aeromonas* Septicemia (MAS) has accounted for production losses of over 20 million pounds of channel catfish across the southeastern United States. The causative agent is a clonal population of highly virulent *Aeromonas hydrophila* (vAh.) The possible route of infection has previously been unknown, but this clonal population has been shown to have a novel O-antigen biosynthesis gene cluster. Further analysis of vAh strains has also indicated that this novel O-antigen cluster was conserved in vAh strains but was unique among described bacterial O-antigens. To determine the role of this novel O-antigen gene cluster in virulence, various single-gene deletions have been induced via the use of λ red recombinase mediated gene deletions. The genes encoding the O-antigen ligase (*waaL*), O-antigen polymerase (*wzy*), and a gene located adjacent to the O-antigen ligase, *gfcD*, were targeted for deletion and evaluated for their effect on *A. hydrophila* virulence. Each mutant was used to challenge fingerling catfish by intraperitoneal injection (IP) at a dose of $1x10^6$ CFU/fish. Both the *waaL* and *wzy* mutants retained virulence, whereas the *gfcD* mutant was completely attenuated in its virulence. The *gfcD* mutant induced an adaptive immune response against wild-type vAh and resulted in significant protection against wild-type vAh. To validate the role of *gfcD* in vAh pathogenicity, the wild-type *gfcD* gene was used to complement the *gfcD* mutant, which resulted in a partial restoration of virulence. GfcD and the other gene products encoded by the *gfcABCD* operon are predicted to be involved in the export and assembly of an O-antigen capsule and the vAh *gfcD* mutant was found to lack an O-antigen capsule, significant reduction in biofilm formation, and reduced

buoyancy compared to wild-type vAh. Furthermore, this *gfcD* mutant was shown to induce an adaptive immune response that protected catfish from subsequent vAh challenges when delivered intraperitoneally into fish. A replicated in-pond raceway trial also revealed that the protection elicited by IP injecting *gfcD* was sufficient at protecting channel catfish from a naturalistic induction of vAh. This protection could also be quantified in blood sera antibody titers six months after vaccination, without the administration of secondary boosters.

Additionally, *Bacillus velezensis* AP193 has been shown to have the ability to reduce mortality associated with the bacterial pathogens *A. hydrophila, Edwardsiella ictaluri*, and *Streptococcus iniae* when fed to fish at a dose of 10^6-10^7 CFU/g of feed in aquaria models. A replicated pond study at the North Fisheries ponds demonstrated that probiotic AP193-fed channel catfish had a moderate increase in growth relative to control channel catfish, and a significant reduction in the pond water phosphorus levels. This led to the hypothesis that the probiotic is degrading phytate present in feed, resulting in improved water quality. Herein is data presenting two alternative forms of infectious disease prevention in channel catfish, an attenuated mutant vaccine delivered through IP injections and a *Bacillus* probiotic that can enhance water quality, reduce disease, and improve growth performance when delivered as a feed amendment.

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Chapter I.

Introduction and Literature Review

1. Introduction

Aquaculture, the farming of aquatic organisms, has become one of the fastest growing food production systems globally (Subasinghe *et al.*, 2009, Subasinghe, 2005, Troell *et al.*, 2014). Unlike capture harvest food production, aquaculture has an ability to be expansive to fit the food production requirements of a given population. It is estimated that nearly half of the world seafood consumption comes from Aquaculture (FUS, 2015). As of 2015, United States per capita consumption of fish and shellfish exceeded 15 pounds, which indicated an increase of 0.9 pounds from 2014 (FUS, 2015). To meet consumer demands and secure reasonable returns on the producer's investment, increased efficiency of aquaculture production facilities is required. Various aquatic species that show rapid rates of growth and development in the wild have been sought after for increased performance in aquaculture systems (Alvarez-Lajonchere and Ibarro-Castro, 2013). Channel catfish is one such species and has become the primary freshwater production species farmed in the US, with 307 million pounds farmed annually during 2015 (FUS, 2015).

To increase production, channel catfish are commonly stocked in extremely high densities. These increased stocking densities, along with the desire for increase in production yields, require increased feeding rates, which in turn leads to the degradation of water quality,

increased stress, and reduced production in fish (Klinger *et al.*, 1983, Ghate *et al.*, 1993, and Tucker and Hargreaves, 2004). Further, these increased feeding rates, degradation of water quality, and increased stocking densities have all influenced the emergence of infectious disease as a limiting factor in catfish production (Tucker and Hargreaves, 2004).

Roughly 45% of channel catfish production losses are due to infectious diseases, with approximately 60% derived from bacterial infections, 30% parasitic, 9% fungal, and 1% viral (Tucker and Hargreaves, 2004). *Edwardsiella ictaluri*, *Flavobacterium columnare*, and *Aeromonas hydrophila* are three bacterial pathogens with significant economic impact on the channel catfish aquaculture industry (Panangala *et al.*, 2007). Traditionally, diseases caused by *Edwardsiella ictaluri* and *Flavobacterium columnare* have been the most common bacterial infections observed in channel catfish production systems, with over 78% of all channel catfish operations experiencing problems with *E. ictaluri* and *F. columnare* (Wagner *et al.*, 2006). Additionally, infections derived from members of genus *Aeromonas* have been observed to be the most common disease found in freshwater fish (Noga, 2010). In channel catfish, *Aeromonas sp.* are traditionally referred to as opportunistic pathogens, which is commonly isolated during mixed infections accounting for the loss of over 3 million pounds of channel catfish annually (Camus *et al.*, 1998 and Zhang *et al.*, 2016). However, the perception of *Aeromonas hydrophila* as a secondary pathogen has been adjusted with the recent emergence of hyper virulent isolates (Hossain *et al.*, 2013). These hypervirulent *Aeromonas hydrophila* isolates (often referred to as vAh) are observed to be the primary form of disease in channel catfish and have directly led to the destruction of over 20 million pounds of channel catfish since their emergence in the United States in 2009 (Rasmussen-Ivey *et al.*, 2016).

Current control methods for treating bacterial diseases in channel catfish generally involve feeding fish with medically top-coated feed (Pridgeon and Klesius, 2012 and USDA APHIS, 2015). However, administering treatments through feed presents a problem due to the requirement of fish to ingest the medication. This is unlikely due to the common occurrence of feed rejection by diseased fish (Pridgeon and Klesius, 2012). Currently, there are three antibiotics approved for the use in catfish: ormetroprim (Romet-30), oxytetracycline (Terramycin), and florfenicol (Aquaflor). These antibiotics are usually expensive and carry withdrawal periods that can delay market timing. Further, evidence has shown increased correlation between the frequent use of antimicrobial compounds and the development of antimicrobial resistance in pathogens (Pridgeon and Klesius, 2012). Thus, additional forms of control are heavily desired. Probiotic and vaccine use in aquaculture have previously been shown as effective forms of bacterial pathogen control and an adequate means of water quality enhancement (Wise *et al.*, 2015, Mohammed *et al.*, 2013, and Cruz *et al.*, 2012). However, until now the efficacy of feed amended probiotics and vaccinations have not been thoroughly tested against *Edwardsiella ictaluri* infections and against natural inductions of *Aeromonas hydrophila* in pond trials, respectively.

This dissertation further demonstrates a potential alternative form of *Edwardsiella ictaluri* disease prevention that can be delivered orally as a probiotic. Further, this probiotic can also increase growth performance and enhance pond water quality. This dissertation also outlines the generation of an effective vaccine candidate against virulent *Aeromonas hydrophila* infections that can be delivered Intraperitoneally to channel catfish.

2. Literature Review Anderson

2.1 Channel catfish industry.

Due to its rapid growth rate, low cost, and proficient reproduction capabilities, *Ictalurus punctatus* (channel catfish) has become one of the most popular and economically important aquaculture species in the United States (USDA, 2003a and USDA, 2003b). Catfish cultivation techniques were first developed in the early to mid-1900s (USDA APHIS, 2015). Since this time, processed catfish production has grown rapidly, increasing tenfold during 1970-1990 (USDA APHIS, 2015). Mississippi, Alabama, Arkansas, and Louisiana have become the leading catfish production states, which make up 46% of the total value of aquaculture in the United States (USDA, 2000). Over the past 30 years, channel catfish has been one of the most successful production species, with a 2,400% increase in production during 1975-1991 (Lewis, 1994). As of 2005, the United States was number three in the world in farmed catfish-production, accounting for 18% of the worldwide production and 551 million pounds of farmed catfish (USDA APHIS, 2015). However, in recent years production has fallen drastically, leading to a loss of 5,365 jobs and a $171 million deficit to Mississippi's channel catfish industry in 2011 (Avery *et al.*, 2013). One factor that could have caused this drastic and rapid decline in the channel catfish industry is outside competition. Importation of lower-priced *Pangasius sp*. (commonly referred to as tra and basa catfish) from Indochina has caused an undercut of the United States catfish by up to $2 a pound, leading to foreign catfish dumping margins that ranged from 37% to 64% of the total United States catfish market (Nixon, 2015, Kaliba and Engle, 2003). In total, Indo-china foreign markets account for roughly 76% of the catfish market, with China (32% of the total market) and Vietnam (25% of the total market) indicating a 50% and 131% increase in production between 2003-2005, respectively (USDA APHIS, 2015). Being an air breather, *Pangasius sp*. can tolerate dissolved oxygen levels that are roughly five times lower than the thresholds of channel catfish. Further these catfish can grow to adult size within

5-6 months, which is substantially more rapid than channel catfish. This creates a production yield commonly exceeding 250-300 tons/ha (FAO, 2016), which is significantly higher to the production potential of channel catfish. Due to this increase in foreign pressure placed on United States catfish production facilities, farmers push water capacity limits by stocking channel catfish in extremely high densities with yields commonly exceeding 6,000 pounds/acre/year (Masser *et al.* 2015). Exceeding stocking densities beyond a production system's carrying capacity frequently results in reduced water quality and significantly lower production yields (Wyatt *et al.*, 2006).

2.2 Water quality requirements for channel catfish production.

Quality and quantity of available water are the primary considerations for channel catfish production facilities (Lewis and Shelton, 2000). Ground water source is preferred; however, watershed ponds are more common and usually come entirely from runoff (Parker, 2011). Surface water, such as rivers and streams, are also common sources, however, these forms are not advised due to the increased likelihood of introducing unwanted fish species, parasites, or diseases into the production system (Lewis and Shelton, 2000). Aspects of water quality of concern in catfish production include, but are not limited to: Temperature, Dissolved Oxygen, pH, Ammonia, Nitrite, Alkalinity, and Hardness (Masser *et al.*, 2015).

Water temperature is one of the most important water quality factors for catfish production (Masser *et al.*, 2015). For most production systems, the overall temperature does not fluctuate rapidly (Masser *et al.*, 2015). The optimum water temperature necessary for optimum growth is between 28°C to 30°C, which is common in production systems in Alabama for 200-250 days during the year (Chapman, 2015 and Masser *et al.*, 2015). Further, optimum spawning and embryo development occurs when water temperatures are between 25°C to 27°C (Chapman,

2015). Temperatures above and below the optimum range reduce metabolic rates, and increase respiration, respectively, leading to reduced feed consumption and growth (Masser *et al.*, 2015).

The measure of dissolved oxygen in the water is extremely important for channel catfish production. For catfish survival, dissolved oxygen levels should be greater than 4 mg/l (Chapman, 2015). Low dissolved oxygen is one of the most common water-quality issues presented in channel catfish production systems (Masser *et al.*, 2015). Low levels of oxygen diffuse into production systems from the air, but these levels can be increased with mechanical agitation of the water by aeration systems. Further, most of the oxygen in production systems comes from photosynthetic organisms living in and on the production ponds (Masser *et al.*, 2015). Thus, it is very common to see dissolved oxygen levels fluctuate throughout the day.

Additionally, pH fluctuations are also common due to respiration (Masser *et al.*, 2015). Generally, the pH is a function of total alkalinity and dissolved carbon released during respiration, with basic water conditions occurring during periods with increased photosynthesis and more acidic water conditions occurring during periods with increased respiration (Tucker, 1991). The optimum pH observed in channel catfish production systems range from 6.5 – 9.0 (Chapman, 2015), however, pH should be closely monitored if ammonia spikes are observed, as pH directly affects the toxicity of ammonia (Masser *et al.*, 2015).

In aquaculture production systems, ammonia (NH_3) exists in equilibrium with dissolved ammonium ions (NH_4^+). Total Ammonia nitrogen (TAN) is the measure of the total amount of nitrogen in the forms of NH_3 and NH_4^+. Ammonia is extremely common in production systems as it is a natural byproduct of fish metabolism. However, the un-ionized ammonia is extremely toxic to channel catfish and can lead to destruction of tissues and even death in low amounts (>0.05 mg/l; Francis Floyd *et al.*, 1990). Ultimately, the un-ionized fraction of the TAN can be

determined by measuring the temperature and pH of the water. Thus, during periods of elevated temperatures and high pH, the higher the un-ionized form. Optimum TAN is observed if the measure is less than 0.5-1 mg/l, however, un-ionized ammonia must also be considered.

Just like ammonia nitrogen, nitrite (NO_2^-) can also be incredibly toxic to catfish due to its inhibition in the ability of hemoglobin to carry oxygen. Nitrite levels above 0.5 mg/l can lead to respiratory stress in channel catfish, however, nitrite generally only reaches extreme levels when nitrification is disrupted during fall and winter months (Masser *et al.*, 2015).

Alkalinity and hardness are typically coupled as one and the same since they are both presented as the measure of $CaCO_3$, but alkalinity is the measure of the capability of water to neutralize acids, or buffering capacity, whereas hardness refers to the relative amounts of calcium and magnesium in the water (Tucker, 1991). Increased Alkalinity levels result in an increased ability of the water to resist rapid pH fluctuations (Tucker, 1991). An increased hardness level indicates increased levels of calcium and magnesium, both of which are important for normal bone and tissue development (Tucker, 1991). Both Alkalinity and Hardness are very important indicators of water quality, and both should be kept between 50-100 mg/l in catfish production systems (Chapman, 2015).

2.3 Maximizing channel catfish production.

To gain productivity maximum with high-density operations, fish feeding efficiency is extremely important. Feeding often exceeds 50% of total operating expenses to the farmer, making it one of the largest costs in raising channel catfish (Engle and Stone, 2002). Generally, catfish should be fed what they will consume in roughly 15 min., however feeding 2-3% of total fish body weight during warmer weather is a more accurate alternative (Lewis and Shelton, 2000). Due to the total operating expenses of feed, the ability for an accurate measure of how

efficiently the fish converts the feed to body mass, also referred to Feed Conversion Ratios (FCR), dramatically affects the profitability of the production facility (Li and Robinson, 2008). Feed conversion ratios fluctuate from pond to pond but are regarded as the amount of feed needed for conversion into one unit (lb/kg) of weight gain by the fish. Hence, the lower the FCR, the more efficiently the fish is using the feed. A high FCR increases production costs (Li and Robinson, 2008). In addition to feeding rate, the FCR of the catfish is also influenced by the overall feed quality. Most commercial food fish rations contain 28-36% crude protein, with 50% crude protein common in fry feed, with the necessary vitamins and minerals (Li and Robinson, 2008 and Masser *et al.*, 2015). In the past this fish meal is essential for high protein diets necessary for more rapid feed conversion in high density systems. Traditionally, forage fisheries have been exploited for the generation of fish meal. The problem lies with heavy depletion of these wild fisheries which are responsible for maintaining the fish meal (Naylor *et al.*, 2009). As these wild stocks are depleted, the prices of fish meal begin to rise. Therefore, a new and economically friendly alternative to fish meal has been heavily sought after.

In recent years, soybean has gained attraction as a necessary replacement to fish meal. Soybeans contain the necessary protein content for efficient feed conversion, along with necessary trace elements such as nitrogen and phosphorous (Tacon, 1987). The problem with soybean used as a source of feed is that it is currently not an ideal additive into fish diets. Feed intake of plant protein sources, like soybeans, is much lower in fish and can lead to an increased deterioration of fish performance by up to 15% (Sales, 2009). Additionally, over two-thirds of the phosphorous found in plant protein sources are unusable by the fish due to a chemical form of phosphorous known as phytate (Zhu *et al.*, 2014). Due to high densities and increased feed rates of production systems, this unused phytate will ultimately be released as fish waste.

Furthermore, the excess influx of phytate into production systems alters aquaculture ecosystems, often leading to eutrophication (Lazzari and Baldisserotto, 2008).

2.4 Eutrophication and the effects of excess plant nutrients in aquaculture.

Eutrophication is the increase of plant nutrients in the water, causing the increase growth of algae and other microorganisms. Hence, with an increase of phosphorus in the system there is a resulting dominance of microbes geared to the degradation of these complex forms of plant nutrients that are unusable by the fish. One bacterial phylum that thrives during eutrophic events is cyanobacteria (Shaw *et al.*, 2003). Members of this phylum play crucial roles in biogeochemical cycling of elements and this elemental cycling is important in structure and biodiversity of aquatic ecosystems. However, due to their extremely rapid growth, and ability to synthesize and release toxins into the water column, they can be devastating to fish production (Rogers, 2008). Amongst these toxins are two very common metabolites known as 2-methylisoborneol (MIB) and geosmin. Both toxins are known to produce off-flavors in catfish, but MIB is the most common cause of flavor issues with catfish raised in production systems in the southeastern United States (Tucker and Ploeg, 1999). The off-flavors caused by MIB are rapidly developing and dissipate slowly from the fish. These off-flavors can occur within a matter of minutes and can reside in the fish for weeks. When production systems need to be operating at maximum, this can cause tremendous delays in harvesting and tremendous production setbacks to aquaculture facilities (USDA APHIS, 2003).

2.5 Phytase and its use in aquaculture to control eutrophication.

One way to combat economic losses from off-flavors is to prevent the over growth of off-flavor producing bacteria and algae that directly lead to eutrophication. One potential solution is by turning unusable chemical forms of phosphorous, that lead to eutrophication, into elemental

forms that can easily be used by the fish. Phytase is a phosphohydrolase that catalyzes the hydrolysis of phytate, allowing for phosphorous availability for absorption (Kumar *et al.*, 2012). To supplement high feed demands, production facilities have been created to exponentially ferment phytase from microorganisms - many of which are already regarded as probiotics (Askelson *et al.*, 2014). For this reason, providing the fish with probiotics can potentially reduce eutrophication and be a viable option for preventing economic loss.

2.6 The use of probiotics in aquaculture.

Probiotics are defined as live microbial adjuncts that can have a beneficial effect on the host by: altering the ambient microbial community, ensuring improved/ enhanced nutritional value as a feed supplement, prevention of infection through enhancing the host response towards disease, or improving the quality of its surroundings (Verschuere *et al.*, 2000a, Hong *et al.*, 2005, and Fuller, 1989). Exploitation of probiotics in Aquaculture were generally used during the production of cultured catfish to study probiotic inoculum concentrations (Cruz *et al.*, 2012). Many of the first probiotics administered to production systems were added directly to culture tanks to reduce disease (Cruz *et al.*, 2012). However, the efficacy of probiotics in aquaculture has relied heavily on ease of delivery and environmental stability as a feed additive (Cutting, 2011). Currently, most probiotics are delivered to aquaculture species as feed additives or through immersion and many have shown to be affective in increasing growth performance, aiding in nutrient digestibility, improving water quality, and at preventing diseases (Cruz *et al.*, 2012).

It is commonly thought that the efficacy of a probiotic as a legitimate form of growth enhancement relies heavily on their ability to colonize the gastrointestinal tract when administered over prolonged periods (Cruz *et al.*, 2012). However, it is unknown whether

probiotics increase the appetite of the production species or if their method of action is solely due to the improvement of feed digestibility (Cruz *et al.*, 2012). Previously, probiotic strains from *Streptomyces sp.*, *Streptococcus sp.*, and Sea Plant Products commercial bacteria strain SY9 have been effective in enhancing weight gain in ornamentals, food fish, shellfish culturing (Cruz *et al.*, 2012, Dharmaraj and Dhevendaran, 2010, and Macey and Coyne, 2005).

The use of probiotics in disease prevention have been fueled by the consumer demand for more natural forms of controlling diseases in animals used for human consumption (Cruz *et al.*, 2012). Probiotic use was also prompted by the desire to prevent diseases, rather than treat them with antimicrobials that can alter animal microbiota and can lead to pathogen resistance. Probiotic isolates of *Lactobacillus rhamnosus, Pseudomonas fluorescens*, *Micrococcus luteus*, and *Vibrio fluvialis* have all been tested for their antagonistic ability against furunculosis in rainbow trout (Cruz *et al.*, 2012, Nikoskelainen *et al.*, 2001, and Irianto and Austin, 2002). Further, lactic acid bacteria (*Lactobacilli sp*.), have also been shown as effective biological control agents against vibriosis in shrimp and streptococcosis and *Staphylococci* infections in African catfish (Verschuere *et al.*, 2000b, Cruz *et al.*, 2012, and Abdullah *et al.*, 2011). Previously, there have been few studies investigating the efficacy of probiotics in disease prevention in channel catfish, however, *Bacillus sp.* have been shown to have antagonistic activity against *Edwardsiella ictaluri* and *Aeromonas hydrophila* in channel catfish aquaria challenges (Ran *et al.*, 2012).

2.7 The use of *Bacillus sp.* as probiotics in aquaculture

One of the first commercialized evaluations of a probiotic in aquaculture was a *Bacillus sp.* preparation called Biostart (Queiroz and Boyd, 1998 and Cruz *et al.*, 2012). Since then, *Bacillus sp.* probiotics have been found effective in increasing growth promotion, reducing

disease, aiding digestion of complex nutrients, and enhancing water quality in aquaculture production systems (Cruz *et al.*, 2012 and Ran *et al.*, 2012). Research has also indicated *Bacillus sp.* to be advantageous in terms of their ease of delivery and environmental stability (Duc *et al.*, 2004). One advantage present in many *Bacillus* species is the ability form spores. Spores are non-vegetative intermediates that can form during extreme environmental conditions. The genus *Bacillus* is made up of gram-positive, endospore-forming rod-shaped bacteria that are ubiquitous in nature and are typically isolated from soils or around plant roots (Mongkolthanaruk *et al.*, 2012). *Bacillus* species have evolved to withstand the environmental extremes during periods when little to no water/nutrients is/are present, during which time, the vegetative cells wall off in a stable capsule (spore) (Nicholson *et al.*, 2000). When nutrients are reintroduced into the environment, the spore degrades, and the vegetative cell emerges, allowing the organism to once again proliferate (Mongkolthanaruk, 2012). From a probiotic standpoint, the sporulation mechanism can be very useful. The ability to form spores can assist *Bacillus* species to surpass the low pH of the gut, permitting the spores to successfully make it to the GI tract where they can safely colonize the intestine (Nayak, 2010 and Clavel *et al.*, 2004). Furthermore, from a consumer standpoint this sporulation can also have a huge advantage over non-spore forming probiotics such as *Lactobacilli*. Forming spores allows for the bacteria to have a tremendous amount of stability, increasing commercial shelf-life (Hong *et al.*, 2005). In addition to being able to survive the GI tract, *Bacillus sp.* can rapidly transform from spore to vegetative cell. One species of *Bacillus* (*B. subtilis*) can be found to grow on minimal media and has a generation time at 37˚C of about 18-20 minutes (Mazza, 1994). This extremely rapid replication time allows it to colonize the gut in a manner that prevents the spores from being totally expelled from the fish gut. Additionally, *Bacillus* species can produce / excrete many antimicrobials, such

as: bacitracin, bacilysin, subtilin, difficidin and surfactin (Baruzzi *et al.*, 2011), which allow them to outcompete and outgrow other bacteria, including many opportunistic pathogens. Most of these antimicrobials target gram-positive bacteria, but some can be used against gram-negative bacteria, molds, and yeasts (Mazza, 1994). Probiotic strains derived from *Bacillus sp.* are also commonly exploited for their use in enhancing water quality due to their efficiency in transforming excess organic matter (Cruz *et al.*, 2012). Thus, it is suggested that by maintaining elevated levels of these *Bacillus* probiotics in production facilities, farmers can minimize the accumulation of dissolved and organic carbon and nitrogen generated by increased bio density and feeding (Cruz *et al.*, 2012). However, evidence supporting the enhancement of water quality by probiotics administered to production systems is limited (Cruz *et al.*, 2012).

2.8 Infectious disease in channel catfish.

Prior to the rapid expansion of production systems between 1970-1990, the channel catfish industry followed management practices with lower bio-densities. Low-intensity management practices hold much lower productivity value; however, these practices result in better water quality and lower stress due to the reduction in fish densities (Wurts and Wynne, 1996). Additionally, this reduction in bio-density also reduces the transmission of disease. However, to increase production yields, profitability, and compete with rapidly expanding foreign markets, farmers began stocking channel catfish in maximum densities. To gain the most production out of the fish available, feeding rates were increased and farmers began multiple-batch management practices in which fingerlings would be stocked into ponds with an existing population of fish (Southworth *et al.*, 2006). As of 2006, 81 % of farms were using multi-batch management (Southworth *et al.*, 2006). The use of carryover fish allows the farmer to provide fish to processors year-round, however the survival of fingerlings stocked with carryover fish is

low and most become diseased early in the production season (Engle, 2003 and Southworth *et al.*, 2006). The high feed inputs associated with maintaining these increasingly large stocking densities, along with extreme stress, stimulated the proliferation of many opportunistic pathogens making them major factors for limiting production (Tucker and Hargreaves, 2004). Infectious diseases are the primary cause of losses during channel catfish production, making up roughly 45% of all recorded losses (USDA APHIS, 1997). Wildlife predation and low dissolved oxygen make up most of the non-infectious losses, with each causing roughly 37% and roughly 12% of the recorded losses, respectively (USDA APHIS, 1997). Most of all recorded losses due to infectious disease are derived from bacterial infections, with parasitic infestations and Channel Catfish Virus Disease (CCVD) also commonly associated with catfish production (USDA APHIS, 1997). However, it is hard to project an exact estimate as to the etiology of infectious diseases in the industry. Multiple or mixed infections are common, and several disease etiologies are still unknown (Tucker and Hargreaves, 2004). Further, it is common for production facilities reporting disease losses not to submit samples for disease testing, and disease samples caused by CCVD have been shown to be infrequently submitted (USDA APHIS, 1997). Diseases due to *Edwardsiella ictaluri* and *Flavobacterium columnare* are the most commonly reported, with over 70% of all operations recording occurrences (USDA APHIS, 1997). Due diligence on farms is variable and many outbreaks go unreported, however, infectious disease is believed to cost the industry millions of dollars (Tucker and Hargreaves, 2004).

2.9 Parasitic diseases in channel catfish

Parasitic diseases are typically observed to be more devastating in younger catfish (Lewis and Shelton, 2000). Protozoan parasites, such as *Ichthyophthirius multifilis*, *Trichodina sp.*, *Trichophrya sp.*, *Ambiphrya sp.*, *Apiosoma sp.*, *Ichthyobodo sp.*, *Epistylis sp.*, *Chilodonella sp.*,

Tetracapsula sp., and *Myxobolus sp.*, are amongst the most common parasitic infections observed in raised fish (Durborow, 2003). These protozoan parasites cause the most deaths out of all the parasitic diseases observed in channel catfish and typical identification and diagnosis of protozoan parasites requires microscopic examination. *Ichthyophthirius multifilis* (Ich) is one of the most common parasitic diseases observed in freshwater fish and virtually all freshwater fish are susceptible. Additionally, channel catfish and other scale less fish are especially vulnerable, due to the increased ability for the parasite to attach to the epithelium (Noga, 2010). Ich is typically observed during periods where the water temperature ranges from 15-25°C, during which it can complete a lifecycle within 3-6 days. Stocking density plays a significant role in the severity of Ich infections, and in high-intensity production facilities, it is possible to observe mortalities reaching 100 %. Additionally, many other protozoan parasites, such as *Trichodina sp.*, *Trichophrya sp.*, *Ichthyobodo sp.*, *Chilodonella sp.* rely on high density farming operations and increased parasitic load before presenting disease (Durborow, 2003). Protozoan parasites are commonly seen associated as ectoparasites located on skin or gills. Protozoan parasites such as *Apiosoma sp.* do not cause distinctive lesions, but their presence on the gills can block the flow of oxygen and lead to deprivation in the fish (Durborow, 2003). Protozoan parasites that do form lessions, such *Epistylus* sp., can lead to ulceration that may make fish more vulnerable to bacterial infections. Red sore disease involves a mixed infection of *Episylus* sp. with *Aeromonas sp.*

Digenean Trematode infections are also incredibly common in fish. Occurrences of these are uncommon in cultured fish, however, there are over 1,700 distinct species of adult digeneans that infect fish (Noga, 2010). Trematodes such *as Clinostomum complanatum* and *Diplostomum spathaceum* have been observed in channel catfish, however these incidences are uncommon

(Terhune *et al.*, 2003). Due to their life cycle, trematodes prevalence in channel catfish is typically increased in production systems under heavy predation of fish-eating birds (Terhune *et al.*, 2003). The life-cycle of digeneans is complex and involves multiple different hosts to complete. Intermediate stages are generally observed in two separate hosts, usually consisting of a snail and fish. Adult stages are observed in a definitive host, which is commonly the predatory bird species. In the past 20 years, trematode genus of *Bolbophorus* has been reported in commercially raised channel catfish, leading to high mortality rates in channel catfish production systems in the southeastern United States (Terhune *et al.*, 2003). This trematode species is commonly associated with facilities containing large populations of ram's horn snails and under predation pressures of the American white pelican.

2.10 Saprolegniasis in channel catfish.

Most fugally derived infections observed in channel catfish production systems come from water molds belonging to the family *Saprolegniaceae*. For this very reason, fungal diseases in channel catfish are commonly referred to as saprolegniasis (Durborow *et al.*, 2003). The most commonly isolated saprolegniasis associated with channel catfish production systems is winter saprolegniasis. The species of the etiological agent responsible for winter saprolegniasis is unknown. This disease is common during periods in which the temperatures are observed below 15°C, however, mortalities are generally not observed until the temperature increases during the spring (Durborow *et al.*, 2003). A chronic mortality pattern is commonly associated with winter saprolegniasis, and the severity of disease is generally higher in fish larger than 2 pounds under high-intensity farming practices. Further, the cause of pathogenesis is somewhat unknown, however, primary factors for winter saprolegniasis include an increased presence of *Saprolegnia*

sp. zoospores and the inability of the catfish to adapt to the more rapidly fluctuating water temperatures observed during the transition for winter to spring (Durborow *et al.*, 2003).

2.11 Channel Catfish Virus Disease.

Channel catfish virus disease (CCVD) is the most important virus of economic importance observed in channel catfish production systems (Lewis and Shelton, 2000). The causative agent of CCVD is a herpesvirus specific to channel catfish (Camus, 2004). CCVD primarily infects fry and fingerlings shortly after transfer from hatcheries into ponds and is typically unseen in fish larger than ten inches (Lewis and Shelton, 2000 and Camus, 2004). During epidemics, the virus is readily transmitted horizontally and is observed in the feces and urine of infected fish (Noga, 2010). Brood stocks of channel catfish are also believed to play a role in transmission (Camus, 2004). CCVD commonly elicits stargazing and erratic swimming in fingerlings and histopathology generally indicates that CCVD can attack all major organ systems, with damage to the kidney's being primary (Camus, 2004 and Noga, 2010). CCVD epidemics present abrupt mortality patterns in which there is a rapid onset of disease and increase in mortalities in fish younger than a year in age. The posterior kidney is the first organ infected and presents the best target for isolating the virus due to the prolonged presence and increase viral titers. Channel catfish cell lines and microscopy can be used to identify the virus (Camus, 2004 and Noga, 2010). Further, CCVD epidemics can also be masked due to an accompanying secondary bacterial disease such as *Edwardsiella ictaluri*, *Flavobacterium columnare*, or *Aeromonas hydrophila*.

2.12 *Edwardsiella ictaluri* in channel catfish.

Edwardsiella ictaluri is an obligate gram-negative pathogen that causes enteric septicemia of catfish (ESC), which is commonly referred to the most economically important

pathogen to the channel catfish industry (Hawke *et al.*, 1998, Tucker and Hargreaves, 2004, Hawke *et al.*, 2013). ESC is more recently recognized bacterial pathogen of catfish, with the first reported cases observed between 1969-1976 (Hawke *et al.*, 1998). Since its emergence it has shown a high predisposition and specificity to channel catfish, with most occurrences being observed in cultured catfish and little to no recorded natural fish kills (Noga, 2010 and Hawke, 1998). As of 1998, 47 % of all reported morbid cases in channel catfish production systems and 30 % of all disease cases submitted to diagnostic labs in Mississippi were derived from ESC. ESC typically occurs in southeastern US production facilities during May-June and September-October, when the temperature ranges between 24°C-28°C (Noga, 2010 and Cunningham *et al.*, 2014). Mortalities can occur outside of this range; however, the incidence of diseased catfish are much lower (Noga, 2010). Traditionally, there have been three different mortality patterns associated with ESC in channel catfish: Acute, Sub-acute, and Chronic (Cunningham *et al.*, 2014 and Tucker and Hargreaves, 2004). The acute mortality pattern is characterized by few clinical signs and extremely high mortality rates occurring over a very limited period (Tucker and Hargreaves, 2004). Fish hit with an acute form of ESC rapidly lose appetite and become lethargic. Star gazing and loss of motion is common immediately prior to death. Bacteremia and septicemia is common, and toxicity can be observed in almost every tissue of the morbid fish. Infections that elicit a sub-acute mortality pattern are characterized by a slower onset of disease with high cumulative mortality rates (Tucker and Hargreaves, 2004). External presentation of this form of infection are much more pronounced than in acute infections. This form is typically associated with shallow small ulcers (white spots) commonly displayed around the head (Tucker and Hargreaves, 2004). Catfish affected by this form behave similarly to that of acute infections, however, these fish will typically halt feeding more gradually. Infections displayed during the

chronic form of ESC can be observed in catfish 30 days after initial infection and are marked by open ulcers present on the front of the head. This form of ESC is where the common name, “hole-in-the-head” disease is derived (Tucker and Hargreaves). The ulcer in the skull can directly lead to damage of the meninges, which correlate with spinning, spiraling, and tail chasing commonly observed in fish with this form of ESC. However, mortalities due to the chronic form are markedly lower to that of the acute and sub-acute forms (Tucker and Hargreaves, 2004).

2.13 *Flavobacterium columnare* in channel catfish.

Flavobacterium columnare is an incredibly common bacterial pathogen observed to affect the skin and gills of channel catfish (Noga, 2010). *F. columnare* is the causative agent of columnaris disease in fish, which was first described in 1922 in fish in the Mississippi River (Tucker and Hargreaves, 2004). The name Columnaris was derived from the presentation of the disease, which commonly involves the formation of column like masses of cells on the gills of fish. Columnaris has traditionally been referred to be second in mortality rate and importance in the channel catfish industry, just behind that of ESC. However, it was shown to be the leading cause of mortality on Mississippi production systems in the year 2000 (Tucker and Hargreaves, 2004). Further, 86 % of documented columnaris cases can be described as mixed infections in which other bacteria also contribute to disease (Tucker and Hargreaves, 2004). This has made the exact economic impact of columnaris on production losses in channel catfish industry hard to determine. Regardless, it is estimated to cost the industry $30-$50 million in production losses annually (Tucker and Hargreaves, 2004 and Noga, 2010, and Declercq *et al.*, 2013). Disease presentation of *F. columnare* is not specific to any age of catfish and has been shown to cause infections across a wide range of water conditions during any season (Tucker and Hargreaves, 2004). However, columnaris is most commonly seen at temperatures above 15°C and has been

shown to be more pathogenic at temperatures around 20°C-35°C, depending on the strain (Declercq *et al.*, 2013 and Noga, 2010). Due to the inability of *F. columnare* to grow on routine laboratory media, traditional diagnosis of columnaris is based on clinical signs and microscopic confirmation of column shaped masses on the gills, along with the confirmation of filamentous gram-negative rods, present on morbid fish. However, selective media specific for *F. columnare*, such as Hsu Shotts and Anacker and Ordal media, have been shown to be effective means of culturing isolates from fish (Bullock *et al.*, 1986). As previously mentioned, the etiology of *F. columnare* in catfish can be variable based on the strain, and this is also true regarding the clinical signs presented. It is common for highly pathogenic strains to cause mortalities within 12-24 h, without presenting any gross tissue damage (Declercq *et al.*, 2013). During more chronic mortality patterns, lesions are generally seen. Mouth ulcers (cigar mouth), skin lesions, and fin and gill rotting are also typically observed with a yellowish-white mucous covering (Declercq *et al.*, 2013). Deeper lesions can also be observed in fish with *Aeromonas sp.* mixed infections (Tucker and Hargreaves, 2004).

2.14 *Aeromonas hydrophila* in channel catfish.

The onset of bacterial infections in channel catfish is almost entirely due to environmental stress (Lewis and Shelton, 2000). This is more important for opportunistic bacteria that are derived from the soil and water, being that they are, for the most part, always present in channel catfish production systems (Lewis and Shelton, 2000). Opportunistic bacteria, such as *Aeromonas hydrophila*, become problematic when the environmental stressors favor their growth. *Aeromonas hydrophila*, is a gram-negative motile bacterium ubiquitous in freshwater environments. Traditionally, *A. hydrophila* is not as commonly reported as ESC or Columnaris in the channel catfish industry, but due to its ubiquitous nature, it is reported to be

the most common pathogen to freshwater fish (Noga, 2010). Even though *A. hydrophila* is ubiquitous in freshwater, it is known as an opportunistic pathogen in channel catfish, causing disease during mixed infections and high stress conditions. *A. hydrophila* have been traditionally observed to be relatively weak pathogens, however, there are zoonotic concerns that have been observed in that make it more than just a pathogen of fish (Noga, 2010). In humans, gastro-intestinal infections have been observed from ingesting infecting fish and systemic infections have also been associated with wounds (Noga, 2010). Recently, zoonosis reports in humans have increased, however, due to the ubiquitous nature of *A. hydrophila* and high exposure of humans to motile aeromonads, infection is still relatively low and commonly only seen in immunocompromised individuals. In channel catfish, during stages of increased stress and poor water quality, *A. hydrophila* can lead to Motile *Aeromonas* Septicemia (MAS) (Harikrishnan *et al.*, 2003). The symptoms of MAS include swelling of tissues, red sores, necrosis, and ulceration (Karunasagar *et al.*, 1989 and Azad *et al.*, 2001). Disease due to *A. hydrophila* are most commonly associated with high-intensity production systems and warmer water observed in the mid to late summer, when temperatures range from 20°C-30°C. The first isolates of Motile aeromonads were observed in animals prior to the onset of high-intensity aquaculture practices. The first documented case of MAS in fish was published in 1930, which describes infections in carp caused by *Aeromonas punctata* (Tucker and Hargreaves, 2004). Over the next 30 years, *Aeromonas* was observed to cause hemorrhagic septicemia in other aquatic species, such as frogs, goldfish, and catfish. In 1961, motile aeromonad species of *Aeromonas punctata*, *Aeromonas hydrophila*, and *Aeromonas liquefaciens* were found to be variants of the same species and taxonomically changed to *Aeromonas hydrophila* (Tucker and Hargreaves, 2004). The opportunistic presentation of the fish disease elicited by *A. hydrophila* was recategorized

under Motile Aeromonad Septicemia in 1975, and *A. hydrophila* has since been recognized as an opportunistic pathogen in channel catfish (Tucker and Hargreaves, 2004, Lewis and Shelton, 2000, and Noga, 2010). However, in 2009 a disease outbreak occurred in 48 catfish farms in west Alabama, causing an estimated loss of more than 3 million pounds of food size channel catfish (Hemstreet, 2010). To date, these hyper virulent strains (also referred to as vAh) have caused an estimated loss of over 20 million pounds of catfish (Rasmussen *et al.*, 2016). During the last five years, vAh from the epidemic outbreaks were found to be highly virulent when compared to the more opportunistic isolates that are traditionally seen in aquaculture systems. The exact etiology of these vAh isolates is still unknown, however, comparative analysis of these hyper virulent isolates identified 55 epidemic-associated regions spanning 336,469 bp. Out of the 336 kb of epidemic-associated sequences, 252 kb are contained within genomic islands, suggesting the possible acquisition of many of these epidemic-associated regions were acquired through lateral gene transfer (Hossain *et al.*, 2013) Furthermore, a total of 34 predicted genes hold high similarity with proteins found in the virulence factor database, with 23 of these proteins predicted as toxic extracellular products such as aerolysin and hemolysin (Hossain *et al.*, 2013 and Pridgeon *et al.*, 2014). The means of vAh disease introduction into channel catfish is unclear, but it is believed that the heightened pathogenicity from vAh is multifactorial and the action of several factors are required for disease in the catfish (Hossain *et al.*, 2013). Additionally, one of the isolates that was used in comparative analysis was isolated from Chinese carp many years before the epidemic hit the United States. Based on the timing of the recorded epidemics along with MLST-based phylogeny, the vAh isolates and the Chinese carp isolate could hold a common ancestor of Asian origin (Hossain *et al.*, 2014). Currently, there has been no confirmed evidence of zoonosis of vAh in humans, however, due to the unknown nature of

vAh disease induction in channel catfish and previous zoonotic considerations, controlling these vAh outbreaks is of increasing concern.

2.15 Controlling infectious diseases in channel catfish.

Controlling infectious diseases is a limiting factor for channel catfish production and is considered to cause major economic constraints to the channel catfish industry. There are a wide range of anti-microbials used to combat, avoid, and control losses and disease in the industry. 37% Formaldehyde (Formalin), potassium permanganate, copper sulfate, and chloramine-T are commonly used disinfectants for the treatment of fish diseases (Kumar and Roy, 2017). Diflubenzuron, malathion, and Methyl parathion are the traditional pesticides used to combat disease in the industry (Kumar and Roy, 2017).

Pharmaceutical agents approved by the Food and Drug Administration available for usages in food fish undergo rigorous testing and are regarded as safer and more effective (Kumar and Roy, 2017). However, this process can be time consuming and the choices available are becoming increasingly limited and expensive. Currently, there are nine chemotherapeutants drugs approved by the Food and Drug Administration (FDA) for their use in aquaculture. Five of which are immersible, three that can be amended on feed, one injectable (Kumar and Roy, 2017). Further, there are only three antibiotics that have been approved by the FDA for use in controlling bacterial pathogen in channel catfish production (Benbrook, 2002). Terramycin (oxytetracycline) and Romet® (ormethoprim plus sulfadimethoxine) are the two most commonly used antibiotics to battle MAS, with Terramycin being the only antibiotic approved by the FDA for treatment of MAS (Camus *et al.*, 1998). Terramycin is fed at a rate of 2.5 to 3.75 g per 45 kg of fish. 1.3 grams of Terramycin® per kg of feed formulation is typically administered at a rate of 1 to 1.5 percent of catfish body weight per day. Whereas, .57g of Terramycin® per kg of feed

formulation is administered at 2 to 3 percent body weight per day (Camus *et al.*, 1998). Romet® has also been a traditional treatment against ESC (Hawke *et al.*, 1998). Romet® is generally administered as a feed additive at a concentration of 50 mg/kg of body weight/day for a maximum of 5 days (Kumar and Roy, 2017). Aquaflor® (florfenicol) is a new aquaculture antimicrobial that has been shown to be effective at treating ESC and columnaris when delivered on feed at a concentration of 10-15 mg/kg of feed for 10 days (Kumar and Roy, 2017). However, these three antibiotics all require a veterinary feed directive from a licensed veterinarian. Veterinary directive is an appropriate means to help regulate the use of antibiotics, but it is not an optimal due to the potential delay in treatment. Additionally, these forms of control can be expensive to the farmer and come with withdrawal periods that can potentially delay harvest times. The cost of developing and approving antibiotics, the public concerns of its misuse, along with the emergence of resistant strains of bacteria from its systematic use, can pose problems for the approval process of additional antibiotics by the FDA.

The efficacy in the control of parasitic diseases in the channel catfish industry is variable. As mentioned earlier, protozoan parasites are generally the most common in channel catfish production systems, and they are also the easiest to treat with therapeutics. However, the appropriate agent selection requires identification of the parasite (Lewis and Shelton, 2000). Salt, potassium permanganate, and copper sulfate have all been shown as effective therapeutics in combating protozoan parasites. Un-iodized salt is commonly used in aquaria and hauling tanks at 1-2 parts per thousand (ppt) to treat fish from external protozoa parasites (Durborow, 2003). Higher concentrations with 10-30 ppt can be used but are not recommended for baths lasting more than an hour (Durborow, 2003). Potassium permanganate and copper sulfate have been shown effective at removing ectoparasites, however, regulatory action for these two therapeutics

have been deferred. Potassium permanganates method of action is based on the relative organic matter present (Durborow, 2003). Generally, as much as 10 parts per million (ppm) of potassium permanganate can be administered for up to 20 min. (Durborow, 2003). Copper sulfate has been an effective control for Ich infections. However, the concentration of copper sulfate depends heavily on the alkalinity of the water. The rate administered is calculated by dividing the total alkalinity by 100 and using the resulting amount in ppm for the copper sulfate treatment (Durborow, 2003). The efficacy of treating digeneans with chemotherapeutics is low, and there are currently no FDA-approved treatments for food fish infested with trematodes. However, control and prevention of these parasitic infections can be accomplished by breaking the life cycle (Terhune *et al.*, 2003). This is commonly performed by eliminating or reducing the numbers of the final or first intermediate hosts.

Just as with the treatment of digeneans, the treatment of winter saprolegniasis primarily focuses on prevention of the disease. Treatment of the disease is expensive, and its efficacy has not yet been documented. The most common means of prevention is by initiating low-intensity management practices during the late summer and early fall (Durborow *et al.*, 2003).

Additionally, there are no effective treatments for CCVD. A customary practice of the industry is to reduce the environmental stress and practice low-intensity production while the fingerlings are below 4 inches in length (Lewis and Shelton, 2000 and Camus, 2004).

Chapter II

Bacillus velezensis AP193 exerts probiotic effects in channel catfish (*Ictalurus punctatus*) and reduces aquaculture pond eutrophication.

1. Abstract

Ictalurus punctatus (channel catfish) is an economically important farmed fish particularly in the southeastern United States. Aquaculture sustainability is threatened by disease pressure and the eutrophic conditions resulted from intensive fish farming. Previous research identified *Bacillus velezensis* strains that reduced mortality due to bacterial pathogens when used as a feed amendment. This study was conducted to determine the effects of *B. velezensis*-amended feed on catfish growth performance, disease control, and pond water quality. Previously, four *B. velezensis* strains were evaluated for their probiotic effects and disease reduction due to *Edwardsiella ictaluri* during an aquarium trial. After ten weeks feeding, fish fed with *B. velezensis* AP193 were observed to have the best growth performance (14% increase in mean growth) and best survival rates after *E. ictaluri* challenge, compared to control fish. *B. velezensis* AP193 was therefore selected for evaluation in a ten-week pond trial, with four replicate ponds per probiotic treatment or control group. Feed amended with *B. velezensis* AP193 induced a 40.4% or 32.6% increase in growth relative to control feed in fingerling catfish that originated from aquaria or raceways, respectively. Additionally, the water quality was improved

in ponds in which fish were fed with the probiotic-amended feed, as significant reductions were found in total phosphorus (19%), total nitrogen (43%) and nitrate (75%). This data suggests that *B. velezensis* AP193 can promote catfish growth, improve pond water quality, and reduce disease when used as a feed amendment.

2. Introduction

For maximized productivity of the aquaculture system, fish feeding efficiency is extremely important. Traditionally, forage fisheries have been exploited for the production of fish meal, but the rapid depletion of wild fisheries (Naylor *et al.*, 2009) has led to the use of soybean meal as an alternative (Da *et al.*, 2013). However, feed conversion ratios (FCRs) are much lower in fish with the use of plant protein resulting in up to a 15% deterioration of fish growth performance compared to a fish meal-based diet (Sales, 2009). Phosphorous found in plant protein sources are unusable by fish, and phytate can serve as an anti-nutrient in chelating iron, resulting in anemia (Zhu *et al.*, 2014). Unused phytate and other feed-derived nutrients will ultimately be released as fish waste and contribute to the eutrophication of the aquaculture pond ecosystem (Cho and Bureau, 2001).

Phytase is a phosphohydrolase that catalyzes the hydrolysis of phytate, allowing for phosphorous availability for absorption (Kumar *et al.*, 2012). This enzyme is found in many microorganisms, which are being exploited for supplementation in feed. To supplement high feed demands, production facilities have been created to ferment phytase from microorganisms, many of which are already regarded as probiotics (Askelson *et al.*, 2014). For this reason, providing the fish with probiotics can potentially reduce eutrophication, induce weight gain, and be a viable option to promote sustainable aquaculture management practices.

Another factor responsible for significant economic losses in aquaculture is due to disease (Stentiford *et al.*, 2017). One traditional treatment for disease is the use of antibiotics, and there are currently only three antibiotics approved by the FDA for their use in food channel catfish (Schnick, 2007). However, with growing concern over the use of antibiotics due to the development of pathogen multi-drug resistance (Patil *et al.*, 2016), it is important to seek alternative means of treatment. Probiotics can reduce fish mortality due to pathogens by direct antagonism via synthesis of secondary metabolites, by competitive exclusion, and/or by activation of the innate immune system (Balcazar *et al.*, 2006, Macfarlane and Cummings, 1999, and Wang *et al.*, 2008). *Bacillus* spp. have the potential for aquaculture application due to their ability to form endospores, allowing for a long shelf life and survival from exposure to gastric acid (Casula and Cutting, 2002 and Hong *et al.*, 2005). Furthermore, strains within the *B. subtilis* group, which includes *B. velezensis* (previously described as *B. amyloliquefaciens* subsp. plantarum (Dunlap *et al.*, 2016)), have not been associated with disease.

In addition to preventing disease, probiotic strains may promote the growth of fish by improving feed nutrient quality and by removing anti-nutrients, such as phytate. Some microorganisms express phytase activity, catalyzing phytate hydrolysis and allowing for phosphorous absorption (Kumar *et al.*, 2012). Purified microbial phytases have been used as a feed additive in livestock to promote growth and reduce eutrophication (Askelson *et al.*, 2014). For this reason, feeding fish with a phytase-expressing probiotic could be a sustainable management practice to reduce eutrophication, induce weight gain, and result in an altered aquaculture pond ecosystem with reduced incidence of disease and off-flavor. Eutrophication due to feed-derived phytate and other nutrients can result in blooms of algae and cyanobacteria (Anderson *et al.*, 2002). Due to the ability of cyanobacterial taxa to synthesize and release toxins

into the water column, they can be devastating to fish production (Sevrinreyssac and Pletikosic, 1990). In addition to hepatotoxins and neurotoxins, some cyanobacteria and other bacterial taxa produce the metabolites 2-methylisoborneol (MIB) and geosmin that result in unwanted off-flavors in catfish (Vanderploeg *et al.*, 1992).

Previous research evaluated a collection of 160 *Bacillus* spp. strains for their antimicrobial activity against bacterial and fungal fish pathogens (Ran *et al.*, 2012). The 21 *Bacillus* spp. strains that showed production of secondary metabolites that inhibited the growth of fish pathogens, *Edwardsiella ictalur*i, *Streptococcus iniae*, and *Aeromonas hydrophila* were then tested for their survival and persistence in the catfish intestine and protection against infections caused by *Edwardsiella ictaluri* (Ran *et al.*, 2012). In Nile tilapia (*Oreochromis niloticus*), a specific strain *B. velezensis* AP193 showed protection against infection by *Aeromonas hydrophila* (Addo *et al.*, 2017) or *Streptococcus iniae* (Addo *et al.*, 2016). This study evaluated the efficacy of specific probiotic strains when used as a feed additive in reducing disease due to *E. ictaluri*, promoting the growth of channel catfish, and their effects on pond water quality.

3. Materials & Methods

Animal welfare statement. All channel catfish challenges were conducted under the approval of the Animal Care and Use Committee (IACUC) of Auburn University in compliance with U.S. regulatory standards for the humane care and use of laboratory animals.

Bacterial strains and growth conditions. *B. velezensis* strains used in this study were from a collection of soil and catfish intestine-isolated bacteria (Ran *et al.*, 2012) (Table 1). As described previously, each *B. velezensis* strain was grown in tryptic soy broth (TSB) or on tryptic

soy agar (TSA) at 28°C. *E. ictaluri* S97-773 was grown in TSB or on TSA at 26°C (Table 1; Ran *et al.*, 2012).

Spore-amended feed preparation. *B. velezensis* spores were prepared with some modification by the method described by Kenny and Couch (Kenney and Couch, 1981). *B. velezensis* strains were grown in TSB overnight at 28°C. The cell suspension was then spread onto spore preparation agar (peptone 3.3 g/L, beef extract powder 1.0 g/L, NaCl 5.0 g/L, K2HPO4 2.0 g/L, KCl 1.0 g/L, $MgSO_4 \cdot 7H_2O$ 0.25 g/L, $MnSO_4$ 0.01 g/L, lactose 5 g/L, agar 15 g/L), and incubated at 28°C for 5 to 7 days. To collect the spores, 5 mL of sterile water was added to the plate, then scraped using a sterile loop, and placed into a sterile tube. The spore suspension was then incubated at 80°C for 15 min to kill any vegetative cells. The concentration of the spore suspension was determined by serial dilution and plating onto TSA followed by incubation at 30°C overnight. The final concentration of spores was altered to ~$1x10^9$ colony forming units (CFUs)/mL. Then the spore suspension was added to fish feed at 8% (v/w) for a final concentration of $4x10^7$ CFU/g of feed, which was consistent with previous published literature and has been regarded as the appropriate dose to elicit probiotic effects in previously performed channel catfish trials (Ran *et al.*, 2012). Feed was spray-coated at 8% of the dry weight with the spore suspension described above, as well as coated with 5% menhaden fish oil at 3% of the dry weight of feed. Prepared feed stocks were stored at 4°C until use. Spore concentration was verified through random pulls of feed through the duration of the coating. 1 g of feed from replicate pulls was placed into 9 ml of sterile 1x PBS and homogenized at 4°C using a RotoFlex Benchtop Tube Rotator (Argos Technologies, Inc., Vernon Hills, IL). Following homogenizing, the resulting supernatant was subjected to serial dilutions, in triplicates, and plate counting onto TSA following an incubation at 30°C overnight.

Aquaria study conditions. Channel Catfish fingerlings were obtained from Auburn University North Fisheries Unit. Each fish was roughly 4 months old, weighed approximately 20 g, and had no immediate history of infection or previous history of *E. ictaluri* infections. The fingerlings were batched-weighed and randomly assigned to their designated feed treatment with five replicate tanks per treatment and 30 fish per replicate tank. Experimental grow-out tanks included the same 60 L aquaria used during the previously conducted *Bacillus* trials (Ran *et al.*, 2012), each containing 45 L of de-chlorinated Auburn, Alabama, city water supplied through a flow through system at a rate of 0.6 liter/min. The water temperature was kept at 25-28ºC for the duration of the trial. Fish were acclimated in experimental tanks while being fed standard catfish fingerling feed for three days prior to initial feeding with experimental feed. During the feeding trial, fish were fed once a day at approximately 3% of the total biomass. After acclimation, fish from each unit were counted and batch-weighed biweekly during the feeding trial. The amount of feed applied to each tank was adjusted every two weeks by the updated weight information. The feeding lasted for 10 weeks, which was deemed necessary based on previously performed challenges (Data not shown). At the end of the trial, fish were batch weighed and the body weight gain and FCR were calculated as:

$$Weight\ gain = (Wf - Wi) / Wi$$

$$FCR = F / (Wf - Wi)$$

Where Wf and Wi were final and initial mean weight (g) per fish in a tank. F is the cumulative amount of feed (g) given to one fish in a tank.

The fish were also evaluated for their levels of protection against *E. ictaluri* infection. After the last batch weighing, fish were further fed with respective diet for 2 days. Then aliquots of the fish were challenged by *E. ictaluri* 18 h post feeding. Each treatment group consisted of five replicates of 60 L aquaria containing 16 fish, except the control group which only contained 4 replicate aquaria. Fish were challenged by immersion for 1 h in 10 L of water containing 5.2 x 10^6 CFU/mL of *E. ictaluri* S97-773. *B. velezensis* spore-amended feeding was not interrupted and was continued for one-day post-challenge due to the cessation of feeding by the fish. Fish mortality was recorded daily for seven days and the final mean mortality for each treatment was used to determine the effects of feeding with each *B. velezensis* strain. Representative dead fish were dissected and the presence of *E. ictaluri* was confirmed by microbiological examination of kidney and liver swabs on TSA. The identity of the recovered *E. ictaluri* was further confirmed through 16S rRNA gene sequencing. All remaining fish were euthanized after day 7 of the challenge through an overdose of MS-222 (>250 mg/l) and incinerated. The strain that indicated the best protective effects against ESC, along with the highest enhancement in growth performance, was further tested for its efficacy in ponds.

Pond study conditions. An average of 861 fingerling catfish with a total average weight of 26.28 kg were released into each of the eight 0.04 ha ponds. Each randomized pond represented one replicate for each of the two treatments used in the study. Therefore, four replicate ponds were stocked for feeding the probiotic and four replicate ponds were stocked for feeding a control diet. Each pond contained two separate groups of fish that were grown to fingerlings in either pond raceway systems or in aquaria. To distinguish the two batches of fish from one another the adipose fins were clipped from the fish that were raised in aquaria prior to release into each replicate pond. For each pond, an average of 461 fish were released from the

raceway origin and an average of 400 fish were released from the aquaria origin. The aquaria derived fish were roughly 3.5 months old and weighed roughly 15 g and the raceway derived fish were roughly six months old weighed roughly 40 g. To determine fish growth performance, 100 fish were randomly pulled from the overall population at each time point and weighed.

Once fish were stocked in each replicate pond, fish were fed once daily at approximately 2% of the total fish biomass for ten weeks. Based on the average biomass that was calculated for each group of four ponds, a volumetric 1% feed amount per pound was determined. The fish were acclimated to the ponds for two weeks prior to the trial and fed approximately 0.5% of average biomass of control feed a day. Each pond was fed 0.5% of average biomass at a time and if all the feed was eaten quickly then another 0.5% was given (up to 2%) until satiation was observed in the catfish. To determine fish growth performance, 100 fish were randomly pulled from the overall population at 10 weeks and weighed. All fish were starved for 48 h prior to harvesting. The remainder of the fish were euthanized with an overdose of MS-222 (>250 mg/L) and incinerated.

Pond water quality analyses. Pond water samples were collected every two weeks from time zero to week ten, for a total of six-time points and stored at -80°C for later use. However, due to loss of product from prolonged storage, a total of 24 samples were used during the final analyses. Samples were placed within 50 mL conical tubes and were immediately stored at -80°C until further analysis. Water samples were analyzed using standard protocols as follows: total ammonia nitrogen (TAN) by the salicylate method (Bower and Holmhansen, 1980 and Le and Boyd, 2012); nitrite-nitrogen by the diazotization method (Boyd and Tucker, 1992); Nitrate-nitrogen was measured by the Szechrome NAS reagent method (Van Rijn, 1993); and total nitrogen (TN) and total phosphorus (TP) were analyzed by ultraviolet spectrophotometric

screening method with Aquamate Model AQA 2000E (Thermo Fisher Scientific, Suwanee, GA, USA) and ascorbic acid methods, respectively, following digestion in potassium persulfate solution (Gross *et al.*, 1999).

Cultivation of *B. velezensis* AP193 from pond water. To assess the presence of strain AP193 within pond water samples, a 15 mL sample was taken from each pond from surface water selected randomly, on a bimonthly basis. Two separate 100 µL aliquots were removed from each pond sample, with one sample heat-treated at 80°C for 10 min. before serial dilution and plating onto Tryptic Soy Agar (TSA), while the other replicate sample was serially diluted and plated onto TSA without heat inactivation. The inoculated plates were incubated for 24 h at 30°C. After 24 h the CFUs/ml of pond water were determined through plate counting and any colonies with a colony morphology indicative of *B. velezensis* AP193 were selected for molecular confirmation. Each pure culture was used for DNA isolation using a E.Z.N.A.® Bacterial DNA Isolation kit (Omega Bio-Tek, Inc., Norcross, GA). A *B. velezensis* AP193-specific primer set was designed that targeted a genetic locus not found within any of the other *B. velezensis* strains with an available genome sequence (n=32), with the C20_157F primer (5'-ATCGCATTTGGATGTGGATT) and the C20_157R primer (5'-CGTTTCTGAATGGCGCTTAT). The PCR thermalcycling conditions consisted of 5 min at 94°C, followed by 25 cycles of a touchdown PCR with 30 secs at 94°C, 30 secs at 68°C to 60°C (5 cycles at 2°C decreasing increments) and 1 min at 72°C. The PCR results were resolved by agarose gel electrophoresis and any PCR amplicon was purified using an E.Z.N.A Cycle Pure kit (Omega Bio-Tek) and Sanger sequenced using the C20_157F primer at the Auburn Sequencing and Genomics laboratory to confirm the identity of the recovered bacterial isolate as *B. velezensis* AP193.

Statistical Analyses. To analyze the differences between probiotic fed and control fed treatments the aquaria and pond growth performance and mortality data were subjected to one-way analysis of variance (ANOVA) and two-way ANOVA, respectively, followed by Tukey for multiple comparison procedure by mean. A two-way ANOVA was conducted to determine the significance of origin and treatment effects and their interaction during the pond trials. The ANOVA and Tukey comparisons were carried out using Statistical Analysis System version 9.3 (SAS Institute, Inc., Cary, NC, USA) and R version 3.4 (R foundation for Statistical Computing, Vienna, Austria). Water quality data were analyzed for means and standard deviation by ANOVA on ranks followed by Tukey for multiple comparison procedure by means of SigmaPlot version 11.0 statistical software (Aspire Software International, Ashburn, VA, USA).

4. Results

Catfish growth and disease susceptibility in aquaria

Four *B. velezensis* strains (AB01, AP79, AP143 and AP193) that had been previously observed to have the best efficacy in inhibiting *E. ictaluri* infections and in persisting within the catfish intestine were selected for a ten-week aquaria study to assess their effects on catfish growth performance (Figure 1, Panel A). Of the four strains, only the diet amended with *B. velezensis* AP143 indicated little to no effects on fish weight gain or FCR compared to the control (Figure 1, Panel A). Fish fed with a diet amended with the other three *Bacillus* strains did show an improved weight gain and FCR compared to the control (Figure 1, Panel A). The weight gain in fish fed a *B. velezensis* AP193-amended feed showed the most weight gain at 6.78 g per fish and had an equivalent FCR to AP79 which was 1.04 (Figure 1, Panel A). The one-way ANOVA and Tukey Multiple Comparison Test showed that the differences between all the treatment groups were marginally insignificant ($P > 0.05$).

Following ten weeks of control or probiotic-amended feeding, fish were challenged by immersion with *E. ictaluri*. Fish that were fed with feed amended with *B. velezensis* AB01 or AP143 showed a 6% or 11% increase in percent mortality, respectively, when compared with the control. On the other hand, fish that were fed with feed amended with *B. velezensis* AP79 were observed to have a 12% decrease in percent mortality when compared to the control. Among all the treatment groups, the fish fed with feed amended with *B. velezensis* AP193 had the lowest mean mortality of 47.8%, which was 23% lower than the control group mortality of 62.1% (Figure 2; $P = 0.07$). While these results did not reveal a statistically significant change in fish growth performance or disease control in aquaria, *B. velezensis* AP193 had been previously observed to provide significant decreases in fish mortality due to infection with *E. ictaluri* (Ran *et al.*, 2012) or with *A. hydrophila* (Addo *et al.*, 2017), and was therefore selected for evaluation in a replicated pond study.

Catfish growth in ponds

Fish fed with the feed amended with *B. velezensis* AP193 spores were observed to have a significant increase in their weight gain compared to the control fish in the pond study (Figure 1, Panel B; $P = 0.04$). The aquaria-origin fish in the AP193-fed ponds had an average weight gain of 40.08 g compared to the average weight gain for fish in control ponds of 28.55 g, which was a 40.4% increase relative to control fish. The raceway-origin fish had an overall better growth relative to aquaria-origin counterparts, with the fish in control ponds exhibiting an average weight gain of 44.78 g and the fish in ponds fed with AP193 observed to have an average weight gain of 59.37 g, which was a 32.6% increase relative to control fish. There was a significant difference in weight gain between the two fish populations ($P = 0.01$). Fish losses due to bird predation precluded reporting FCRs from the pond study. The differences in average weight in

fish in the control ponds and the AP193-fed ponds at week zero and at week ten were determined and revealed that there was no difference in average weight initially, but that there was an increase in the average weight of the AP193-fed fish relative to control fish by week ten (Figure 1, Panel B; $P = 0.04$).

Detection of strain AP193 from pond water

While no AP193-like colonies were detected in pond water samples at earlier time points, at the eight-week time point the probiotic fed ponds and the control ponds had means of 25 CFU/mL and 10 CFU/mL for colonies with an AP193-like colony morphology, respectively. Of the four colonies tested from each pond, 100% of the colonies isolated from the probiotic fed ponds were positively identified as strain AP193, whereas only 50% of the control pond isolated colonies were positive with the AP193-specific primer set (Data not shown). There was no observable difference in AP193 colony detection after week 8.

Pond Water Quality

Significant differences were observed between the control and probiotic fed ponds for total phosphorus, total nitrogen, and nitrate-nitrogen (Table 2). Total phosphorus was lower in concentration in treated ponds than control ponds, with means of 0.110 mg/L and 0.136 mg/L, respectively ($P = 0.014$). Total nitrogen was also lower in treated ponds, 0.195 mg/L, then in control ponds, 0.344 mg/L ($P = 0.025$). Nitrate-nitrogen also followed this trend with the greatest difference with 0.013 mg/L in treated ponds and 0.051 mg/L in control ponds. There were no differences found between the treatments for total ammonia nitrogen and nitrite-nitrogen ($P > 0.05$).

5. Discussion

In previous research, a collection of *Bacillus* spp. strains was observed to have *in vitro* antagonistic activity against multiple aquatic pathogens and to persist within channel catfish intestines after the cessation of spore-amended feeding. Furthermore, when *Platydoras armatulus* (striped catfish) were fed with spore-amended feed significant reductions in mortality relative to the control group was observed after challenge with *E. ictaluri* (Ran *et al.*, 2012). This study was initiated to evaluate the capacity of four specific strains when delivered as a feed additive to 1) enhance the growth of channel catfish under aquaria and pond conditions, 2) reduce mortality due to disease and 3) improve water quality.

The four best-performing *Bacillus* spp. strains (AB01, AP79, AP143 and AP193) from previous studies were selected for further study for their potential as probiotics for use in aquaculture. All four of these strains were found to be affiliated with *B. velezensis* based on phylogenetic analyses (Hossain *et al.*, 2015a), without any predicted virulence factors (Data not shown). It was previously observed that strain AP193 expresses the antibiotic difficidin and that the production of this polyketide is critical for AP193-mediated biocontrol activity in plants (Hossain *et al.*, 2015a). The previous study performed by Hossain and colleagues observed that strain AP193 mutants deficient in difficidin synthesis (Δ*sfp* or Δ*dfnD*) were also completely lacking in the ability to inhibit the *in vitro* growth of *E. ictaluri*, further supporting the hypothesis that difficidin production is important for *E. ictaluri* disease control while leaving open the possibility that other mechanisms (e.g. competitive exclusion, stimulation of fish immune competence) were also involved.

In this study, a significant increase was observed in the growth performance of catfish fed with AP193-amended feed in pond trials, with 40.4% and 32.6% increases in average weight gain for the two populations of fish (aquaria or raceway sourced, respectively) used in the pond

study (P = 0.04). Furthermore, multiple comparisons between the two populations of fish indicated that the raceway-reared fish had a significantly higher weight gain compared to the aquaria reared fish (P = 0.01); However, the difference in weight gain may be due to the differences in starting weights of the two different fish populations. Future research will explore the mechanism(s) by which *B. velezensis* AP193 and other probiotic strains may improve fish growth, including potentially by enhancing feed conversion efficiency and/or by decreasing the levels of the anti-nutrient phytate within the plant-based diet. In support of this latter hypothesis, *B. velezensis* AP193 was observed to express phytase activity that was greater than, or comparable to that of, the other *B. velezensis* strains (Data not shown). The hydrolysis of the phosphate groups associated with phytate, mediated by a probiotic-expressed phytase, could result in more iron availability to support fish growth as well as less phosphate excreted from fish.

Significant reductions in total phosphorus, total nitrogen, and nitrate-nitrogen levels were observed in ponds containing channel catfish fed with AP193 that indicate beneficial, pond-wide effects on water quality. Nitrogen is required for plant growth, but excessive concentrations of nitrate-nitrogen and ammonium in ponds can contribute to dense phytoplankton blooms containing cyanobacterial algal blooms leading to toxic eutrophication and fish “off-flavor” (Boyd, 1982). While a previous study did not show any efficacy in the application of a bacterial amendment directly to pond water (Li and Boyd, 2016), in this study the probiotic applied via feed indicated an improvement in water quality. Soy-based fish feed contains high levels of phytate, which is inositol-hexaphosphate (Cao *et al.*, 2007; Storebakken *et al.*, 1998). Previous research has determined that a hypervirulent strain of *A. hydrophila* causing epidemic outbreaks of motile *Aeromonas* septicemia has the ability to use *myo*-inositol as a sole carbon source,

which suggests that the presence of high levels of inositol in the diet could contribute to *A. hydrophila* pathogenesis (Hossain *et al.*, 2013). Additionally, genomic analysis of *B. velezensis* AP193 indicates that this strain contains a phytase gene (Hossain *et al.*, 2015a), and has been observed to express phytase activity. Thus, *B. velezensis* AP193 has the capacity to degrade the phytate present within feed, potentially resulting in uptake of phosphorus by the intestinal microbiota. Reduced phosphorus excretion into the pond water lessens pond water eutrophication, and presumably also reduces levels of bioavailable inositol that may contribute to *A. hydrophila* pathogenesis. This suggests that *B. velezensis* AP193 could reduce the severity of *A. hydrophila* outbreaks by means of competitive exclusion. Subsequent studies will investigate the benefit of feeding fish with feed amended with *B. velezensis* AP193 in reducing mortality associated with virulent *A. hydrophila*.

Previously, gut microbiota analysis was performed during the aquaria trials conducted by Chao Ran and colleagues. This study indicated that the addition of *B. velezensis* AP193 as a probiotic resulted in no significant changes in the intestinal microbiota. This could indicate that despite the addition of elevated levels (4×10^7 CFU/g feed) of AP193 amended to channel catfish feed there appeared to be stability in the fish intestinal microbiota throughout the ten-week study, in contrast to that observed with the control diet or even with the AP143 treatment (Thurlow *et al.*, 2018a). The absence of a significant alteration to gut microbiota suggests that the probiotic does not interfere with bacteria already present within the catfish intestine, and that AP193 may have a stabilizing effect on the structure of the intestinal microbial assemblage. Further, the fish that were delivered a control diet indicated a decrease in in their relative gut Fusobacteria over time for control samples, as well as an increase of *Proteobacteria* over the ten-week study for control and AP143 treatments, respectively (Data not shown). *Fusobacteria* are gram-negative

anaerobic, rod-shaped bacteria that produce butyrate and are known to be commensal microbiota in the channel catfish intestine (Larsen *et al.*, 2014). Since butyric acid has been observed to inhibit fresh water fish pathogens (Nuez-Ortin *et al.*, 2012), maintaining the level of *Fusobacteria* taxa within the channel catfish intestine may be beneficial. A high relative abundance of *Fusobacteria* taxa within the fish intestine was observed previously (Data not shown; Larsen *et al.*, 2014). Interestingly, during a similar analysis performed on fish gut samples extracted during this pond study the *Fusobacteria* abundance was shown to be reduced within the control fish intestinal microbiota over the course of ten-week trial. Additionally, the probiotic-fed fish had levels of *Fusobacteria* taxa that were only moderately decreased at the ten-week time point. The high relative abundance of *Fusobacteria* was not observed during similar microbiota analyses performed on the pond water (Data not shown; Thurlow *et al.*, 2018a), further indicating that *Fusobacteria* taxa are natural inhabitants of the catfish gastrointestinal tract. Additionally, no significant differences were observed during the pond water microbiota based on a culture-independent based analysis of 16S rRNA gene amplicon relative abundance (Data not shown; Thurlow *et al.*, 2018a).

Due to lower resolution of this genetic marker, no determination could be made regarding the relative abundance of *B. velezensis* or strain AP193. Therefore, I used a culture-dependent approach to determine the levels of *B. velezensis* AP193 in pond water at different time points. The low levels of AP193 (25 CFU/ml) detected in water from ponds in which fish were fed with an AP193-amended diet indicates that some level of the probiotic is present within the pond ecosystem. The observation that strain AP193 was isolated from a control pond, albeit at low levels (5 CFU/ml), could be due to cross-contamination of ponds or due to PCR primer cross-reactivity with other *B. velezensis* strains. These results collectively indicate that feeding channel

catfish feed amended with *B. velezensis* AP193 did not significantly alter the pond water microbial assemblages and that low levels of the probiotic are present in pond water after prolonged feeding.

In conclusion, the addition of AP193 to channel catfish feed resulted in an observed stabilization of the intestinal microbiota composition over time. Additionally, the probiotic AP193 resulted in improved water quality parameters in ponds. These results suggest that AP193 is a viable candidate as a channel catfish probiotic to promote fish growth and reduce aquaculture pond eutrophication, warranting further study in larger scale production ponds over longer time periods.

Table 1. List of bacterial strains used in this study.

Bacterial strains	**Description**	**References**
B. velezensis AB01	Probiotic previously shown to have biocontrol activity	(Ran *et al.*, 2012)
B. velezensis AP79	Probiotic previously shown to have biocontrol activity	(Ran *et al.*, 2012)
B. velezensis AP143	Probiotic previously shown to have biocontrol activity	(Ran *et al.*, 2012)
B. velezensis AP193	Probiotic previously shown to have biocontrol activity	(Ran *et al.*, 2012)
E. ictaluri S97-773	Pathogenic isolate from diseased catfish	(Walakira *et al.*, 2008)

Table 2. Mean concentrations (mg/L) of water quality parameters in control ponds and ponds with catfish fed with control or AP193-amended feed.

Water Quality Parameter	***P* value**	**Control (Mean ± SE)**	**AP193 Treatment (Mean ± SE)**
Total Phosphorus (mg/L)	0.014	0.136 ± 0.010a	0.110 ± 0.013b
Total Nitrogen (mg/L)	0.025	0.344 ± 0.051a	0.195 ± 0.025b
Total Ammonia Nitrogen (mg/L)	0.829	0.142 ± 0.014a	0.137 ± 0.012a
Nitrite-Nitrogen (mg/L)	0.945	0.004 ± 0.001a	0.004 ± 0.001a
Nitrate-Nitrogen (mg/L)	0.044	0.051 ± 0.019a	0.013 ± 0.005b

Labeled means in a column without a common letter differ ($P < 0.05$) ($N = 24$).

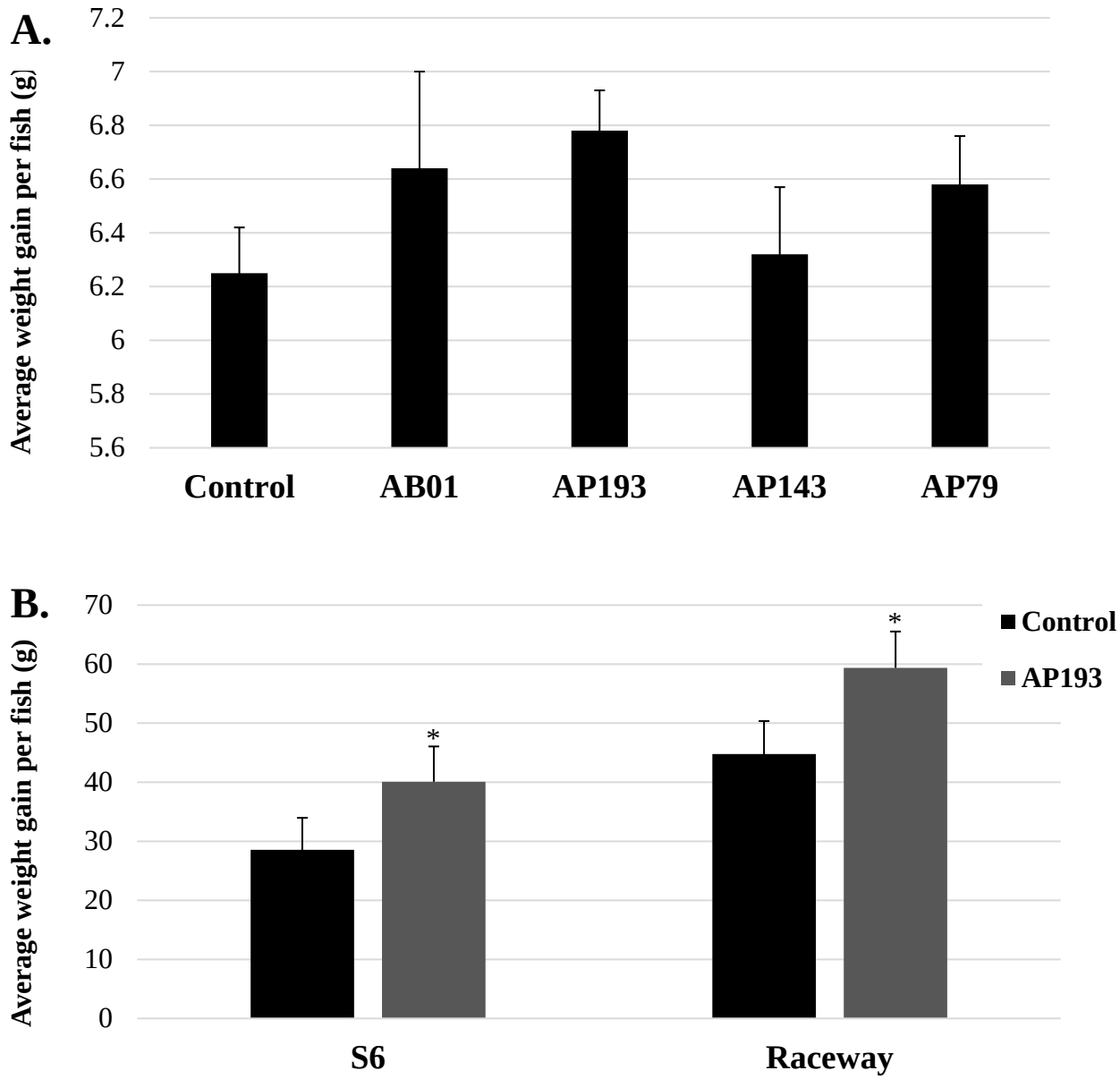

Figure 1. Growth performance of channel catfish after 10 weeks of probiotic feeding in (**Panel A**) aquaria or (**Panel B**) ponds. For the pond study fish were obtained from either aquaria or from a raceway, and fins were clipped to distinguish between the two origins. All data is presented as the average weight gain per fish ± SE of 5 replicate tanks or 4 replicate ponds for Panel A and Panel B, respectively. Asterisk labeled means ($P < 0.05$ compared to 10 weeks control time point).

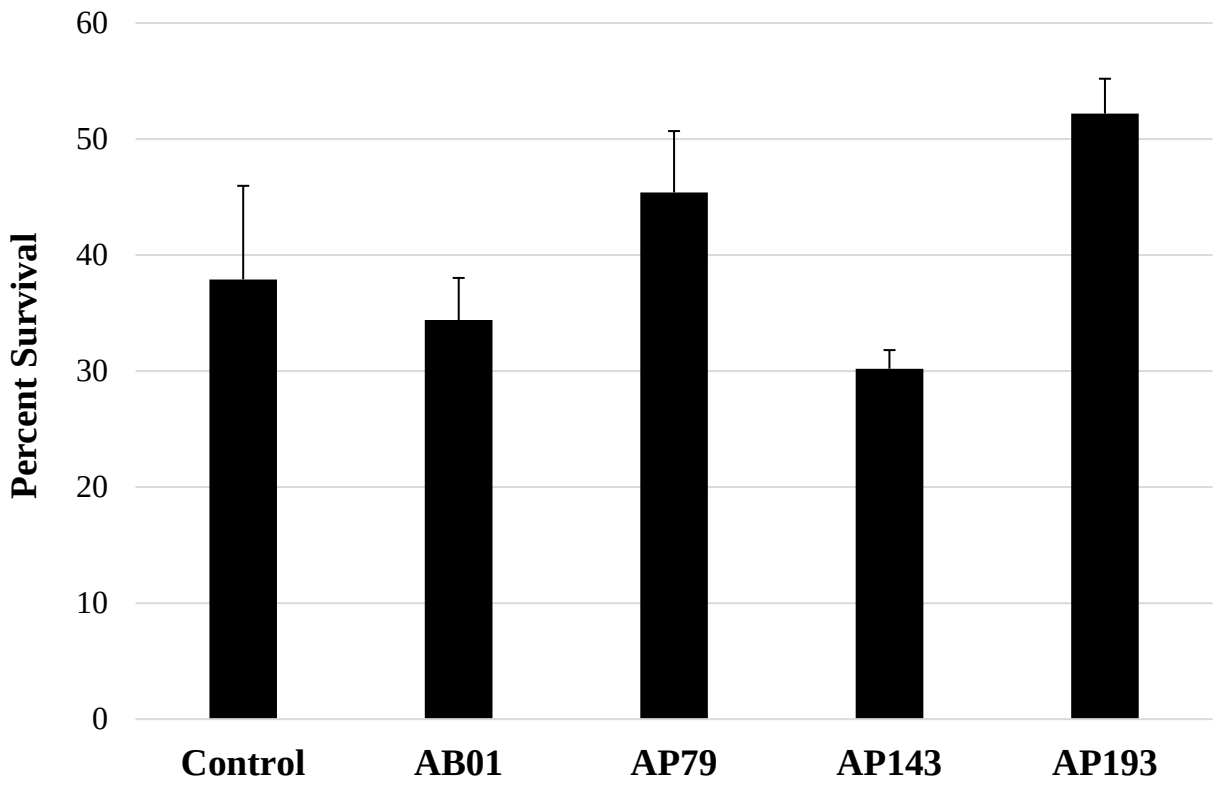

Figure 2. Percent Survival of fingerlings challenged with *E. ictaluri* S97-773 following a 10-week probiotic or control feeding regiment. The percent survival of fingerlings delivered feed amended with AP193 indicated the highest survival rate which was 23% higher than fingerlings delivered a control diet ($P = 0.07$). All data is presented as the average percent survival ± SE of 5 replicate tanks.

Chapter III

Genome modifications of virulent *Aeromonas hydrophila* (vAh) strain ML09-119 using a conjugally transferable recombineering system.

1. Abstract

Generating attenuated bacterial mutants for their use as appropriate vaccines against bacterial pathogens is challenging due to the lack of effective tools for targeted gene deletion. Traditionally, most mutants generated to combat bacterial diseases in aquaculture have been carried out through serial passaging on antibiotic plates. This has been shown to be affective at altering the virulence of the pathogen but presents safety concerns due to the likelihood of genetic reversion, leading to the bacteria reverting to its pathogenic form. One potential alternative to serial passaging is using double cross-over deletion events. This method is labor intensive, due to the requirement of suicide plasmid constructs for each deletion event. Previously, an efficient means of targeted gene deletion was carried out using a modified PCR-based λ Red-mediated recombineering system. This system allows for rapid, site-specific deletion event through homologous recombination of small PCR generated amplicons. These amplicons generally contain 30 to 50 base pairs of homologous regions to the gene of interests, flanking an antibiotic cassette for adequate selection of mutants. This site-specific integration is efficient, but due to the presence of antibiotic cassettes, presents safety concerns with vaccine candidates. Previously, a dual expression plasmid was generated that contains the λ Red-

recombinase system for the initial homologous recombination event and Flp recombinase for removal of the homologous recombined antibiotic cassette, leaving behind a marker-less mutant. The expression of these two recombinase systems are under the control of arabinose and rhamnose inducible promotors, allowing for each system to be turned on at various times. This was shown to be effective, however, due to the size of the vector, it held a low efficiency for conjugation. Here I present a small temperature sensitive *flp* recombinase plasmid that has a high conjugation efficiency in *A. hydrophila*. This plasmid can be conjugally transferred to mutants generated post λ Red-mediated deletion for the generation of marker-less mutants.

2. Introduction

Many early approaches for attenuating virulent bacteria included methods that lacked specificity for targeted genetic manipulation (Ranallo *et al.*, 2006). Traditional approaches that were site specific, such as double-crossover methods, are labor intensive and often very difficult to achieve due to low transformation frequencies (Hossain *et al.*, 2015a, Hirayama *et al.*, 2012, Jost *et al.*, 1997, and Li *et al.*, 2013). Due to lacked specificity and labor needed for classical methods of genetic manipulation, many of these methods have been replaced by genetic engineering techniques exploiting homologous recombination, commonly referred to as "Recombineering" (Murphy and Campellone, 2003).

One recombineering technique is derived from the homologous recombinase activity of the λ phage, which has been shown to function in many gram-negative bacteria, such as *E. coli* and *Salmonella* sp. (Jost *et al.*, 1997, Court *et al.*, 2002, Datsenko and Wanner, 2000, Murphy, 1998, and Yu *et al.*, 2000). λ phage has been shown to shut down host repair and exonuclease machinery to successfully integrate its own genome into the bacterial chromosome for the continuation of its lifecycle. Through the study of the λ phage in *Escherichia coli*, three very

important genes have been shown to play a crucial role in the phage's ability to recombine into the bacterial chromosome. The *gam* gene shuts down the RecBCD complex, preventing the destruction of the incoming dsDNA. The *exo* gene holds a 5'-3' dsDNA exonuclease activity which degrades the dsDNA into ssDNA. The *bet* gene plays a key role in annealing the ssDNA, degraded by the *exo* gene, to complementary regions of the bacterial chromosome for homologous recombination (Murphy and Campellone, 2003). The function of exo, bet, and gam is vital to the integration and proliferation of the λ phage, but this machinery can also be exploited for mutant generation, commonly used for pathogen virulence studies and vaccine creation (Ranallo *et al.*, 2006 and Hossain *et al.*, 2015b). This is commonly accomplished by the creation of PCR derived selectable markers, flanked with homologous regions to the target gene on the bacterial chromosome. Therefore, by exploiting the λ red recombinase system, a site-specific integration of a selectable marker into a target gene can disrupt the genes function and allow for mutant selection. Disrupting virulence genes through the integration of a selectable marker is a vital form of mutant selection. However, due to stringent regulatory approval of live vaccines (Gudding and Goodrich, 2014) and the potential of antibiotic resistance from horizontal gene transfer, it is advantageous to remove the selectable marker.

The Flp recombinase is one recombineering system that could assist in generating marker-less mutants. The mechanism of the Flp/FRT recombinase system relies heavily on flanking FRT sites that are located on the 5' and 3' ends of the target gene on the bacterial chromosome (Ellermeier *et al.*, 2002). These FRT sequences can be placed in the genome through λ Red-mediated deletions. Subsequently, the Flp recombinase can be introduced into bacteria using a mobilizable plasmid (Hossain *et al.*, 2015b and Ellermeier *et al.*, 2002). The FLP recombinase recognizes and binds to the FRT sites, causing for site specific excision of genetic

elements contained between the flanking FRT regions. To select for FRT mutants lacking the integration of a selectable marker, *sacB* gene (along with other antibiotic resistance genes) are commonly placed between the FRT sites. This is very important because this presents a recombinase system that can be used for both marked and unmarked mutagenesis (Ishikawa and Hori, 2013 and Cherepanov and Wakernagel, 1995). Here I present data outlining the development of a temperature sensitive Flp recombinase plasmid that can be used in conjunction with the λ red recombinase to generate marker-less mutants.

3. Materials and Methods

Bacterial strains, plasmids, and culture conditions. The list of bacterial strains and plasmids used in this study is presented in Table 1. *A. hydrophila* ML09-119 (written as vAh in this chapter) and *E. coli* SM10λpir were initially revived from -80°C stocks and streaked for isolation onto TSA at 30°C and 37°C, respectively, for 24 h. Isolated colonies were then placed into sterile TSB overnight, after which time, they were diluted 1:100 in all subsequent cultures. *E. coli* SM10λpir (Simon *et al.*, 1983) was routinely used for conjugation of the plasmid to *A. hydrophila*. When required, growth medium was supplemented with tetracycline (10.0 µg/ml), chloramphenicol (25 µg/ml), and colistin (10.0 µg/ml).

Generation of pCMT-flp. The list of primers used to generate pCMT-*flp* are listed in Table 2. All primers were purchased from Eurofins MWG Operon (Huntsville, AL). PCMT-*flp* was routinely extracted using FastPlasmid Mini Kit (5 PRIME, Inc. Germantown, MD). Restriction enzymes and T4 DNA Ligase (Quick ligase) used for generating pCMT-*flp* were purchased from New England Biolabs (NEB, Ipswich, MD). Restriction digested DNAs with sticky ends were blunt-ended using DNA Terminator kit (Lucigen, WI). The *flp* gene, which is required for FRT mediated site-specific recombination (Cherepanov and Wackernagel, 1995)

was PCR amplified from pCP20 using primers Flp-pRhamF and Flp-pRhamR and was cloned into pRham N-His SUMO vector (Lucigen Corp.) under the control of the rhaPBAD promoter. The resulting plasmid pRham-*flp* was then digested with XbaI and blunt-ended to insert a tetracycline resistance gene (*tetA*) which was PCR amplified from pMJH65 using primers tetAF and tetAR. After cloning this *tetA* cassette into the pRham-*flp* plasmid, resulting in plasmid pRham-*flp-tetA*, the *flp-tetA* cassette was digested with AlwNI and BsaAI, and blunt-ended for cloning into the *repA101-oriR101* cassette which was PCR amplified from pMJH65 using primers UP-F-flp-oriR and DN-R-oriT. After cloning *flp-tetA* into *repA101-oriR101* cassette, the construction of the resulting plasmid pCMT-*flp* was confirmed by Ilumina MiSeq sequencing using sequencing primers FlpFa, FlpR1, FlpF2, and FlpR2. To determine the efficacy of pCMT-*flp* plasmid in excision of an antibiotic resistance cassette flanked by FRT sequences, pCMT-*flp* was transferred into strains of vAh mutants by conjugation (Hossain *et al.*, 2015b).

Conjugal transfer of pCMT-flp. pCMT-*flp* was introduced into *E. coli* SM10λpir through electroporation according to methods previously published with slight modifications (Sambrook *et al.*, 1998). For generating electrocompetent cells, *E. coli* SM10λpir was reactivated from -80°C stocks onto sterile TSA plates as described above. One isolated colony was placed into TSB and incubated overnight at 37°C with shaking at 200 rpm. The overnight culture was then diluted (1:100) into 150 ml of Super Optimal Broth (SOB) and incubated at 37°C with shaking for 3 h (OD_{600} = 0.5). Following incubation, the culture was separated into two ~75 ml aliquots and centrifuged at 8,000 x g for 10 min. at 4°C. The bottles were placed on ice immediately after centrifuging and the supernatant was aspirated. The cells were washed with sterile 10% glycerol, prechilled to 4°C and centrifuged at 8,000 x g for 10 min. at 4°C. This spin/wash was repeated three times. After washing, the cells were gently resuspended in 200 µl

of 10% glycerol, taking care not to introduce bubbles. The electrocompetent cells were then placed at -80°C until electroporation. For electroporation of pCMT-*flp* into SM10λpir, electrocompetent SM10λpir was thawed on ice and separated into 50 µl aliquots and combined with 4 µl of pCMT-*flp* (3.80 ng/µl aqueous). This reaction was gently stirred and pipetted into a prechilled electroporation cuvette, taking care to minimizing the introduction of bubbles. The cuvette was gently tapped to deposit cells across the bottom of the cuvette. The cells were then electroporated at 1600 V for ~ 4.5 ms. After electroporation, 1 ml of SOC (SOB + 20mM glucose) was added directly to the electroporated cells in the cuvette. The SOC with electroporated cells were incubated at 30°C with 200 rpm shaking for 1 h. 100 µl aliquots were placed onto 2x YT agar plates supplemented with tetracycline to select for cells containing pCMT-*flp*. Once inside *E. coli* SM10λpir, pCMT-*flp* was conjugally transferred into vAh according to the methods described previously with modifications (Maurer *et al.*, 2001). VAh mutants containing a chloramphenicol cassette flanked by FRT regions, post λ red mediated deletion event, were incubated in 5 ml cultures supplemented with chloramphenicol. *E. coli* SM10λpir were incubated in 5 ml cultures supplemented with tetracycline to select for transformants containing pCMT-*flp*. Both cultures were incubated at 30°C with shaking at 200 rpm until an OD_{600} = 1.0. 500 µl aliquots of both vAh and *E. coli* SM10λpir cells were centrifuged, separately, at 10,000 x g for 10 min., at which time, the supernatant was aspirated, and the cells washed with 500 µl of LB. This spin/ wash step was repeated twice, and the cells were resuspended in 500 µl of LB. 500 µl of vAh cell solution were added to 125 µl of the SM10λpir cell solution containing pCMT-*flp* (4:1 ratio) and placed onto LB plates and incubated for 12 h at 30°C. 1/10 of the bacteria was removed from the LB plates and spread onto LB plates containing tetracycline and colistin and incubated at 30°C for 24 h. Isolated colonies were tested

for their presence of cytochrome c oxidase and further incubated in LB broth media supplemented with tetracycline and colistin to verify presence of vAh cells containing pCMT-*flp*.

Unmarked mutant generation through Flp-mediated excision by pCMT-flp. Prior to the removal of the antibiotic cassette, the vAh mutants were cured of pMJH65 containing the λ red recombinase machinery. Initially, cells were grown in fresh TSB at 30°C until an OD_{600} = 1.0 The mutants were subjected to heat induction at 43°C for 1 h with shaking at 200 rpm, then grown at 37°C overnight and streaked for isolation onto TSA plates to recover cured cells. The loss of pMJH65 from vAh was confirmed by an inability of cells to grow in the presence of tetracycline. After curing, vAh mutants, containing the integrated chloramphenicol cassette flaked by the FRT regions, were conjugated with SM10λpir containing pCMT-*flp* as described above. Flp recombinase activity was induced for the removal of the chloramphenicol resistance gene cassette through incubating at 30°C until an OD_{600} = 1.0, then shifting the temperature to 37°C for 1 h. The induced cultures were then streaked onto fresh non-selective TSA plates and incubated for up to 36 h at 30°C. Isolated colonies that failed to grow in the presence of chloramphenicol were tested by PCR and sequencing to confirm the Flp-mediated scar. Curing of pCMT-*flp* was performed on all marker-less mutants following the protocol above.

4. Results

Removal of antibiotic resistance cassette by pCMT-flp.

The plasmids with machinery optimized for targeted deletion are displayed in Figure 1. Red recombinogenic plasmid pMJH65 (generated from plasmid pMJH46) was necessary for the introduction of the FRT sites prior to the introduction of Flp recombinase plasmid pCMT-*flp* (Hossain *et al.*, 2015b). Upon generation of both the marked and marker-less mutant, pMJH65 and pCMT-*flp* are easily cured after heat induction at 43°C, respectively, due to the presence of

temperature sensitive *repA101* gene. Post curing of pMJH65 and conjugation with SM10λpir containing pCMT-*flp*, all marked vAh mutants that grew in the presence of chloramphenicol, tetracycline, and colistin on TSA were verified for their ability to grow in TSB in the presence of chloramphenicol, tetracycline, and colistin. This was deemed necessary, as some transconjugants were identified as false positives after growing on TSA. Further, every conjugation event tested with pCMT-*flp* indicated high efficiency of transconjugation (Data not shown). This conjugation efficiency observed seemed to be much higher with pCMT-*flp* when compared to the previously created dual expression plasmid, which often showed over 10^6 transconjugants compared to few or no transconjugants, respectively. After inducing Flp recombinase activity in these vAh transconjugants, over 10% of the mutants tested indicated a lack of an ability to grow in the presence of chloramphenicol (Data not shown; Hossain *et al.*, 2015b).

5. Discussion

The genetic manipulation of pathogenic isolates for virulence studies and vaccines presents many challenges including antibiotic resistance, poor recombination efficiency, and reduced ability to transform plasmids, containing the necessary recombination machinery, into isolates (Murphy, 1998, Matsuda *et al.*, 2005, and Hossain *et al.*, 2015b). Previously, there was an observed inability to electroporate plasmids into vAh, which appeared to be amplified with plasmids of larger size (Hossain *et al.*, 2015b).

Alternatively, conjugation could be used to rapidly and efficiently introduce the machinery necessary for targeted deletion (Hossain *et al.*, 2015b). Further, through the presence of *repA101*, the vector, carrying the antibiotic resistant cassette, could be cured from the mutants. However, the means of removing the selectable marker from the genome of the mutants proved to be problematic. Previously, the introduction of pCP20 was shown to be effective in generating

marker-less *Edwarsiella ictaluri* mutants (Hossain *et al.*, 2015b), however this plasmid was incompatible for conducting FLP/FRT mediated recombination in vAh due to the presence of the chloramphenicol resistance cassette (*cat* gene). Further, the presence of the two separately controlled recombinase machineries found in the dual expression plasmid gave it an increased size which appeared to reduce its conjugation efficiency in vAh. This contributed to delays during targeted mutagenesis, which led to the postponement of aquaria virulence studies.

The above concerns prompted the creation of pCMT-*flp*, a vector a third the size of the previously generated dual expression plasmid and almost half the size of pCP20. Additionally, this vector was compatible with the vAh mutants due to the integration of the *tetA* gene in place of the *cat* gene found in pCP20. PCMT-*flp* and the dual expression plasmid, varied in their induction of the Flp recombinase. As described earlier, the Flp recombinase, and its promotor, found in pCMT-flp was amplified from pCP20. PCP20 was chosen due to its thermal induction of the FLP synthesis at 37°C (Kirill and Wanner, 2000). Alternatively, the pRham promotor (rhaBAD) present in the dual expression plasmid was induced by the presence of rhamnose. Previously, the rhaBAD promotor is controlled by the regulatory cascade of RhaR and RhaS (Meisner and Golberg, 2016 and Egan and Schleif, 1993). RhaR is a transcription factor that binds rhamnose and activates transcription of the *rhaS* gene. Through the accumulation of RhaS, RhaT is activated allowing transport of rhamnose into the cells (Via *et al.*, 1997, Meisner and Golberg, 2016, and Bhende and Egan, 2000). The *rhaSRT* cluster had been added to the dual expression plasmid to induce the FLP recombinase post λ red genetic manipulation. The addition of a rhamnose inducible promotor in the dual expression plasmid allowed for an intelligent plasmid that could be tightly controlled, however, this cluster increased the size of the vector. Alternatively, pCP20 has been shown to be thermally inducible, with the *flp* gene under the

control of the λ cI857 repressor (Merlin *et al.*, 2002). However, there is a basal expression of Flp observed at 30°C, leading to an ample amount of Flp to catalyze the FRT recombination at temperatures between 30°C and 37°C (Merlin *et al.* 2002 and Meisner and Goldberg, 2016).

FRT recombination was observed in pCMT-*flp* while growing the vAh transconjugants at 30°C and due to the similarities in the *flp* promotors, I hypothesize that the Flp recombinase activity of pCMT-*flp* is constitutively active in vAh at 30°C – 37°C, similarly to pCP20. However, this has not been adequately investigated. Theoretically, having a constitutively active promotor could lead to a reduction in control of the Flp recombinase when compared to a recombinase system that holds a tightly controlled promotor (Meisner and Goldberg, 2016). Regardless, due to the addition of the Flp recombinase machinery post λ red deletion, having a vector with a decreased size was of greater importance when compared to the regulation of the recombinase activity.

Table 1. The list of bacterial strains and plasmid used in this study.

Bacterial strains	Relevant features	References
E. coli		
E. coli SM10λpir	*thi-1thr leutonAlacYsupE recA*::RP4-2-TcT::Mu Km^r *λpir*	Simon *et al.*, 1983
A. hydrophila		
A. hydrophila ML09-119	Wild-type virulent *Aeromonas hydrophila* (vAh) isolate	Hemstreet, 2010
Plasmids		
pMJH46	Conjugally transferrable recombinogenic plasmid used to generate pMJH65	Hossain *et al.*, 2015b
pMJH65	Conjugally transferrable recombinogenic plasmid used for targeted gene deletion in vAh and used to create pCMT-*flp*	Hossain *et al.*, 2015b
pCMT-flp	Temperature-sensitive Flp recombinase plasmid used to generate marker-less vAh mutants	This study and Hossain *et al.*, 2015b
pMJH97	*cat-oriT-oriR* backbone plasmid for complementation of mutants	Hossain *et al.*, 2015b

pCP20	Temperature-sensitive Flp recombinase plasmid	Cherepanov and Wackernagel, 1995

Table 2. List of primers used in this study.

Primer Name	Sequence in 5’ to 3’ direction
Flp-pRhamF	CGCGAACAGATTGGAGGTCCACAATTTGGTATATTATGTA
Flp-pRhamR	GTGGCGGCCGCTCTATTATATGCGTCTATTTATGTAGGA
tetAF	CGACAGGAGCACGATCAT
tetAR	TGTAGCACCTGAAGTCAGC
UP-F-flp-oriR	ATGGCTTCCATGTCGGCAGAAT
DN-R-oriT	TTGGTGTATCCAACGGCGTCAGCCGGGCAGGATAGGTGAAG TAGGCCCACCCGCGAGCGGGTGTTCCTTCTTCACTGTCCCTT ATTCGTTCCACTGAGCGTCAGACC
FlpF1	CGCATTCACAGTTCTCCGCAAG
FlpR1	GTGCCTACTAACGCTTGTCT
FlpF2	CTTCGATCATTGGACCGCTG
FlpR2	CGAATCATCGGAAGAAGCAG

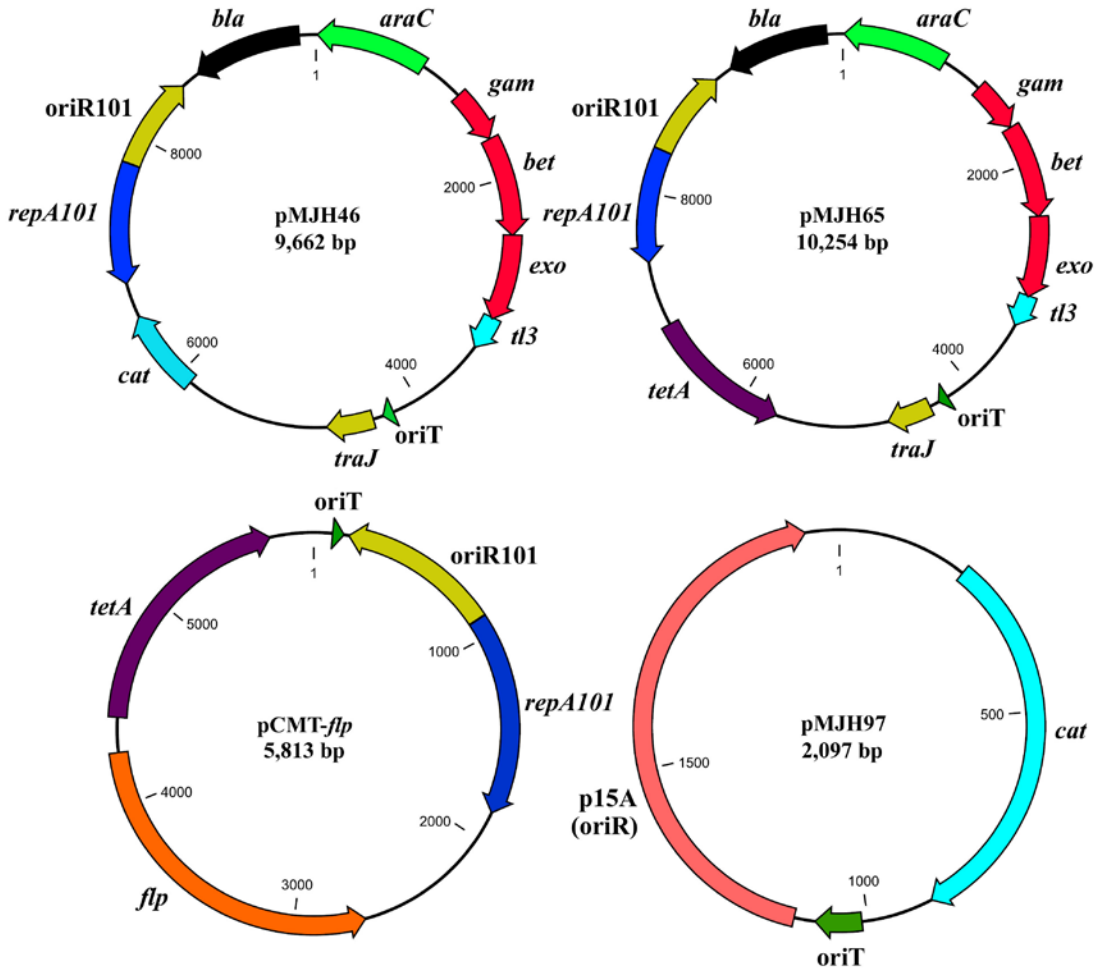

Figure 1. Schematic maps of conjugally transferable λ recombinogenic and Flp recombinogenic plasmids used for generating marker-less single-gene knockouts. pMJH46 was used for generating pMJH65, as pMJH46 was found incompatible with vAh due to the *cat* cassette. PMJH65 was used to induce the primary disruption into the vAh genome. PCMT-*flp* was used post pMJH65 to generate marker-less mutants. PMJH97 is an empty vector used for the complementation of the marker-less vAh mutants (discussed in further detail in Chapter IV of this dissertation). Plasmid maps were generated by CLC Genomics Workbench (version 4.9).

Chapter IV

O-antigen capsule assembly contributes to *Aeromonas hydrophila* virulence in channel catfish.

1. Abstract

A hypervirulent *A. hydrophila* (vAh) pathotype has been identified as the etiologic agent responsible for disease outbreaks in farmed carp species and channel catfish (*Ictalurus punctatus*) in China and the southeastern United States, respectively. Disease isolates from both fish species were found to encode a novel O-antigen biosynthesis and assembly gene cluster that is conserved in vAh strains. Previously, the chemical structure of the vAh O-antigen was determined by chemical analysis, Smith degradation, mass spectrometry, and 2D proton and carbon NMR spectroscopy and found to be unique among described bacterial O-antigens. The O-antigen consists of a hexasaccharide repeating unit featuring a 4)-α-L-Fuc*p*-(1-3)-β-D-Glc*p*NAc-(1-4)-α-L-Fuc*p*-(1-4)-β-D-Glc*p*-(1- backbone, substituted with single residue side chains of α-D-Glc*p* and α-D-Qui*p*3NAc linked to O-3 of the two fucose residues. To assess the role of this novel O-antigen in vAh virulence, genes predicted to be required for O-antigen synthesis and capsule assembly were deleted. Deletion of genes predicted to be required for formation of the LPS-associated O-antigen, such as the O-antigen ligase (*waaL*) and O-antigen polymerase (*wzy*), did not attenuate vAh virulence. However, an adjacent *gfc* (group 4 capsule) genetic operon that is predicted to play a role in group 4 capsule (G4C) assembly was observed

to be required for vAh virulence in channel catfish. Furthermore, the vAh *gfcD* mutant was found to lack significant biofilm formation or buoyancy compared to wild-type vAh and induced an adaptive immune response that protected catfish from vAh challenge. This study indicates the importance of the G4C polysaccharide assembly process in the pathogenesis of this highly virulent *A. hydrophila* pathotype.

2. Introduction

Bacterial pathogen *A. hydrophila* is the causative agent of motile *Aeromonas* septicemia (MAS) in channel catfish. The highly virulent *A. hydrophila* (vAh) isolates affiliated with sequence type 251 (ST251) are responsible for epidemic MAS outbreaks in channel catfish and are highly virulent to channel catfish as compared to typical opportunistic *A. hydrophila* isolates from diseased fish (Pridgeon and Klesius, 2011). Historically, *A. hydrophila* has been considered as an opportunistic pathogen in stressed or immunocompromised channel catfish. However, vAh strains apparently act as a primary pathogen in healthy channel catfish, with high mortality observed in mature, market-sized fish (Bebak *et al.*, 2015).

Comparative genomics (Hossain *et al.*, 2013) and phylogenetic (Hossain *et al.*, 2014) analyses of vAh strains isolated from Asian carp and channel catfish from China and the US, respectively, showed that they shared a recent common ancestor, with MAS outbreaks occurring in China in the late 1980s whereas the first documented occurrence in the US was a case in 2004 in Mississippi (Hossain *et al.*, 2014, Pang *et al.*, 2015). Since that time, MAS due to vAh strains has spread across the southeastern United States and has led to the mortality of over 20 million pounds of channel catfish in the State of Alabama alone (Rasmussen-Ivey *et al.*, 2016). VAh isolates are widespread in the US and China and pose a continuous threat for commercial fish farming in these countries.

There have been a large number of predicted virulence factors seemingly acquired by horizontal transfer in vAh strains (Bebak *et al.*, 2015, Hossain *et al.*, 2013, Pang *et al.*, 2015, and Rasmussen-Ivey *et al.*, 2016); however, their roles in virulence have not been studied experimentally (Hossain *et al.*, 2013). One putative virulence factor is the O-antigen capsule, as the capsular polysaccharides of other bacterial pathogens have been shown to play significant roles in bacterial pathogenesis by aiding host colonization (Magee and Yother, 2001), invasion of host cells (Merino *et al.*, 1997), biofilm formation (Crawford *et al.*, 2008), and/or preventing phagocytosis by host macrophages (Locke *et al.*, 2007). In *E. coli*, capsules are classified in four different groups based on their assembly and secretion to the outer membrane (Whitfield, 2006). The group 4 capsule (G4C) consists of oligosaccharide repeats comparable to that of the LPS-associated O-antigen, hence the name "O-antigen capsule" (Whitfield, 2006, Peleg *et al.*, 2005). The G4C polysaccharides have been previously characterized in *E. coli* (Peleg *et al.*, 2005), *Shigella sonnei* (Caboni *et al.*, 2015), and *Salmonella* (Gibson *et al.*, 2006) . In *S. sonnei* the G4C was found to be responsible for virulence modulation in a rabbit infection model (Caboni *et al.*, 2015).

Previously, the glycosyl composition and structure of the *A. hydrophila* ML09-119 O-antigen was determined and observed to be novel. Presented hereafter is data identifying the composition of the O-antigen capsule further confirming it as a G4C with comparable oligosaccharide repeats to that of the LPS-associated O-antigen. Additionally, presented here is data indicating the role of LPS-associated O-antigen and G4C assembly in vAh pathogenicity to channel catfish.

3. Materials and Methods

Animal welfare statement. All Channel catfish challenges were conducted under the approval of the Animal Care and Use Committee (IACUC) of Auburn University in compliance with U.S. regulatory standards for the humane care and use of laboratory animals.

Bacterial strains, plasmids and Culture Conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Oligonucleotide primers used for mutant construction, complementation and sequencing are listed in Table 2. Primers were purchased from Eurofins Operon (Huntsville, AL). *A. hydrophila* strain ML09-119, which was isolated from a diseased catfish derived from an epidemic outbreak of MAS in channel catfish in Alabama in 2009 (Hemstreet, 2010, Hossain *et al.*, 2013, and Tekedar *et al.*, 2013), was used in this study as a well characterized vAh strain. *A. hydrophila* ML09-119 and its respective mutant strains were routinely grown on trypticase soy broth (TSB) and trypticase soy agar (TSA) at 30°C with and without shaking at 200 rpm. *E. coli* strains SM10λpir and CC118λpir were grown on 2×YT medium at 37°C. When required, growth media were supplemented with sucrose (15%), chloramphenicol (12.5 µg/ml or 25.0 µg/ml), tetracycline (10 µg/ml), and/or colistin (10 µg/ml).

Structural Analysis of the O-antigen Capsule. *Extraction of the O-antigen capsule (G4C).* The capsule was extracted from *A. hydrophila* ML09-119 following previously described methods with modifications (Peleg *et al.*, 2005). For preparation of the total cellular polysaccharides, ML09-119 was grown for overnight in TSB at 30°C with shaking. The overnight grown culture of *A. hydrophila* ML09-119 was diluted 1:100 into 300 ml of fresh TSB and incubated statically at 30°C for 48 h and then the culture was subjected to centrifugation at 8,000 ×*g* for 10 min and resuspended in 3 ml of sterile 1× phosphate-buffered saline. An equal volume of saturated phenol (pH = 8.0) was added and the mixture was incubated for 30 min at 70°C with occasional mixing. After 30 min, the mixture was subjected to centrifugation at

10,000 ×*g* for 1 h. The aqueous layer was aspirated and mixed with 2 volumes of 100% ethanol and incubated at 70°C for 1 h to precipitate polysaccharides. The mixture was then subjected to centrifugation at 12,000 ×*g* for 30 min. The supernatant was aspirated, and the pellet was washed with 500 µl of 70% ethanol, subjected to centrifugation at 12,000 ×*g*, dried, and stored at -80°C until further separation of the capsule polysaccharide. To separate the capsule polysaccharide, the dried total polysaccharide preparations were resuspended in 500 µl of sterile nuclease free water and subjected to ultracentrifugation at 86,000 ×*g* for 1 h. Following the ultracentrifugation, the supernatant containing the capsule polysaccharide was aspirated. To remove any residual LPS contamination from the supernatant, a phase partition was performed with Triton X-114. Triton X-114 was added to the supernatant to a final concentration of 1%. This mixture was incubated at 4°C for 1 h with constant inversion to ensure a homogenous solution. To separate the Triton X-114 and LPS micelles, the mixture was then incubated at 37°C for 10 min and subjected to centrifugation and 18,000 ×*g* at 25 °C for 1 h. The resulting aqueous phase containing the capsule was centrifuged, lyophilized, and submitted to the CCRC (Complex Carbohydrate Research Center, Athens, GA) for NMR analysis.

NMR Spectroscopy. Samples were deuterium exchanged by dissolving in D_2O (99.9% D, Sigma) and lyophilizing. The samples were then dissolved in 0.28 ml D_2O (99.996% D, Cambridge Isotope Laboratories) and transferred to 5-mm precision NMR tubes with D_2O-matched magnetic susceptibility plugs (Shigemi). 1D Proton and 2D TOCSY and NOESY NMR spectra, run with water presaturation, and gradient enhanced COSY and HSQC spectra were acquired on a Varian Inova-600 MHz spectrometer, equipped with a cryoprobe, at 50°C. Chemical shifts were measured relative to internal acetone (δ_H =2.218 ppm, δ_C=33.0 ppm).

Phylogenetic analysis of *gfc* operon. The evolutionary history of each of the genes in the *gfc* operon was inferred using the Maximum Likelihood method based on the JTT matrix-based model using MEGA7.0 (Kumar *et al.*, 2016). The tree with the highest log likelihood was used for inference. The percentage of trees in which the associated taxa clustered together is shown next to the branches of each tree. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016).

Generation of Lipid A-Core ligase (*waaL*) and O-antigen polymerase (wzy) mutants by allelic exchange. Non-polar targeted gene deletions in the *A. hydrophila* ML09-119 O-antigen ligase gene *waaL* were constructed using suicide plasmid pDMS197 (Edwards *et al.*, 1998). To generate a *waaL* deletion mutant, two primer sets (LiupF/LiupR and LidnF/LidnR) were used to amplify approximately 400 bp upstream and downstream sequences of the *waaL* gene, respectively, using ML09-119 genomic DNA as template. The chloramphenicol acetyltranferase gene (*cat*) was amplified from plasmid pMJH46 (Figure 1, Chapter 3 and 15) using primers catF and catR. Primers LiupR and LidnF contained reverse complemented sequences of catF and catR primers, respectively, at their 5′-ends. The two PCR fragments were fused to the flanking *cat* cassette by splicing by overlap extension PCR (SOE) (Horton *et al.*, 1989) using primers Liup-intF and Lidn-intR. The suicide plasmid pDMS197 was digested with XbaI and blunt ended using the DNA terminator end repair kit (Lucigen, Middleton, WI). The PCR product was ligated to pDMS197 using quick ligase (NEB, Ipswich, MA) and

electroporated into *E. coli* SM10λpir to generate plasmid pDMS197waaL that contained sequences upstream and downstream of the *waaL* gene and therefore replaced *waaL* with a *cat* cassette. The suicide plasmid pDMS197waaL was introduced into *A. hydrophila* ML09-119 by conjugation with *E. coli* SM10λpir bearing plasmid pDMS197waaL. Single cross-over mutants of *A. hydrophila* Ml09-119 were selected on TSA plate supplemented with chloramphenicol, tetracycline and colistin. Double cross-over mutants were obtained by plating isolates onto LB agar plates supplemented with 15% sucrose and 12.5 µg/ml chloramphenicol. Mutants grown on this selective medium were subjected to phenotypic and genotypic characterizations. The complete deletion of *waaL* was confirmed by PCR followed by Sanger sequencing at the Auburn University Genomics and Sequencing Laboratory. The *wzy* gene from vAh strain ML09-119 was deleted according to the methods described above using primer listed in Table 2. The presence of the O-antigen was determined by LPS extraction using a LPS Extraction Kit (Portsmouth, NH).

Generation of *waaL* and *gfc* operon mutants by recombineering. *A. hydrophila* isogenic mutants *waaL*, *gfcBCD*, *gfcD*, *gfcC* and *gfcB* were generated by recombineering according to methods described previously (Hossain *et al.*, 2015b). Briefly, a genome-integrated *cat* cassette with flanking FRT sequences was PCR amplified from the genomic DNA of mutant *A. hydrophila* ML09-119*vgr3::cat* using a primer pair (Table 2) that contained 60 bp homologous sequence of targeted genes at their 5′ and 3′-ends. Purified PCR amplicons were introduced by electroporation into electrocompetent *A. hydrophila* ML09-119, which harbored recombinogenic plasmid pMJH65 and was grown in the presence of L-arabinose. Electroshocked *A. hydrophila* ML09-119 cells were recovered in SOC medium after overnight incubation at 30°C with shaking at 200 rpm. Mutants were selected on 2×YT plates supplemented with 25 µg/ml chloramphenicol after growth overnight at 37°C. Mutants were streaked onto TSA plates and incubated overnight

at 37°C for the removal of the temperature sensitive plasmid pMJH65. Mutants were transferred to TSB broth with 25 µg/ml chloramphenicol and grown for overnight. The removal of plasmid pMJH65 from mutant strains was confirmed by their lack of growth in TSB supplemented with to tetracycline (10 µg/ml). Markerless mutants of *A. hydrophila* were generated using *flp* recombinase plasmid pCMT-flp according to methods described in chapter 3 (Hossain *et al.*, 2015b). The genotypes of the mutants were verified by PCR followed by Sanger sequencing using pair of primers specific to immediate upstream and downstream of the insertion site of the chloramphenicol-resistant cassette.

Complementation of *waal* and *gfc* operon mutants. To complement the avirulent phenotype of *A. hydrophila waaL* mutant, wild type *waaL* gene was cloned at HindIII restriction digestion site of pGNS-BAC after PCR amplification of *waaL* using primers Ligase F-HindIII and Li234R-HindIII (Table 2). *A. hydrophila gfcABCD, gfcB, gfcC* and *gfcD* mutants were complemented with the complete *gfcABCD* operon cloned into the plasmid pMJH97 (Figure 1, Chapter 3 and Hossain *et al.*, 2015b). The resulting plasmid pBBC2 was introduced into each of the *gfcABCD, gfcB, gfcC* and *gfcD* mutants by conjugation with *E. coli* SM10λpir (pBBC2) according to the methods described previously (Hossain *et al.*, 2015b). Transconjugants of *A. hydrophila* mutants complemented with respective plasmids were selected on 2×YT plates supplemented with chloramphenicol and colistin. The mutant colonies were identified as *A. hydrophila* by a positive oxidase test to distinguish from oxidase-negative *E. coli* SM10λpir. The introduction of complementing plasmid into the *A. hydrophila gfcBCD gfcB, gfcC* and *gfcD* mutants was confirmed by PCR using primer pairs CCatR and gfcD-CM-1R, and pABC-R and p15AF.

Purification of LPS and SDS-PAGE analysis. The LPS was extracted from bacterial cultures of wild-type *A. hydrophila* ML09-119 and the *gfcD* mutant according to methods described previously (Marolda *et al.*, 2007). Briefly, *A. hydrophila* cultures were grown for 16 h at 30°C. After 16 h, all cultures were adjusted to an optical density at 600 nm (OD_{600}) = 2.0, then 1.5 ml was aspirated from each of the bacterial cultures and the bacterial suspension was subjected to centrifugation at 13,000 x *g* for 3 min. The cells were then washed with phosphate-buffered saline (PBS, pH 7.5). This process was repeated twice, at which time the pellets were resuspended with 150 µl of lysis buffer [2% sodium dodecyl sulphate (SDS), 4% β-mercaptoethanol (2-ME), and 0.5 *M* of Tris-HCl, pH 6.8] and boiled for 10 min. The samples were then treated with 10 µl of proteinase K (20 mg/ml, G Science) for 2 h at 37°C. To remove proteins, an equal volume of 90% phenol was added to each culture and incubated at 70°C for 15 min. Each culture was then subjected to centrifugation at 10,000 x *g* for 10 min. After centrifugation, the aqueous layer was aspirated and treated with Tris-EDTA saturated diethyl ether to remove any residual phenol. The LPS was separated with 15% Tricine-SDS-polyacrylamide gel electrophoresis (Tricine-SDS-PAGE) and visualized using a Pierce silver staining kit (Thermo Scientific, USA).

Capsule visualization by bright field microscopy. *A. hydrophila* strains ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97), and ML09-119 *gfcD* (pBBC2) were grown overnight in TSB with 25 µg/ml of chloramphenicol at 30°C with shaking at 200 rpm. The empty vector pMJH97 was introduced into the wild-type ML09-119 and the *gfcD* mutant so that capsule visualization could be compared in the presence of chloramphenicol as with the complemented mutant. Following overnight incubation, the three strains were adjusted to an OD_{600} = 1.0 (~1×10^9 CFU/ml) and diluted 1:100 into fresh TSA agar plates containing 0.25% agar and

chloramphenicol (25 µg/ml). Each plate was incubated statically for 48 h at 30°C. After 48 h, 10 µL of bacteria were aspirated from the soft agar plates and stained following a previously described capsule staining technique (Croxatto *et al.*, 2007). Each of the three replicate bacterial strains were spread onto a fresh microscope slide (No.1.5). After drying, each smear was negatively stained using 1% Congo Red solution (unfiltered). After drying, the negative stain was flooded with Maneval's modified stain. Following 1 min positive stain, the slides were then gently washed with sterile distilled water, dried, and viewed at 100× using an Olympus BX61 QI Imaging Microscope using CellSens Dimensions Imaging software (Olympus America Inc., PA).

Biofilm assay. To quantify differences in biofilm formation between ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) and the *gfcD* complemented mutant (ML09-119 *gfcD* (pBBC2)), a biofilm assay was conducted following methods previously described with modifications (O'Toole, 2011). The empty vector was introduced into ML09-119 and ML09-119 *gfcD* following the conjugation protocol in Chapter III of this dissertation (Hossain *et al.*, 2015b). This was deemed necessary to place all strains under similar growth conditions in the presence of chloramphenicol (Figure 4). Three isolates were incubated in 2 ml TSB containing 25 µg/ml of chloramphenicol at 30°C for overnight with shaking at 200 rpm. Each strain was adjusted to an OD_{600} = 1.0 and diluted (1:100) into 6 ml of fresh TSB containing chloramphenicol (25 µg/ml). The 6-ml aliquot was then separated into three replicate tubes containing 2 ml of TSB with chloramphenicol and incubated statically for 24 h at 30°C. Each replicate tube was then standardized to an OD_{600} = 1.5 and diluted (1:100) into a 1 ml master mix containing fresh TSB with chloramphenicol. Four replicates of 100 µl of the master mix was placed into 4 wells of a Greiner 96-well flat bottom polystyrene plate. The plate was incubated statically at 30°C for 48 h. Unbound cells were removed from the plates through gentle washing

(2×) with 300 µl of sterile ultrapure water. 125 µl of 0.1% crystal violet was placed into each well of the 96 well plate and incubated at RT for 15 min. To remove unbound crystal violet, each well was washed three times with 300 µl of sterile ultrapure H_2O and dried. 125 µl of 30% acetic acid in sterile dH_20 was placed into each well to solubilize the crystal violet bound to the attached cells. Absorbance on each plate was quantified at OD_{550} using a Tecan Infinite M1000Pro plate reader.

Fluorescent microscopy of biofilm formation. Biofilm visualization was carried out on wild-type strain ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) and ML09-119 *gfcD* (pBBC2) following methods previously described with modifications (Shibata *et al.*, 2006). Each strain was grown overnight in TSB containing 25 µg/ml chloramphenicol at 30°C. The cultures were standardized to an OD_{600} = 1.5 and each strain was diluted (1:100) into 25 ml fresh TSB with chloramphenicol (25 µg/ml). The 25 ml cultures were placed into sterile petri dishes containing UV sterilized coverslips (22 mm x 22 mm x 1 mm) and were incubated statically at 30°C for 48 h. The coverslips were then extracted from the broth and gently washed of all non-bound cells (3×) with 25 ml of sterile dH_2O. Coverslips were then stained in the dark with 400 µl of SYBR Gold nucleic acid stain (Invitrogen, CA) (1:1000 dilution) for 15 min, at which time, they were rinsed gently with sterile H_2O and dried. The coverslips were fixed using a drop of SlowFade Gold Antifade (supplied concentration) (Thermo Fisher Scientific, MA). Fixed cells were viewed using a FITC filter (excitation from 465 to 505 nm; emission from 515 to 565 nm) at 40× and 100× using an Olympus BX61 QI Imaging Microscope using CellSens Dimensions Imaging software (Olympus America Inc., PA).

Virulence studies in channel catfish. Channel catfish fingerlings were obtained from Auburn University North Fisheries Unit. Each fish was roughly 3.5 months old and weighed

approximately 15 g. Each treatment contained three replicates of 50-liter tanks in a randomized block design with 10 fish/ replicate tank. The fish were acclimatized for at least 12 days prior to challenge with the wild type *A. hydrophila* ML09-119 strain or its respective mutants. The water temperature was maintained at 28°C during the acclimatization and the course of the experiment. All tanks were on flow-through at a rate of 0.6 liter/min throughout the experiments to maintain optimal water quality. The fish were fed daily at 2% BW daily up until the challenge. Cryopreserved (-80°C) stocks of *A. hydrophila* strains were streaked on TSA plates and incubated overnight at 30°C to obtain well isolated colonies. A single isolated colony for each strain was grown in TSB separately overnight and 50 µl of each culture was transferred to fresh TSB (1:100 dilution) to grow until OD_{600} = 0.8 - 1.0. Spent growth media was removed by subjecting the culture to centrifugation at 8,000 ×g for 10 min. and resuspended with fresh TSB to adjust OD_{600} to ~1.0 for each strain. For each treatment, three groups of ten fish each were injected intraperitoneally (IP) with 200 µl of bacterial cells adjusted to the approximate concentration of 1.0×10^6 CFU/fish. The fish were monitored for mortality for seven days and percent mortalities were calculated. Dead fish were subjected to necropsy for determining the presence of *A. hydrophila* in kidney, and liver tissues.

Prevention of MAS disease in catfish by vaccination with *A. hydrophila* ML09-119 *gfcD*. Survivors of the initial challenge with ML09-119 *gfcD* were housed in their respective tanks (under the same aquaria conditions as stated above) for 21 days. Following the 21-day incubation period, one fish from each replicate tank was sacrificed for bleeding. All blood serum was coagulated for 12 h at 4°C and the plasma was aspirated and stored at -20°C until analysis. The remaining survivors were challenged via IP with wild-type ML09-119 at a concentration of 5×10^6 CFU/fish. Fish in the negative control treatment group were injected with sterile TSB,

whereas the positive control fish were naïve fish that were housed in adjacent tanks during the acclimation period, initial challenge, and 21-day incubation period.

Statistical analyses. Statistical analysis was performed using Statistical Analysis System (SAS) software (SAS Institute Inc., Cary, NC, USA) and R (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance was determined by one-way analysis of variance (ANOVA) and Tukey post hoc multiple comparisons tests. Quantitative data are presented as means ± standard error. Differences were considered statistically significant if a P value is <0.05.

4. Results

Determining the structure of the *A. hydrophila* ML09-119 O-antigen (OPS).

Partial purification of the capsule was conducted and analyzed by 1D proton NMR (Figure 7). Although some residual contaminants obscure some of the polysaccharide peaks, it is apparent that the same peaks are present in CPS as in OPS, suggesting that CPS repeats the same polysaccharide structure as LPS. The degree of O-acetylation seems to be higher in the CPS, but this may be since the CPS fractions were not subjected to acid hydrolysis as was OPS, which may have led to partial removal of O-acetyl groups in OPS.

Virulence of *A. hydrophila* ML09-119 O-antigen ligase and O-antigen polymerase mutants in channel catfish.

To test whether the LPS-associated O-antigen plays a significant role in vAh virulence to a catfish host, an O-antigen deficient mutant ML09-119Δ*waaL::cat* and O-antigen deficient mutant Δ*wzy::cat* was generated by deleting the predicted O-antigen ligase gene *waaL* and O-antigen polymerase gene wzy respectively. Allelic exchange methods were used to generate a *waaLup:cat:waaLdn* and a *wzyup:cat:wzydn* cassette to replace *waaL* and *wzy* with *cat* gene in

each of their respective mutants. There was an observed reduction in o-antigen presence in both the ML09-119Δ*waaL::cat* mutant and the ML09-119 Δ*wzy::cat* (data not shown, conducted by Dr. Mohammad J. Hossain). An experimental IP challenge of channel catfish with wild-type ML09-119 and its O-antigen deficient mutant demonstrated that the O-antigen ligase mutant was significantly attenuated in its virulence in channel catfish, whereas the O-antigen polymerase mutant was insignificantly different in its virulence when compared to wild-type ML09-119 (Figure 1, Panel A; $P = 0.0001$ and $P = 0.9$ respectively). However, complementation of ML09-119*waaL::cat* with the wild-type *waaL* gene did not restore virulence of ML09-119*waaL*::*cat* mutant in channel catfish (data not shown). This suggested that there was a polar effect of the ML09-119 *waaL*::*cat* mutant on adjacent genetic loci. To evaluate this hypothesis, an O-antigen deficient mutant was generated using recombineering to precisely delete the *waaL* coding region of ML09-119 without affecting its flanking sequences (Hossain *et al.*, 2015b). The experimental challenge of channel catfish with the *waaL* mutant generated by recombineering demonstrated that the O-antigen deficient mutant was equally virulent to channel catfish as compared to wild-type ML09-119 (Figure 1, Panel B). These findings suggested that the *waaL* mutant constructed by allelic exchange interfered with *gfc* operon transcription, particularly *gfcD* that is located 122 bp upstream of *waaL* (Figure 2). Sequence analysis of the *waaL* flanking region in the mutant generated by allelic exchange revealed that a predicted *gfcD* transcriptional termination site (AGATAATAAAAGGGCGAGCAGTTTGCAACTGCTCGCCCTTTTTGATGAAAA) was deleted. Therefore, it was deemed necessary to evaluate the contribution of the *gfc* operon to vAh virulence.

Annotation of the *A. hydrophila* ML09-119 *gfc* operon.

The annotated genome for *A. hydrophila* ML09-119 deposited in GenBank (accession # CP005966.1, Jan. 2014) predicts three open reading frames (ORFs) upstream of *waaL* with homology to *gfcD, gfcC,* and *gfcB* (synonymous with *ymcA, ymcB* and *ymcC*, respectively) (Tekedar *et al.*, 2013). In *Escherichia coli* K-12 substrain MG1655 there are two operons *yjbEFGH* and *gfcABCD* that have homology with the *A. hydrophila* ML09-119 *gfc* operon and have been implicated in exopolysaccharide production (Ferrières *et al.*, 2007 and Peleg *et al.*, 2005). For each of the predicted genes in the *gfc* operon a phylogenetic analysis was conducted using MEGA7.0 (Kumar *et al.*, 2016), with 1000 iterations of a maximum likelihood analysis indicating that the predicted amino acid sequences from *A. hydrophila* ML09-119 were more closely related to the GfcB, GfcC and GfcD sequences from *E. coli* K12 compared to their paralogs YjbF, YjbG and YjbH, respectively (Figure 10 and data not shown). In conducting multiple alignments of the GfcD amino acid sequences it was observed that the *A. hydrophila* ML09-119 GfcD sequence annotated in GenBank (AHML_15465) had 266 amino acids truncated from its amino-terminus (Figure 9). A full-length GfcD sequence was obtained using GeneMark.hmm to predict ORFs from the genomic region between *waaL* and *gfcC* (Lukashin and Borodovsky). The subsequent generation of *A. hydrophila* ML09-119 mutants and assessment of their respective virulence was therefore conducted for each of the predicted *gfcBCD* loci that were hypothesized to play a role in G4C assembly and potentially contribute to *A. hydrophila* virulence.

Later, *in silico* searches were conducted to identify *A. hydrophila* ML09-119 homologs of the other genetic loci implicated in G4C assembly in *E. coli*, specifically the genetic loci *gfcA* (synonymous with *ymcD*), *yccZ, etp* and *etk* (Peleg *et al.*, 2005).

A search of the region upstream of *gfcB* with SeqBuilder (DNAStar) revealed an 84-amino acid reading frame with characteristics typical of GfcA homologs from diverse bacterial species: a predicted signal peptidase 1 cleavage site between residues 20 and 21 (SignalP 4.1); high percentage of Ala, Gly, Ser, and Thr residues; and a basic residue at the C-terminus. Although not predicted in the annotation in the original genome entry (CP005966.1, 2014) it is annotated now as a hypothetical protein (NC_021290.1, Apr. 2017). Based on these observations, it is predicted that *A. hydrophila* ML09-119 encodes a *gfcABCD* operon.

Later, *in silico* searches were conducted to identify *A. hydrophila* ML09-119 homologs of the other genetic loci implicated in G4C assembly in *E. coli*, specifically the genetic loci *yccZ* (*gfcE*), *etk*, and *etp* but did not return any significant BLAST hits. A search of GenBank using the *gfcABCD* operon sequence revealed that this operon is present in all sequenced *A. hydrophila* isolates of epidemic disease outbreaks in channel catfish in the United States (Hossain *et al.*, 2013, Pridgeon *et al.*, 2014a, Pridgeon *et al.*, 2014b) and carp isolates in China (Hossain *et al.*, 2014) (data not shown). A search of the Conserved Domain Database predicted that the GfcD protein is homologous with the bacterial putative lipoprotein pfam06082 in the pfam database for *Escherichia coli* K12 and EPEC 0127:H6 strains (Figure 2).

Assessing the virulence of *A. hydrophila* ML09-119 with mutations in the *gfc* operon.

To determine the contribution of gene products in the *gfc* operon to capsule biosynthesis and *A. hydrophila* virulence, markerless in-frame deletion mutants were constructed for *gfcB, gfcC* and *gfcD as* described previously (Table 1 and Hossain *et al.*, 2015b). The cumulative percent survival of channel catfish fingerlings intraperitoneally injected with different *A. hydrophila* mutants revealed that each of the genes inactivated in the *gfc* operon contributed to vAh virulence (Figure 5). Catfish challenged with wild-type *A. hydrophila* ML09-

119 indicated an average survival rate of 27% ± 22% post intraperitoneally injection with 1.0×10^6 CFU/ fish. Based on Tukey posthoc multiple comparisons, a significant difference was observed between the survival rates of the fish challenged with the wild-type compared to each of the *gfc* operon mutants ($P < 0.05$), each was observed to have survival rates between 89-100%. All the fish that were challenged with ML09-119 *gfcD* survived when monitored for at least 15 days. Due to this, ML09-119 *gfcD* was chosen for further evaluation in subsequent experiments. Mutant complementation indicated that virulence could be at least partially restored upon introduction of the *gfcABCD* operon into each of the respective mutants, with a decrease in survival rate (~56%) observed in the complemented mutants (Figure 5). Tukey pairwise comparisons indicated insignificant differences between the average survival rate of the fish challenged with the wild-type and the fish challenged with the *gfcBCD* (pBBC2) complemented mutant ($P = 0.44$). The survival rate for all other *gfc* mutants complemented with pBBC2 were significantly higher than the wild-type ($P < 0.05$). In all cases, data are expressed as mean ±SE of three replicate tanks each with 10 channel catfish fingerlings.

The *A. hydrophila gfcD* mutant lacks a group 4 capsule.

Bright field microscopy was conducted to visualize G4C in the wild-type *A. hydrophila* ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) and the *gfcD* complemented mutant ML09-119 *gfcD* (pBBC2). 48-h growth kinetics indicated that there was no significant difference in growth rates between the wild type and GfcD deficient mutant (Figure 12). The clearing, or halo, around each cell was observed in the wild-type and the *gfcD* complement but appeared to be absent in the *gfcD* mutant (Figure 3). No other phenotypic differences were observed.

The *A. hydrophila gfcD* mutant lacks buoyancy.

Because homologs of *gfcB*, *gfcC* and *gfcD* have been found responsible for O-antigen capsule assembly and the lack of those homologs have been shown to result in decreased buoyancy in Percol (Caboni *et al.*, 2015), the buoyancy of *A. hydrophila* ML09-119, ML09-119 *gfcD* and the complemented mutant ML09-119 *gfcD* (pBBC2) was determined. Buoyancy was reduced in the absence of GfcD when compared to the wild-type strain (Data not shown, conducted by Dr. Dawei Sun). The buoyancy was restored in ML09-119 *gfcD* upon complementation (Data not shown, conducted by Dr. Dawei Sun).

The *A. hydrophila gfcD* mutant exhibits a reduction in its ability to form a biofilm.

To test whether the absence of *gfcD* directly affected bacterial biofilm formation, the wild-type ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) and the *gfcD* complemented mutant ML09-119 *gfcD* (pBBC2) were quantified for their ability to form a biofilm (Figure 4). Both the wild-type strain and the complemented mutant exhibited significantly higher biofilm formation compared to the GfcD deficient mutant after 48 h when incubated statically at 30°C ($P < 0.001$) (Figure 4, Panel A). The wild-type and the complemented mutant were observed to have similar levels of biofilm formation ($P = 0.72$).

To visualize differences in biofilm formation observed in the biofilm assay, the wild-type ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) and the complemented mutant ML09-119 *gfcD* (pBBC2) were grown on glass coverslips for 48 h statically at 30°C and stained with SYBR Gold (Figure 4, Panel B). Fluorescent microscopy indicated that there was a significant reduction in the number of *A. hydrophila gfcD* cells attached to the coverslip after 48 h. In contrast, there were no observable differences in bacterial cell attachment between the wild-type and *gfcD* complemented mutant. These fluorescent micrographs correlate with the microtiter biofilm assay indicating a reduction in biofilm formation by the GfcD-deficient mutant.

LPS-PAGE.

To identify the effect *gfcD* played on the LPS-associated O-antigen, LPS was extracted from the reference strain AL06-06, wild type strain ML09-119, ML09-119 *gfcD* mutant, ML09-119 *wzy* mutant, and ML09 *waaL::cat* mutant and visualized via silver staining, which showed a reduced presence of LPS from the wzy mutant, a reduced O-antigen from the *waaL* mutant, and an apparent moderate decrease in intensity of LPS stained in the *gfcD* mutant (Data not shown and Figure 11).

Prevention of MAS in channel catfish by the ML09-119 *gfcD* mutant.

To determine the vaccination efficacy of *A. hydrophila* ML09-119 *gfcD* in channel catfish in preventing fish from developing MAS, the survivors of the initial challenge with ML09-119 *gfcD* were maintained for 21 days post-vaccination (Figure 6). Following the 21-day incubation period, survivors were sub-challenged with wild-type ML09-119 at a dose of $1x10^6$ CFU/fish. *A. hydrophila* ML09-119 *gfcD* protected channel catfish from MAS with an 89% ± 11% survival rate, which was significantly higher than the 25% ± 14% survival rate of naïve fish challenged with the wild-type to have ($P = 0.006$). There was no significant difference observed between the survival rates of fish challenged with a sham broth and ML09-119 *gfcD* protected channel catfish ($P = 0.62$). In all cases, data is expressed as mean percent survival ± SE of three replicate tanks.

Adaptive immune response in vaccinated fish.

To determine the relative antibody titers found in the blood serum in each of the five dilutions (1/10, 1/20, 1/40, 1/80, 1/160), serum absorbance levels in all three treatments were standardized against the blank. Averages and standard error of each standardized absorbance levels in the replicate fish serums of each treatment indicated that ML09-119 specific IgM

immunoglobins in the fish that were vaccinated with the *gfcD* mutant were substantially higher than both the positive control, fish that survived the initial encounter with ML09-119, and Naïve fish (Data not shown, conducted by Dr. Dawei Sun). In addition, the naïve fish showed an average decrease of 93 ± 0.75% in their relative absorbance levels when compared to the absorbance levels of the *gfcD* vaccinated fish. Fish that survived the initial encounter with ML09-119 indicated a 54 ± 4% decrease in absorbance when compared to the fish that were delivered the *gfcD* mutant.

Serum Resistance

A comparison of serum resistance between a typical opportunistic *A. hydrophila* AL06-06 and vAh ML09-119 showed no significant differences. Furthermore, results showed that ML09-119 and ML09-119 *gfcD* were completely resistant to complement-mediated killing with ML09-119 observed to have a 170.9% survival rate and ML09-119 *gfcD* mutant observed to have a 98.3% survival rate (Data not shown, conducted by Luke Foshee). In contrast, the ML09-119 *wzy* and ML09-119 *waaL* mutants were completely sensitive to complement- mediated killing showing zero survival (Data not shown, conducted by Luke Foshee). Complement sensitive *Escherichia coli* strain HB101 was used as a positive control and showed 100% killing due to serum complement.

5. Discussion

Based on a comparative genome analysis, the genome of vAh isolates was found to contain 55 unique regions that were absent in rAh isolates (Hossain *et al.*, 2013). Among the ORFs that were vAh-specific were 25 ORFs predicted to encode a 26.5 Kb O-antigen biosynthesis gene cluster. The significant role that the O-antigen has been shown to play in virulence in other bacterial pathogens (Goebel *et al.*, 2008, Lerouge and Vanderleyden, 2001,

Nagy *et al.*, 2006, DeShazer *et al.*, 1998, West *et al.*, 2005, Janda, 2001, Beaz-Hidalgo, 2013, Naharro *et al.*, 2010, Merino *et al.*, 1996a, and Merino *et al.*, 1996b) prompted a further examination of the role of the vAh O-antigen biosynthesis gene cluster in virulence. The annotation of the ML09-119 O-antigen biosynthetic cluster predicted the presence of gene products required for the synthesis and transport of activated sugars to the outer membrane and ligation of these sugars to the growing O-antigen chain (Hossain *et al.*, 2013). Further, the O-antigen biosynthesis gene cluster contained genes required for the synthesis of nucleotide sugars D-Rhamnose, D-mannose, GDP-L-Fucose, 3-acetamido-3, and 6-dideoxy-d-galactose (D-FucP3NAc). Additionally, a total of five different glycosyltransferace genes and one acetyltransferase genes were also discovered, which were predicted to assemble the nucleotide sugar repeat onto the membrane lipid undecaprenol pyrophosphate (Und-PP). The O-antigen flippase, O-antigen polymerase (*wzy*) and O-antigen ligase (*waaL*) gene products were predicted to be present in the ML09-119 gene cluster, which predicts that ML09-119 relies on the Wzy-dependent pathway to synthesize the O-antigen, attach it to lipid A and export the LPS to the outer leaflet of the outer membrane (Whitfield, 2006, Raetz and Whitfield, 2002, Han *et al.*, 2011).

Previously performed structural analysis of the vAh O-antigen by the CCRC, which included NMW, GC-MS, and methylation analyses, revealed a structure that has not been previously observed in any other bacterial species. The identification of this novel O-antigen structure and its predicted dependency on the Wzy-pathway led to the hypothesis that *waaL* and *wzy* were critical for the formation of a fully functional LPS-associated O-antigen.

Upon deleting both *waaL* and *wzy* there was an observed lack of LPS-associated O-antigen to the outer membrane, and a ML09-119 *waaL* mutant showed an attenuation of

virulence. However, complementation with the wild-type *waaL* did not restore virulence to ML09-119 *waaL* despite multiple attempts (data not shown). Further analysis of this mutant indicated that the transcription termination site of an upstream operon had been functionally altered and suggested that there was a polar effect of the *waaL* mutation. The annotated genome for *A. hydrophila* ML09-119 deposited in GenBank predicted three open reading frames, *gfcB*, *gfcC*, and *gfcD* that were upstream of *waaL*. CDD analysis of this operon indicated homology to gene products of the *gfc* operon (Figure 8), which has been identified in at least 29 genera of gram negative bacteria and been shown in *Escherichia coli* O127 and O157 pathogenic strains to play a role in capsule assembly (Sathiyamoorthy *et al.*, 2011). The crystal structure of GfcB, an outer membrane-anchored lipoprotein, showed that it has a flattened β-barrel structure (PDB id 2in5, unpublished). GfcC is a soluble periplasmic protein containing two β-grasp domains (PDB id 3P42) (Sathiyamoorthy *et al.*, 2011), and because it holds similar physical characteristics like Wza, may function as an export protein, however, this has not been thoroughly investigated. GfcD is predicted to be an outer membrane 22-strand beta-barrel lipoprotein that may function as a transport protein for the formed polysaccharide chain (Sathiyamoorthy *et al.*, 2011). GfcA has been observed in many of these genera to encode a short inner membrane protein but was previously unidentified in *Aeromonas hydrophila* ML09-119. However, further investigation showed that the genome of ML09-119 deposited in GenBank had an unannotated region upstream of *gfcB*, which is predicted to be *gfcA*.

Due to the expected requirement of the *gfc* operon on G4C assembly, ML09-119 wild-type and the *gfcD* mutant were characterized for capsule formation, biofilm formation, buoyancy, resistance to serum, and virulence. As expected, the *gfcD* mutant lacked an observable G4C under bright field microscopy compared to wild-type ML09-119. Previously, there was a

reduction in buoyancy observed in the *gfcD* mutant when compared to the WT, which can be reversed upon complementation. This buoyancy reduction has been observed in other bacteria that lack capsules and could further indicate the absence of a formed capsule in the *gfcD* mutant (Peleg *et al.*, 2005). Additionally, previously performed serum resistance studies indicated that the *waaL* and *wzy* mutants held an increased susceptibility to fish serum, which was not observed in ML09-119 or ML09-119 *gfcD*. This is expected to be attributable to increased complement-mediated killing in the *waaL* and *wzy* mutants that is due to increased deposition of complement in cells that have reduced presence or length of O-antigen chains (Goebel *et al.*, 2008, Shiller *et al.*, 1989). Additionally, there were observed reductions in both the presence of the O-antigen and shortened O-antigen chains in both the vAh *waaL* and *wzy* mutants (data not shown). This reduction was not observed in ML09-119 or ML09-119 *gfcD*, supporting the idea that GfcD does not play a role in the formation of the LPS-associated O-antigen. Further, there was no observed virulence attenuation in the *waaL* or *wzy* mutants generated by recombineering, which indicates that expression of the vAh LPS-associated O-antigen is not critical to vAh virulence when IP injected into fish, however, more testing is needed to justify the role of the O-antigen in complement resistance and avoidance of the innate immune response (Goebel *et al.*, 2008). This correlates with the serum resistance data, in which the O-antigen deficient mutants were more sensitive to complement mediated killing when compared to their wild-type form, however, the exact role of the vAh O-antigen in the avoidance of the innate immune response needs to be further investigated. Further, the *gfcD* ortholog in *E. coli*, *yjbH*, has also been shown to play a role in secretion of exopolysaccharides (Ferrières *et al.*, 2007). Based on the rapid onset of mortality and infections during aquaria trials, it is likely that the *gfc* operon could also play a role in secreted virulence factor(s). However, no quantitative analysis on exact levels of EPS

production have been carried out. Previously, G4C formation has been shown to play a role in biofilm formation and attachment to host tissues (Caboni *et al.*, 2015, Croxatto *et al.*, 2007, Huang *et al.*, 2014, Balestrino *et al.*, 2008, Barak *et al.*, 2007, Boddicker *et al.*, 2006, Wang *et al.*, 2004). This data correlates with my observations, indicating that *gfcD* is important for biofilm production and attachment to a substrate. Future studies should explore the role of the G4C and biofilm formation in vAh attachment to host tissues and to substrates in aquatic environments.

The pathogenic role of the group 4 capsular (G4C) polysaccharide and *gfcABCD* operon gene products has not been previously determined in *A. hydrophila* but has been shown to play a role in virulence in other gram-negative bacteria (Caboni *et al.*, 2015, Croxatto *et al.*, 2007, Goebel *et al.*, 2008, Nadler *et al.*, 2012, Attridge and Holmgren, 2009, Shifrin *et al.*, 2008, Nakhamchik *et al.*, 2007, Gibson *et al.*, 2006). G4C have been shown to assist bacteria in an increased ability to resist innate immune killing and prevent phagocytosis and other immune modulating machinery, however, this could not be confirmed during serum resistance assays. To determine the role for the *gfc* operon in vAh virulence, aquaria challenges were carried out with mutants in *gfcB*, *gfcC*, *gfcD* and *gfcBCD*. All mutants were observed to have an attenuation of virulence in channel catfish, with ML09-119 *gfcD* showing a total attenuation of virulence whereas mutants in *gfcB* and *gfcC* also had significant reductions in virulence compared to wild-type ML09-119 (Figure 5). Initial *in silico* examination of the *gfc* operon predicted the presence of *gfcBCD* in vAh. However, further bioinformatic analyses revealed an 84-amino acid reading frame directly upstream of *gfcB* that has homology with GfcA found in other bacterial species. This ORF was not predicted in the annotation in the original ML09-119 genome and was therefore not targeted for genetic deletion or complementation experiments. Genetic

complementation was carried out to confirm that the attenuated virulence was due to disruption of the targeted gene(s), by introducing the intact *gfcABCD* operon. Partial restoration of virulence was observed after introduction of the plasmid-borne *gfcABCD* operon into each of the mutants, which was anticipated due to the lack of antibiotic selection for the complementing plasmid *in vivo* (Figure 5).

It is important to note that during the initial disruption in the *gfcD* mutant, the FRT-cat-FRT integration contributed to a four-nucleotide disruption in *gfcC* (indicated by the scar in *gfcC* in Figure 2). However, the presence of stop codons for all six reading frames were still observed in the marker-less mutant post pCMT-*flp*. Further, this indicates that even though there could be a reduction in the last one or two amino acids from the C-terminal of the protein, this may not influence the functionality of a protein, under most situations (Datsenko and Wanner, 2000). Additionally, due to the in-frame deletions of pMJH65, each mutant generated through recombineering may have removed the RBS binding site for downstream genes. However, having an FRT site present in every marker-less mutant should address the lack of an RBS site. FRT has been shown to hold its own RBS binding site, therefore, its presence should assist in the expression of downstream genes (Datsenko and Wanner, 2000).

Due to the full attenuation of virulence observed for ML09-119 *gfcD*, it was of interest to challenge surviving fish with wild-type ML09-119 after 21 days of initial mutant challenge. Fish that had previously received an IP dose of ML09-119 *gfcD*, showed much higher survival rate compared to naïve fish (Figure 6), indicating that the fish had mounted an adaptive immune response post vaccination. This was further supported by the observation of a significant increase in systemic blood serum IgM levels specific to the wild-type ML09-119 antigens (data not shown). The serum IgM levels observed from ML09-119 *gfcD* vaccinated fish were

significantly higher than the titers found in naïve fish or from fish that survived the initial encounter with the wild-type. The enhanced adaptive immune response observed for the *gfcD* mutant is hypothesized to result from the absence of the G4C resulting in greater surface antigen exposure to complement and other immune modulating cells (Goebel *et al.*, 2008 and Attridge and Holmgren, 2009).

The lack of *gfcE*, *etp*, and *etk* homologs in the ML09-119 G4C operon is puzzling. These three genes, encoding an outer membrane helical pore, a tyrosine phosphatase, and tyrosine kinase, follow *gfcABCD* in the G4C operon of *E. coli* & *Shigella* species but also are paralogous to *wza*, *wzb*, and *wzc* elsewhere on the *E. coli* chromosome and implicated in group 1 capsule (colanic acid) secretion, *E. coli* K30 capsule secretion, and other capsules (Nadler *et al.*, 2012 and Sathiyamoorthy, 2011). In *E. coli* and other species, a Wzc octamer (CapB in *Staphylococcus aureus*) undergoes cyclic autophorphosylation and dephosphorylation (with Wzb) which may aid in synthesis or secretion of group 1 EPS or capsule through the Wza pore (Olivares-Illana *et al.*, 2008, Temel *et al.*, 2013, Nadler *et al.*, 2012). Although the ML09-119 *gfcBCD* genes are more homologous to *gfcBCD* in *E. coli*, the lack of *wza*, *wzb*, and *wzc* homologs makes the operon more comparable to *yjbEFGH* present in many organisms, which has been shown to be responsible for secretion of an unknown exopolysaccharide (Ferrières *et al.*, 2007). However, even with an observed lack of *wza*, GfcD, shown to be a large outer membrane β-barrel protein, may provide the exit portal for ML09-119 capsule in lieu of Wza. Understanding how the ML09-119 O-antigen capsule is secreted will help clarify why the *wzc* gene is missing in *Aeromonas* genomes.

Table 1. List of bacterial strains and plasmids used in this study.

Bacterial strains and plasmids	Relevant features	References
Bacterial strains		
A. hydrophila		
A. hydrophila ML09-119	Wild-type virulent *Aeromonas hydrophila* (vAh) isolate	Hemstreet, 2010
A. hydrophila AL06-06	Reference *Aeromonas hydrophila* (rAh) isolate	Hossain *et al.*, 2013
ML09-119 *gfcD::cat*	Marked deletion of *gfcD* through the integration of cat cassette	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcC::cat*	Marked deletion of *gfcC* through the integration of cat cassette	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcB::cat*	Marked deletion of *gfcB* through the integration of cat cassette	Hossain *et al.*, 2015b
ML09-119 *gfcD::cat::FRT*	Marked deletion of *gfcD* containing cat cassette flanked by FRT regions	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcC::cat::FRT*	Marked deletion of *gfcD* containing cat cassette flanked by FRT regions	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcB::cat::FRT*	Marked deletion of *gfcD* containing cat cassette flanked by FRT regions	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcBCD::cat::FRT*	Marked deletion of *gfcD* containing cat cassette flanked by FRT regions	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcD*	Unmarked deletion of *gfcD* gene	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcC*	Unmarked deletion of *gfcC* gene	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcC*	Unmarked deletion of *gfcB* gene	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcBCD*	Unmarked deletion of *gfcBCD* operon	Thurlow *et al.*, 2018b/ this study

ML09-119 *gfcBCD* (pBBC2)	A. hydrophila *gfcBCD* operon mutant with pBBC2	Hossain *et al.*, 2015b
ML09-119 (pMJH97)	*A. hydrophila* with pMJH97	Thurlow *et al.*, 2018b/ this study
ML09-119 *gfcD* (pMJH97)	*A. hydrophila gfcD* gene mutant with pMJH97	Thurlow *et al.*, 2018b/ this study
ML09-119 (pBBC2)	*A. hydrophila* with pBBC2	Hossain *et al.*, 2015b
ML09-119 *waaL::cat*	Replacement of *waaL* gene with *cat* gene	Hossain *et al.*, 2015b
ML09-119 *wzy::cat*	Replacement of *wzy* gene with *cat* gene	Thurlow *et al.*, 2018b/ this study
ML09-119 *vgr3::cat*	Replacement of *vgr3* gene with *cat* gene	Hossain *et al.*, 2015b
E. coli		
E. coli SM10λpir	*thi-1thr leu tonA lacY supE recA*::RP4-2-TcT::Mu Km^r *λpir*	Simon *et al.*, 1983
E. coli CC118λpir	*Δ(ara-leu) araD ΔlacX74 galE galKphoA20 thi-1 rpsE rpoB argE(Am) recA1 λpir*	Herrero *et al.*, 1990
E. cloni 10G	F^-*mcrAΔ(mrr-hsdRMS-mcrBC) endA1 recA* φ80d*lacZ*ΔM15Δ*lac*X74 *araD*139 Δ (ara,leu)7697 *galU galK rpsL* (StrR) *nupG* λ^- *tonA*	Lucigen (Middleton, WI)
Plasmids		
pDMS197	Suicide vector, *sacB*, Tet^R	Edwards *et al.*, 1998
pDMS197waaL	Suicide vector contains cat cassette flanked by upstream and downstream regions of *waaL*	Thurlow *et al.*, 2018b/ this study
pDMS197wzy	Suicide vector contains cat cassette flanked by upstream and downstream regions of *wzy*	Thurlow *et al.*, 2018b/ this study
pGNS-BAC-waaL	*waaL* cloned into plasmid pGNSBAC	Thurlow *et al.*, 2018b/ this study

pMJH97	*cat-oriR-oriT* backbone plasmid for pBBC2	Hossain *et al.*, 2015b
pBBC2	*cat-oriR-oriT-gfcABCD* complement vector	Hossain *et al.*, 2015b
pMJH46	Conjugally transferrable recombinogenic plasmid	Hossain *et al.*, 2015b
pMJH65	Conjugally transferrable recombinogenic plasmid	Hossain *et al.*, 2015b
pCMT-flp	Temperature-sensitive Flp recombinase plasmid	Hossain *et al.*, 2015b

Table 2. List of primers used in this study.

Primer Name	Sequence in 5′ to 3′ direction
LidnF	TTAGCTCACTCATTAGGCAAGATCGGCTCTATGCAACT
LidnR	TGATTATGATGTAATGACTGG
Lidn-intR	GGCAGTTACCATTCATGAGT
Liup-intF	AGAAGCGGTGCTGATAACG
LiupF	ACTTAAGCTCGCCGAACTC
LiupR	AGTTCAACGGAAGTCTACGCTGTCGAGGCCATGTG
Ligase F-HindIII	AGTCTAAGCTT GACCAGCGCATTGAGAGAGAGG
Li234R-HindIII	AGTCTAAGCTT GCTCAAGCCAACAACCGCGAA
WzydnF	TTAGCTCACTCATTAGG**C**CTAGCTGTGGTGCCAGAATA
Wzydn-intR	CTGATGTTATTATTGACCAAG
WzydnR	CATTCAATATAGTGTCTGGA
WzyupF	CCGCGACAACAACTCCTT
Wzyup-intF	GTGACGCCACCGATGATA
WzyupR	AGTTCAACGGAAGTCTA**C**GCACTTCCTGTATCAAGATT
CatF	GTAGACTTCCGTTGAACT
CatR	GCCTAATGAGTGAGCTAA
CCatR	GGCCGTCGACCAATTCTCATGTT
gfcD-CM-1F	GCGACAAAAATAAGGCTGCCA
pABC-R	TGAGTCGCAAGAATGGCCT
p15AF	TCACATATTCTGCTGACGCACC
CatF	GTAGACTTCCGTTGAACT
CatR	GCCTAATGAGTGAGCTAA
LipoRecF	T*A*G*A*GATATCAATATTCGTATTGCCAATCTCCTTGCTAATCG AGTACCAGAGTAGACTTCCGTTGAACT
LipoRedR	C*A*A*C*TGCTCGCCCTTTTTGATGAAAAAAGATCGGCTCTATG CAACTTTTGAGCCTAATGAGTGAGCTAA
Lipo-upF	CCGAATGGTAATCCACAGTT
Lipo-dnR	TAGAACAGCTGGTCACGAGA
LipocmF-notI	GACGGCGGCCGCTCACTCTGGTCAATTGGTCT
Lipo-FRT-F	C*A*A*C*TGCTCGCCCTTTTTGATGAAAAAAGATCGGCTCTATG CAACTTTTGA GTGTAGGCTGGAGCTGCTTC
Lipo-FRT-R	T*A*G*A*GATATCAATATTCGTATTGCCAATCTCCTTGCTAATCG AGTACCAGA CATATGAATATCCTCCTTAGT
gfcB-FRT-F	C*G*T*T*TACATCAGCTTGAGCCAAAGCAAAAAATGGTATTAGA CTAACCATCAGGTATCTTT GTGTAGGCTGGAGCTGCTTC
gfcB-FRT-R	T*T*G*G*CAGCCTTATTTTTGTCGCTTTGCAATCATTTTCCTATTC CACTGAGTAACCCATCT CATATGAATATCCTCCTTAGT

gfcB-upF	GTCCAGCTCTTGTAGTAACTGC
gfcB-dnR	TAATGCGAATGACGGCTCCACC
gfcC-FRT-F	ATGAAAAGATACCTGATGGTTAGTCTAATACCATTTTTTGCTTTG GCTCAAGCTGATGTA GTGTAGGCTGGAGCTGCTTC
gfcC-FRT-R	CATTCTGGTACTCGATTAGCAAGGAGATTGGCAATACGAATATT GATATCTCTATACTG CATATGAATATCCTCCTTAGT
gfcD-tetA-dnR	CTTGCCAGAGACGGAACTTGAA
gfcB_TetA-uF	GGCCTGATCCATTGCAGAA
gfcB-FRT-Fn	CCATCAGGTATCTTTTCATAATGCGTACGGCTTTAATATTATCAT TTCAATAACCGGTAA GTGTAGGCTGGAGCTGCTTC
Liga-F	ATGACCAGGCTGGCGAAGATCC

** represents nucleotides with a phosphorothioate linkage*

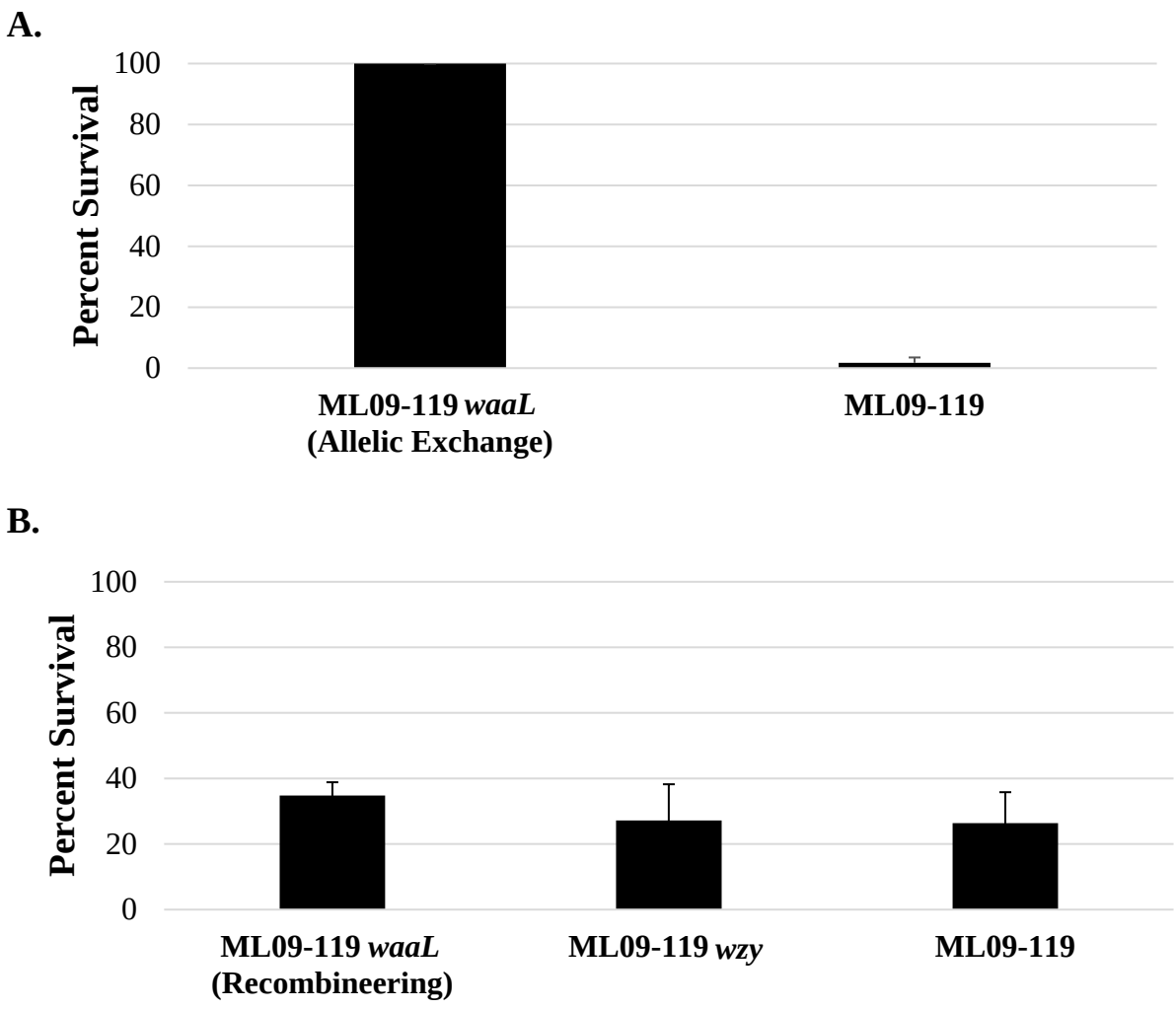

Figure 1. Percent survival of channel catfish after challenged with vAh strain ML09-119, O-antigen ligase mutants (*waaL*), and O-antigen polymerase mutant (*wzy*). **Panel A**. Percent survival of fish challenged with the *waaL* mutant generated by allelic exchange was significantly different when compared to the percent survival of fish challenged with the wild-type (ML09-119) ($P = 0.0001$). **Panel B.** Percent survival of fish challenged with the *waaL* mutant generated by recombineering and *wzy* mutant were insignificantly different when compared to the percent survival of fish challenged with the wild-type ($P = 0.75$). Error bars represent the standard error between the replicates of each treatment.

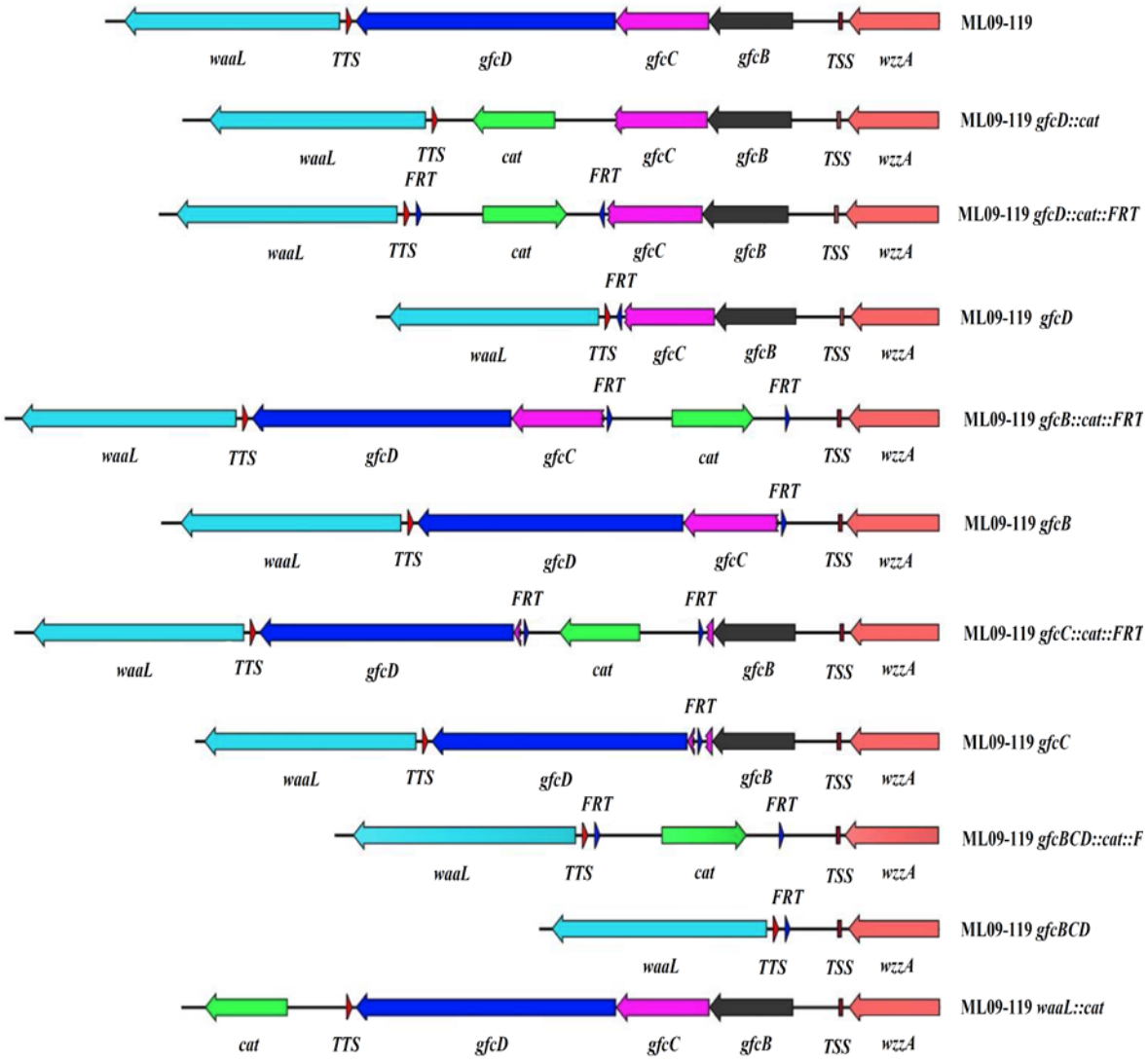

Figure 2. Genetic organization of the lipoprotein, O-antigen, and LPS core biosynthesis gene cluster. The O-antigen capsule (G4C) assembly cluster contains a predicted *gfcABCD* operon. The mutant strains used for studying the role of *gfcABCD* operon in virulence and other vAh phenotypes are also depicted.

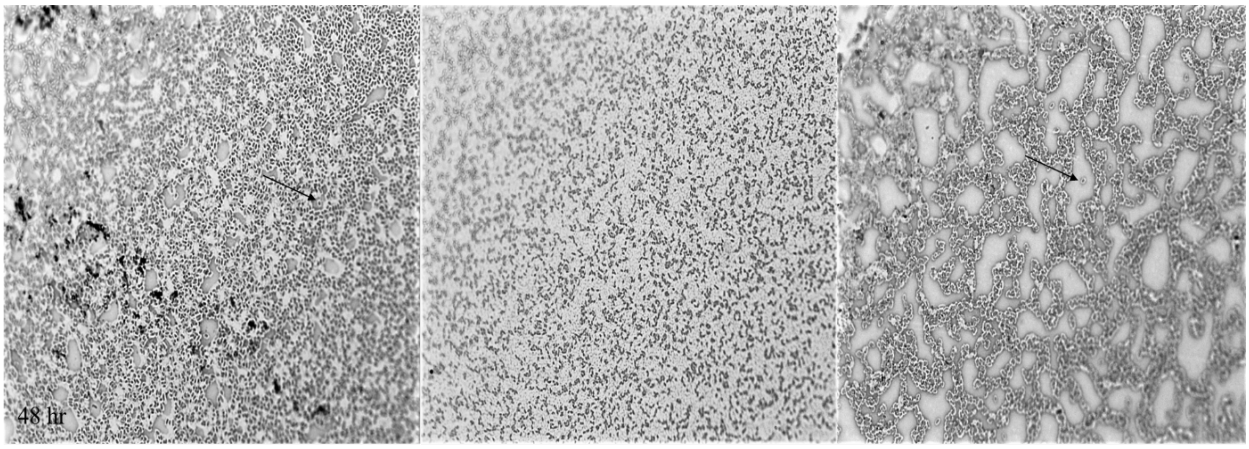

Figure 3. Modified maneval's capsule staining of the *A. hydrophila* ML09-119 (pMJH97), ML09-119 *gfcD* (pMJH97) mutant, and complemented ML09-119 *gfcD* (pBBC2) mutant, respectively. Cells producing capsules result in a halo around each cell after positive Maneval's staining (black arrow). No halo was observed around ML09-119 *gfcD* cells after 48 h. All cells observed at 40×.

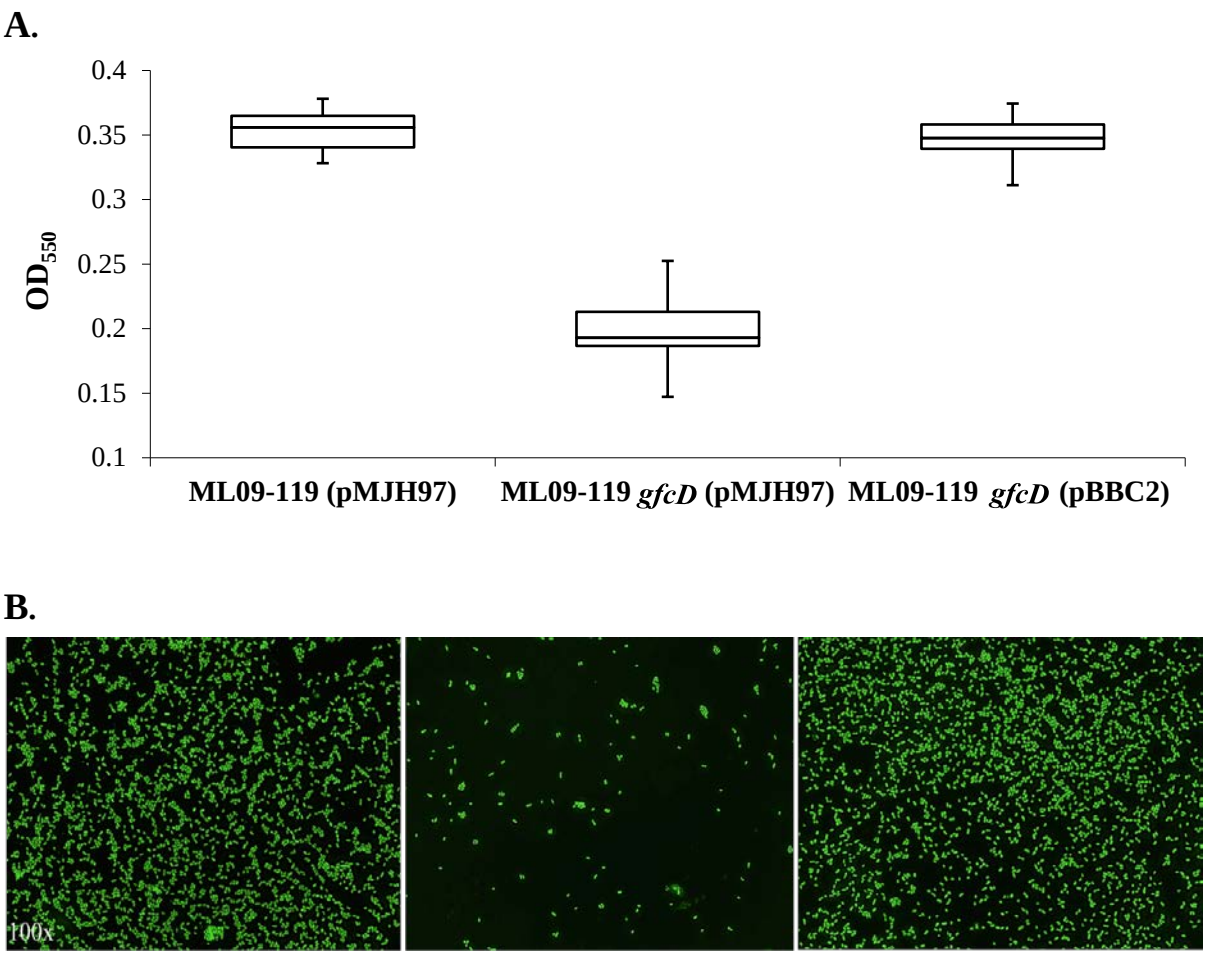

Figure 4. Biofilm formation was reduced in the *gfcD* mutant relative to wild-type vAh. **Panel A.** Biofilm quantification was carried out to identify the differences between strain ML09-119, the ML09-119 *gfcD* mutant, and the ML09-119 *gfcD* complement. The wild-type strain ML09-119 and the *gfcD* complement were observed to have significantly higher levels of biofim formation compared to the gfcD deficient mutant ($P < 0.001$). There were no significant differences between the wild-type and the *gfcD* complement ($P = 0.72$). **Panel B.** Fluorescent microscopy of biofilms formed by the wild-type ML09-119 (pMJH97) (A), ML09-119 *gfcD* (pMJH97) mutant (B), and ML09-119 *gfcD* (pMJH97) complemented mutant (C). Observable cell density in each micrograph represents relative attachment post wash.

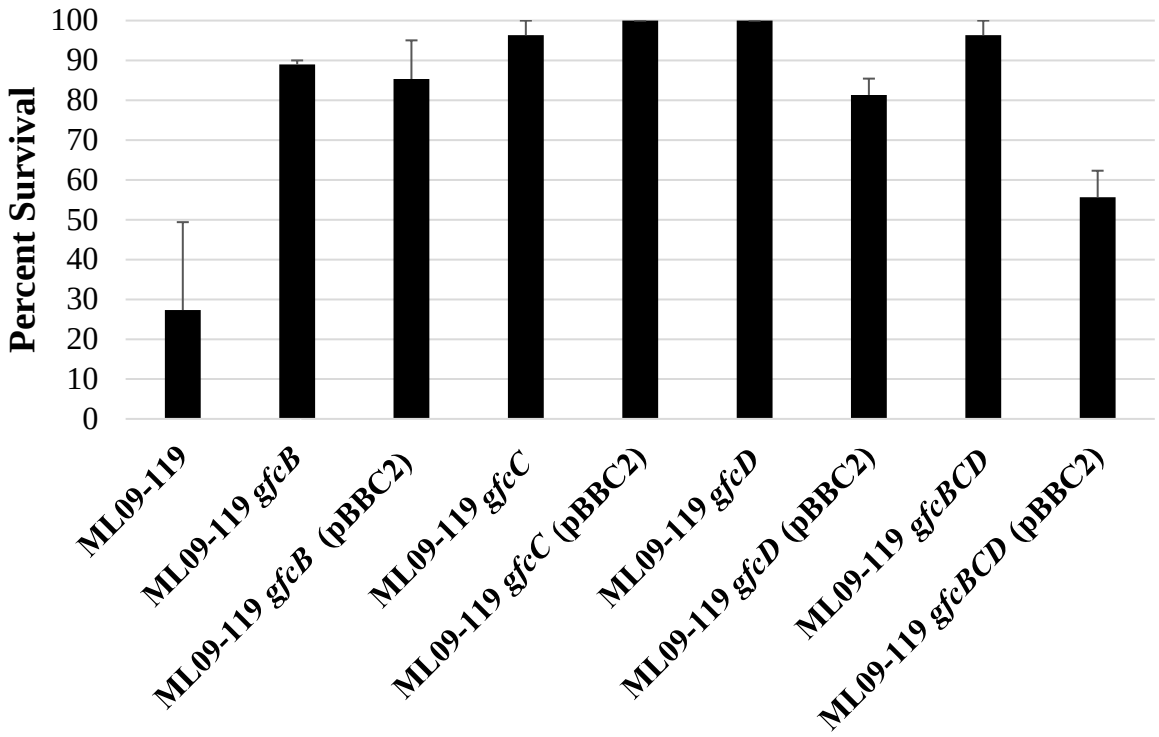

Figure 5. Percent survival of channel catfish after challenged with vAh strain ML09-119, its mutants and complemented mutant. Percent survival of fish challenged with all *gfcBCD* operon mutants were significantly increased compared to the wild-type ($P < 0.05$). Error bars represents the standard error between the replicates of each treatment.

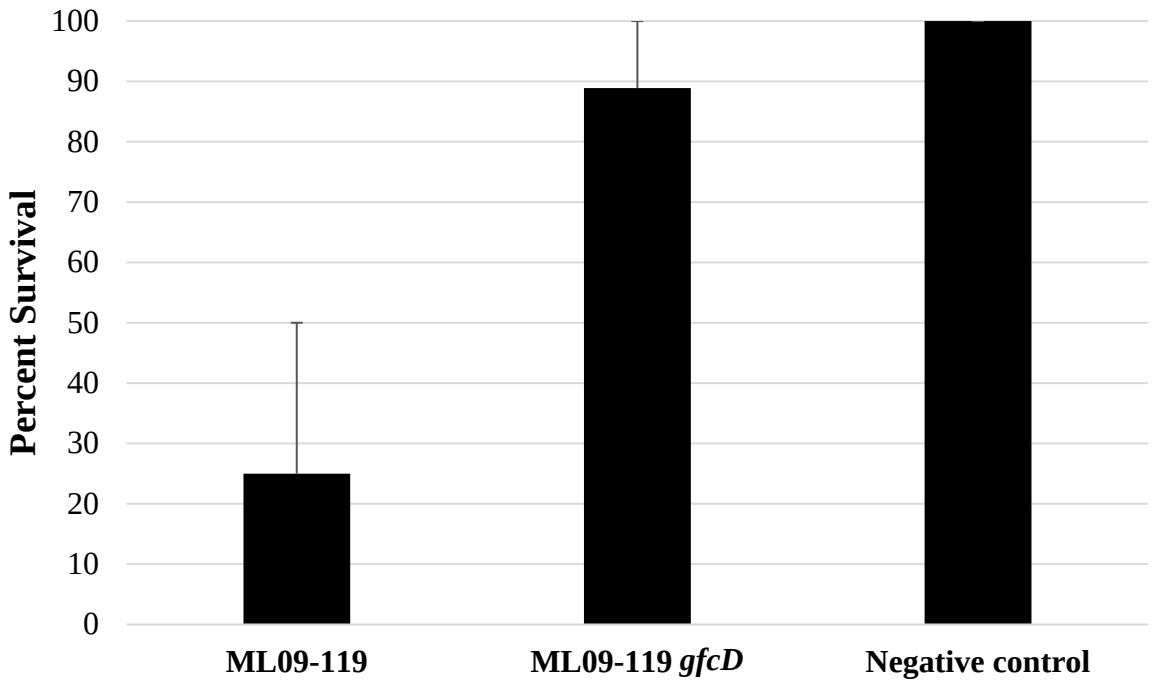

Figure 6. Protective effects of ML09-119 *gfcD* mutant. Naïve fish and survivors of the initial challenge with ML09-119 *gfcD* were sub-challenged against the wild-type after 21 days. Survival rates of fish previously challenged with ML09-119 *gfcD* were significantly higher than that of naïve fish ($P = 0.006$). The negative control represents a population of naïve fish that were challenged with sterile TSB (sham). No significant difference was observed between the survival rates of the negative control and ML09-119 *gfcD* ($P = 0.62$).

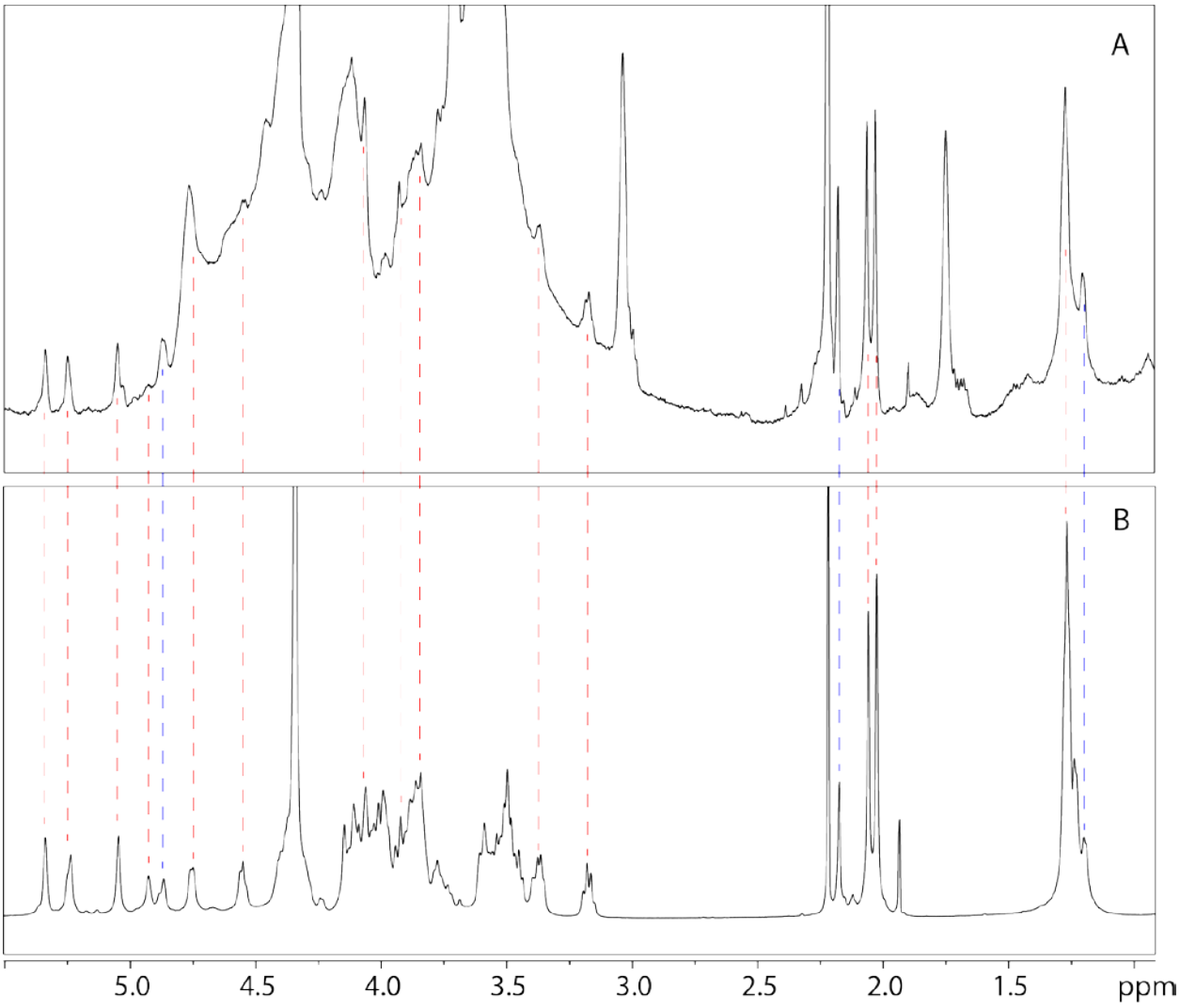

Figure 7. Comparison of the 1D proton NMR spectra of CPS (A) and OPS (B). Red and blue dashed lines indicate common signals; blue dashed lines indicate common signals from O-acetylated repeats.

*Asterisks indicate positions which have a fully conserved residue and colors indicate conservation between amino acids.

Figure 8. Alignment of *Aeromonas hydrophila* GfcB (A), GfcC (B), and GfcD (C) amino acid sequence data to with their closest non-vAh-derived orthologs from the Conserved Domain Database (CDD). GenBank annotation is missing from the hypothesized GfcA from *Aeromonas hydrophila* ML09-119. This region also held no hits to any related orthologs on the CDD and was not annotated in the ML09-119 genome.

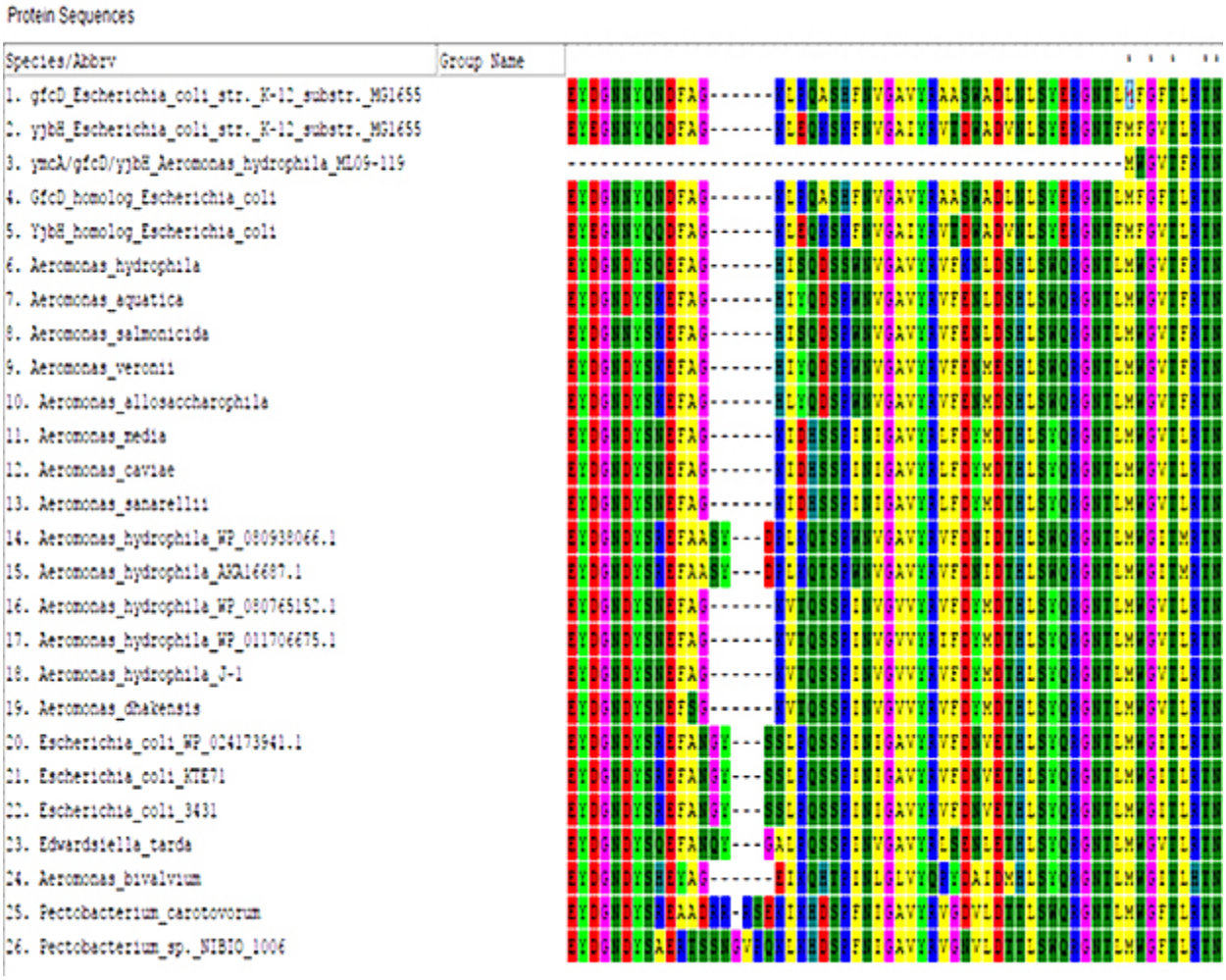

Figure 9. Alignment of *Aeromonas hydrophila* ML09-119 *gfcD* sequence taken from the annotated *gfc* operon in GenBank. Upon alignment to homologous sequences found in 26 additional strains, 266 amino acids were found to be absent from the deposited ML09-119 sequence.

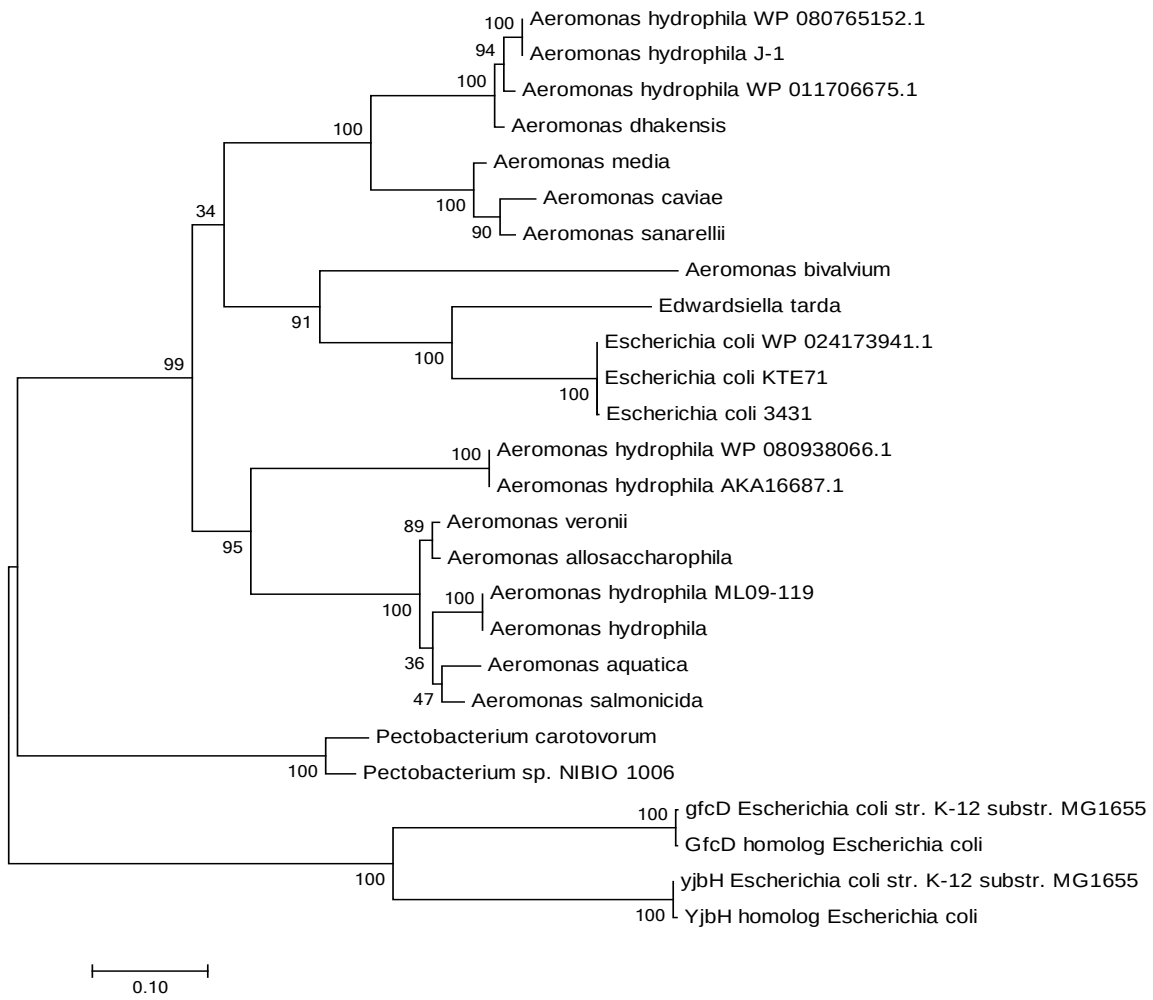

Figure 10. Molecular Phylogenetic analysis of GfcD by Maximum Likelihood method. There was a total of 646 positions in the final dataset.

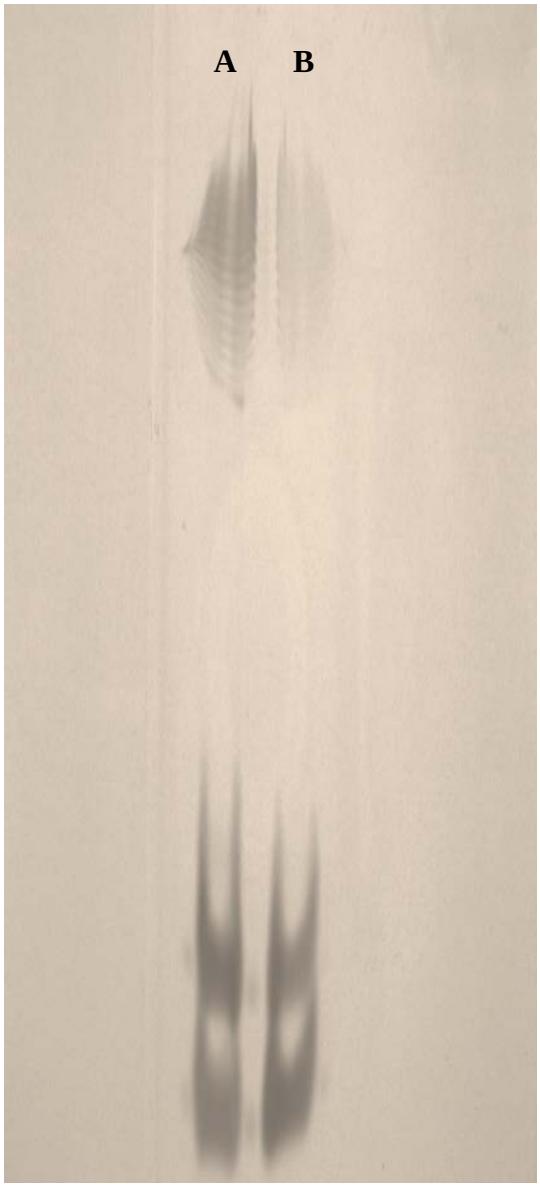

Figure 11. PAGE gel analysis of the LPS from ML09-119 (A) and ML09-119 *gfcD* (B), with LPS revealed by silver staining.

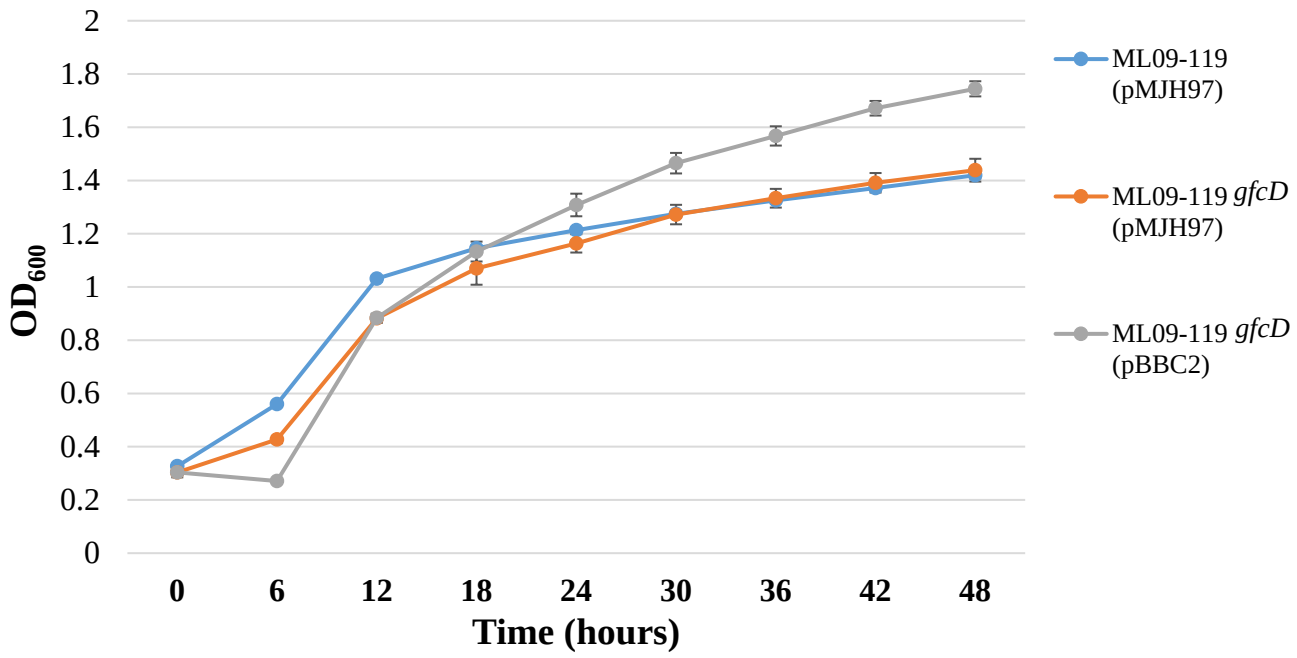

Figure 12. 48-h growth kinetics of ML09-119, ML09-119 *gfcD*, and ML09-119 *gfcD* complemented mutant. ML09-119, ML09-119 *gfcD* carry an empty vector that contains the chloramphenicol acetyltransferase (*cat*) gene (reference to manuscript). ML09-119 *gfcD* (pBBC2) contains the same empty vector with the *gfcBCD* operon. The wild type and O-antigen deficient mutant containing the empty vector showed no significant differences in growth after 48 h (P = 0.7). All error bars represent the standard error between the replicate cultures of each strain.

Chapter V

Chapter V. Protection of channel catfish (*Ictalurus punctatus*) from MAS in ponds by vaccination or probiotic supplementation.

1. Abstract

Motile Aeromonas Septicemia (MAS) is one of the big three bacterial diseases known to contribute to economic losses in channel catfish production systems across the southeastern United States. One of the most commonly observed causative agent contributing to MAS in channel catfish, *Aeromonas hydrophila*, is a gram-negative bacterium that is typically referred to as opportunistic, causing disease in channel catfish as a secondary pathogen. In 2009, highly virulent *Aeromonas hydrophila* (vAh) isolates began emerging as primary infections across the southern United States. Since 2009, vAh has contributed to the loss over 20 million pounds of channel catfish. The forms of treatments currently approved to combat against MAS in channel catfish production systems are extremely limited. The development of an effective vaccine against MAS has had success, but only in aquaria trials through intraperitoneal (IP) delivery. Additionally, a *Bacillus* probiotic has been shown effective at reducing disease due to *Aeromonas hydrophil*a, when delivered as a feed amendment. However, the protective effects derived from supplementation of this *Bacillus* probiotic has only been thoroughly tested in tilapia reared in aquaria. Providing a vaccine in conjunction with a probiotic has been shown effective

in other animal models, however, the synergistic effects of vaccination with simultaneous supplementation with a probiotic has never been tested against MAS and has not been thoroughly tested in channel catfish. Here is data presenting the protective effects elicited by IP vaccinating catfish with an attenuated mutant or through orally supplementing a bacillus probiotic on feed against MAS in ponds. Further, this study presents data testing the synergistic effect of probiotic supplementation to IP vaccinated catfish in eliciting protection against MAS.

2. Introduction

Aeromonas hydrophila has long been recognized to contribute to mortalities in aquaculture production systems, leading to Motile Aeromonas Septicemia (MAS) in channel catfish production facilities in the United States (Figueiredo and Plumb, 1977). However, infections due to *A. hydrophila* are typically regarded as opportunistic in nature, causing infections in fish during mixed infections, decrease in water quality, or overstocking (Tekedar *et al.*, 2013, Harikrishnan *et al.*, 2003, and Peatman *et al.*, 2018).

Beginning in 2009, epidemic outbreaks of MAS due to hypervirulent isolates of *A. hydrophila* (also known as v*Ah*) emerged in channel catfish production ponds in the southeastern United States, leading to tremendous economic losses to the aquaculture industry (Hossain *et al.*, 2014 and Hemstreet, 2010). To date, vAh has contributed to an estimated loss of over 20 million pounds of channel catfish (Rasmussen-Ivey *et al.*, 2016).

Due to the unknown nature of vAh disease induction in channel catfish (Peatman *et al.*, 2018), controlling these vAh outbreaks is of increasing concern. Currently, the most effective form of control is through antibiotic therapy, with Terramycin (oxytetracycline) and Romet® (ormethoprim plus sulfadimethoxine) being the two most commonly used antibiotics to battle MAS. Additionally, Terramycin is the only antibiotic approved by the FDA for treatment of

MAS in food channel catfish (Camus *et al.*, 1998). Aquaflor® (Florfenicol) is another antimicrobial alternative used in aquaculture to combat *Edwardsiella ictaluri* infections in channel catfish, and its efficacy has been shown to combat mixed infections of *A. hydrophila* and *Flavobacterium columnare* when given in conjunction with copper sulfate (Darwish *et al.*, 2012). However, these three antibiotics require a veterinary feed directive. This directive is appropriate in regulating the use of antibiotics, but due to potential delays it can cause in treatment, it is not an ideal form of control of vAh. There are also concerns with the increase prevalence of bacteria resistant to the antimicrobials used to combat infectious diseases in the industry (Petersen *et al.*, 2002). Additionally, each of these forms of treatment are expensive to the farmer and carry withdrawal times that can delay harvest. Due to the excessive cost of the current treatments against MAS, and the lack of effective treatment methods against these highly virulent isolates, alternative forms of control are needed.

Vaccines have been shown to be critical components of disease prevention in farmed fish (Sommerset *et al.*, 2005 and Hastein *et al.*, 2005). Attenuated bacterial mutant vaccines have been shown to confer protection against their wild-type form in farmed fish, making them effective vaccine candidates (Wise *et al.*, 2015, Chatakondi *et al.*, 2018, Shoemaker *et al.*, 2011, Mohammed *et al.*, 2013, and Marana *et al.*, 2017). Previously, attenuated vAh mutants created to combat MAS in channel catfish have been generated through serial passaging of vAh isolates on antibiotic-containing media and delivered to fish through intraperitoneal injection (IP) (Pridgeon *et al.*, 2012). These mutants were observed to protect fish from MAS under controlled aquaria trials, but this approach has limitations due to the potential of genetic reversion in serially passaged mutants, and the inability to vaccinate fish in high-throughput via IP injection.

IP injection of bacterins is very common in mammalian-based vaccinations and typically confers the best protection when given in conjunction with an adjuvant (Hastein *et al.*, 2005). IP vaccinations have been shown to be an effective form of disease prevention in channel catfish (Wise *et al*., 2015 and Thurlow *et al.*, 2018b). However, vaccine efficacy has not been thoroughly tested in preventing MAS in ponds and channel catfish production systems.

Previously, a vAh *gfcD* mutant was generated that prevented O-antigen capsule assembly and indicated efficacy as a form of prevention of MAS in aquaria trials (Hossain *et al.*, 2015b and Thurlow *et al.*, 2018b). A strong adaptive immune response was observed in fish vaccinated with the *gfcD* mutant relative to wild-type vAh (Thurlow *et al.*, 2018b); however, all previous testing of the protective effects elicited by this vAh mutant were performed in aquaria.

In addition to vaccines, probiotics have also been shown effective at preventing infections derived from *Aeromonas hydrophila* (Addo *et al.*, 2017). Further, *Bacillus velezensis* AP193 has been shown as a viable alternative to controlling infections in channel catfish in aquaria due to *Edwardsiella ictaluri* (Thurlow *et al.*, 2018a). However, the efficacy of these probiotics in preventing *Aeromonas hydrophila* infections have only been verified in aquaria challenges in tilapia and have not yet been thoroughly evaluated in channel catfish in ponds.

This study presents data evaluating both *Bacillus velezensis* AP193 and the attenuated *gfcD* mutant for their use as an orally administered probiotic and IP delivered vaccine, respectively, in channel catfish in ponds to combat MAS.

3. Materials and Methods

Animal welfare statement. All animal challenges were conducted under the approval of the Institutional Animal Care and Use Committee (IACUC) of Auburn University, or the USDA-ARS Aquatic Animal Health Research Unit, in compliance with U.S. regulatory standards for the

humane care and use of laboratory animals. All field trials were conducted with approval by the Alabama State veterinarian and USDA APHIS Center for Veterinary Biologics (Ames, IA).

Bacterial strains and culture conditions. Virulent *A. hydrophila* strain ML09-119 was isolated from a diseased catfish derived from an epidemic outbreak of MAS in channel catfish in Alabama in 2009 (Hossain *et al.*, 2013, Tekedar *et al.*, 2013). The vaccine strain used in this study to prevent MAS in channel catfish was generated by attenuating ML09-119 through targeted gene deletion of *gfcD* (*A. hydrophila* ML09-119 *gfcD*) (Hossain *et al.*, 2015b and Thurlow *et al.*, 2018b). *Bacillus velezensis* AP193 was previously isolated from a soil and gut library (Ran *et al.*, 2012). All isolates were initially revived from -80°C stocks and streaked for isolation onto TSA and incubation at 30°C for 24 h . Isolated colonies were then placed into sterile TSB overnight, after which time, they were diluted 1:100 in all subsequent cultures. All ML09-119 cultures used during IP challenges were incubated to an OD_{600} = 1.0 at 30°C with shaking at 200 rpm.

ML09-119 *gfcD* formalin inactivation. Inactivation of ML09-119 *gfcD*, referred to as vaccine in the duration of this study, was carried out through the fixation of cultures in 10% formalin. Bacterial strains were grown in TSB overnight at 30°C with shaking at 200 rpm. Cultures were then diluted into fresh TSB (1:100) and grown to an OD_{600} = 1.0 ($1x10^9$ CFU/ ml predetermined through previously performed growth kinetic experiments). The cells were then subjected to centrifugation at 10,000 x *g* for 10 min, at which time they were washed with sterile ultrapure water. Centrifugation and washing was repeated 3x and the cells were resuspended and quantified through serial dilutions and plate counting. All cell counts were performed in quadruplicates to increase significance. After plating onto TSA, cells were inactivated in formalin (10% final concentration in sterile ultrapure water) at 4°C for 24 h . After inactivation,

the cells were again subjected to centrifugation at 10,000 x *g* for 10 min. and washed (3x), then plated onto TSA to test for cell viablility. Verifying inactivation via cell plating was performed in triplicates.

Spore-amended feed preparation. The commercial feed was amended with *Bacillus velezensis* AP193 spores following a previously described protocol with modifications (Ran *et al.*, 2012, Thurlow *et al.*, 2018a, and Chapter II of this dissertation). *B. velezensis* AP193 were plated onto TSA at 30°C for 24 h, at which time they were submitted for large scale fermentation of spores (Osprey Biotechnics, Sarasota, FL). Prior to submission, *B. velezensis* AP193 colonies were subjected for molecular confirmation to verify the presence of *B. velezensis* AP193 following a protocol described previously with slight modifications (Thurlow *et al.*, 2018a and Chapter II of this dissertation). Isolated colonies were inoculated into TSB at 30°C with 200 rpm shaking for 24 h. Following incubation, 3 ml of the bacterial solution was used for DNA isolation using a E.Z.N.A.® Bacterial DNA Isolation kit (Omega Bio-Tek, Inc., Norcross, GA). A *B. velezensis* AP193-specific primer set was designed that targeted a genetic locus not found within any of the other *B. velezensis* strains with an available genome sequence (n=32), with the C20_157F primer (5'- ATCGCATTTGGATGTGGATT) and the C20_157R primer (5'- CGTTTCTGAATGGCGCTTAT). The PCR thermalcycling conditions consisted of 5 min at 94°C, followed by 25 cycles of a touchdown PCR with 30 secs at 94°C, 30 secs at 68°C to 60°C (5 cycles at 2°C decreasing increments) and 1 min at 72°C. The PCR results were resolved by agarose gel electrophoresis and the pure cultures of the PCR positive colonies were submitted for large scale fermentation. After the large-scale fermentation, the resulting spore preparation, containing AP193 at a concentration of $6.0x10^{11}$ CFU/g of spore preparation powder, were mixed into corn oil and spray coated onto feed at .5% of total weight of feed at the Charles C.

Miller Jr. Poultry Research and Education Center (Auburn University, AL), resulting in a final concentration equal to $1x10^6$ CFU/g of feed. During the spray coating, three random pulls of 200 g aliquots of feed were extracted, and their respective cells counts were quantified through homogenizing feed, replicated serial dilutions, and plate counting onto TSA following an incubation at 30°C overnight. Once confirmed of the relative density of spores, the feed was trucked to West Alabama and administered to fish in each of the 16 randomized raceways. For the duration of this manuscript, this feed is referred as the feed supplemented with the probiotic.

Challenge conditions for evaluating vaccine efficacy in ponds. 18,000 channel catfish fingerlings, weighing about 40g, were delivered to E.W. Shell Experiment station at Auburn University, acclimated for 10 days, and vaccinated. During acclimation, all fish were placed into recirculating systems with constant aeration. Dissolved oxygen levels were greater than 5 ppm and fish were fed at a rate of 1.5% of total bodyweight twice daily. The vaccine was grown in 1L of TSB to a final OD_{600} = 1.0. After OD adjusting and plate counting, the cells were washed and inactivated following the protocol outlined above. Post inactivation, cells were washed and suspended into 0.6 L of sterile 1xPBS. 12 h prior to vaccination, the cells were emulsified with 1.4 L of adjuvant (Montanide ISA 763 AVG, SEPPIC Inc. Fairfield, NJ) through sonication at 50% amplitude for 30 seconds. The day of vaccination, fish were anesthetized with clove oil (100 mg/l of water; Coyle *et al.* 2004) and IP delivered 200 µl of the vaccine adjuvant solution (final cell concentration equal to $1.0x10^6$ CFU worth of antigens per fish). During the vaccination, a random subpopulation of naïve fish and vaccinated fish were transported to 45-liter aquaria. Each of the naïve fish and vaccinated fish were randomly distributed into aquaria at a stocking density of 15 fish/tank and were fed a control-based diet at 2.5% total body weight for 35 days. All fish were reared in static tanks supplemented with 1 h daily water exchanges

followed by the addition of NaCl at 1 ppt. Water temperature was kept at 28 ± 2°C for the duration of the experiment. Following the 35-day rearing, each fish was anesthetized with MS-222 (50 mg/L) and challenged with 100 µl of vAh strain ML09-119 (final concentration of $1x10^6$ CFU/ml). All remaining fish were reared in 10.5 m^3 tanks (about 10,000 L) at E.W. Shell Fisheries Center for 21 days. These fish were reared under recirculating water supplemented with 8 ppt NaCl and 2 ppm $MgSO_4$ twice daily for 21 days. Fish were fed 2.0% of total body weight for 21 days. After 21 days, the fish were delivered to in pond raceways at West Alabama. During the field trials, three in pond raceways units were placed into three separate ponds. Each raceway unit consisted of four separate raceways containing naïve fish (roughly 1,200 fish/ raceway) and three separate raceways that contained the IP vaccinated fish (roughly 1,000 fish/raceway) in a randomized block design. Additionally, each pond contained >80,000 production fish that were not confined to raceways and were marketed at the end of the study. Mortalities due to enteric septicemia, *Edwardsiella ictaluri*, exceeded 40% in the vaccinated fish during the 21-day rearing period. Due to these losses, only two of the raceway units could be stocked with vaccinated fish (three replicate raceways/ unit containing 1,000 fish/replicate raceway). All fish were fed the same commercial diet at 2% – 4% total body weight/ day for the duration of the 6-month study, spanning from June-November. Dissolved oxygen (DO) and water temperature was recorded in each raceway daily and bi-weekly water quality was assessed on each unit to record: total ammonia nitrogen (TAN), NO_2^-, pH, Alkalinity, and Hardness. Mortalities were recorded for the duration of the trial, and all fish exhibiting clinical signs of MAS were plated onto M9 + inositol media to verify vAh as the causative agent. Preparation of 1 L M9 minimal salts + inositol medium (Hossain *et al.*, 2013) is as follows: 790 ml of ultra-pure water and 15 g of agarose were autoclaved for 15 min. at 121°C and cooled to 55°C. After cooling, 200 ml of sterile 5X M9

minimal salts, 2 ml of sterile 1M $MgSO_4$, 0.1 ml of sterile 1M $CaCl_2$, and 5.5 ml sterile 1M Myo-inositol were added to 790 ml autoclaved water + agarose. M9 minimal salts, MgSO4, CaCl2, and Myo-inositol were filter sterilized using 0.22 µm filters prior to adding. Following the 6-month trial, a random subset of 10 fish from each of the control and vaccinated fish of two raceway units were sacrificed and bled for enzyme linked immunosorbent assay (ELISA). All remaining fish were harvested, weighed for their relative growth performance, and sent to market.

Enzyme linked immunosorbent assay.

Wild-type (WT) plate coating. ML09-119 was sub-cultured overnight at 30°C shaking in 5 ml TSB. Cells were subjected to centrifugation at 10,000 × *g* for 10 min at room temperature (RT). The supernatant was aspirated, and cells were diluted in 1x coating solution (KPL SeraCare, Milford, MA) at a final cell concentration of ~1×10^7 CFU/ml. 100 µl of cells in coating solution were placed into each of the 96 well plates and incubated for 1 h at RT. The coating solution containing unbound bacteria was aspirated from the plate and 300 µl of 1% blocking solution (KPL SeraCare, Milford, MA) was placed into each well and incubated at RT for 15 min. The plate was then washed 3 times with 1× wash solution and dried.

IgM reactivity in fish blood serum. IgM anti-*Aeromonas* antibodies found in the blood serum of the catfish post-vaccination were quantified through indirect ELISAs using the HRP Anti-mouse IgG (H+L) Protein Detector ELISA Kit following the manufacturer's recommendations and as previously described (KPL SeraCare, Milford, MA;Thurlow *et al.*, 2018b). Blood samples were clotted for 12 h at 4°C. Clotted blood was removed by centrifugation at 3,000 x g for 5 min. The resulting supernatant, designated as serum here, was removed and stored at -20 C for later use. For the ELISA, 100 µl of fish serum diluted 1/40 in

1% blocking solution was placed into each well of the 96 well plates coated with the WT. The plate was covered and allowed to incubate for 1 h at RT. The plate was dumped and each well of the plate was washed 3 times with 300 µl of 1× wash solution. The plate was dried and 100 µl of the 9E1 mouse immunoglobin (IgG) anti-catfish IgM antibody (University of Mississippi, Jackson, MS, USA) was diluted 1/35 in 1% blocking solution, placed into each well, and incubated for 1 h at RT. Unbound antibodies were removed from wells and each well was then washed 3× with 1× wash solution and dried. 100µl of 1/1000 diluted HRP conjugated anti-mouse goat IgG (supplied in kit) was placed into each well and incubated for 1 h at RT. The plate was dumped, washed 3 times with 1× wash solution and dried. The color reaction was initiated using 100 µl of the ABTS peroxidase substrate system at the manufacturer recommended concentration. The reaction was stopped after 15 min using 100 µl of 1× stop solution and recorded at OD_{405} using a Tecan Infinite M1000Pro plate reader. Titers were reported as positive if the serum measured at least 2× negative control (Naïve fish) spectrophotometer readings.

Raceway challenge conditions evaluating *Bacillus velezensis* AP193 supplementation. A total of 18,000 fingerlings weighing roughly 40 g were placed into raceways located in the three in pond raceway units, which also containing the vaccinated fish. The fish were separated into 16 raceways with each raceway containing roughly 1,200 fish. These raceways were adjacent to the raceways containing vaccinated fish, as described in the IP vaccination raceway trial above, in a randomized block design, yielding one replicate raceway/ quad or 4 total replicate raceways/ unit. These fish were fed the same commercial diet delivered to the raceways containing the naïve fish, however this feed was supplemented with *B. velezensis* AP193 following the protocol described above. For the raceways containing fish that were supplemented with this probiotic to be comparable to the challenge conditions for evaluating vaccine efficacy

in this field trial, all fish administered this probiotic were delivered feed at a varied rate of 2%-4% of total body weight/ day for the duration of the 6-month study. Further, due to the proximity of the fish supplemented with this probiotic amended feed, the daily and bi-weekly water quality parameters gained in each pond, containing the raceway units, were deemed relevant for the raceways containing the control fish (Naïve fish fed a control diet), vaccinated fish, and fish supplemented with the probiotic. Mortalities were also recorded in the raceways containing fish supplemented with the probiotic, and all fish exhibiting clinical signs of MAS were plated onto the M9+inositol medium. Following the 6-month trial, all remaining fish were harvested, weighed, and sent to market.

Synergistic effects of vaccination and probiotic supplementation against vAh. As previously mentioned, a subset of both the naïve fish and vaccinated fish were taken from the E.W. Shell Experiment Station at Auburn University and transferred into in 45-liter aquaria. Each of the two populations of fish were separated into replicate tanks containing 10 fish/tank. The fish were placed onto a control diet (commercial diet) at 2.5% bodyweight/day for 21 days. Each of the four treatments contained four randomly assigned replicates of 45-liter tanks. Each tank was maintained at 28°C during the experiment. All tanks were kept under static conditions with constant aeration with the introduction of 1 h of flow-through at a rate of 0.6 liter/min. daily. Following the 21-day acclimation period, half of the replicate tanks from each of the vaccinated and non-vaccinated populations were placed onto a diet amended with the probiotic at $1x10^6$ CFU/g of feed. The remaining fish were continued on a control diet with no feed amendment. All fish were fed their respective diets at 2.5% of total body weight for 14 days, which was deemed as the necessary time for probiotic feeding based on previous performed challenges (Ran *et al.*, 2012). Following the 14-day feeding period, all fish were anesthetized

with MS-222 (50 mg/L) and intraperitoneally challenged against vAh WT strain ML09-119 at a concentration of $1x10^6$ CFU/ fish (200 µl injections at $5x10^6$ CFU/ml) following the protocol previously described (Thurlow *et al.*, 2018b and Chapter IV of this dissertation). Mortalities were monitored for seven days, at which time the fish were euthanized and incinerated.

Statistical analyses. Statistical analyses were performed using R (R Foundation for Statistical Computing, Vienna, Austria). Statistical significance for mortality data in raceway trials was determined by one-way analysis of variance (ANOVA) followed by Tukey post hoc multiple comparisons tests. The significance for all ELISA data was determined by one-way ANOVA followed by Tukey post hoc multiple comparisons. All quantitative data is presented as means ± standard error. Differences were considered statistically significant if a P-value < 0.05.

4. Results

Efficacy of vaccine administered by IP during raceway trial.

During the 21-day acclimation, the vaccinated fish housed at the E.W. Shell Experiment Station were hit with an ESC outbreak which knocked out roughly half of the vaccinated population. This prevented the use of a third raceway unit for testing the vaccinated fish, and the remaining two raceway units were reduced to 3 replicate raceways containing 1,000 fish. Fish were stocked on June 18th, and three days after stocking, the fish in both units began to feed at around 1% total bodyweight/ day. During the first week of the trial, the feeding was increased to 2% of total bodyweight/day and for the duration of the study, the fish were delivered 2%-4% of total bodyweight/day, which was based on satiation of fish. Dissolved oxygen (DO) varied from 1.5-5 mg/L during the morning to 5-21.5 mg/L during the late afternoon. The average water temperature was 27.5°C, with temperatures ranging from 26°C – 35°C during June – September. The temperature decreased at the end of September, in which it fell below 21°C. Bi-weekly

water quality parameters indicated the following ranges: TAN (0.5-3mg/L), NO_2^- (0-0.44 mg/L), pH (7.7-8.5), Alkalinity (120-140 mg/L), and Hardness (51-85 mg/L).

During the study, two of the three raceway units were hit with vAh. VAh was first isolated in the production fish in the open ponds on July 2nd. VAh mortalities were confirmed in the raceway units on July 24th and mortalities due to vAh continued through September 10th. VAh was not isolated in morbid fish in the raceway units until September 4th and the outbreak in the two raceway units lasted roughly 10 days and resulted in over 200 mortalities. Mortalities due to vAh exceeded 1,000 fish/pond in the catfish in the open ponds containing the raceway units. During the outbreak, vAh was only isolated from one of the units containing vaccinated fish. However, vAh was not isolated from any of the vaccinated fish in this raceway unit during the outbreak (Figure 1). One-way ANOVA followed by Tukey post hoc indicated significant differences between the number of mortalities observed in the naïve fish when compared to the vaccinated population ($P < 0.0001$).

Further, Fish blood sera ELISAs indicated highly significant differences in IgM reactivity to the WT between these fish in these two populations 6 months after vaccination (Figure 2; $P < 0.0001$). Tukey post hoc also indicated that there was a significant difference between IgM reactivity to the WT in fish that were vaccinated when compared to naïve fish in both raceway units that contained vaccinated fish ($P < 0.0001$). However, there was no observed significant difference in IgM reactivity to the WT between naïve fish in the unit that was confronted with vAh when compared to blood serum IgM reactivity observed in naïve fish reared in the unit that had no reported confrontation with vAh ($P > 0.9$). Further, the two naïve populations in the two units were highly insignificant in their differences in IgM reactivity to the WT when compared to the reactivity of naïve fish reared in aquaria not used during this study ($P > 0.9$). There were no

significant differences in IgM reactivity observed between the vaccinated populations reared in separate units ($P > 0.9$).

Efficacy of probiotic administered orally to fish in raceways trial.

As previously discussed, fish were stocked into all the raceway units on June 18th, and three days after stocking, the fish in the units began to feed at around 1% total bodyweight/ day. During the first week of the trial, the feeding was increased to 2% of total bodyweight/day and for the duration of the study, the fish were delivered 2%-4% of total bodyweight/day. Due to the proximity of the raceways containing both vaccinated and non-vaccinated populations of fish, daily and bi-weekly water quality parameters observed in the raceways containing non-vaccinated fish supplemented with the probiotic correlated with the vaccine raceway water quality parameters previously discussed.

Outbreaks due to vAh occurred in two out of the three raceway units. During the outbreak, all the raceways containing non-vaccinated (naïve) fish delivered a control diet were slightly reduced in their percent survival by roughly 2% when compared to raceways containing fish that were fed a diet supplemented with the probiotic (Figure 3; $P > 0.05$). When compared to raceways containing vaccinated fish, there was a significant decrease in their survival, exceeding 4%, observed in the non-vaccinated fish that were supplemented with the probiotic ($P < 0.001$).

When comparing the differences in growth performance after the 6-month feeding period, the fish that were delivered a control diet indicated an average weight gain that was lower than the weight gain of fish supplemented with the probiotic (Figure 4; $P = 0.07$). Albeit the marginal insignificance, there was a reduction in average weight gain exceeding 10 g in the fish fed a control diet when compared to fish fed a diet supplemented with the probiotic. Further, the

relative weight gain observed in fish that were supplemented with the probiotic still indicated over a 10% reduction when compared to vaccinated fish ($P < 0.01$).

Synergistic effects against vAh when supplementing vaccinated fish with the probiotic.

The aquaria IP challenge conducted on vaccinated and non-vaccinated fish (naïve) populations indicated significant differences in their percent survival when confronted with the wild type after the two-week feeding with a control diet or a diet amended with the probiotic (Figure 5). When confronted with the wild-type, the naive fish that were delivered a control diet indicated the lowest percent survival, which was 65% and 61% lower than the relative percent survival of vaccinated fish delivered a control diet and naïve fish that were delivered a diet supplemented with the probiotic, respectively ($P < 0.001$). There was a reduced percent survival in the naïve fish that were administered the diet supplemented with the probiotic when compared to vaccinated fish administered a control diet, however, this difference was insignificantly different ($P > 0.2$). There were no mortalities due to vAh observed in either of the vaccinated treatment groups that were delivered feed amended with or without the probiotic ($P = 1.0$).

5. Discussion

VAh has led to the destruction of over 20 million pounds of channel catfish in production systems across the Southeastern United States, however, there has been no confirmation as to how vAh infects channel catfish in production systems. Further, it is predicted that infections derived from vAh appear to be multifactorial (Hossain *et al.*, 2013). Despite the unknown nature of vAh pathogenicity, it had been previously observed that the attenuation of *Aeromonas hydrophila* virulence in channel catfish can be achieved through the deletion of a group 4 capsular assembly gene (*gfcD*) (Thurlow *et al.*, 2018b). Further, there has been no recorded case of MAS in channel catfish due to IP injecting the GfcD deficient mutant (referred to as vaccine

in this study). Previous aquaria trials have indicated that IP injecting the live version of this mutant leads to increased protection against sub challenges with the WT 21 days after the initial vaccination. However, due to the complexities in regulatory approval of field trials with live attenuated mutant vaccines, there was an inability to test the vaccination efficacy of the live version during the raceway trial. Presented here is data that correlates with previous aquaria vaccination trials, indicating that IP injecting the inactivated form of the vaccine into channel catfish can protect them against vAh in aquaria and production systems months after initial vaccination.

There were no recorded mortalities due to vAh observed in any of the IP vaccinated fish populations reared in aquaria and the in-pond raceways. The data derived from this trial provides the first recorded evidence of a viable vaccine candidate that can elicit protective effects against vAh infections in production systems. However, during the field trial heavy mortalities were observed due to *Edwarsiella ictaluri* and *Flavobacterium columnare*, which made these diseases limiting factors in the trial. Thus, it is advantageous for future work to evaluate the prevention of infections from ESC and columnaris by using additional vaccine(s) or even a, "Vaccine Cocktail" containing the appropriate vaccines against these pathogens (Wise *et al.*, 2015, Chatakondi *et al.*, 2018, Shoemaker *et al.*, 2011, Mohammed *et al.*, 2013).

The vaccinated fish also contained systemic blood-based IgM that was reactive to the WT and this reactivity was significantly higher than the IgM reactivity observed in naïve fish blood sera reared in the raceways under similar conditions. Further, this reactivity was observed in fish sera 6 months after the initial IP vaccination. This protection against gram-negative pathogens has been shown before in channel catfish, however, this was the first record of an adaptive immune response against vAh observed in a field trial more than 35 days post vaccination with

the GfcD deficient mutant (Wise *et al.*, 2015, Mohammed *et al.*, 2013, and Thurlow *et al.*, 2018b).

In addition to protective effects, there was also an observed increase in growth performance in fish that were delivered the vaccine. Fish delivered the inactivated vaccine via IP held an average weight that was 38% higher than the respective naïve fish reared in the same raceway unit ($P < 0.0001$). It is important to note that these two populations were separated during the 21-day period post vaccination, however, these two groups of fish were divided from the same initial population and genetic line. Therefore, more testing needs to be conducted to verify a correlation between a heightened growth performance and the vaccine.

The fish delivered the probiotic amended on feed also indicated an increase in relative weight gain when compared to fish delivered a control diet. Even though this increase in weight gain was marginally insignificant, it correlates with previously reported data testing the average weight gain of channel catfish from supplementation with a probiotic in ponds (Thurlow *et al.*, 2018a and Chapter II). Just as with the average weight gain in fish fed a control-based diet when compared to the weight gain of vaccinated fish, there was an observed increase in average weight gain in vaccinated fish when compared to fish supplemented with the probiotic. As previously discussed, the vaccinated fish were housed at a separate facility for the first 21 days of the trial. Therefore, there is not sufficient evidence to prove that this observed weight gain was a direct effect of the vaccine.

During the outbreaks, there was also a slight increase in survival rate observed in the raceway reared fish that were administered the probiotic when compared to fish that were delivered a control diet. However, the increased survival of roughly 2% observed in the fish

supplemented with the probiotic was insignificantly different than the survival rate of naïve fish fed a control diet ($P > 0.05$).

Even though the differences in mortality observed during the raceway trial was insignificant, the aquaria trial conducted with a subsample of the same population of probiotic fed naïve fish indicated significant separation in the percent survival when compared to naïve fish after being challenged against the WT two-weeks after oral administration of the probiotic. Further, the survival rate observed in naïve fish that were fed a control diet was 61% lower than that of naïve fish fed the probiotic during the aquaria trial ($P < 0.001$). This was a substantially significant difference in percent survival compared to the 2% difference observed in the similar populations during the pond trial. In both the raceway trial and aquaria trial, the vaccinated fish outperformed the fish supplemented with the probiotic in their relative percent survival against vAh. This difference in percent survival was deemed significant during the vAh outbreak ($P < 0.001$), however, there was no significant differences observed between the vaccinated fish population and the fish supplemented with the probiotic when challenged against vAh during the aquaria trial ($P > 0.2$). These differences in significance could be due to the low mortality rate and short duration of the vAh outbreak observed during the raceway trial. Further, there was an inability to accurately identify the number of fish responding to feed in the raceway units, which could also lead to the differences observed in the pond and aquaria trials. What is important to note about the aquaria challenge, is that the wild-type was IP injected into the catfish after feeding the probiotic for two-weeks. Based on the mortality data, it is a surprising result to see such a substantial increase in systemic protective effects from a gut derived probiotic. Previous studies have indicated that the effectiveness of an orally administered probiotic as a protective measure against infection is often attributed to an enhanced immunity in the fish (Addo *et al.*,

2017 and Welker and Lim, 2011). The innate immunity has been shown to play a significant role in the protection of teleost from infectious diseases, as the adaptive immune response has been greatly hindered in teleost due to their poikilothermic nature (Uribe *et al.*, 2011 and Gao *et al.*, 2012). Innate immunity modulators such as phagocytes, cytokines, complement, peroxidase, lysozyme activity have been stimulated in tilapia through probiotic supplementation (Addo *et al.*, 2017). However, the stimulation of innate immune modulators through probiotic supplementation has not been thoroughly tested in channel catfish in ponds. Future work looks to identify the immune modulators stimulated in channel catfish during supplementation of *Bacillus velezensis* AP193.

The testing of synergistic protective effects that could be elicited against vAh through vaccination and oral supplementation of the probiotic did not indicate any significant differences when compared to solely vaccinating fish during the aquaria trial. It is important to note that there were no mortalities observed due to vAh in both the vaccinated populations with and without probiotic supplementation ($P < 0.05$).

This study presents data confirming the effectiveness of intraperitoneally delivered vaccinations in preventing vAh infections in ponds, however, this form of vaccination presents logistical issues when trying to mass vaccinate fish. Further, IP vaccinations in conjunction with adjuvants can also lead to adverse reactions at the site of injection, indicating safety concerns in young fish due to the invasive nature of the vaccination and the heightened localized immune response (Embregts and Forlenza, 2016 and Hastein *et al.*, 2005). Oral administration of vaccines has been found to be effective alternative form of large scale vaccine delivery in aquaculture when compared to IP injections (Wise *et al.*, 2015 and Rombout *et al.*, 2014). This form of vaccine administration offers one of the most attractive forms of immunization currently

available for aquatic production species, due to the ease of delivery, applicability in young and old fish, and an efficient means of administering bacterial boosters (Mutoloki *et al.*, 2015, Chettri *et al.*, 2015, Nakanishi and Ototake, 1997, Wise *et al.*, 2015, and Sommerset *et al.*, 2005). However, the efficacy of orally administered vaccines has never been previously demonstrated as effective forms of prevention against MAS in channel catfish. Further, the synergistic protective effects through simultaneous oral vaccination and probiotic supplementation has not been thoroughly tested in channel catfish. Therefore, it is of extreme importance to carry out future studies testing the synergistic capabilities of oral vaccination and probiotic supplementation in channel catfish production systems.

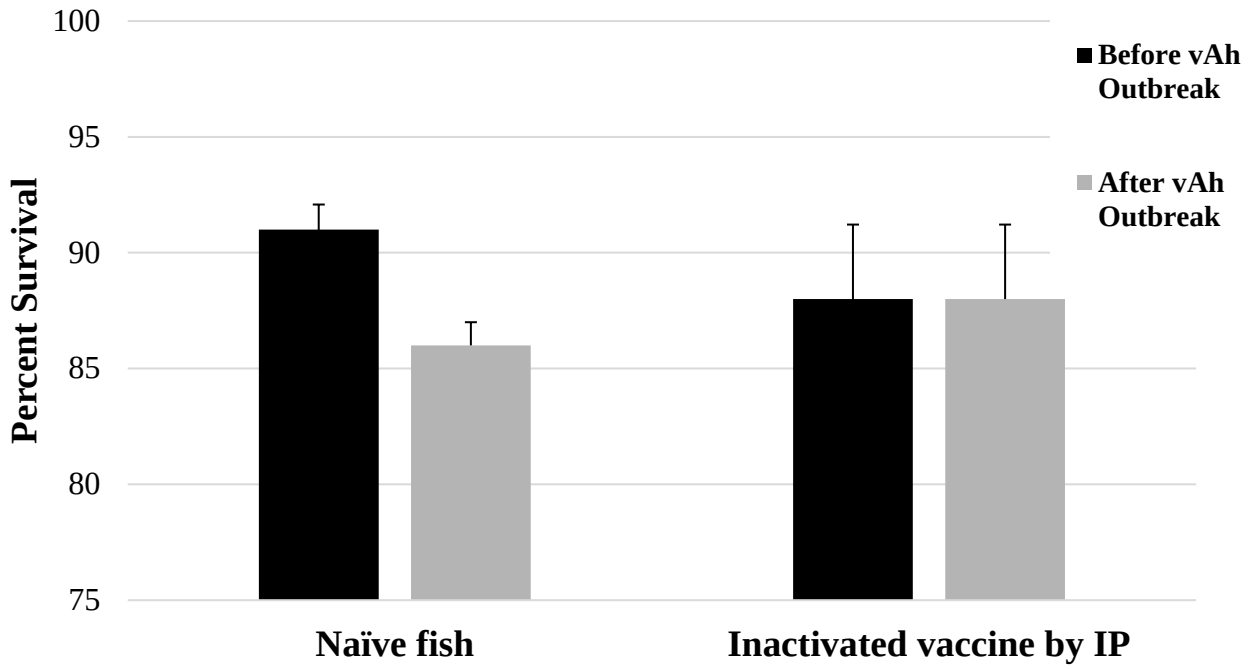

Figure 1. Survival of fingerling catfish confronted with vAh after IP delivery of the vaccine or naïve fish. Data is presented as the percent survival ± SE for 4 replicate raceways and 3 replicate raceways containing naïve fish and vaccinated fish, respectively, in Unit 1.

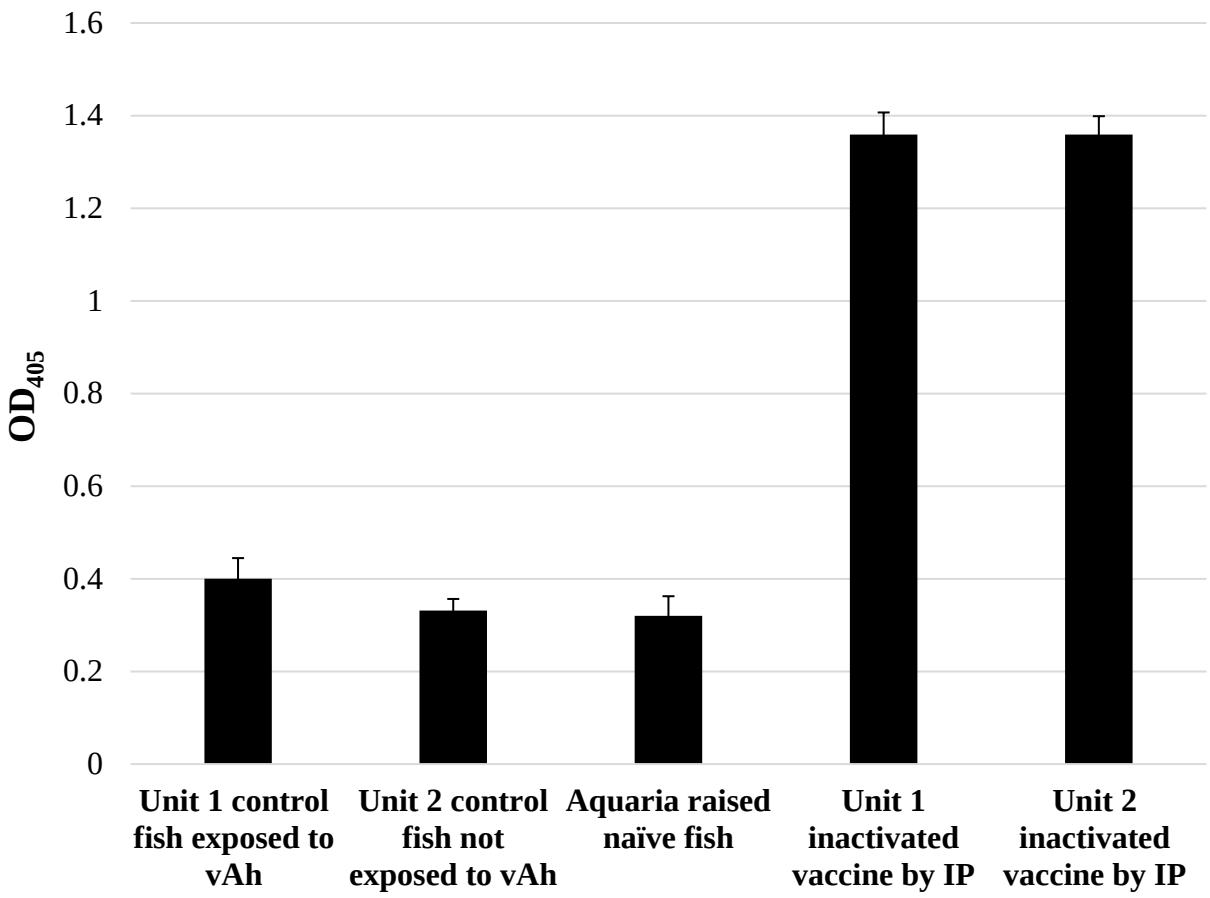

Figure 2. Average IgM antibody titers in fish sera 6 months after IP vaccination. Fish in Unit 1 were exposed to vAh 4 months prior to bleeding. All field trial data is presented as the absorbance at 405nm ± SE of 5 replicate fish serums from 3 raceways/ unit. The average of 5 naïve fish sera, removed from fish not used in this study, were compared.

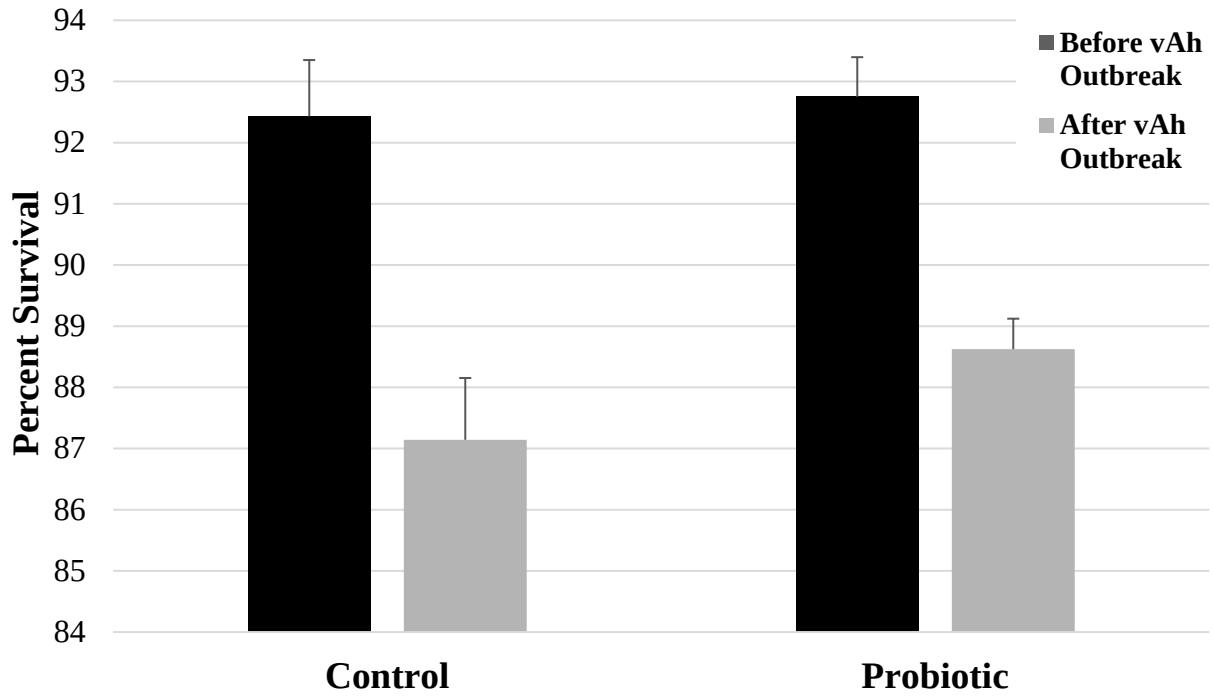

Figure 3. Survival of fingerling catfish confronted with vAh after delivery of control-based diet or diet amended with the probiotic *Bacillus velezensis* AP193. Data is presented as the percent survival ± SE for 8 replicate raceways across the two units with reported vAh infections.

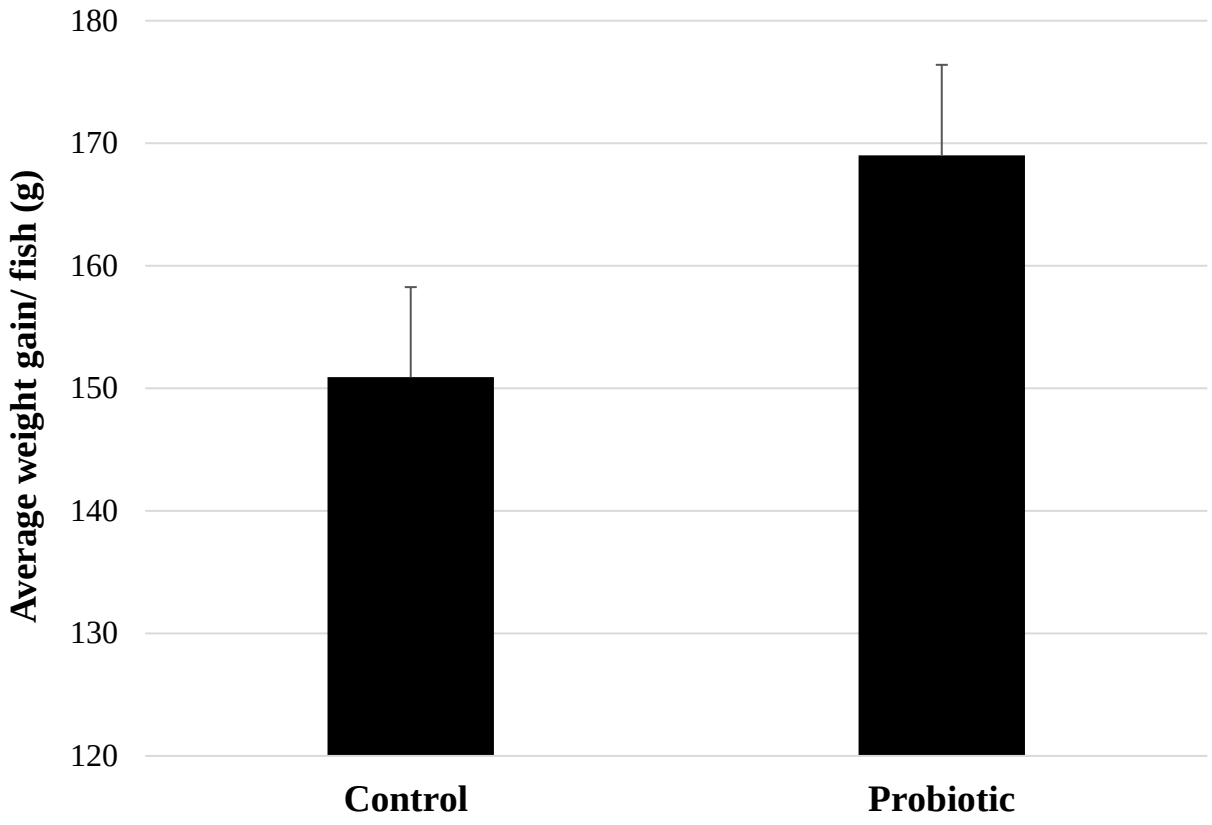

Figure 4. Growth performance of channel catfish after 6 months of probiotic feeding in raceways. All data is presented as a compiled average between all raceways containing fish fed a control-based diet and a diet amended with the *Bacillus velezensis* AP193 probiotic strain. All data is presented as the average weight gain per fish ± SE of 12 replicate raceways from across three separate ponds.

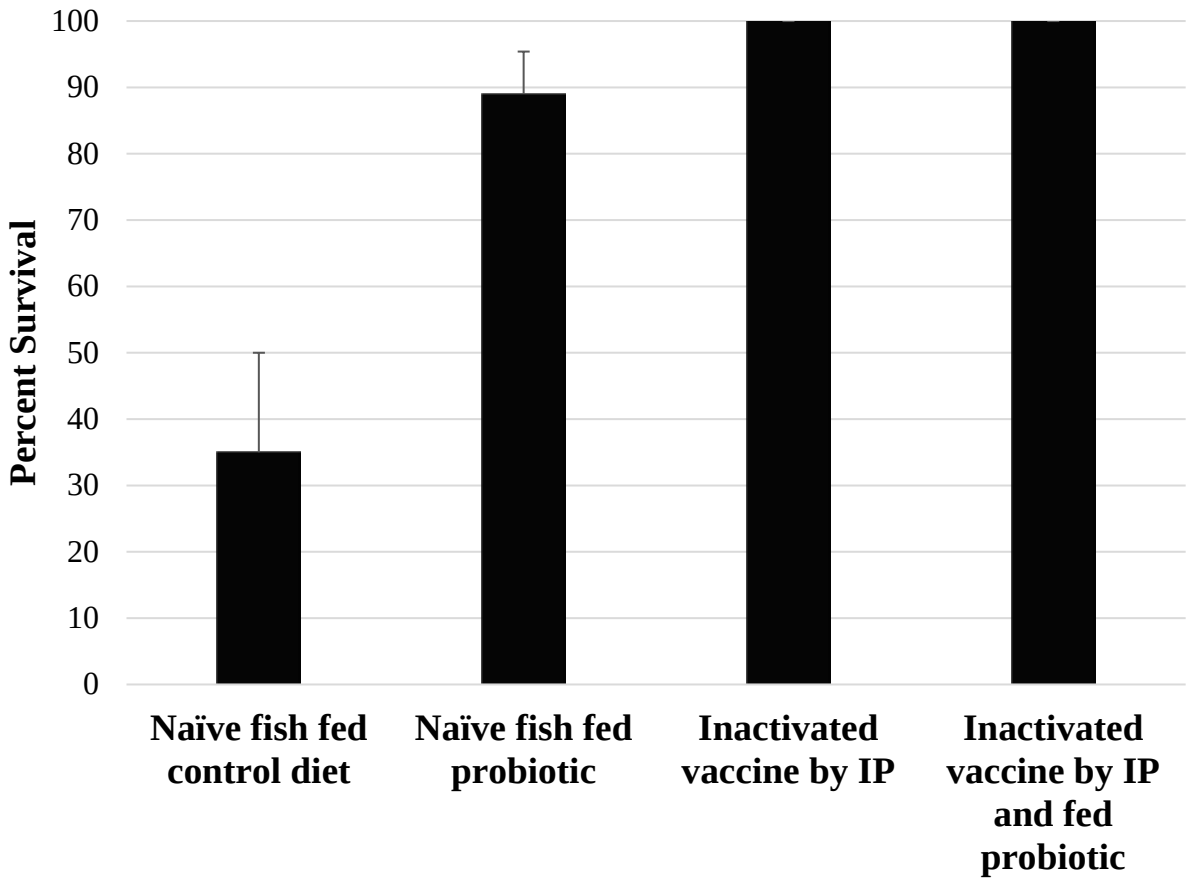

Figure 5. Percent survival of channel catfish challenged with vAh strain ML09-119 after vaccination with and without probiotic feeding or naïve fish. Error bars represent the average percent survival ± SE of 3 replicate tanks for each treatmen

Chapter VI

Conclusions

This dissertation presents data evaluating two potential forms of infectious disease control in channel catfish production systems. The first in the form of a probiotic that could elicit protective effects against *Edwardsiella ictaluri* infections. This probiotic also indicated efficacy at enhancing growth performance and water quality. Four *Bacillus* strains were originally tested when delivered as a feed additive to 1) enhance the growth of channel catfish under aquaria conditions, 2) reduce mortality due to disease, and 3) improve water quality. While two of the *Bacillus* strains indicated a moderate decrease in fish mortality due to *E. ictaluri* infection, feed amended with *B. velezensis* AP193 provided the greatest reduction in fish mortality of 23%, however the differences observed in each of the strains tested were insignificant ($P > 0.05$).

Additionally, each of the four *B. velezensis* strains were observed to enhance fish growth performance. *B. velezensis* AP193-amended feed resulted in the greatest observed growth performance (~8%) compared to control feed. Interestingly, there was an increase in the growth performance of catfish fed with AP193-amended feed in pond trials, with 40.4% and 32.6% increases in average weight gain for the two populations of fish (aquaria or raceway sourced, respectively) used in the pond study. *B. velezensis* AP193 was observed to express phytase activity that was greater than, or comparable to that of, the other *B. velezensis* strains.

The presence of phytase could help to explain the significant reductions in total phosphorus, total nitrogen, and nitrate-nitrogen levels that were observed in ponds containing channel catfish fed with AP193 that indicate beneficial, pond-wide effects on water quality. *B. velezensis* AP193 also indicated the capacity to degrade the phytate present within feed, potentially resulting in uptake of phosphorus by the intestinal microbiota.

Further, this dissertation presents data indicated that the addition of *B. velezensis* AP193 as a probiotic resulted in no significant changes in the intestinal microbiota. The low levels of AP193 (25 CFU/ml) detected in water from ponds in which fish fed with an AP193-amended diet indicates that some level of the probiotic is present within the pond ecosystem. Albeit the marginal insignificance, the addition of AP193 to channel catfish feed could reduce the disease severity of *Edwardsiella ictaluri*. Prolonged feeding of AP193 may also result in the stabilization of the intestinal microbiota composition over time. Additionally, AP193 resulted in improved water quality parameters in ponds. Taken together, these results suggest that AP193 could be a viable candidate as a channel catfish probiotic to prevent disease, promote fish growth, and reduce aquaculture pond eutrophication, warranting further studies in larger scale production ponds over longer time periods.

The second form of infectious disease control explored the protective effects elicited by an attenuated bacterial vaccine against virulent *Aeromonas hydrophila* (vAh) infections in channel catfish. Previously, an efficient system was exploited for generating single gene knockout mutants for virulence studies. However, attempts at generating marker-less mutants were cumbersome and conjugally inefficient when generating large numbers of mutants. The above concerns prompted the creation of pCMT-*flp*, a plasmid that was substantially smaller than the current vector used for generating marker-less mutants. This mutant indicated an increased

conjugation efficiency, which often elicited 10^6 transconjugants during conjugation events between *E. coli* SM10λpir and vAh strain ML09-119 (hereafter presented as WT). Post conjugation, the Flp recombinase activity of pCMT-flp could be induced for the removal of the chloramphenicol resistance gene cassette through incubating at 30°C until an OD_{600} = 1.0, then shifting the temperature to 37°C for 1 h. After verifying the absence of the selectable marker, pCMT-*flp* can be cured from the marker-less mutant by heat induction at 43°C. Ultimately, this plasmid allowed for a more efficient form of generating marker-less mutants for subsequent virulence studies and vaccine generation.

Previously performed genome analysis indicated that the genome of vAh isolates contained 55 unique regions that were absent in rAh isolates. Among the ORFs that were vAh-specific were 25 ORFs predicted to encode a 26.5 Kb O-antigen biosynthesis gene cluster, which had not been previously observed in any other bacterial species. The annotation of the WT O-antigen biosynthetic cluster predicted the presence of gene products required for the synthesis and transport of activated sugars to the outer membrane and ligation of these sugars to the growing O-antigen chain. Further, the O-antigen flippase, O-antigen polymerase (Wzy) and O-antigen ligase (WaaL) gene products were predicted to be present in the WT gene cluster, which indicates that WT relies on the Wzy-dependent pathway to synthesize the O-antigen, attach it to lipid A and core of the LPS, and export the LPS to the outer leaflet of the outer membrane.

Upon deleting both *waaL* and *wzy* through allelic exchange double crossover mutations there was an observed lack of LPS-associated O-antigen to the outer membrane and a ML09-119 *waaL* mutant showed an attenuation of virulence. However, complementation with the wild-type *waaL* did not restore virulence to the *waaL* mutant despite multiple attempts. Further analysis of this mutant indicated that the transcription termination site of an upstream operon had been

functionally altered and suggested that there was a polar effect of the *waaL* mutation. The annotated genome for *A. hydrophila* ML09-119 deposited in GenBank predicted three open reading frames, *gfcB, gfcC,* and *gfcD* that were upstream of *waaL,* with *gfcD* residing directly upstream of *waaL*. CDD analysis of this operon indicated homology to gene products of the *gfc* operon, which has been identified in at least 29 genera of gram negative bacteria and been shown in *Escherichia coli* O127 and O157 pathogenic strains to play a role in capsule assembly.

Due to the expected requirement of the *gfc* operon on G4C assembly, the WT and the *gfcD* marker-less mutant, generated through pMJH65 and pCMT-*flp*, were characterized for capsule formation, biofilm formation, buoyancy, resistance to serum, and virulence. *gfcD* was chosen based on its proximity to *waaL* and the hypothesis that is had been directly affected by the allelic exchange deletion event performed in *waaL*. As expected, the *gfcD* mutant lacked an observable G4C under bright field microscopy compared to the WT. Previously, there was a reduction in buoyancy observed in the *gfcD* mutant when compared to the WT, which can be reversed upon complementation. Additionally, previously performed serum resistance studies indicated that the *waaL* and *wzy* mutants held an increased susceptibility to fish serum, which was not observed in the WT or *gfcD* mutant. There were also observed reductions in both the presence of the O-antigen and shortened O-antigen chains in both the vAh *waaL* and *wzy* mutants. This reduction was not observed in WT or the *gfcD* mutant, supporting the idea that GfcD does not play a role in the formation of the LPS-associated O-antigen. Additional *waaL* or *wzy* mutants were generated by recombineering and indicated no reduction in virulence during aquaria trials. This indicates that expression of the vAh LPS-associated O-antigen is not critical to vAh virulence when IP injected into fish, however, this may indicate the role the O-antigen could play in complement resistance and avoidance of the innate immune response. Based on the

rapid onset of mortality and infections during aquaria trials, it is likely that the *gfc* operon could also play a role in secreted virulence factor(s). However, this has not been thoroughly investigated. Previously, G4C formation has been shown to play a role in biofilm formation and attachment to host tissues, and the *gfcD* mutant indicated significant reductions in biofilm production and substrate attachment when compared to vAh strain WT. Further, biofilm production and substrate attachment could be restored in the *gfcD* mutant through complementation.

To determine the role for the *gfc* operon in vAh virulence, aquaria challenges were carried out with marker-less mutants in *gfcB, gfcC, gfcD,* and *gfcBCD.* All mutants were observed to have an attenuation of virulence in channel catfish, with the *gfcD* mutant showing a total attenuation of virulence whereas mutants in *gfcB* and *gfcC* also held significant reductions in virulence compared to wild-type WT. Further, partial restoration of virulence was observed in each of the marker-less mutants. Due to the full attenuation of virulence observed for the *gfcD* mutant, it was of interest to challenge surviving fish with the WT after 21 days of initial mutant challenge. Fish that had previously received an IP dose of the *gfcD* mutant, showed a significantly higher survival rate compared to naïve fish, indicating that the fish had mounted an adaptive immune response against vAh after the initial confrontation with the *gfcD* mutant. This was further supported by the observation of a significant increase in systemic blood serum IgM levels specific to the WT antigens. The serum IgM levels observed from *gfcD* vaccinated fish were significantly higher than the titers found in naïve fish or from fish that survived the initial encounter with the WT (data not shown).

Further testing on the *gfcD* mutant (hereafter referred to as vaccine) was carried out through field trials examining the protection elicited through IP injecting the vaccine to combat

vAh disease in production systems. Through IP injecting the inactivated form of the vaccine into channel catfish there was an observed reduction in vAh disease, indicating a protective effect against vAh in aquaria and production systems that could be observed months after initial vaccination. Additionally, the vaccinated fish contained systemic blood-based IgM that was reactive to the WT and this reactivity was significantly higher than the IgM reactivity observed in naïve fish blood sera reared in the raceway units under similar conditions. Further, this reactivity was observed in fish sera 6 months after the initial IP vaccination. There was also no indication that the naïve fish that confronted vAh during the outbreak were significantly different in their blood sera IgM reactivity when compared to naïve fish that had no recorded history of vAh confrontation. Fish delivered the inactivated vaccine via IP also held an average weight that was 38% higher than the respective naïve fish reared in the same raceway unit, however, these fish had been house in separate systems for 21 days prior to the start of the field trial. Vaccinating fish through IP injecting the vaccine was effective at reducing artificially induced and natural inductions of vAh in both aquaria trials and a pond trial, respectively. This was the first recorded evidence of an effective vaccine against a natural induction of vAh in channel catfish production ponds.

The *Bacillus* probiotic strain AP193 that was tested in the pond study at North Auburn, was also delivered as a feed amendment during this field trial. Albeit the marginal insignificance during this study, there was an increased survival rate against MAS observed when the probiotic was orally administered to channel catfish in aquaria when compared to fish that were delivered a control diet. More testing is needed to confirm the protective effects against a natural induction of MAS in ponds through prolonged supplementation of the probiotic, however, this is the first

evidence that oral administration of a probiotic may increase protection against MAS in catfish in production ponds.

The raceway trial also presented growth performance data that correlated with the previously performed pond study, in which there was an observed increase in weight gain in the fish supplemented with the probiotic. It is important to note that even though this data correlated with previously performed trials, the difference in weight gain observed in the fish supplemented with the probiotic was marginally insignificant when compared to fish delivered a control diet throughout the 6-month study.

Lastly, this dissertation presents data evaluating the synergistic effects elicited against MAS in vaccinated fish during two-week supplementation of the probiotic. During the aquaria trial, there was an inability to resolve a difference in survival rate in vaccinated populations with and without probiotic supplementation. However, it is important to note that this inability was due to the 100% survival rate in both populations of fish when challenged against WT vAh. Due to the logistical concerns presented during IP vaccinations in channel catfish, future studies will be directed towards further testing the synergistic capabilities of simultaneous oral vaccination and probiotic supplementation against MAS in channel catfish production systems.

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