Evaluating Efficacy of Plant Growth Promoting Rhizobacteria for Promoting Growth and Preventing Disease in both Fish and Plants

by

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Abstract

Plant growth promoting rhizobacteria (PGPR), are bacteria residing within the rhizosphere of a plant, that elicit health benefits to the plant (Kloepper and Schroth, 1978; Kloepper *et al.*, 2004). To understand the growth promoting and disease-inhibiting activities of PGPR strains, the genomes of 12 different PGPR strains affiliated with the *B. subtilis* group were sequenced. These *B. subtilis* strains exhibited high genomic diversity, whereas the genomes of *Bacillus amyloliquefaciens* strains (a member of the *B. subtilis* group) and *B. velezensis* strains (formerly *B. amyloliquefaciens* subsp. *plantarum*, now a part of the *B. amyloliquefaciens* clade (Fan *et. al.*, 2017)) are highly conserved. A total of 2,839 genes were consistently present within the core genome of *B. velezensis*. Comparative genomic analyses of *B. amyloliquefaciens* and *B. velezensis* strains identified conserved genes that have been linked with biological control and colonization of roots or leaves. There were 73 genes uniquely associated with *B. velezensis* strains with predicted functions related to signaling, transportation, secondary metabolite production, and carbon source utilization. Genes involved in secondary metabolite biosynthesis were deleted in B. velezensis strain AP193 to evaluate their role in plant pathogen biocontrol, revealing that difficidin expression is critical and solely sufficient for reducing the severity of bacterial spot caused by *Xanthomonas axonopodis* pv. vesicatoria in tomato plants. A root extract medium was used as a growth substrate along with extended incubation times to obtain new diverse rhizobacteria isolates. The antagonistic potential of these isolates against a broad range of plant pathogenic bacteria, fungi and oomycetes was observed. Among the isolates tested, *Burkholderia gladioli* C101 produced the most potent and heat-stable active secondary metabolites that were

active against root or foliar fungal, oomycete or bacterial pathogens. A cell-free formulation of *B. gladioli* C101 was used as a foliar spray to suppress bacterial spot disease in tomato; resulting in a significant reduction in bacterial spot disease severity in greenhouse experiments when the cell-free supernatants were applied prior or after inoculation with *Xanthomonas perforans* (*Xp*). *Bacillus* strains isolated from soil or channel catfish intestine were screened for their antagonism against *Edwardsiella ictaluri* and *Aeromonas hydrophila,* the causative agents of enteric septicemia of catfish (ESC) and motile aeromonad septicaemia (MAS), respectively. The top 21 strains expressed antagonistic activity against multiple aquatic bacterial pathogens including *Edwardsiella tarda, Streptococcus iniae, Yersinia ruckeri, Flavobacterium columnare,* and/or the oomycete *Saprolegnia ferax.* Survival of the 21 *Bacillus* strains in the intestine of catfish was determined as *Bacillus* CFU/g of intestinal tissue of catfish after feeding *Bacillus* spore-supplemented feed for seven days followed by normal feed for three days. Five *Bacillus* strains were selected to incorporated into feed in spore form at a dose of 8×10^7 CFU/g and fed to channel catfish for 14 days before they were challenged by *E. ictaluri* in replicate. Two *Bacillus subtilis* strains conferred significant benefit in reducing catfish mortality ($P < 0.05$). The effects of probiotic-amended diets fed to juvenile Nile tilapia, *Oreochromis niloticus* on growth and susceptibility to Streptococcus iniae infection was determined. Fish (average weight 16.5±0.2 g) were fed five diets formulated with *Bacillus subtilis* group strains SB3086, SB3295, SB3615 or AP193 either individually or in combination for strains SB3086 and SB3615 at a targeted concentration of approximately 4 x 107 CFU/g of feed, or with a basal control diet with no additives for 21 days. After the 21-day growth trial, no significant difference in growth performance

was observed with any probiotic-amended diet. However, results from the challenge showed significantly lower mortalities between treatments as compared to the control ($P <= 0.0001$). An additional study was conducted to evaluate the individual and combined effects of long-term feeding of diets containing two probiotic Bacillus subtilis group strains (Aqua NZ and AP193) and the prebiotic Previda®, a commercial hemicellulose extract, on growth performance, immune parameters and *Aeromonas hydrophila* susceptibility of juvenile Nile tilapia, *O. niloticus*. Nile tilapia of average weight 7.47 ± 0.11 g were fed diets formulated with the probiotics and/or the prebiotic, or a control diet for 8 weeks and, subsequently, challenged with A. hydrophila by intragastric gavage at a dosage of 3.9×10^7 CFU/fish. Fish attained a mean weight of 59.5 ± 0.99 g at the end of the growth period. None of the diets significantly improved mean percent weight gain ($P = 0.70$), thermal growth coefficient ($P=0.88$) or feed conversion ratio ($P = 0.87$) of Nile tilapia. Except for the diet containing the prebiotic Previda® only ($P = 0.17$), all other diets resulted in significantly higher fish survival compared to the control ($P < 0.05$) when challenged *A. hydrophila*. The prebiotic and probiotic strains used in combination emerged as the most important diet with respect to mortality reduction. Four *Bacillus velezensis* strains were evaluated for their probiotic effects and disease reduction due to *E. ictaluri* in an aquarium study. 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. No significant differences were observed in the catfish intestinal microbiota or the pond microbiota due to probiotic-amended feed. 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%).

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Table of Contents

List of Tables

List of Figures

Chapter I

Introduction and Literature Review

1. Introduction

Both plants and animals depend on microbes for many biological functions. A few key examples include, nutrient utilization by gut microbiota, and nutrient exchange between plant host and microbes. Microbes and their eukaryotic hosts have evolved together and rely on each other for survival. Therefore, plant and animal species have evolved host-microbe relationships that can be exploited. This dissertation explores the use of beneficial bacteria to improve production of crop plants and aquaculturally important fish.

Biological control is the use of living organisms to reduce harmful effects of a pest or pathogen on a host organism (Eilenberg *et al.*, 2001). Other methods of pest control involve the use of pesticides, herbicides, and or nematicides in crops, which can leach into ground water, kill beneficial species in addition to the target pest, as well as a range of other unintended consequences (Steichen *et al.*, 1986) Additionally, the control of pathogens involves the use of antibiotics which adds cost to production, potentially introduces antibiotics into the human food supply, and contributes to the development of antibiotic-resistant bacteria (Chang *et al.*, 2015). Biological control provides an alternative or a supplement to tradition control methods. Although it is unlikely that

traditional control methods, such as pesticide and antibiotic treatments, will be eliminated from use in agriculture and aquaculture, the use of beneficial bacteria can mitigate their use.

Probiotics, active microbes ingested for a health benefit (Gatesoupe, 1999), and Plant growth promoting rhizobacteria (PGPR), plant beneficial microbes living in the soil around plant roots (Kloepper *et al.*, 1978), are studied within to determine their efficacy as growth promoters, disease control agents, and water quality improvers. This dissertation involves the use of *Bacillus* spp. as probiotics and PGPR. In addition to being well established as PGPR (PGPR; Kloepper *et al.*, 1978) *Bacillus* spp. can form endospores, a dormant and more resistant state, allowing for long-term storage.

2. Literature Review

2.1 16S rDNA in microbial ecology

The 16S rRNA gene serves as a basis for the separation of life into three domains. Ribosomes are essential for life; they translate the genetic code to construct the proteins necessary to carry out life's functions. Due to this, there is a commonality across all organisms, allowing for comparison and grouping. Ribosomes are composed of two subunits, one larger and one smaller, which consist of proteins and RNA. The 16S rRNA is part of the small ribosomal subunit in bacteria (Woese *et al.*, 1990).

Starting in the 1990s, microbiologists began using 16S rDNA as a molecular ecological tool for analyzing a complex bacterial assemblage (Giovannoni *et al.*, 1990). It has since become the standard marker for molecular microbial ecology. Because 16S rDNA has conserved and variable regions, this gene can be compared and contrasted with all bacteria (Ibal *et al.*, 2019). The phylogenetic use of the small ribosomal subunit RNA

gene, in addition to established large databases of sequences to compare make 16S rDNA a useful ecological tool (Case *et al.*, 2007).

Microbial analysis based on 16S rDNA sequences has inherent biases. Bacteria can have multiple copies of the 16S rRNA gene, thus their numbers would be over-estimated in analysis. In addition the taxonomic resolution of 16S rDNA analysis is limited with closely related organisms sometimes being indistinguishable, such is the case with the *Bacillus subtilis* group witch contains 8 separate species identical based on their 16S rRNA gene (Wang *et al.*, 2007). This deficiency can be overcome by analysis of a second, less conserved gene, alongside 16S rRNA gene analysis; such as the use of gyrB to distinguish between members of the *B. subtilis* group (Wang *et al.*, 2007).

2.2 Endospores

Certain genera within the Firmicutes phylum, including *Bacillus*, can form endospores, dormant cells that are more resistant to environmental stresses than active cells (Fritze, 2004). The process of sporulation is prompted by a lack of nutrients in the environment (Driks, 2002; Piggot and Hilbert, 2004). A vegetative cell undergoes asymmetrical cell division forming a mother cell and a forespore. The mother cell then supports the development of the forespore into an endospore (e.g., Coppolecchia *et al.*, 1991; Meisner, 2008). An endospore can remain viable for long periods of time with no access to nutrients. Endospores show no detectable metabolism, have low enzymatic activity, and store only small amounts of high energy compounds such as ATP (Setlow, 1983; Setlow, 1994; Cowan *et al.*, 2003). The endospore will germinate into a vegetative cell when nutrient levels increase beyond a designated threshold. An endospore can survive harsh physical, chemical, and radioactive damage (Setlow, 2005). Endospores

have been shown to survive the vacuum, radiation, and extreme temperatures of space (Nicholson *et al.*, 2000).

2.3 Rhizosphere

The rhizosphere is the area surrounding a plant root. This nutrient rich area supports a microbial community that depends on chemicals released from the plant (Hiltner, 1904). The microbes residing in the rhizosphere can have beneficial, neutral, or even damaging effects on the plant (Newton *et al.*, 2010). Plants influence the microbiota of the rhizosphere in various ways, such as supplying carbon rich nutrients to the microbiota, altering pH, providing oxygen, as well as releasing antimicrobial compounds (Derek *et al.*, 2012). Typically, the rhizosphere supports a less diverse but much larger population of bacteria than root-less soil (Marilley *et al.*, 1998; Marilley and Aragno, 1999; Lynch and Whipps, 1990; Semenov *et al.*, 1999). This increase in microbial numbers is attributed to the release of carbon containing compounds from the plant roots. The rhizosphere can be divided into three zones: the endorhizosphere, which includes the endodermis and cortex of the root; the rhizoplane, which is the middle zone that includes the epidermis; and the ectorhizosphere, which is the outer zone from the rhizoplane into the surrounding soil (McNear, 2013). Microbes can live as endophytes in the endorhizosphere, residing in the apoplastic space (area between cells). In addition, microbes can be found as epiphytes on the rhizoplane and in the ectorhizosphere (Knief, 2014; McNear, 2013).

2.4 Plant disease management

Yield losses due to pathogens is estimated to be 16% globally (Ficke *et al.*, 2018). Plant disease management using heavy metals such as copper or other biocides may be

damaging to the environment, and pathogens have developed resistance to some traditional disease control approaches such as copper-based biocides (Richard *et al.*, 2017). It is important to control disease in order to mitigate losses. Using biological control is one tool that can be used to control disease and improve yield. Living microorganisms are applied to seeds, and/or surrounding soil in order to promote plant health (Kloepper *et al.*, 1989; Kloepper *et al.*, 2004; Yang *et al.*, 2009; Zehnder *et al.*, 2001).

2.5 PGPR

Plant growth promoting rhizobacteria (PGPR) is a term defined by Kloepper *et al.* (1978) as soil bacteria that colonize plant roots and promote plant growth. Many PGPRs have been shown to be useful in agriculture, such as the use of *Pseudomonas putida* and *Serratia marrcescens* to protect from angular leaf spot, a disease caused by *Pseudomonas syringae* (Liu *et al.*, 1995). *Pseudomonas fluorescens* has also been used as a PGPR, its use showing yield increases for potato, sugar beet, and radish (Burr *et al.*, 1978; Kloepper *et al.*, 1980). Additionally, many *Bacillus* spp. have been shown to be helpful PGPRs, such as the use of *Bacillus licheniformis* and *Bacillus pumilus* to promote the growth of *Pinus pinea* plants (Probanza *et al.*, 2001). *B. pumilus* has been shown to induce resistance to cucumber beetle feeding, as well as wilt disease transmitted by the beetle (Zehnder *et al.*, 1997). In addition, *B. subtilis,* and *B. pumilus* control cucumber pathogens (Raupach and Kloepper, 1998). Furthermore, *B. amyloliquefaciens* and *B. subtilis* PGPR induce systemic resistance (ISR) to a variety of plant diseases (Kloepper *et al.*, 2004). Induced systemic resistance (ISR) involves a signaling cascade, started by an infecting microbe, that leads to the increased ability of a plant to fight off pathogens even

in locations different from the original infection (Heil and Bostock, 2002). PGPR Bacillus spp. strains produce diverse antimicrobial compounds that include antibiotics (Emmert *et al.*, 2004), volatile organic compounds (VOCs) (Yuan *et al.*, 2012), and lipopeptides (Ongena *et al.*, 2007) that are associated with biocontrol activity against plant pathogens.

The diverse microbial assemblages associated with plants are a resource from which PGPR can be isolated, many of which produce bioactive secondary metabolites; however, the majority of soil microbiota are recalcitrant to laboratory cultivation, and this precludes identification of many potentially useful metabolites (Roesch *et al.*, 2007; Handelsman, 2005).

2.6 PGPR colonization

PGPR strains typically need to colonize plant roots extensively to exert their beneficial effects via direct and indirect mechanisms (Lugtenberg and Kamilova, 2009), with the exception that extensive root colonization is not required for induced systemic resistance (ISR) (Kamilova *et al.*, 2005). In some PGPR strains, root colonization is a prerequisite for their biocontrol activity against plant pathogens through antibiosis (Chin *et al.*, 2000). For example, *B. velezensis* FZB42 exerts growth promoting activities through efficient colonization of plant roots (Fan *et al.*, 2011).

2.7 Pectin

Pectin is a polysaccharide composed of D-galacturonic acid polymers combined with methylgalacturonic acid residues. It is a component of plant cell walls, the middle lamella, and the intine of pollen grains; it can also be found in roots. (Howe, 1921; Vercauteren *et al.*, 2002). Pectin is biological broken down as follows: demethoxylated

into polygalacturonate, enzymatically cleaved into digalacturonate, and further enzymatically broken down into galacturonate. Galacturonate is a hexuronate like glucuronate and fructuronate (Hugouvieux-Cotte-Pattat and Robert-Baudouy, 1987). Organisms cable of breaking pectin down can utilize it as a carbon source. Previous research on PGPR strains isolated from plant rhizospheres indicated that all the best-performing *B. velezensis* strains (formerly known as *B. amyloliquefaciens* subsp. *plantarum*) had the ability to use pectin as a sole carbon source (Popovici, 2016).

2.8 Discovery and isolation of new PGPR

The diverse microbial assemblages associated with plants are a resource from which plant growth promoting rhizobacteria (PGPR) strains can be isolated, many of which produce bioactive secondary metabolites; however, the majority of soil microbiota are recalcitrant to laboratory cultivation, and this precludes identification of many potentially useful metabolites (Roesch *et al.*, 2007; Handelsman, 2005). The cultivation of novel bacterial lineages from soils has been accomplished using dilute growth media (Joseph *et al.*, 2003) and with diffusion chambers allowing nutrients available *in situ* to promote bacterial growth with extended cultivation times appropriate for K-selected populations (Nichols *et al.*, 2010; Lynch and Neufeld, 2015). These approaches have expanded the phylogenetic breadth of microbial culture collections and led to the discovery of the novel antibiotic teixobactin (Ling *et al.*, 2015). In addition, plant root extracts have been used as a sole carbon source to cultivate rhizobacteria or to enrich for archaeal populations (Jacoby *et al.*, 2018 and Simon *et al.*, 2005); however, in these previous reports of the use of plant root extracts as a growth substrate to cultivate

rhizosphere-associated microbes, root-derived complex carbohydrates have been removed either by centrifugation, filtration and/or extraction.

2.9 Aquaculture disease and treatment

Disease outbreaks have become a major challenge to the profitable culture of fish and shellfish as aquaculture operations increase. Globally, total annual losses from disease outbreaks have reached billions of dollars (US) and have been identified as a threat to the sustainability of the industry (Pridgeon and Klesius, 2011a). Feed inputs associated with high-density fish culture stimulate the proliferation of opportunistic bacteria (Austin *et al.*, 1995). The combination of high-density fish culture with rapidly changing water temperature and chemical composition of aquaculture ponds places stress on fish, thereby resulting in favorable conditions for the onset and spread of disease.

Chemotherapy by oral administration of antibiotics in fish feeds is the most common treatment for bacterial diseases. There are currently only three FDA-approved treatments for use in aquaculture production facilities (Schnick, 2007). Anabolic steroids, growth promoters and some antibiotics, such as oxytetracycline (OTC), sulfadimethozine and ormetoprim among others, are commonly administered in feed to improve growth performance and to control the outbreak of diseases in aquaculture (Defoirdt *et al.*, 2011). However, the indiscriminant application of antibiotics may result in many problems including the spread of drug-resistant pathogens, environmental hazards and food safety problems. This has fostered an increased interest in alternatives to antibiotics. Probiotics, which have various health-promoting properties and minor adverse side effects, are gaining an increasing scientific and commercial interest in aquaculture practice. The beneficial effects of probiotics involve improvement of feed utilization, modulation of

intestinal microflora, enhancement of immune responses and antagonism to pathogens. The most commonly used probiotics in aquaculture are lactic acid bacteria and Bacillus spp. (Wang *et al.*, 2008a). *Bacillus* spp. have advantages as probiotics in that their spore-forming ability allows greater viability after pelleting and high survival rates after exposure to gastric acid (Casula and Cutting, 2002; Hong *et al.*, 2005; Hyronimus *et al.*, 2000). *Bacillus* spp. have been reported to have various beneficial attributes when applied to fish (Ai *et al.*, 2011; Aly *et al.*, 2008a; Kumar *et al.*, 2008; Nayak *et al.*, 2007; Newaj-Fyzul *et al.*, 2007; Salinas *et al.*, 2005; Salinas *et al.*, 2008).

2.10 Water quality in aquaculture

Phytase is a phosphohydrolase that catalyses 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. 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).

2.11 Aquaculture farming of channel catfish

Aquaculture farming of the channel catfish, *Ictalurus punctatus*, has been one of the most successful animal production industries in North America in the past 30 years and currently represents the largest aquaculture industry in the United States. Due to its rapid growth rate, low cost, and proficient reproduction capabilities, the channel catfish is an economically important aquaculture species, particularly in the southeastern United States (Agriculture, 2003). Over 90% of all catfish produced in the U.S. are raised in Alabama, Arkansas, Louisiana, and Mississippi and are primarily grown in earthen ponds ranging in size from 2 to 10 ha (USDA, 2003a, 2003b).

Enteric Septicemia of Catfish (ESC), caused by the Gram negative bacterium *E. ictaluri* (Hawke, 1979), is an important endemic infectious disease in the channel catfish aquaculture industry (Hawke and Khoo, 2004). Losses resulting from ESC were reported in over 78% of all operations with outbreaks being reported in 42% of catfish production ponds, with an economic loss between $20 and $30 million yearly (USDA, 2003a, 2003b; Wagner *et al.*, 2002).

Another important pathogen in channel catfish is *A. hydrophila*, which is the primary causative agent of motile aeromonad septicaemia (MAS) (Harikrishnan *et al.*, 2003) and can infect multiple fish species including tilapia, catfish, goldfish, common carp, and eel (Pridgeon *et al.*, 2011). In 2009 and 2010, *A. hydrophila* was identified as the etiologic agent of a disease epidemic in farmed channel catfish, resulting in higher mortality rates than typical for MAS with over five million pounds of catfish lost in the Alabama commercial catfish industry. The *A. hydrophila* strains (e.g., strain AL09-119)

isolated from diseased fish during this epidemic are highly virulent in aquaria disease challenge trials compared to *A. hydrophila* reference strains (Pridgeon *et al.*, 2011).

2.12 Aquaculture farming of tilapia

Tilapia production is of increasing importance in aquaculture globally and is second only to carp by production volume estimates (FAO, 2012). Among the several species of tilapia cultured commercially, Nile tilapia, *O. niloticus* (Linnaeus, 1758) is the most abundant and important species. Global aquaculture production of this species has increased from around 200,000 metric tons in 1990 to about 2.8 million metric tons in 2010 (FAO, 2012). The current trend in tilapia aquaculture development is towards increased intensification and commercialization (Goncalves *et al.*, 2011); however, disease is a primary constraint to the growth of the industry and severely impedes both economic and socio-economic development in many producer countries (Austin and Austin, 2007). Diseases caused by bacterial pathogens are responsible for heavy mortalities and annual losses. Among the major bacterial pathogens is Aeromonas hydrophila, which continues to plague the culture of this animal resulting in decreased survivability and profitability.

As mentioned above, tilapia are susceptible to *A. hydrophila* infection (causative agent of motile aeromonad septicemia (MAS)), often being associated as a secondary pathogen with a number of other diseases affecting Nile tilapia, such as epizootic ulcerative septicemia (EUS). Primarily, however, *A. hydrophila* is usually a primary opportunistic pathogen, causing disease outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry (Fang *et al.*, 2004). In recent years in the western region of Alabama, U.S.A, a MAS disease outbreak caused by

a highly virulent strain of *A. hydrophila* has resulted in the loss of millions of kg of food size channel catfish (USDA, 2010; Pridgeon and Klesius, 2011b). This virulent *A. hydrophila* genotype corresponds to sequence-type 251, and is affecting farmed fish in China and the United States (Hossain *et al.*, 2014; Pang *et al.*, 2015).

Streptococcal infections in fish, caused by *Streptococcus iniae,* have become one the most important bacterial disease of cultured Nile tilapia, *O. niloticus* causing mass mortality and severe economic losses (Shoemaker *et al.*, 2001). According to Shoemaker *et al.* (2010), the estimated economic impact of S. iniae outbreaks on the US aquaculture industry is approximately US $10 million and greater than US $100 million globally. This bacterium has also been discovered as a zoonotic pathogen with the confirmation of a number of cases involving the elderly or immuno-compromised humans (Weinstein, 1997; Koh *et al.*, 2004; Facklam *et al.*, 2005; Lau *et al.*, 2003; Agnew and Barnes, 2007). Thus, the need for an effective control method is not only limited to the economic loss in aquaculture, but also to protect the health of fish farmers and processers. Conventionally, antibiotics are used to control S. iniae infection in aquaculture; however, reported cases of lack of efficacy and resistance of bacteria to antibiotics (Stoffregen *et al.*, 1996; Shoemaker and Klesius, 1997; Locke *et al.*, 2008; Gaunt *et al.*, 2010) have heightened the need for alternative disease control methods. An alternative to prevent and control pathogenic bacteria is the use of probiotics. These are biologically active components of single or mixed cultures of live microorganisms, which when administered in adequate amounts are capable of improving the growth and health of the host (Salminen *et al.*, 1999; Lara-Flores *et al.*, 2010). Due to their reported benefits, probiotics have been commercialized and sold in the aquaculture industry as feed additives. Prevention of

disease by inclusion of individual probiotic bacteria strains and/or their mixtures in the diet of fish have become preferential to antibiotic therapy (Boyd and Gross, 1998; Shelby *et al.*, 2006; Welker and Lim, 2011).

2.13 Fish gut microbiota

The fish gut microbiota plays a major role in many host physiological processes (Sekirov *et al.*, 2010). As soon as a fish hatches, microbes begin colonizing the gastrointestinal tract. Once established, bacteria can influence host gene expression, leading to a more favorable environment for the colonizing bacteria, and perhaps deleterious for subsequent bacteria (Balcazar *et al.*, 2006a). The establishment of a symbiotic microbiota is critical for excluding pathogens and maintaining fish health (Balcazar *et al.*, 2006a). For channel catfish specifically, the gut microbiota is dominated by Fusobacteria (Bledsoe, 2016; Larsen, 2014).

2.14 Probiotics in aquaculture

Probiotics are living microbes supplemented to food that provide a health benefit to the host (Gatesoupe, 1999). Probiotics are available to aquaculture farmers in powder or liquid form. Production is carried out in batch fermentations, then concentrated to desirable numbers (Lacroix, 2007; Martinez Cruz *et al.*, 2012). A few commercial probiotics for aquaculture are currently available for use, such as Sporolac (*Saccharomyces boulardii*) and Lactobacil (*Lactobacillus acidophilus*). These products have been shown to induce resistance to lymphocystis disease virus (LCDV) in olive flounder (Harikrishnan *et al.*, 2010). They were also shown to increase immune activity by enhancing innate immune factors such as phagocytic activity, complement activity, superoxide production, and lysozyme activity. Fish treated with these probiotics and then

challenged with LCDV showed a significant decrease in mortality over control groups (Harikrishnan *et al.*, 2010).

Similarly, studies on probiotic *B. subtilis, B. licheniformis,* and *Enterococcus faecium* showed that they increased leucocyte levels as well as growth rate in trout. Additionally, high levels of the probiotics were detectable in the gastrointestinal tract of studied trout (Merrifield *et al.*, 2010). A variety of *Bacillus* spp. probiotics increase growth rate and improve immune response in tilapia (Zhou *et al.*, 2010). In addition, *Bacillus* spp. probiotics have been shown to promote growth in fish such as *Penaeus monodon* (Rengpipat *et al.*, 1998), catfish (Queiroz and Boyd, 1998), and *Macrobrachium rosenbergii* (Rahiman *et al.*, 2010). Furthermore, *Bacillus* spp. control disease in Penaeids (Moriarty, 1998) and *Farfatepenaeus brasiliensis* (Moreira *et al.*, 2011); improve nutrient digestibility in *Macrobrachium rosenbergii* (Rahiman *et al.*, 2010); improve water quality while being fed to *Penaeus monodon* (Wang *et al.*, 2008; Shishehchian *et al.*, 2001), *Macrobrachium rosenbergii* (Rahiman *et al.*, 2010), *Pannaeus vannamei* (Zhou *et al.*, 2009); increase stress tolerance in *Paralichthys olivaceus* (Taoka *et al.*, 2006a); and improved reproduction in *Poecilia reticulata* and *Xiphophorus maculatus* (Ghosh *et al.*, 2007).

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.*, 2006a; Macfarlane and Cummings, 1999; Wang *et al.*, 2008a). *Bacillus* spp. have good potential as probiotics 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; 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.

Chapter II

Deciphering the conserved genetic loci implicated in plant colonization and disease control through comparative genomics of *Bacillus velezensis*.

1. Abstract

To understand the growth promoting and disease-inhibiting activities of plant growth promoting rhizobacteria (PGPR) strains, the genomes of 12 different PGPR strains affiliated with the *Bacillus subtilis* group were sequenced. These *B. subtilis* strains exhibited high genomic diversity, whereas the genomes of *Bacillus amyloliquefaciens* strains (a member of the *B. subtilis* group) and *Bacillus velezensis* strains (formerly *B. amyloliquefaciens* subsp. *Plantarum*, now a part of the *B. amyloliquefaciens* clade (Fan *et. al.*,2017)) are highly conserved. A pairwise BLASTp matrix revealed that gene family similarity among *Bacillus* genomes ranges from 32% to 90%. A total of 2,839 genes were consistently present within the core genome of *B. velezensis*. Comparative genomic analyses using a large number of *B. amyloliquefaciens* and *B. velezensis* strains identified conserved genes that have been linked with biological control and colonization of roots or leaves. There were 73 genes uniquely associated with *B. velezensis* strains with predicted functions related to signaling, transportation, secondary metabolite production, and carbon source utilization. Although *B. velezensis* strains contain gene clusters that

encode many different secondary metabolites, only polyketide biosynthetic clusters encoding difficidin and macrolactin were found conserved within this subspecies. Genes involved in secondary metabolite biosynthesis were deleted in *B. velezensis* strain AP93 to evaluate their role in plant pathogen biocontrol, revealing that difficidin expression is critical and solely sufficient for reducing the severity of bacterial spot caused by *Xanthomonas axonopodis* pv. Vesicatoria in tomato plants. This study provides insight into genomic features of PGPR strains involved in biocontrol activity and host colonization.

2. Introduction

Bacteria associated with plant roots that exert beneficial effects on plant growth and development are referred to as plant growth–promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978; Kloepper *et al.*, 2004). *Bacillus* and *Pseudomonas* spp. are predominant among the diverse bacterial genera that have been identified as PGPR (Podile and Kishore, 2006). Members of the *B. subtilis* group, including *B. subtilis*, *B. licheniformis*, *Bacillus pumilus*, *B. amyloliquefaciens*, *B. velezensis*, *Bacillus atrophaeus*, *Bacillus mojavensis*, *Bacillus vallismortis*, *Bacillus sonorensis*, and *Bacillus tequilensis* have been identified as PGPR strains for their capacity to stimulate plant growth and suppress pathogens within rhizosphere and phyllosphere (Kloepper *et al.*, 2004; Hao *et al.*, 2012; Kim *et al.*, 2012). Strains of *B. amyloliquefaciens* and *B. velezensis* are widely used for their positive effects on plant growth (Idriss *et al.*, 2002). Reva *et al.* (Reva *et al.*, 2004) reported that seven *Bacillus* isolates from plants or soil were closely related but distinct from *B. amyloliquefaciens* type strain DSM7^{T}. In addition, these strains were more proficient for colonization of the rhizosphere than other members of the *B. subtilis*

group. GB03 (Nakkeeran *et al.*, 2005), INR7 (Kokalis–Burelle *et al.*, 2002) and FZB42 (Chen *et al.*, 2007) are PGPR strains within the *Bacillus subtilis* group that have been widely used in different commercial formulations to promote plant growth.

In addition to promoting plant growth, many PGPR strains exhibit biological control of plant diseases. Antibiosis, through the production of inhibitory bioactive compounds, and induced systemic resistance are widely reported biological control mechanisms of *Bacillus* spp. PGPR strains (Ryu *et al.*, 2004). PGPR *Bacillus* spp. strains produce diverse antimicrobial compounds that include antibiotics (Emmert *et al.*, 2004), volatile organic compounds (VOCs) (Yuan *et al.*, 2012), and lipopeptides (Ongena *et al.*, 2007) that are associated with the observed biocontrol activity against plant pathogens. *B. amyloliquefaciens* NJN-6 produces 11 different VOCs that provide antifungal activity against *Fusarium oxysporum* f. sp. *cubense* (Yuan *et al.*, 2012). Similarly, lipopeptides, such as surfactin and fengycin, are produced by *B. subtilis* which induces systemic resistance in bean plants (Ongena *et al.*, 2007).

PGPR strains usually need to colonize plant roots extensively to exert their plant growth promoting effects using both direct and indirect mechanisms (Lugtenberg and Kamilova, 2009), with the exception that extensive root colonization is not required for induced systemic resistance (ISR) (Kamilova *et al.*, 2005). In some PGPR strains, root colonization is a prerequisite for their biocontrol activity against plant pathogens through antibiosis (Chin *et al.*, 2000). For example, *B. velezensis* FZB42 exerts growth promoting activities through efficient colonization of plant roots (Fan *et al.*, 2011). Previously, it has been demonstrated that over-expression of genes involved in phosphorylation of DegU, a two-component response regulator, of *B. amyloliquefaciens* strain SQR9 positively

influences plant root colonization as well as other growth promoting activities by PGPR strains for controlling cucumber wilt disease (Xu *et al.*, 2014). Moreover, the root colonization capacity of a poor root colonizer can be improved by cloning genes required for efficient root colonization by PGPR (Dekkers *et al.*, 2000). Since competitive root colonization by PGPR are controlled by many genes or genetic clusters (Dietel *et al.*, 2013), identification of those clusters involved in competitive root colonization are challenging if genome sequences of those PGPR strains are not available (Lugtenberg and Kamilova, 2009). Understanding the mechanisms of competitive root colonization, antibiosis and ISR of PGPR strains may open the possibility for genetic engineering of PGPR strains that would increase the plant-growth promoting capacity of these bacteria.

In this study, the genomes of 12 different *Bacillus subtilis* group isolates from diverse locales were sequenced. Comparative genomic analyses of PGPR strains and representative strains of the *B. subtilis* group without any reported biocontrol activity against plant pathogens provided insights into the genomic features involved in biocontrol activity and host colonization of PGPR strains. PGPR strain AP193, which has been previously shown to have the capacity to inhibit the growth of plant and animal bacterial pathogens (Ran *et al.*, 2012), was targeted for the deletion of *dfnD*, *srfAA* and *sfp* genes, which are predicted to be involved in the biosynthesis of difficidin, surfactin, and all polyketide and lipopeptides produced by non-ribosomal peptide synthesis, respectively. Mutants were then tested for their ability to exert inhibitory activities against plant pathogens *in vitro* and their ability to control bacterial spot disease in tomato caused by *X. axonopodis* pv. vesicatoria.

3. Materials and Methods

Bacterial strains, plasmids and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1. Both *Escherichia coli* and *Bacillus* strains were grown in Luria-Bertani (LB) medium; however, for electrocompetent cell preparation, *Bacillus velezensis* AP193 was grown in NCM medium (17.4 g K_2HPO_4, 11.6 g NaCl, 5 g glucose, 5 g tryptone, 1 g yeast extract, 0.3 g trisodium citrate, 0.05 g $MgSO_4 \cdot 7H_2O$ and 91.1 g sorbitol in 1 L deionized water, pH 7.2). For production of secondary metabolites, *Bacillus* cultures were grown for 48 h at 30°C in Tryptic Soy broth (TSB). In addition, ampicillin (100 µg/mL), chloramphenicol (12.5 µg/mL) or erythromycin (200 µg/mL for *E. coli* or 5µg/mL for *Bacillus*) were used as selective agents in the growth media when required. Agar plates were made by adding 15 g/L agar to liquid medium.

Sequencing, assembly and annotation. Next-generation sequencing of *Bacillus* spp. genomes was performed using Illumina and Roche 454 sequencing platforms. Indexed Illumina libraries were prepared for strains AP71, AP79, and AB01 using Nextera DNA Sample Prep Kit (Epicentre, Madison, WI) and sequences were generated using an Illumina MiSeq with a 2 × 250 paired end sequencing kit. Barcoded Illumina libraries for strains AP143, AP193, and AP254 were constructed using a NxSeq® DNA Sample Prep Kit (Lucigen, Middleton, WI) and sequenced at EnGenCore (Univ. of South Carolina) using a 454 sequencing platform. Genomic DNA library construction and sequencing (using Illumina HiSeq 2000 platform and 2 × 101 paired end sequencing kit) for *B. subtilis* GB03, *Bacillus pumilus* INR7, *B. mojavensis* KCTC 3706T, *B. tequilensis* KCTC 13622T, *Bacillus siamensis* KCTC 13613T and *B. sonorensis* KCTC 13918T

were conducted at the National Instrument Center for Environmental Management (Seoul, Republic of Korea). Sequence reads were trimmed for quality and *de novo* assembled using the CLC Genomics Workbench (CLCBio, Cambridge, MA). Gene prediction and annotation were performed using GeneMark (Lukashin and Borodovsky, 1998) and RAST annotation server (Aziz *et al.*, 2008), respectively. The identity of individual ORFs from secondary metabolite biosynthesis gene clusters was confirmed by BLASTx against the GenBank database. Genome sequence reads for strains AB01, AP71, AP79, AP143, AP193, AP254, GB03 (Choi *et al.*, 2014), INR7 (Jeong *et al.*, 2014), KCTC 3706T, KCTC 13613T (Jeong *et al.*, 2012), KCTC 13918T and KCTC 13622T were deposited into the Short Read Archive (SRA) at NCBI under the accession numbers SRR1176001, SRR1176002, SRR1176003, SRR1176004, SRR1176085, and SRR1176086, SRR1034787, SRR1141652, SRR1141654, SRR1144835, SRR1144836 and SRR1144837, respectively.

Determination of average nucleotide identity (ANI). Average nucleotide identities between genomes were calculated using ANI calculator that estimates ANI according to the methods described previously (Goris *et al.*, 2007).

Phylogenetic analysis of *Bacillus* species. For phylogenetic analysis, the *gyrB* gene sequence for each of the 25 strains (a list of strains is presented in Figure 1) was retrieved from their respective genome sequences. Strains AS43.3, FZB42, YAU B9601-Y2, CAU B946 and 5B6 were used as representative strains of *B. velezensis*; strains DSM7, LL3 and TA208 were used as representative strains of *B. amyloliquefaciens* subsp. *amyloliquefaciens*. The *gyrB* phylogenetic tree was inferred with MEGA5.05 (Tamura *et al.*, 2011) using both the Neighbor-Joining (Saitou and Nei, 1987) and

Maximum Likelihood (ML) methods (Felsenstein, 1981). All positions that contained gaps or missing data were eliminated from the final dataset that contained a total of 1911 bp positions of *gyrB* sequence. We used 729,383 bp of DNA, representing the conserved core genome found in 25 strains of *B. subtilis* group, to generate a phylogenomic tree using RAxML (v 7.2.7) (Pfeiffer and Stamatakis, 2010). The phylogenomic tree was visualized with iTOL (http://itol.embl.de) (Letunic and Bork, 2011).

BLAST matrix. The BLAST matrix algorithm was used for pairwise comparison of the proteomes of *Bacillus* PGPR strains using methods described previously (Friis *et al.*, 2010). The BLAST matrix determines the average percent similarity between the proteome of each two strains by measuring the ratio of the number of conserved gene families shared between the two strains to the total number of gene families of the two strains. The absolute number of shared and combined gene families for each two strains was displayed in the matrix output. In addition, the matrix showed the number of proteins shares within each proteome against each other.

Core- genome analysis. The core-genome of 13 *Bacillus* spp. strains was generated using their coding and non-coding sequences. Whole genome sequences from these strains were aligned using progressive Mauve (Darling *et al.*, 2004) which identifies and aligns locally collinear blocks (LCBs) in the XMFA format. LCBs from alignments were collected using stripSubsetLCBs (http://gel.ahabs.wisc.edu/mauve/snapshots/), using minimum lengths of 500bp. All LCBs were concatenated and converted to multifasta format using a perl script. The same protocol was used to obtain all core sequences, with the exception that the minimum lengths of LCBs were 50 bp instead of 500 bp. *Bacillus* spp. core genomes were obtained from the comparative alignment of all

81 complete *Bacillus* spp. genomes available in the GenBank as of August 2014. The core genome of the *B. subtilis* group was obtained from comparative analysis of 53 whole genomes of *B. subtilis* strains that included 41 genomes obtained from GenBank and 12 PGPR genomes sequenced in this study. The *B. amyloliquefaciens* group core genomes were generated from 32 *B. amyloliquefaciens* and 28 *B. velezensis* genomes, respectively. Core genomes were exported to the CLC Genomics Workbench (v 4.9) for evaluation of alignments and were annotated using the RAST server (Aziz *et al.*, 2008). The list of *Bacillus* spp. strains used for core genome determination is available (data not shown; Hossain *et al.*, 2015). Additionally, to identify PGPR-specific core genes, the raw sequence reads of PGPR strains sequenced in this study were sequentially reference mapped against the genome sequence of the non-PGPR strain *B. subtilis* subsp. *subtilis* str. 168 according to methods described previously (Hossain *et al.*, 2013).

Identification of core genes uniquely present in *B. velezensis* strains. The aligned genome sequences of 32 *B. amyloliquefaciens* strains and 28 *B. velezensis* strains (which were included within the *B. amyloliquefaciens* strains) were uploaded into the CLC Genomics Workbench to obtain the respective species- and subsp.-level core genomes. Trimmed sequence reads of *B. velezensis* strain AP193 were reference mapped against the *B. velezensis* core genome to obtain core genome-specific sequence reads. The parameters of reference mapping were as follows: mismatch cost =2, insertion cost =3, deletion cost =3, length fraction = 0.5 and similarity = 0.8. Sequence reads mapped to the *B. velezensis* core genome were then mapped against the species *amyloliquefaciens* core genome to obtain unmapped sequence reads. These unmapped sequence reads, representing the *B. velezensis* core genome but absent in *B. amyloliquefaciens* group core

genome were assembled *de novo* using CLC Genomics Workbench and the resulting contigs were uploaded to the RAST server for gene prediction and annotation. Each of the ORFs exclusively encoded by the *B. velezensis* core genome was further confirmed for their uniqueness using BLASTn analysis against the genome sequences of 28 *B. velezensis* and four *B. amyloliquefaciens* subsp. *amyloliquefaciens* strains (data not shown; Hossain *et al.*, 2015).

Prediction of secondary metabolite biosynthesis gene clusters in PGPR strain AP193. Secondary metabolite biosynthesis gene clusters for strain AP193 were predicted using the secondary metabolite identification tool antiSMASH (Blin *et al.*, 2013). Primer-walking PCR was used to fill the gaps between contigs containing gene clusters encoding secondary metabolite biosynthesis. Gene prediction and annotation were carried out by GeneMark (Lukashin and Borodovsky, 1998) and BLASTx (NCBI), respectively.

DNA manipulation and plasmid construction for PGPR strain AP193 mutagenesis. Chromosomal DNA was isolated with E.Z.N.A. Bacterial DNA Isolation Kit (Omega Biotek, Atlanta, GA). All plasmids were isolated with E.Z.N.A. Plasmids Mini Kit II (Omega Biotek). All primers used in this study are listed in Table 2. The gene knock-out construct was assembled using splicing by overlap extension (SOE) PCR (Horton *et al.*, 1989). The assembled product was gel purified with Gel/PCR DNA Fragments Extraction Kit (IBI), digested with appropriate restriction enzymes and cloned into pNZT1 to construct the delivery plasmids for gene replacement.

***In vitro* plasmid methylation using cell free extract of *B. velezensis* AP193.** To methylate plasmids prior to transformation into *B. velezensis* AP193, the method developed for *Lactobacillus plantarum* was used with minor modifications (Alegre *et al.*,

2004). Cells from a 100 mL overnight culture of strain AP193 (OD_{600} = 1.3-1.5) were pelleted by centrifugation (8000 ×*g*), washed with 100 mL of chilled PENP buffer (10 mM potassium phosphate, 10 mM EDTA, 50 mM NaCl and 0.2 mM PMSF, pH 7.0) and then re-suspended in the same buffer to a final volume of 4 mL. The cells were disrupted by performing two bursts (amplitude 50, pulse 3 and watts 25-30) for 5 min each with a pause of 2 min using Vibra-Cell sonicator. The sample was maintained on ice during disruption to prevent overheating. Cell debris was removed by centrifugation (8000 × *g*) at 4°C and extract was collected by decanting. Three mL aliquots of extract were mixed with 3 ml of glycerol (100% v/v) and 0.6 mL of BSA (1 mg/mL), and stored at -20°C.

The DNA modification assay was performed in a final volume of 100 µl of the following: 53 µl TNE buffer [50 mM Tris (pH 7.5), 50 mM NaCl, 10 mM EDTA], 10 µl S-adenosylmethionine (0.8 mM), 2 µl BSA (5 mg/mL), 25 µl cell free extract derived from strain AP193 and 10 µl plasmid DNA extracted from *E. coli* K12 ER2925 (0.5-1 µg/µl). The mixture was incubated at 37°C for 16 h. Methylated DNA was extracted with a DNA Clean & Concentrator Kit (Zymo Research, CA), re-suspended in water and stored at -20°C.

Preparation of *B. velezensis* AP193 electrocompetent cells and electrotransformation. For preparation of electrocompetent cells, strain AP193 was grown overnight in TSB and then diluted 100-fold in NCM to inoculate a subculture. The culture was grown at 37°C on a rotary shaker until the OD_{600} reached to 0.7. The cell culture was cooled on ice for 15 min and subjected to centrifugation at 8000 × *g* for 5 min at 4°C. After washing four times with ice cold ETM buffer (0.5 M sorbitol, 0.5 M mannitol, and 10% glycerol), electrocompetent cells were re-suspended in 1/100 volume

of the original culture (Zhang *et al.*, 2011). For electroporation, 100 µl of cells was mixed with 100 ng of plasmid DNA in an ice-cold electroporation cuvette (1 mm electrode gap). Cells were exposed to a single 21 kV/cm pulse generated by Gene-Pulser (Bio-Rad Laboratories) with the resistance and capacitance set as 200 Ω and 3µF, respectively. The cells were immediately diluted into 1 mL of recovery medium (NCM plus 0.38M mannitol) (Zhang *et al.*, 2011) and shaken gently at 37°C or 30°C for 3 h to allow expression of the antibiotic resistance genes. Aliquots of the recovery culture were then spread onto LB agar plates supplemented with appropriate antibiotics.

Two-step replacement recombination procedure for the modification of the strain AP193 genome. The two-step replacement recombination procedure was performed as previously described with minor modifications (Zakataeva *et al.*, 2010). To initiate integration of the entire plasmid into the strain AP193 chromosome through a single crossover between the target gene and its homologous sequence on the plasmid, strain AP193 bearing a delivery plasmid with a specific gene deletion construct was cultured in LB broth at 37°C (a non-permissive temperature for plasmid replication) for 24 h. The culture was then serially diluted, plated onto LB agar plates with erythromycin, and incubated at 37°C. Clones were screened by colony PCR using two sets of primers. Each set of primers anneals sequences specific to one of the homologous fragments and to the chromosomal region just outside of the other homologous fragment (Table 2). PCR products with reduced size relative to the wild-type genotype for either primer set indicated successful chromosomal integration of the plasmid. In the second step, separate clones of the integrant were cultured with aeration in LB at 30°C for 24-48 h to initiate the second single-crossover event, resulting in excision of the plasmid which yields

erythromycin sensitive (EmS) clones with either a parental or a mutant allele on the chromosome. Colony PCR was used to examine the presence of desired mutations by primer sets that flank the deleted sequence (Table 2).

Construction of strain AP193 mutants defective in secondary metabolite biosynthesis. A summary of all of the mutant strains generated in this study is given in Table 1. The disruption of the *dfnD* gene was achieved as follows: DNA fragments corresponding to positions -867 to +247 and +643 to +1570 with respect to the *dfnD* translation initiation site were PCR amplified using AP193 genomic DNA as template. The two fragments were assembled by fusion PCR. A frameshift mutation was introduced during the fusion to ensure complete disruption of the gene. The assembled knock-out construct was digested with XhoI and SpeI and cloned into pNZT1, yielding pNZ-dif. The plasmid was methylated *in vitro* as described above and introduced into strain AP193 by electroporation. Once introduced into strain AP193, plasmid pNZ-dif generated the isogenic mutant AP193Δ*dfnD* by a two-step replacement recombination.

To generate the *sfp* deletion mutant, DNA fragments corresponding to positions -781 to +29, with respect to the *sfp* translation initiation site, and +95 to + 935, with respect to the *sfp* translation termination site, were PCR amplified using AP193 genomic DNA as template, assembled by fusion PCR, digested with HindIII and PstI, and cloned into pNZT1 to construct pNZ-sfp. This plasmid, pNZ-sfp, was used to generate mutant AP193Δ*sfp* using procedures described above.

The Δ*srfAA* mutant was obtained as follows: DNA fragments corresponding to positions +5375 to +6091 and +6627 to +7366, with respect to the *srfAA* translation initiation site, were PCR-amplified, fused by fusion PCR, digested with HindIII and PstI

and cloned into pNZT1 as pNZ-srf. Similarly, a frameshift mutation was introduced during the fusion of the upstream and downstream fragments of the target deletion sequence to ensure complete disruption of the gene. The plasmid pNZ-srf was used to generate mutant AP193Δ*srfAA* using procedures described above.

***In vitro* antimicrobial activities of PGPR strain AP193 and its mutants against plant pathogens.** Plant pathogens *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter, X. axonopodis* pv. vesicatoria, and *Xanthomonas axonopodis* pv. campestris were grown in TSB until the OD_{600} reached 1.0. The wild type strain AP193, as well as the three isogenic mutants Δ*dfnD,* Δ*sfp,* and Δ*srfAA* developed in this study, were grown at 30°C in TSB for 48 h at 220 rpm. Those cultures were then centrifuged at 10,000 × *g* for 2 min and supernatants were filtered through 0.2 µm nylon filter (VWR, PA). For antibiosis assays, 100 µl of an overnight culture for each of the plant pathogens were spread onto TSA plates (Thermo Scientific, NY) separately, then a sterile cork borer of 10 mm diameter was used to bore wells in those agar plates. Filtered supernatants of AP193 and its three mutants were separately added to fill wells. The plates were allowed to dry and then incubated at 30°C overnight. Zones of inhibition were measured and compared between mutants and wild-type strain AP193 to determine their antimicrobial activities against plant pathogens.

***In vivo* antibiosis of strain AP193 and its mutants against a plant pathogen.** Rutgers tomato seeds (Park Seed, USA) were sown in Styrofoam trays and three weeks after planting seedlings were transplanted into a 4.5 inch square pot with a commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, Maine). Three days after transplanting, plants were sprayed with PGPR suspensions (10^6 CFU/mL) or sterile

distilled water. PGPR-inoculated plants were placed into a dew chamber at 100% humidity in the dark for two days at 24°C and then transferred to the greenhouse. One day later, plants were challenge-inoculated with *X. axonopodis* pv. vesicatoria by spraying approximately 10 mL of a 10^7 CFU/mL pathogen suspension over each plant. Those pathogen-inoculated plants were placed in the dew chamber for two days and then placed in the greenhouse. Plants were watered once daily. Disease severity ratings and harvest were conducted after 14 days of challenge-inoculation. For disease severity rating, four compound leafs were selected from the bottom of each plant. The disease severity of each of the compound leaves was determined by rating the disease severity of each leaflet and calculating the average rating for the compound leaf. The leaflet was rated using a 0-4 rating scale, where 0=healthy leaflet, 1= <20% necrotic area of the leaflet, 2= 20-50% necrotic area of the leaflet, 3= 51-80 % necrotic area of the leaflet, 4= 80-100 % necrotic area of the leaflet, or fully dead leaflet. In addition, the dry shoot and root weights were determined. The experimental design was a randomized complete block with ten replications per treatment. The experiment was conducted twice.

Data analysis. All data were analyzed by analysis of variance (ANOVA), and the treatment means were separated by using Fisher's protected least significant difference (LSD) test at P=0.05 using SAS 9.3 (SAS Institute, Gary, NC, USA).

4. Results

Genome statistics and genetic relatedness of *Bacillus* species

Genome sequences of 12 different PGPR *Bacillus* spp. strains were determined using next-generation sequencing. The summary statistics for each *Bacillus* spp. genome sequences and their assemblies are presented in Table 3. The approximate sizes of the 12

Bacillus spp. genomes ranged from 2.95 Mbp to 4.43 Mbp with an average genome size of 3.93 Mbp, which is similar to the 4.09 Mbp average genome size of the 26 complete *B. subtilis* genomes available in GenBank (April, 2015). The percent G+C contents of the 12 PGPR *Bacillus* spp. strains ranged from 41.3% to 46.6%, averaging 45.15% which is close to the average percent G+C content (43.72%) of the 73 *B. subtilis* genome sequences available in GenBank (March, 2015). Pairwise average nucleotide identities (ANI), a newly proposed standard for species definition in prokaryotes (Richter and Rosselló-Móra, 2009), were calculated for 13 *Bacillus* PGPR strains to determine their interspecies relatedness among *Bacillus* species. The ANI values for each of the six PGPR *Bacillus* spp. strains AB01, AP71, AP79, AP143, AP193 and GB03 against *B. velezensis* FZB42 (Chen *et al.*, 2007) were greater than 98% (data not shown), indicating that each of these six PGPR strains are affiliated with the *B. amyloliquefaciens* group. The 98.88% ANI of PGPR strain AP254 to *B. subtilis* subsp. *subtilis* strain 168 suggests that AP254 affiliates with *B. subtilis* (data not shown). The pairwise ANI comparison of PGPR strains INR7, KCTC 3706T, KCTC 13613T, KCTC 13918T and KCTC 13622T against each other with ANI values less than 95% (data not shown) suggests that they are distantly related to each other and represent diverse *Bacillus* species.

Phylogenetic relationship of *Bacillus* strains

A phylogenetic analysis based on *gyrB* gene sequences showed sufficient resolution among *Bacillus* taxa and was consistent with ANI comparisons. Strains AP71, AP79, AP143, AP193, AB01 and GB03 were grouped together with the reference strains of the *B. amyloliqufaciens group* with high bootstrap support, indicating that they are affiliated with *B. velezensis*. The three strains of *B. amyloliquefaciens* subsp.

amyloliquefaciens DSM7, TA208 and LL3 clustered as a single clade separated from strains of *B. velezensis,* supporting the division of the two species within *B. amyloliquefaciens* group (Borriss *et al.,* 2011). The placement of strain AP254 with *B. subtilis* subsp. *subtilis* strain 168 as a single clade with strong bootstrap support, suggesting its affiliation with members of the *B. subtilis* group (figure not shown; Hossain, 2015). A *gyrB* gene based phylogenetic tree constructed using Maximum Likelihood (ML) methods was concordant with the phylogeny constructed using Neighbor-Joining methods (data not shown). In addition to the *gyrB*-based phylogeny, we constructed a phylogenomic tree using 729,383 bp of core genome sequences present within the genome of 25 *B. subtilis* group isolates to provide a more refined phylogenetic placement of PGPR strains. The topology and allocation of strains to clades in the *gyrB* phylogeny was similar to the phylogenomic tree except a few minor differences (figure not shown; Hossain, 2015). The topology of the tree regarding the position of strain *B. siamensis* KCTC13613 differs significantly between the *gyrB*-based tree and the phylogenomic tree, with the *gyrB* based phylogeny placing KCTC13613 in a separate clade whereas the phylogenomic tree included it within a monophyletic group that includes strains of *B. velezensis.*

BLAST matrix

Genome wide proteome comparisons of 13 PGPR *Bacillus* strains using an all-against-all BLASTp approach demonstrated that PGPR *Bacillus* spp. strains are highly diverse, as indicated by gene family similarity between PGPR *Bacillus* spp. genomes ranging from 32% to 90%. Consistent with the phylogenetic analysis, high similarity was

found among strains AP71, AP79, AP193, AB01, GB03, and FZB42, with proteomic similarity ranging from 70% to 90%.

Core- genome analysis

An analysis of genome sequence alignment using progressive Mauve determined that the core genome of the 13 PGPR *Bacillus* spp. strains contains 1,407,980 bp of genomic DNA which encodes a total of 1,454 ORFs (data not shown). Comparison of the core genome sequences of the genus *Bacillus*, subgroup *B. subtilis, B. amyloliquefaciens* group, and *B. velezensis* demonstrated that as the number of genomes increases that the number of different subsystems within each respective core genome decreases (figure not shown; Hossain, 2015). The highest numbers of subsystems in each of the core genome categories, except for the genus *Bacillus* core genome, were devoted to carbohydrate metabolism. These findings suggest that strains from the genus *Bacillus* use diverse carbon sources. The core genome for the genus *Bacillus* has more subsystems devoted to RNA, DNA, and protein metabolism compared to carbohydrate metabolism (figure not shown; Hossain, 2015).

The genome alignment from 28 different *B. velezensis* strains, including six strains sequenced in this study, identified 2,550,854 bp of core genome sequence predicted to encode 2,839 ORFs (data not shown; Hossain *et al.*, 2015). The genome alignment of 32 *B. amyloliquefaciens* strains, including 28 *B. velezensis* strains, identified 2,418,042 bp of core genome sequence predicted to encode 2,773 ORFs (data not shown; Hossain *et al.*, 2015).

The genome alignment of 53 strains of *B. subtilis* group, including 12 strains sequenced in this study, identified 578,872 bp of core genome sequence predicted to

encode 674 ORFs (data not shown; Hossain *et al.*, 2015). The relatively higher number of protein coding genes, approximately 4,000 present within the genome of *Bacillus* spp. used in this study and the lower number of ORFs (674) encoded by their core genomes, suggests a large amount of genomic plasticity among *Bacillus* genomes that have experienced frequent gene acquisitions and losses. It was observed that the *B. amyloliquefaciens* group core genome was devoid of mobile genetic elements, such as prophages, transposable elements, and plasmids (data not shown). Furthermore, the *B. subtilis* core genome was also devoid of genes or genetic clusters involved in iron acquisition and metabolism, secondary metabolite biosynthesis, signal transduction and phosphorus metabolism (figure not shown; Hossain, 2015).

In this study, the genus *Bacillus* core genome was also determined by analyzing all complete genome sequences from the genus *Bacillus* (n=81) currently available in GenBank. Our study determined that the genus *Bacillus* contains 194,686 bp of core sequence predicted to encode 201 different ORFs (data not shown; Hossain *et al.*, 2015). The predicted functions present in all *Bacillus* strains are limited to the following subsystem features: cofactor synthesis, vitamin synthesis, prosthetic groups and pigments biogenesis, cell wall and capsule biogenesis, membrane transport, RNA metabolism, nucleoside metabolism, protein metabolism, regulation and cell signaling, DNA metabolism, respiration, amino acids and derivatives, sulfur metabolism, and carbohydrate utilization.

Comparative analysis of core genes uniquely present in *B. velezensis*

The comparison of PGPR-specific genomes with that of non-PGPR *B. subtilis* subsp. *subtilis* str. 168 did not identify any genes other than essential housekeeping genes

that were conserved within the genome of PGPR strains (data not shown). The comparative analysis of core genomes from 28 *B. velezensis* and 32 *B. amyloliquefaciens* species identified 193,952 bp of sequences that are present within the *B. velezensis* core genome but absent in the *B. amyloliquefaciens* core genome. Among these genetic loci there were 73 genes shared by all 28 *B. velezensis* strains but were not present in any strains of *B. amyloliquefaciens* (data not shown; Hossain *et al.*, 2015). The putative functions of these genes includes transportation (7 genes), regulation (7 genes), signaling (1 genes), carbon degradation (10 genes), synthesis of secondary metabolites (19 genes), and hypothetical proteins (12 genes) (figure not shown; Hossain, 2015). Many of these gene products are known to be involved in interactions with plants and rhizosphere competence of *B. velezensis* strains (e.g., pectin utilization). For instance, the core genomes of 28 *B. velezensis* strains share several different ORFs that are required for uptake and the use of D-galacturonate and D-glucuronate. In addition, genes required for biosynthesis of the polyketides difficidin and macrolactin were consistently found in PGPR *B.velezensis* strains, suggesting their relevance in the biocontrol activities of these strains.

Gene clusters encoding secondary metabolite biosynthesis and natural competency in strain AP193

Due to our observations of beneficial interactions between PGPR strain AP193 and both plant and animal hosts (Ran *et al.*, 2012), we selected this strain for more intensive genome analysis. Assembly of strain AP193 genome sequences *de novo* resulted in 152 contigs larger than 1 kb, with a combined length of 4,121,826 bp. Analysis of AP193 contig sequences, using the antiSMASH secondary metabolite

prediction program, suggests that gene clusters are present that are responsible for synthesis of three different polyketides: bacillaene, macrolactin and difficidin. In order to provide complete sequences for these biosynthesis pathways, the gaps between contigs 5 and 6, contigs 33 and 38, as well as contigs 27 and 28 were filled using PCR, followed by DNA sequencing. Each of the gene clusters in AP193 are collinear to their counterparts in *B. velezensis* FZB42; a naturally competent plant root-colonizing *B. amyloliquefaciens* clade isolate with the ability to promote plant growth and suppress plant pathogens (Chen *et al.*, 2007). The percent amino acid identities of the proteins encoded by those clusters were within the range of 98% and 100% when compared with those of FZB42. Secondary metabolite biosynthesis gene clusters involved in non-ribosomal synthesis of cyclic lipopeptides surfactins, fengycin and bacillomycin D and of the antimicrobial dipeptide bacilysin present in FZB42 were also detected in the AP193 genome. The percent amino acid identities of the AP193 proteins encoded on those clusters to the FZB42 homologs ranged from 98% to 100%. The lack of natural competency of the PGPR strain AP193 prompted us to determine the presence of competence-related genes within this strain. We searched the AP193 genome sequences for the presence of competence related genes found within the genome of FZB42, and observed that all of the genes required for encoding the structural components of the competence system found in strain FZB42 are present within the genome of AP193 with 98 to 100% identity (data not shown); however, genes *comQ*, *comX* and *comP* that are involved in regulating the competence quorum-sensing system of *B. velezensis* FZB42 (Chen *et al.*, 2007) were absent within the genome of strain AP193 (data not shown). The absence of *comQ*, *comX* and *comP* may be responsible for the lack of natural competency for strain AP193.

AP193 secondary metabolites inhibit the growth of multiple bacterial plant pathogens *in vitro*

The antimicrobial activities of strain AP193 and its mutants AP193Δ*dfnD* (deficient in the production of difficidin), AP193Δ*srfAA* (deficient in surfactin production) and AP193Δ*sfp* (unable to produce any polyketide or lipopepetide due to a deletion of *sfp* gene encoding 4'-phosphopantetheinyl transferase) were tested against plant pathogens *P. syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria, and *X. axonopodis* pv. campestris. The AP193 wild type strain demonstrated strong antimicrobial activity, whereas the AP193Δ*sfp* mutant was devoid of an inhibitory effect against those plant pathogens (Fig. 1), underlining the contribution of lipopeptides and polyketides in the biocontrol activity of AP193. This also indicates that the dipeptide bacilysin, whose synthesis is independent of Sfp, was not involved in antagonistic activity expressed *in vitro*. The AP193Δ*srfAA* mutant conferred antimicrobial activity similar to wild-type to *P. syringe* pv. tabaci, *R. radiobacter*, *X. axonopodis* pv. vesicatoria, and *X. axonopodis* pv. campestris (Fig. 1), suggesting that surfactin has no putative role in the antibacterial activity of AP193 against those plant pathogens under the conditions tested in this study. These findings also demonstrated that surfactin neither influences the antimicrobial compound biosynthesis in AP193 nor does it inhibit antibacterial activities of the antibacterial compounds produced by AP193. Difficidin acts as the major antibiotic in antagonism of AP193 against plant pathogens *P. syringe* pv. tabaci, *R. radiobacter*, *X. axonopodis* pv. vesicatoria, and *X. axonopodis* pv. campestris as indicated by the lack of the inhibitory effect of the AP193Δ*dfnD* mutant

against those plant pathogens (Fig. 1). This finding demonstrates the importance of difficidin in the biocontrol activity of subsp. *plantarum* strains against plant pathogens.

Strain AP193 secondary metabolites control bacterial spot caused by *X. axonopodis* pv. vesicatoria in tomato plants

To determine the role of bioactive compounds produced by strain AP193 in providing protection against plant diseases, the AP193 wild-type strain and its AP193Δ*dfnD*, AP193Δ*sfp* and AP193Δ*srfAA* mutants were applied to tomato plants several days before those plants were subsequently inoculated with plant pathogen *X. axonopodis* pv. vesicatoria. The AP193 wild-type and AP193Δ*srfAA* mutant significantly ($P < 0.05$) reduced disease severity of bacterial spot on tomato plants compared to the disease control (Table 4). Additionally, the application of strain AP193 significantly increased the root dry weight of the plants (Table 4). Unlike AP193 wild-type and its AP193Δ*srfAA* mutant, strains AP193Δ*sfp* and AP193Δ*dfnD* neither protected tomato plants from severe bacterial spot caused by *X. axonopodis* pv. vesicatoria nor improved the plants' growth parameters (Table 4), further supporting the importance of difficidin for plant disease protection. These findings are in agreement with the *in vitro* antibiosis pattern of AP193 wild-type strain and its AP193Δ*dfnD*, AP193Δ*sfp* and AP193Δ*srfAA* mutants demonstrated against plant pathogen *X. axonopodis* pv. vesicatoria.

5. Discussion

PGPR *Bacillus* spp. strains are being extensively used worldwide to improve crop yields and to protect against plant diseases. In this study, the genomes were sequenced for 13 different PGPR strains of *B. subtilis, B. pumilus, B. amyloliquefaciens, B. velezensis, B. mojavensis, B. siamensis, B. sonorensis*, and *B. tequilensis* representing diverse

members of *B. subtilis* group. This study used ANI, *gyrB*-based and core genome-based phylogenies to resolve the taxonomic affiliation of all available sequenced *Bacillus* spp. strains. Our findings demonstrated that six of the 12 strains sequenced in this study are affiliated with *B. velezensis*. This includes strain GB03 that was formerly designated as *B. subtilis*. Previously *B. siamensis* type strain KCTC 13613T had been proposed as a novel species (Sumpavapol *et al.*, 2010), but the core genome-based phylogenomic analysis (figure not shown; Hossain, 2015) revealed that *B. siamensis* KCTC 13613T is instead affiliated with *B. velezensis*. This finding is in agreement with the findings of Jeong et al (Jeong *et al.*, 2012) that determined the close affiliation of *B. siamensis* type strain KCTC 13613T to *B. velezensis* based on ANI. These findings support the use of a core genome-based phylogenomics approach in providing better phylogenetic resolution compared to a single housekeeping gene (e.g., *gyrB*) based phylogeny. Though phylogenies based on *gyrB* and core genome sequences demonstrated that *B. velezensis* are highly similar, the comparisons of their proteomes demonstrated that they are closely related but distinct from each other and may exert their plant growth promoting activities through different mechanisms.

B. velezensis strain AB01 was isolated from the intestine of channel catfish (Ran *et al.*, 2012), and its affiliation with other plant-associated strains may suggest its transient presence within a fish gastrointestinal tract; conversely, it may be that there are commonalities among *B. velezensis* strains that link their ability to associate with plants as well as animal hosts. Similarly, *B. siamensis* type strain KCTC 13613T, found to be closely affiliated with *B. velezensis* in this study, was isolated from salted crab rather than a plant-associated source. The efficacy of strains AB01, AP193 and other plant-

associated strains as probiotics in fish shows the common capacity for biocontrol of animal and plant pathogens as well as overlap in host colonization (Ran *et al.*, 2012).

With rapid advances in sequencing technologies it is now possible to extend genomic analysis beyond individual genomes to analyze core genomes (Medini *et al.*, 2008). In this study, core genomic analyses were conducted on PGPR strains from species affiliated with the *B. subtilis* group. This analysis identified 73 genes exclusively present among all subsp. *plantarum* but absent in the subsp. *amyloliquefaciens* strains sequenced to date. This small number of subsp. *plantarum*-specific genes is in agreement with a previous report that identified 130 subsp. *plantarum*-specific genes using limited number of genome sequence of subsp. *plantarum* (He *et al.*, 2012). Of these 73 *plantarum*-specific genes identified in this study, many are predicted to be important for plant- and soil-associated functions. For example, genes that are required for the utilization of D-galacturonate and D-glucuronate were found in the pool of *B. velezensis*-specific core genes. This observation is consistent with the absence of these genes in the genome of *B. amyloliquefaciens* subsp. *amyloliquefaciens* DSM7 (Ruckert *et al.*, 2011), a strain without any reported PGPR activity. The presence of D-galacturonate and D-glucuronate utilization genes could be advantageous for *B. velezensis* for their plant growth promoting activity through efficient root colonization. Pectin, a complex polymer found in plant tissues, is broken down to D-glucuronate and D-galacturonate which then serves as a carbon source for bacterial growth (Nemoz *et al.*, 1976). This pectin could potentially serve as a nutrient source for efficient root colonization of PGPR through competitive nutrient uptake.

Since many of the PGPR strains are from the *B. subtilis* group, the core genome estimation was expanded to include a larger number of *B. subtilis* group strains. Increasing the number of *Bacillus subtilis* genomes analyzed to 53 resulted in a 579,166 bp core genome that is predicted to encode 674 ORFs. This much smaller number of predicted genes reflects significant genomic diversity among *B. subtilis* group. This finding demonstrated that the number of ORFs found in the *B. subtili*s group core genome is close to the number of 610 ORFs of *B. subtilis* considered as indispensable for growth in a complex medium (http://www.minibacillus.org/project#genes).

To validate the function of any particular bacterial gene and its involvement in plant-related processes, it is essential to construct isogenic mutants that are devoid of those genes. Therefore, we deleted genes from PGPR strain AP193 to evaluate the role of several secondary metabolites biosynthesis gene clusters in the biological control of plant diseases. Gene deletion methods for naturally transformable *B. subtilis* and *B. amyloliquefaciens* clade strains through linearized plasmids or PCR fragments are well established (Fabret *et al.*, 2002; Koumoutsi *et al.*, 2004; Liu *et al.*, 2008; Yan *et al.*, 2008; Zhang *et al.*, 2011). However, our repeated attempts failed using these well-established methods. The lack of natural competence and genetic amenability for strain AP193 prompted us to adopt a different approach for the modification of its genome. A methylated shuttle vector pNZT1 (Zakataeva *et al.*, 2010) with gene deletion constructs were employed to deliver targeted AP193 genetic modifications. Three different mutants generated in this study demonstrated the efficacy of *in vitro* methylation of plasmids by cell-free extract in circumventing the restriction system that was presumed to have prevented transformation through electroporation.

Difficidin, first detected in the broth of two *B. subtilis* strains, is a highly unsaturated 22-membered macrocylic polyene lactone phosphate ester with broad-spectrum antibacterial activity (Zimmerman *et al.*, 1987). The molecular structure of difficidin (Wilson *et al.*, 1987) and genetic organization of its biosynthetic pathway have been reported previously (Chen *et al.*, 2006). Previously it has been demonstrated that difficidin expressed by strain FZB42, together with its dipeptide bacilysin, are antagonistic against *Erwinia amylovora* which is responsible for fire blight disease of orchard trees (Chen *et al.*, 2009). This study using an isogenic mutant AP193Δ*dfnD* demonstrated for the first time that difficidin solely, not in conjunction with any other polyketide or dipeptide, exerts *in vitro* antibacterial activity against multiple plant pathogens such as *P. syringe* pv. tabaci, *R. radiobacter*, *X. axonopodis* pv. vesicatoria and *X. axonopodis* pv. campestris. We also demonstrated, by using isogenic mutant AP193Δ*dfnD*, that difficidin expression is solely responsible for control of bacterial spot disease in tomato plants caused by *X. axonopodis* pv. vesicatoria. Taken together, these findings demonstrate that difficidin is the most important strain AP193 secondary metabolite for biological control of plant diseases due to bacterial pathogens. In addition, the construction of the *sfp* gene deletion allowed investigation of the contribution of multiple secondary metabolites produced by AP193 for its biocontrol activity. The *sfp* deletion mutant completely lost the antagonistic activity against each pathogen that was susceptible to the AP193 wild-type strain. Since the *sfp* deletion is expected to have a loss of difficidin synthesis, in addition to other metabolites, the lack of antimicrobial activity of AP193Δ*sfp* is consistent with that of the AP193Δ*dfnD* mutant and therefore suggests that difficidin is the primary metabolite responsible for *in vitro* inhibition of bacterial

pathogens. In contrast, the surfactin mutant retained antimicrobial activity against all plant pathogens tested, demonstrating that surfactin is neither critical for *in vitro* antibiotic activity nor influences the synthesis or secretion of other secondary metabolite biosynthesis in this *Bacillus* spp. strain. However, this finding does not rule out the role of surfactin in plant growth promoting activity since it has been observed that surfactin of *B. subtilis* elicits ISR in plants (Ongena *et al.*, 2007) and is expressed in the plant cells colonized by FZB42 (Fan *et al.*, 2011).

By studying the contributions of other genetic loci that are conserved among top-performing PGPR strains we may uncover the relative significance of their respective functions for plant colonization, growth promotion and/or pathogen biocontrol. In particular, future investigation of those genes related to the uptake and utilization of pectin-derived sugars will allow us to determine the relative importance of these gene products for colonization of their respective plant and persistence within this microbiome. A better understanding of the role of the gene products indicated by the comparative genomic analysis of *Bacillus* spp. PGPR strains could help in developing application strategies that result in higher activity of the strains.

Table 1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source or reference
E. coli K12 ER2925	*dcm-6 dam13::Tn9*	New England Biolabs
B. velezensis strain AP193 +	Wild type	Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University)
AP193Δ*sfp*	deficient in lipopeptides and polyketides	This study
AP193Δ*srfAA*	deficient in surfactin production	This study
AP193Δ*dfnD*	deficient in difficidin production	This study
Bacillus velezensis FZB42	Wild type	(Chen *et al*., 2007)
pMK4	*E. coli-Bacillus* shuttle plasmid, rolling circle replicative, Cm^R	BGSC
pNZT1	Replication thermosensitive derivative of the rolling-circle plasmid pWV01 (pG^+ replicon, Em^R)	Xiaozhou Zhang, Virginia Tech
pNZ-sfp	pNZT1 with upstream and downstream sequences of gene *sfp*	This study
pNZ-srf	pNZT1 with knock-out construct of *srfAA*	This study
pNZ-dif	pNZT1 with knock-out construct of *dfn*D	This study

Table 2. Primers used in this study.

Name	Sequence (5' to 3')
HindIII*SfpLL*	ATCAAAGCTTATACGCTGCTTCTGCCTGAT
SfpLR	CAGATCCGCGATGTGTTCTT
SfpRL	AAGAACACATCGCGGATCTGCGGTCCATATATACTCCGT
PstI*SfpRR*	ATCCTGCAGTGGCGGTTATGCTACAATGA
SfpUp	CGCTTTAACACACGGACTGA
SfpDn	TTTGTAGGAGCGGGAGAAGA
SfpDL	AAAGAGAGGAATCGGGACGA
SfpDR	TGTTTTGACGGGGCTGAT
HindIII*SrfLL*	ATCCAAGCTTATATGTACGGTCCGTCGGAA
SrfLR	GTTCCATTTGCAGCACTTCA
SrfRL	TGAAGTGCTGCAAATGGAACACTGGTCAAGCTGGCTGAAC
PstI*SrfRR*	ATCCCTGCAGGGTGCTTCAGCTCAATTCCT
SrfUp	GCGAAAGAGCGTCTGTAGAA
SrfDn	AGCCGTCATTGTCAGGTCAA
SrfDL	TCGGTCACAGGGAAATCTCT
SrfDR	CTGCTTGCGGTACTGCTCT
XhoI*DifLL*	TCAACTCGAGGGCGATTCTCGGTTTATCTC
DifLR	GATGGAGGATGCCGGTTAC
DifRL	GTAACCGGCATCCTCCATCCAAGAACGCTTTCGGGATT
SpeI*DifRR*	ATCCACTAGTGCCATATCAGATACCGCAGA
DifUp	TGGCTGATAAGCACCTACGA
DifDn	AAATCCGATTACAGGCGAGA
DifDL	ATAAGAAACCCGGTTCGGA
DifDR	TGGCGTGACGTCTCTCATC

Note: Restriction digestion sites are underlined within the sequence of the primers.

Table 3. Summary of draft genomes of *Bacillus* species sequenced used in this study.

Isolates	Number of Contigs (>1kb)	Size (total bp in assembly)	%G+C	NCBI BioProject Number	NCBI Short Read Archive Accession No.	Approx. sequence coverage (×)	Number of predicted ORFs
AB01	20	3,903,296	46.4	PRJNA239317	SRX475739	44	3944
AP71	198	4,278,192	45.7	PRJNA239317	SRX475740	15	4531
AP79	47	4,236,770	45.8	PRJNA239317	SRX475741	31	4368
AP143	146	2,956,670	46.6	PRJNA239317	SRX475742	24	3324
AP193	152	4,121,826	46.3	PRJNA239317	SRX475807	37	4159
AP254	59	4,048,419	43.8	PRJNA239317	SRX475808	29	4717
GB03	26	3,849,547	46.5	PRJNA227787	SRX380920	560	3928
INR7	44	3,681,709	41.3	PRJNA227786	SRX447924	750	3857
KCTC 3706T	17	3,935,582	43.7	PRJNA227789	SRX447926	895	4140
KCTC 13613T	23	3,779,696	46.3	PRJNA161489	SRX450083	500	3915
KCTC 13918T	32	4,428,962	45.5	PRJNA227788	SRX450084	1000	4704
KCTC 13622T	33	3,981,302	43.9	PRJNA227791	SRX450086	1000	4299

Table 4. Effects of plant growth promoting rhizobacteria (PGPR) strains on severity of bacterial spot disease and plant growth.

Strain [ab]	Disease severity [c]	Shoot Dry Weight (g)	Root Dry Weight (g)
Disease Control	2.11 a	2.07 bc	0.378 c
AP193	1.30 b	2.18 b	0.453 a
AP193Δs*rfAA*	1.48 b	2.16 b	0.423 abc
AP193Δ*sfp*	2.31 a	2.18 b	0.405 abc
AP193Δ*dif*	2.06 a	2.00 c	0.389 bc
Healthy Control	0.00 c	2.38 a	0.435 ab
LSD	0.35	0.15	0.050

Note:

a. The experimental design was a randomized complete block with ten replications per treatment. The experiment was conducted twice. Values followed by the same letter were not significantly different (P=0.05) according to Fischer's protected LSD.
b. One plant was in each replication. Plants were sprayed with PGPR suspension (10^6 CFU/mL) one week after transplanting, and were challenge-inoculated with pathogen solutions (10^7 CFU/mL) three days after inoculating PGPR.
c. Disease severity ratings and harvest were done 14 days later. For disease severity rating, four compound leafs were selected from the bottom of each plant. The disease severity of each of the compound leaves was determined by rating the disease severity of each leaflet and calculating the average rating for the compound leaf. The leaflet was rated using a 0-4 rating scale, where 0=healthy leaflet, 1= <20% necrotic area of the leaflet, 2 = 20-50% necrotic area of the leaflet, 3= 51-80 % necrotic area of the leaflet, 4 = 80-100 % necrotic area of the leaflet, or fully dead leaflet.

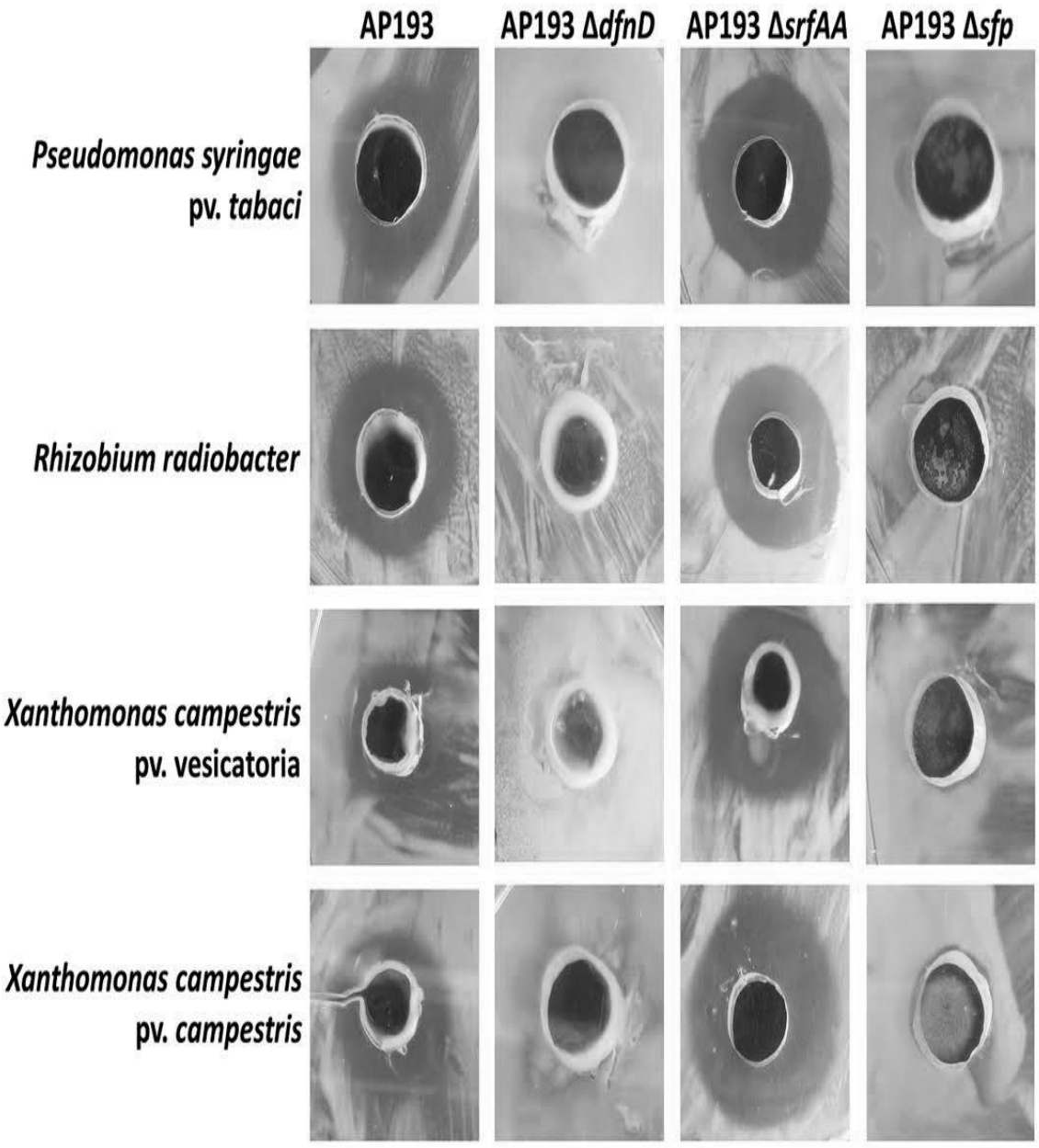

Figure 1. Antimicrobial activities of *Bacillus* sp. AP193 and its mutants Δ*srfAA*, defective in surfactin expression, Δ*dfnD,* defective in difficidin expression, and Δ*sfp,* defective in the expression of multiple secondary metabolites (including difficidin) against plant pathogens *Pseudomonas syringe* pv. tabaci, *Rhizobium radiobacter*, *Xanthomonas axonopodis* pv. vesicatoria and *Xanthomonas axonopodis* pv. campestris as demonstrated with an agar diffusion assay.

Chapter III

Burkholderia gladioli C101 metabolites protect tomato plants against *Xanthomonas perforans* infection.

1. Abstract

While metagenomic studies have broadened the known diversity of microbiota associated with agricultural soils and their potential in producing secondary metabolites, cultivation-based approaches have routinely recovered only a small percentage of extant soil microbial diversity. In this study, a root extract medium was used as a growth substrate along with extended incubation times to obtain diverse rhizobacteria isolates. The antagonistic potential of these isolates against a broad range of plant pathogenic bacteria, fungi and oomycetes was observed. Among the isolates tested, *Burkholderia gladioli* C101 produced the most potent and heat-stable active secondary metabolites that were active against root or foliar fungal, oomycete or bacterial pathogens. This study explored the potential of cell-free formulations of *B. gladioli* C101 as a foliar spray to suppress bacterial spot disease in tomato. A significant reduction in bacterial spot disease severity was observed in greenhouse experiments when cell-free supernatants were applied prior to or after inoculation of tomato plants with *Xanthomonas perforans*. Reduced disease severity was observed in a dose-dependent manner for application of *B. gladioli* C101 secondary metabolites that have utility for their *in planta* antagonism of *X.*

perforans and potentially other phytopathogens.

Importance. The study used soybean root extract as a medium to cultivate diverse rhizobacteria that use plant-derived growth substrates and would otherwise not be isolated when using traditional media. These rhizobacteria were identified based on their 16S rRNA sequences. Some of the rhizobacteria isolated had *in vitro* antagonism toward pathogenic microbes, particularly the *Burkholderia* species that showed antagonistic activity toward multiple phytopathogens, including *X. perforans*. Furthermore, in an in-planta pathogenicity assay, tomato plants treated with bioformulated cell-free supernatant from *B. gladioli* C101 resulted in significant antagonistic activity on *X. perforans* with reduced disease severity observed in tomato plants.

2. Introduction

Bacteria inhabiting the rhizosphere produce diverse secondary metabolites that mediate microbial and plant interactions and can have pharmaceutical and agricultural applications. Common plant disease management practices using heavy metals such as copper or other biocides may be damaging to the environment, and pathogens have developed resistance to some of theses disease control approaches, including copper-based biocides (Richard *et al.*, 2017). The exceptionally diverse microbial assemblages associated with plants are a resource from which plant growth promoting rhizobacteria (PGPR) strains can be isolated, many of which produce bioactive secondary metabolites; however, the majority of soil microbiota are recalcitrant to laboratory cultivation, and this precludes identification of many potentially useful metabolites (Roesch *et al.*, 2007 and Handelsman, 2005). The cultivation of novel bacterial lineages from soils has been accomplished using dilute growth media (Joseph *et al.*, 2003) and with diffusion

chambers allowing nutrients available *in situ* to promote bacterial growth with extended cultivation times appropriate for K-selected populations (Nichols *et al.,* 2010 and Lynch and Neufeld, 2015). These approaches have expanded the phylogenetic breadth of microbial culture collections and led to the discovery of the novel antibiotic teixobactin (Ling *et al.*, 2015). In addition, plant root extracts have been used as a sole carbon source to cultivate rhizobacteria or to enrich for archaeal populations (Jacoby *et al.*, 2018 and Simon *et al.*, 2005); however, in these previous reports of the use of plant root extracts as a growth substrate to cultivate rhizosphere-associated microbes, root-derived complex carbohydrates have been removed either by centrifugation, filtration and/or extraction. Our previous research on PGPR strains isolated from plant rhizospheres indicated that all of our best-performing *B. velezensis* strains (formerly known as *B. amyloliquefaciens* subsp. *plantarum*) had the ability to use pectin as a sole carbon source (Popovici, 2016). Therefore, this study was initiated to cultivate diverse rhizobacteria using a root extract medium (REM) that included plant root-derived complex carbohydrates, such as pectin, and to evaluate these rhizobacteria for their ability to antagonize plant pathogens.

Rhizobacteria can contribute in many ways to plant health, including nutrient uptake and biological control of soil-borne plant diseases. Rhizobacteria including *Bacillus*, *Burkholderia,* and *Pseudomonas* species have been described as PGPR strains that can also control plant diseases and pests through various mechanisms, including the production of secondary metabolites such as polyketides (Svoboda *et al.*, 1954, Burkett-Cadena *et al.*, 2008, Chowdhury *et al.*, 2015, Kloepper *et al.*, 2004, Kloepper *et al.*, 1981, Rojas-Rojas *et al.*, 2018, and Taylor, 1953). The *Burkholderia* and *Bacillus* spp. strains that were antagonistic toward plant pathogens were evaluated to determine their

effectiveness in colonizing endophytically or epiphytically within the phylosphere and were found to be poor colonizers within the phylosphere. However, the antagonistic compounds produced by these strains had activity against phyllosphere-colonizing pathogens. The goal of this study was to exploit this antagonism to minimize phyllosphere disease severity. Therefore, cell-free formulations of selected isolates were used as foliar sprays to explore their antagonistic potential against foliar pathogens. Tomato and other vegetable crops are jeopardized by many bacterial diseases, including bacterial spot disease caused by *Xanthomonas* species, namely *Xanthomonas euvesicatoria, Xanthomonas vesicatoria, X. perforans and Xanthomonas gardneri* (Allareddy *et al.*, 2015). In the absence of viable management strategies, there is a need for an effective solution against this endemic foliar pathogen (Richard *et al.*, 2017).

3. Materials and Methods

Soil sampling and rhizobacteria isolation on REM agar. In this study three soil types representative of Alabama soils were selected, including a sandy soil from the Cullars Rotation, a clay-rich soil from the E.V. Smith Agricultural Experiment Station, and an organic “black belt” soil from the AU Arboretum (originally from the shipment of soil during AU-Montgomery construction) and representative forest soil type. Each soil was used to grow soybean plants from seed in a greenhouse for 21 days, then the entire root system including loosely-adherent soil was removed and homogenized in 1x phosphate-buffered saline using an IKA T10 Ultra-turrax homogenizer (Wilmington, NC) at 50% power for 10 sec in order to obtain microorganisms from the rhizosphere, rhizoplane, and endophytic populations. A REM agar was produced by sampling additional soybean roots (grown in EV Smith soil for 21 days), washing the roots with

distilled, deionized water and then homogenizing the roots in sterile water at maximum power for at least five periods of 30 sec each until the root material was thoroughly homogenized to sub-micron particulates. The root homogenate was mixed with M9 minimum salts media (Difco) plus 1.5% agar at different inclusion rates, ranging from 0.1 to 5% (w/v), then allowed to solidify in petri dishes. Root-associated bacteria were cultivated by homogenization of soybean roots containing associated soil aggregates in sterile water as described above, followed by 10-fold serial dilution and plating 100 μL of the dilutions (10^{-1} through 10^{-8} used for range-finding experiments, 10^{-4} to 10^{-6} used for the culture collection) onto REM agar. Plates were sealed with parafilm and incubated at room temp for 2.5 months, with weekly monitoring for microbial growth. Colonies were used to inoculate both REM agar and tryptic soy agar (TSA), and due to the better growth observed on TSA, this growth medium was used for all subsequent cultivation steps subsequent to the primary cultures. Each rhizobacteria culture was cryopreserved using 20% glycerol in tryptic soy broth (TSB) at -80 ^{o}C.

Phylogenetic affiliation of rhizobacteria based on 16S rRNA gene sequences. DNA from rhizobacteria cultures were isolated using E.Z.N.A genomic DNA isolation kit (Omega Bioteck, Atlanta, GA). Genomic DNA was used as a template to amplify 16S rRNA genes using the primers 27F and 907R (Gerardo *et al.*, 2014). Each PCR amplicon was purified using the E.Z.N.A cycle pure kit (Omega Bioteck, Atlanta GA) and Sanger sequenced in both directions to generate a consensus sequence for each bacterial isolate. Consensus sequences were generated after trimming for quality and assembly using Geneious R 1.1 and compared against the databases at GenBank (nr/nt) and EzTaxon databases to identify bacterial ribotypes and their respective nearest neighbors.

Phylogenetic analysis was carried out using 16S rRNA genes from each isolate and its respective nearest type strain with MEGA7.0 (Pu *et al.*, 2014) using maximum likelihood with 1000 iterations (DeLorenze *et al.*, 2013).

Screening rhizobacteria for inhibition of plant pathogen growth.

Rhizobacteria isolates were cultured on TSA and incubated at 30 ^{o}C for 48 h prior to assay for production of secondary metabolites that inhibited the growth of different plant pathogens (data not shown; Shantharaj *et al.*, 2019). The phytopathogens in this study, namely *Xanthomonas campestris pv. malvacearum, Pseudomonas savastanoi pv. glycinea, Erwinia carotovora, Clavibacter michiganensis subsp. michiganensis, Clavibacter xyli subsp. xyli, Rhodococcus fascians, Streptomyces scabies, Pythium ultimum, Rhizoctonia solani, Fusarium oxysporum f. sp. lycopersici, Fusarium oxysporum, f. sp. vasinfectum, Fusarium virguliforme, Bacillus velezensis* AP193 for the inhibition assay were collected from Phytobacteriology laboratory (Dr. Joseph W Kloepper) at Auburn University. Bacterial spot pathogen *X. perforans,* a tomato isolate from Alabama was collected from Phytobacteriology laboratory (Dr. Neha Potnis) at Auburn University.

In anti-phytobacteria assays, rhizobacteria colonies were suspended in sterile molecular-grade water, and their optical density at 600 nm (OD_{600}) was normalized to 0.3, and 5 μL of each normalized bacterial suspension was dropped on to TSA plate and incubated for 48 h at 30^{o} C. The rhizobacterial culture on the plate was killed by exposing it to UV light (1200 x 100 mJ/ cm^2) for 5 min. Phytobacteria populations at approximately 10^6 CFU/ mL in TSA soft agar (0.3%) at 37^{o} C were used for a soft agar overlay and were subsequently incubated at 30^{o}C for 24h to 48h and observed for the

zone of inhibition. For the anti-phytofungal assay, fungal cultures were grown on cornmeal agar at 30^{o} C for seven days. The rhizobacterial culture was suspended in sterile DI water and normalized to an OD_{600} = 1.0. Ten μL of the normalized rhizobacteria suspension was inoculated onto a potato dextrose agar (PDA) plate. The center of the PDA plate was then inoculated with a 5 mm size corn meal agar plug of a confluent fungal culture. The PDA plates were incubated at 30^{o}C for 5-7 days then observed for zones of inhibition. The antagonistic activity was measured by the inhibitory zone and represented as strong, moderate, and nil.

Greenhouse biocontrol study. The rhizobacterial strain, *B. gladioli* C101 was selected for evaluation of biocontrol activity against copper resistant *X. perforans* strain from Alabama. The greenhouse experiment was conducted at the Plant Science Research Center at Auburn University. Greenhouse temperature was maintained at 25^{0}C day/ 21^{o}C night throughout the year. Tomato variety Bony best (susceptible to *X. perforans* infection) was used in the greenhouse study. Tomato seeds were germinated in the commercial potting substrate (Sunshine mix, Sun Gro Horticulture, Agawam, MA 01001), and ten-day-old seedlings were transplanted to 8x8x5 cm plastic inserts and placed in trays. The transplants were fertilized with 15-5-15 N-P-K every week and watered daily. *X. perforans* strain from Alabama was cultured on TSB medium overnight at 30^{o}C on a shaking incubator; cells were pelleted by centrifuging at 5000 x *g* for 10 min. *X. perforans* titer was adjusted of 10^{7} CFU/ mL in sterile tap water by serial dilution and used for plant infection. Greenhouse trials were conducted using a randomized complete block design with 3-5 plants per treatment.

Bioformulation. B. gladioli was cultured in 1% TSB for 72 h until OD_{600}= 1.0. The cell-free supernatant was collected by centrifuging at 5000 x g for 15mins at 25^0C, and this bio-formulated concentration was considered as 1X. The 1X bioformulation is diluted to 0.75X, 0.5X, 0.25X and used for in planta assay. As a positive control, *B. velezensis* AP193 that had previously demonstrated bacterial spot biocontrol under greenhouse conditions was also included in the experiment (Mukhtar *et al.*, 2013). *B. velezensis* was cultured in 1% TSB for 72 h until OD_{600} = 1.0. The cell-free supernatant was collected by subjecting the cultures to centrifugation at 5000 x *g* for 15 min at 25^0C.

In-planta assay. In the initial in-planta study, *B. gladioli* formulation and dosage requirement to manage *X. perforans* were standardized. The ten-day-old tomato transplants were tested with the range of *B. gladioli* dilution with the following experimental treatments: 1) Water control, 2) *X. perforans*, 3) *B. gladioli +X. perforans*, 4) *B. gladioli* were applied as a foliar spray; these were applied two days prior and seven days post-*X. perforans* challenge. The standardized 1X concentrated *B. gladioli* formulation was used for further studies. The 10-day old tomato transplants were subjected to the following experimental treatments: 1) Water control, 2) *X. perforans*, 3) *B. velezensis*, 4) *B. gladioli*, 5) *B. velezensis+X. perforans*, 6) *B. gladioli+X. perforans*. When either *B. gladioli* or *B. velezensis* supernatants were applied as a foliar spray, these were applied two days prior and seven days post-*X. perforans* challenge. Treatment groups that included foliar sprays of *B. gladioli* or *B. velezensis* supernatants without *X. perforans* challenge were applied at the same time as the treatment groups that did receive a challenge. Disease severity ratings was carried based on Horsfall-Barratt method with 5-point rating scale, 0= 0% lesions, 1=10-25% lesions, 2=25-50-% lesions,

3= 50-75% lesions, 4=75-100% lesions. *X. perforans* population is calculated as Log_{10} CFU/ cm^2 leaf area. *X. perforans* colonies are counted from serially diluted leaf extracts from experimental treatments and cultured on TSA plates.

***B. gladioli* C101 secondary metabolite extraction and antagonistic stability.** *B. gladioli* was cultured in TSB medium with starting inoculum of $OD_{600=}$ 0.1, and the TSB medium without inoculating the bacteria served as a control in 30 ^oC incubator rotated at 200rpm for 72 h to reach OD_{600}= ~ 1.0. The culture and control TSB media alone were centrifuged at 5,000 x *g* for 15min, and the supernatant was added with three volumes of ethyl acetate and agitated for 10 min and transferred to a separating funnel, the aqueous layer is discarded, and the solvent layer is collected and evaporated using rotary evaporator at room temperature. The extracted compound is reconstituted in methanol for in-vitro assay. The 1mL of methanol extract containing compound and control extract is added to an eppendorf along with 5 mm Whatman filter discs (5 in number) and rotary vacuum evaporator at 30 ^oC room temperature. The absorbed antibiotic compound to the filter disc and the control disc was used in the plate assay. TSA plate is overlaid with 3 mL of *X. perforans* (10^6 CFU/ mL) in top agar (TSA with 0.3% agar), and one filter disc is adsorbing *B. gladioli* antibiotic compound was placed on the overlay. The plate was incubated at 30 ^oC for 48h and observed for inhibition zone.

Viability and stability of the *B. gladioli* C101 secondary metabolite. *B. gladioli* rhizobacterial strains are cultured in 1% TSB for 72 h until OD_{600}= 1.0 (1X concentration). 1.5 mL of culture is aliquoted in triplicates into two mL eppendorf tubes. The bacteria culture is treated at different temperatures. Bacteria culture is treated with 10 ^oC temperature increment starting from 30, 40, 50, 60, 70, 80, 90, 100 ^oC for 30 min with

intermittent mixing. The temperature treated *B. gladioli* culture is cooled at room temperature for 10 min and centrifuged at 5000 x *g* for 5 min. The supernatant is transferred to another eppendorf tube and stored at 4 ^{o}C. The *B. gladioli* cell viability is tested by treating the cell pellet with 25 mM TTC solution and incubated for 30min in a shaker incubator set for 30 ^{o}C. The viable cells that have reduced TTC to formazan were extracted in DMSO and absorbance was read at 510 nm using a spectrophotometer. Ten µL of the supernatant from different temperature treated *B. gladioli* culture is spotted on a TSA plate and dried till is absorbed in the medium and overlaid with *X. perforans* 10^6 CFU/ mL. Plates were incubated at 30 ^{o}C and observed for inhibition zone.

Sequencing, Assembly and Annotation. The *B. gladioli* C101 genome was sequenced using an Illumina MiSeq sequencer. Indexed libraries were prepared using Nextera DNA Sample PrepKit (Epicentre, Madison, WI) and sequences were generated with a 2 × 250 paired-end sequencing kit (Illumina, San Diego, CA). Sequence reads were trimmed for quality then assembled de novo using the bacterial bioinformatics database and analysis resource from the PATRIC (Pathosystems Resource Integration Center). The assembled contigs were used for predicting the open reading frames (ORFs) for secondary metabolite biosynthesis gene clusters using antiSMASH 4.0 (Blin *et al.*, 2017). The *B. gladioli* C101 genome sequence was deposited in GenBank as the BioProject and Accession # PRJNA551879, VIYR00000000.

Statistical analysis. The data were analyzed by statistical software JMP 12.0.1 copyright 2015 SAS Inst. Inc. Greenhouse experiments were analyzed using the experimental trial with mean ± SE with significance calculated according to Tukey-Krammer HSD at 0.01. The data represented in graphs and tables within were analyzed

using three experimental replicates and represented by mean ± SE, as well as significance using Tukey-Krammer HSD at 0.01.

4. Results

Ribotyping of rhizobacteria cultivated on root extract agar growth medium

REM agar plates with varying concentrations of soybean root extract were used to cultivate bacteria derived from soybean roots grown in agricultural soil (EV Smith Agricultural Experiment Station) with long-term incubation for 2.5 months, and the plates were periodically monitored for the presence of bacterial colony forming units (CFUs). Isolated colonies were obtained at the 10^{-4} to 10^{-6} serial dilutions, indicating that REM agar resulted in the cultivation of over 10^6 CFU/g of soil collected from the inclusion of 1% (w/v) root extract. Reduced numbers of CFUs were observed at lower amounts of root extract added, and no significant CFU increase was seen at >1% (w/v) root extract additions. Therefore, a 1% (w/v) root extract was selected for the final REM agar composition. After inoculating agar plates with serially diluted soybean, rhizosphere samples from three different agricultural soils as well as a representative forest soil collectively produced 184 unique colony morphologies after three months of incubation at 25^{o}C. After primary isolation of multiple representatives of each colony morphology on REM agar, each of the cultures was found to grow more rapidly on complex media such as tryptic soy agar (TSA) plates, so TSA was routinely used for subsequent bacterial cultivation.

Rhizobacteria were ribotyped based on consensus ~1000 bp 16S rRNA gene sequences and were found to include representatives from the genera *Arthrobacter, Bacillus, Bosea, Burkholderia, Cabelleronia, Chitinophaga, Ensifer, Flavobacterium,*

Microbacterium, Paenibacillus, Paraburkholderia, Pseudomonas, Ralstonia, Rhizobium, and *Rhodococcus* (Table 1). These rhizobacteria were affiliated with type strains from each of their respective genera based on a maximum likelihood phylogenetic analysis (Fig. 1).

Screening for rhizobacteria that produce bioactive secondary metabolites that inhibit phytopathogen growth

Rhizobacterial isolates were tested for their antagonistic activity against bacterial, fungal and oomycete pathogens using *in vitro* plate assays. Zones of inhibition were measured for each combination of UV-inactivated suspension of rhizobacteria isolate and phytopathogen. This study focused on rhizobacteria isolates that displayed potent and broad-spectrum activity against a wide range of phytopathogens. Among the REM isolates, 8% of these strains showed the production of metabolites with antibacterial activity against one or more gram-negative bacterial plant pathogens. In particular, REM isolates affiliated with the genera *Bacillus, Burkholderia* and *Paraburkholderia* were among the most efficient producers of antagonistic compounds under *in vitro* antagonistic assay conditions (data not shown ; Shantharaj *et al.*, 2019). Antagonistic activity was tested against the gram-positive plant pathogens *Clavibacter michiganensis* subsp. *michiganensis, Clavibacter* subsp. *xyli, Rhodococcus fascians* and *Streptomyces scabies;* against fungal and oomycete plant pathogens, namely *Pythium ultimum, Rhizoctonia solani, Fusarium oxysporum* f.sp. *lycopersici, Fusarium oxysporum* f. sp. *vasinfectum* and *Fusarium viruliforme,* as well as the gram-negative bacterial pathogens, *Xanthomonas, Erwinia* and *Pseudomonas* spp. (data not shown ; Shantharaj *et al.*, 2019). All rhizobacteria isolates, except *Pseudomonas beteli* C32, showed weak or strong

inhibitory activity for the *in vitro* plate assay. Two rhizobacteria isolates, *B. gladioli* C101 or *B. velezensis* AP193, were further selected for *in vivo* biocontrol evaluation given their broad-spectrum activity.

Testing cell-free supernatants of rhizobacteria isolates as a foliar spray in reducing bacterial spot disease severity

The ability of *B. gladioli* or *B. velezensis* to control copper-resistant *X. perforans* was evaluated as a foliar application on tomato. *B. velezensis* AP193 was previously demonstrated to reduce bacterial spot due to *Xanthomonas* spp. infection of tomato (Murillo *et al.*, 2010), and served as a positive control in these experiments. Due to the concerns of using viable cells of *Burkholderia* spp. due to opportunistic infections caused by members of the *Burkholderia cepacia* complex (Vardakas *et al.*, 2009), and the poor phyllosphere colonization by these rhizobacteria (data not shown), the efficacy of cell-free supernatants from *B. gladioli* or *B. velezensis* cultures to control bacterial spot disease were evaluated as foliar sprays. The application of *B. gladioli* or *B. velezensis* culture supernatants prior to *X. perforans* infection (figures not shown ; Shantharaj *et al.*, 2019), or after *X. perforans* infection (figures not shown ; Shantharaj *et al.*, 2019), was observed to control bacterial spot disease resulting in significant reductions in disease severity ($P < 0.001$) with comparably low disease severity levels (disease severity < 0.5 in each case). Interestingly, there was an approximately 10,000-fold reduction in *X. perforans in planta* population when *B. gladioli* supernatant was used as a pre-treatment ($P < 0.001$), whereas *B. velezensis* supernatant pre-treatment only resulted in an approximately 100-fold reduction in *X. perforans in planta* population, although this was statistically insignificant ($P = 0.1$; figure not shown ; Shantharaj *et al.*, 2019). In contrast,

foliar application of *B. gladioli* or *B. velezensis* supernatants after *X. perforans* infection reduced the *X. perforans in planta* population by 75 to 100-fold compared to *X. perforans* treatment alone ($P < 0.0001$; figure not shown ; Shantharaj *et al.*, 2019).

A dose response was conducted to evaluate different concentrations of cell-free supernatant on disease incidence. Cell-free supernatants from *B. gladioli* or *B. velezensis* cultures was applied at different concentrations (25%, 50%, 75%, and 100% strength) to the leaves of 4-5-week old tomato plants prior to *X. perforans* inoculation. Bacterial spot disease severity was significantly reduced to a mean score of 0.6 or 0.8 for disease severity index (on a 5 point disease rating scale) after foliar application of 75% or 100% strength *B. gladioli* or *B. velezensis* supernatants, respectively ($P < 0.0001$; figures not shown ; Shantharaj *et al.*, 2019), whereas control plants inoculated with *X. perforans* and without application of *B. gladioli* or *B. velezensis* supernatants showed disease severity score of 3.2 by 21 days after inoculation.

***B. gladioli* secondary metabolite stability and durability**

The secondary metabolite(s) expressed by *B. gladioli* was extracted in ethyl acetate, reconstituted with methanol, and showed significant *in vitro X. perforans* growth inhibition (data not shown). Given the human safety concerns of introducing viable *Burkholderia* cells into crop production, the heat susceptibility of *B. gladioli* cultures was assessed along a temperature gradient, which revealed that *B. gladioli* cell viability was completely lost when incubated at 60 ^{o}C or higher (figure not shown ; Shantharaj *et al.*, 2019). The same heat-treated culture supernatants were then assessed overtime for their *in vitro* antagonistic effects on *X. perforans*, which revealed 100% of the anti-*X. perforans* activity was detected at time zero even when cultures were incubated at 100 ^{o}C

($P < 0.0001$), but with progressive loss of activity over time of incubation post-heating that was more pronounced with samples that had the highest temperature exposure ($P < 0.0001$). The greatest stability of the anti-*X. perforans* active secondary metabolites was observed in the samples treated at 60 ^{o}C, with the majority of activity retained even after two weeks of storage (figure not shown ; Shantharaj *et al.*, 2019). These results suggest that conditions can be obtained in which *B. gladioli* cellular viability is lost while retaining the activity of the bioactive metabolite(s) that provide anti-*X. perforans* biocontrol.

Prediction of secondary metabolite clusters encoded in the genome of *B. gladioli*

A *B. gladioli* C101 draft genome sequence was determined using the Illumina MiSeq platform, which generated a predicted genome size for *B. gladioli* C101 of 8.3 Mb, which is similar to the 8.4 Mb genome size of *B. gladioli* ATCC 10248 (NCBI BioProject PRJNA238809). The number of contigs > 1 kb was 168 with an average 68.0% G+C content, which is similar to the % G+C content of other *B. gladioli* strain genomes available in GenBank. The *B. gladioli* C101 contigs were screened for the presence of biosynthetic gene clusters (BGCs) using antiSMASH 5.0. The BGCs encoded by *B. gladioli* C101 that were predicted to have homology with BGCs that encode secondary metabolites (in decreasing order of similarity) including rhizomide A/B/C (100% of genes in BGC show similarity; expressed by many *Burkholderia* spp. with cytotoxic activity; Kunakom and Eustaquio, 2019), jessenipeptin (66% of genes in BGC show similarity; found previously from a *Pseudomonas* spp. that was resistant to amoeba grazing, and encoding a metabolite with activity against *Staphylococcus aureus*; Arp *et al.*, 2018), ralsolamycin (60% of genes in BGC show similarity; a metabolite that induces

fungal chlamydospore formation and was discovered from *Ralstonia solanacearum*), enacyloxin (58% of genes in BGC show similarity; polyketide with activity against gram-negative bacteria produced by hybrid *cis*-AT/*trans*-AT PKS; Mahenthiralingam *et al.*, 2011), barbamide (41% of genes in BGC show similarity; a chlorinated lipopetide with molluscicidal activity derived from a marine cyanobacterium (Chang *et al.*, 2002); a predicted barbamide BGC was deleted in *Burkholderia mallei* but its metabolite was not characterized; Ong *et al.*, 2004), microsclerodermin (28% of genes in BGC show similarity; reported from Myxobacteria with anti-fungal activity; Hoffmann *et al.*, 2013), iso-migrastatin (27% of genes in BGC show similarity; anti-tumor compound produced by *Streptomyces* sp. MK929-43F1; Ju *et al.*, 2005), xenoamicin (25% of genes in BGC show similarity; found previously from *Xenorhabdus doucetiae* with anti-*Plasmodium* activity), desotamide (9% of genes in BGC show similarity; a cyclohexapeptide with potent activity against gram-positive bacteria derived from *Streptomyces scopuliridis* (Li *et al.*, 2015); a draft genome of *Burkholderia anthina* XXVI was observed to have a BGC with homology to that of the desotamide BGC; de Los Santos-Villalobos *et al.*, 2018), lasalocid (7% of genes in BGC show similarity; produced by *Streptomyces lasaliensis* (Migita *et al.*, 2009), used in cattle for increased feed efficiency and with activity against gram-positive bacteria) and phosphinothricin (6% of genes in BGC show similarity; a herbicide produced by *Streptomyces viridochromogenes* (Schwartz *et al.*, 2005); this BGC contains predicted ORFs for a phosphonoacetaldehyde dehydrogenase and a PEP-phosphomutase; Schwartz *et al.*, 1998). In addition, there were predicted BGCs encoding the siderophores cupriachelin (11% of genes in BGC show similarity) and taiwachelin (11% of genes in BGC show similarity). There were also several predicted BGCs

including NRPS, PKS and hybrid NRPS/PKS clusters that lacked any significant similarity to known BGCs, indicating that additional metabolites with unknown bioactivity may be expressed by *B. gladioli* C101.

5. Discussion

Phylogenetically diverse bacterial isolates were obtained on a soybean root extract medium, which allowed the isolation of bacterial isolates that would otherwise have been missed on more traditional media due to the presence of complex carbohydrates found within the roots as well as a prolonged incubation time allowing for the isolation of slower growing bacteria. Moreover, among these rhizobacteria isolates around 8% were found to have antagonistic activity against a panel of plant pathogens. Despite the use of a specialized media, there are likely many more rhizobacteria present within the soil not cultured in this study. Studies like the present one as well as other culture-based approaches such as the isolation chip (iChip) expand our collection of cultured isolates.

Among the rhizobacteria isolates obtained on REM agar, the genera *Bacillus*, *Burkholderia* and *Paraburkholderia* include many species that produce secondary metabolites with potential to antagonize plant pathogens. *Bacillus* spp. have in particularly shown antagonism against plant pathogens and have been studied as biocontrol agents *in planta* on crop plants (Fowler *et al.*, 2013 and Matsumoto *et al.*, 2012). Many *Burkholderia* species are well-known for their ability to promote plant growth and produce specific bioactive metabolites, such NRPS and PKS compounds such as rhizoxin, thailanstatin, burkholdac, spliceostatin, thailandamide, bactobolin and enacyloxin (Intrakamhaeng *et al.*, 2012 and Gessmann *et al.*, 2012).

Some *Burkholderia* species, include members of the *B. cepacia* complex, have been associated with human disease, particularly patients with cystic fibrosis, thus *Burkholderia* spp. have not been developed commercially as biocontrol agents for agricultural crops. This concern may be alleviated through the use of cell-free supernatants or heat-treated cultures in which no viable *Burkholderia* cells remain. In support of the efficacy of this treatment, *X. perforans* biocontrol was observed to be more effective when using cell-free supernatants from *B. gladioli* C101 compared to the application of living cells. While the production of bacterial secondary metabolites would be expected to be very different *in vitro* compared to *in planta* (McCullough *et al.*, 2011), the as-yet-uncharacterized metabolite(s) produced by *B. gladioli* C101 under laboratory conditions had demonstrated activity against *X. perforans* as well as other phytopathogens.

The use of biocontrol agents has shown promise in managing disease in greenhouse crops in recent decades. Soilborne pathogens such as *Sclerotinia, Pythium, Rhizoctonia,* and *Fusarium* have been controlled through the use of *Coniothyrium minitans, Gliocladium, Trichoderma, Bacillus, Pseudomonas, Streptomyces* and nonpathogenic *Fusarium* spp. (Ong *et al.*, 2004). For biocontrol of primary foliar diseases in greenhouses caused by *Botrytis* as well as powdery mildew, *Trichoderma, Ampelomyces quisqualis, Bacillus,* and *Ulocladium* have shown efficacy in reducing disease severity (Lee *et al.*, 2010 and Fellas *et al.*, 2011). This study revealed that tomato plants treated with cell-free supernatants from lab-grown cultures of *B. gladioli* C101 or *B. velezensis* AP193 significantly reduced the severity of bacterial spot disease as compared to a negative control. The ultimate bioformulation used for crop protection

would need to be cost-effective, safe and useful for the management of disease due to *X. perforans* or other plant phyllosphere diseases. The liquid formulation of *B. gladioli* C101 supernatant that lacked viable cells after heat treatment at 60 ^{o}C had a shelf life of at least one month when stored at 4^{o}C. Further research on the specific metabolite(s) responsible for *X. perforans* biocontrol and their effectiveness in different formulations (e.g., heat-treated and lyophilized supernatants) could extend shelf life as well as improve distribution to growers.

The *B. gladioli C101* genome was predicted to contain many different BGCs that encode for bioactive secondary metabolites that could be responsible for inhibition of the growth of bacterial, fungal and oomycete phytopathogens. Analysis of the BGCs predicted from *B. gladioloi* C101 revealed many clusters that have not been previously associated with *Burkholderia* spp., including several BGCs that produce secondary metabolites that were characterized from *Streptomyces* spp. including desotamide, iso-migrastatin and lasalocid among other BGCs that have been characterized in non-*Burkholderia* spp. In fact, we observed so many BGCs with a putatively non-*Burkholderia* origin that we conducted a phylogenetic analysis of the sequences derived from the *B. gladioli* draft genome, which matched exactly with the previously determined sequences obtained from the pure culture (data not shown). Among these predicted BGCs, only the cluster responsible for the biosynthesis of enacyloxin is known to produce a metabolite that inhibits the growth of gram-negative bacteria. The enacyloxin BGC, a hybrid *cis*-AT/*trans*-AT polyketide synthase (PKS) identified from *Burkholderia ambifaria* AMMD (Mahenthiralingam *et al.*, 2011), had 58% of the genes in this cluster similar to that of the predicted BGC from *B. gladioli* C101, suggesting that there may be

differences in enacyloxin structure and/or bioactivity in this strain. Lastly, several BGCs were predicted from the *B. gladioli* C101 genome that lacks a known characterized secondary metabolite, which could contribute to phytopathogen inhibition. Future studies will determine the chemical structure and function of the metabolites produced by *B. gladioli* C101 and evaluate the best formulations to prevent disease due to black spot and other phytopathogens.

In conclusion, this study demonstrated that a root extract medium could be used to cultivate phylogenetically diverse rhizobacteria. The efficacy of rhizobacteria isolates in inhibiting plant pathogen growth and disease was determined both *in vitro* and *in planta*. The rhizobacteria isolates did not efficiently colonize tomato phyllosphere; therefore, their ability to inhibit disease due to the foliar pathogen *X. perforans* was explored and indicated that *B. gladioli* and *B. velezensis* cell-free supernatants could prevent or treat *X. perforans* infection and induce a significant reduction in black spot disease severity. These results are of significant interest for integrated pest management of endemic bacterial spot disease for which there is currently no viable management option. Further studies will characterize the structure and function of the secondary metabolite(s) that reduce disease due to *X. perforans* and evaluate the most cost-effective and safe formulation for plant disease biocontrol.

Table 1. Rhizobacteria isolates and their closest phylogenetic affiliated type strain from GenBank and EzTaxon databases based on consensus 16S rRNA gene sequences. Each of the isolate IDs includes multiple strains that had identical or similar 16S rRNA gene sequence ribotypes based on a maximum likelihood analysis.

Taxon	**#Isolate ID**	*** % Identity**	**# Type Strain Accession**
Arthrobacter nanjingensis	GHP1-1*, GHP1-2	100*, 99.0	KF479547
Bacillus siamensis	C68a, C76a, C77a, C78a	100, 99.9, 100, 99.9	KCTC 13613
Bacillus albus	F19	100	MAOE01000087
Bosea thiooxidans	GHP4-3, GHP4-5	99.1, 98.9	jgi.1048902
Burkholderia anthina	GHP2-2*	99.7*	AJ420880
Burkholderia dabaoshanensis	F11	98.8	FJ210816
Burkholderia gladioli	C101	99.9	BBJG01000151
Burkholderia territorii	GHP2-1	99.8	JJOA01000042
Burkholderia contaminans	A11	100	LASD01000006
Caballeronia zhejiangensis	GHP4-2, GHP4-4, GHP6-6	100, 100, 100	JFHD01000081
Chitinophaga polysaccharea	C6, C19, C22, C23, C50, C54*	100, 100, 100, 99.3, 100, 98.8*	KC430923
Cupriavidus pauculus	C40	98.9	ALOU01000103
Ensifer adhaerens	C5, C36, C39, C41, C44, C47, C48, C53, C52, C73, C124	100, 99.8, 100, 100, 100, 100, 100, 100, 100, 100	JNAE01000171
Flavobacterium anhuiense	C1, C4, C7, C14*, C18, C25, C26, C43, C78, C77, C76	100,100, 100, 100*, 100, 100, 100, 100, 100, 99.8, 100	EU046269
Microbacterium azadirachtae	C34*, C45	100*,100	JYIT01000023
Mycolicibacterium cosmeticum	GHP2-6*	100	CCBB010000001

Mycolicibacterium llatzerense	GHP2-4	99.6	AJ746070
Paenibacillus taichungensis	C2, C49a, C49b	99.8, 100, 99.6	EU179327
Paraburkholderia sediminicola	F15*, F34	99.4*, 100	EU035613
Psuedomonas beteli	C32, C57, C58, C59, C60, C64a, C68, C99, C127	99.3, 99.3, 99.3, 99.3, 99.3, 99.7, 99.3, 99.3, 99.3	AB021406
Pseudomonas montelli	C8, C21	100, 100	OP-1
Ralstonia mannitolytica	GHP2-8	98.9	AJ270258
Ralstonia Pickettii	A1, A2*, F10, F13, F14, F16, GHP3-2, GHP3-1	100, 98.9*, 100, 99.6, 100, 100, 99.8, 100	JFZG01000019
Rhizobium pusense	C120*	100*	jgi.1102370
Rhizobium radiobacter	C3, C35, C37, C42, C46, C55, C51, C64, C94, C127a	100, 100, 100, 100, 100, 100, 100, 100, 100, 100	LT009724
Rhizobium tropici	A3, A14, C17	100, 100, 100,	CP004015
Rhodococcus degradans	A10*	100*	JQ776649
Rhodococcus pedocola	C72	100	KT301938
Rhodococcus wratislaviensis	C123	100	BAWF01000105
Rhodococcus jostii	C126	99.9	FNTL01000001

Note: Four soil types were sampled, including a sandy soil from the Cullars Rotation (C), a clay-rich soil from the E.V. Smith Agricultural Experiment Station (GH), an organic "black belt" soil from the AU Arboretum (A) and Forest soil (F). The unique ribotype is represented in (*)

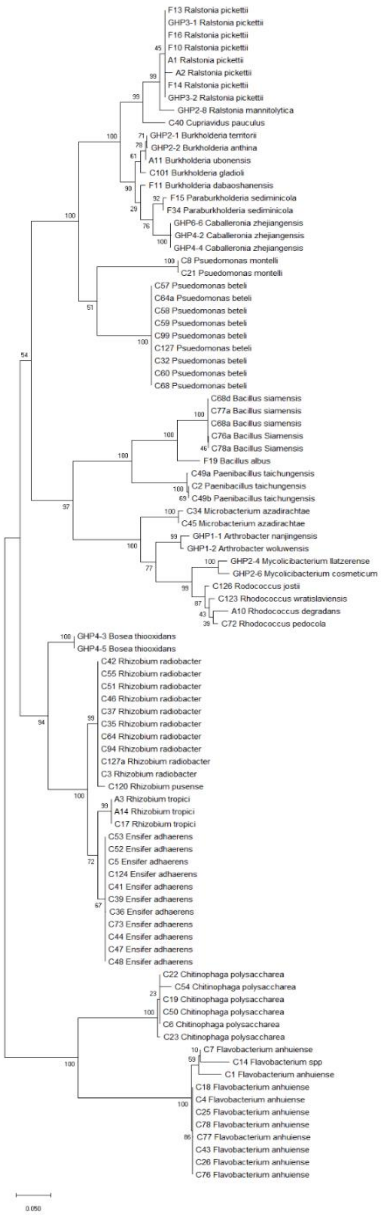

Figure 1. Identifying the rhizobacteria inhabiting Alabama soils, including Cullar's Rotation agricultural soil (C), Donald E. Davis Arboretum (A), EV Smith- Greenhouse (GH) or a Forest (F) soil. 16S rRNA gene consensus sequences were determined and compared to type strains from the EzTaxon database using a maximum likelihood analysis, with bootstrap percentages based on 1000 replications. Bar is equivalent to 0.05 substitutions per nucleotide position.

Chapter IV

Disease control in warm-water fish species using *Bacillus* probiotic strains.

1. Abstract

Bacillus strains isolated from soil or channel catfish intestine were screened for their antagonism against *Edwardsiella ictaluri* and *Aeromonas hydrophila,* the causative agents of enteric septicemia of catfish (ESC) and motile aeromonad septicaemia (MAS), respectively. Twenty-one strains were selected and their antagonistic activity against other aquatic pathogens was also tested. The top 21 strains expressed antagonistic activity against multiple aquatic bacterial pathogens including *Edwardsiella tarda*, *Streptococcus iniae*, *Yersinia ruckeri*, *Flavobacterium columnare*, and/or the oomycete *Saprolegnia ferax.* Survival of the 21 *Bacillus* strains in the intestine of channel catfish, *Ictalurus punctatus,* was determined as *Bacillus* CFU/g of intestinal tissue of catfish after feeding *Bacillus* spore-supplemented feed for seven days followed by normal feed for three days. Five *Bacillus* strains that showed good antimicrobial activity and intestinal survival were incorporated into feed in spore form at a dose of 8×10^7 CFU/g and fed to channel catfish for 14 days before they were challenged by *E. ictaluri* in replicate. Two *Bacillus subtilis* strains conferred significant benefit in reducing catfish mortality ($P < 0.05$). A similar challenge experiment conducted in Vietnam with four of the five *Bacillus* strains also showed protective effects against *E. ictaluri* in striped catfish, *Pangasianodon*

hypophthalmus. Safety of the four strains exhibiting the strongest biological control *in vivo* was also investigated in terms of whether the strains contain plasmids or express resistance to clinically important antibiotics. The *Bacillus* strains identified from this study have good potential to mediate disease control as probiotic feed additives for catfish aquaculture. Another study was conducted to evaluate the effects of probiotic-amended diets fed to juvenile Nile tilapia, *Oreochromis niloticus* on growth and susceptibility to *S. iniae* infection. Fish (average weight 16.5±0.2 g) were fed five diets formulated with *B. subtilis* group strains SB3086, SB3295, SB3615 or AP193 either individually or in combination for strains SB3086 and SB3615 at a targeted concentration of approximately 4 x 10^7 CFU/g of feed, or with a basal control diet with no additives for 21 days. After the 21-day growth trial, no significant difference in growth performance was observed with any probiotic-amended diet. In a serum bactericidal activity assay SB3086, SB3615, and AP193 showed significant higher activity than the control ($p=0.0002$). Lysozyme activity was also significantly higher in fish fed probiotic diets than those fed a control diet ($p=0.0001$). After 21 days of feeding, fish were challenged with *S. iniae* by intraperitoneal (IP) injection at a dosage of 8 x 10^6 CFU/fish. Results from the challenge showed significantly lower mortalities for probiotic fed fish as compared to the control ($P <= 0.0001$). Overall, fish fed with strain SB3615 showed the lowest percent mortality (44.0 ± 7.2%) and the highest mortality occurred in the control diet (77.3 ± 7.0%). The combined feeding with strains SB3086 and SB3615 did not result in any significant difference in reducing mortality due to *S. iniae* infection in juvenile Nile tilapia as compared with the individual probiotic treatments. A third and final study was conducted to evaluate the individual and combined effects of long-term feeding of

diets containing two probiotic *B. subtilis* group strains (Aqua NZ and AP193) and the prebiotic Previda®, a commercial hemicellulose extract, on growth performance, immune parameters and *A. hydrophila* susceptibility of juvenile Nile tilapia.. Nile tilapia of an average weight of 7.47 ± 0.11 g were fed diets formulated with the probiotics and/or the prebiotic, or a control diet for 8 weeks and, subsequently, challenged with *A. hydrophila* by intragastric gavage at a dosage of 3.9×10^7 CFU/fish. Fish attained a mean weight of 59.5 ± 0.99 g at the end of the growth period. Under the conditions of the present trial, none of the diets significantly improved mean percent weight gain ($P = 0.70$), thermal growth coefficient ($P=0.88$) or feed conversion ratio ($P = 0.87$) of Nile tilapia. Except for the diet containing the prebiotic Previda® only ($P = 0.17$), all other diets resulted in significantly higher fish survival compared to the control ($P < 0.05$). The combined effect of the prebiotic and probiotic strains emerged as the most effective diet with respect to mortality reduction. The mean lysozyme and respiratory burst activities did not show any significant differences between treatments and control ($P = 0.14$ and 0.32, respectively). Thus, the probiotic strains used in this study have the potential to prevent disease due to *A. hydrophila* in *Nile Tilapia.*

2. Introduction

Disease outbreaks have become a major challenge to the profitable culture of fish and shellfish as aquaculture operations intensify. Globally, total annual losses from disease outbreaks have reached billions of dollars (US) and have been identified as a threat to the sustainability of the industry (Pridgeon and Klesius, 2011a). Feed inputs associated with high-density fish culture stimulate the proliferation of opportunistic bacteria (Austin *et al.*, 1995). The combination of high-density fish culture with rapidly

changing water temperature and chemical composition of aquaculture ponds places stress on fish, thereby resulting in favorable conditions for the onset and spread of disease.

Chemotherapy by oral administration of antibiotics in fish feeds is the most common treatment for bacterial diseases. However, the indiscriminant application of antibiotics may result in many problems including the spread of drug-resistant pathogens, environmental hazards and food safety problems. This has fostered an increased interest in alternatives to antibiotics. Probiotics, which have various health-promoting properties and minor adverse side effects, are gaining an increasing scientific and commercial interest in aquaculture practice. The beneficial effects of probiotics involve improvement of feed utilization, modulation of intestinal microflora, enhancement of immune responses and antagonism to pathogens. The most commonly used probiotics in aquaculture are lactic acid bacteria and *Bacillus* spp. (Wang *et al.*, 2008a). *Bacillus* spp. have advantages as probiotics in that their spore-forming ability allows greater viability after pelleting and high survival rates after exposure to gastric acid (Casula and Cutting, 2002; Hong *et al.*, 2005; Hyronimus *et al.*, 2000). *Bacillus* spp. have been reported to have various beneficial attributes when applied to fish (Ai *et al.*, 2011; Aly *et al.*, 2008a; Kumar *et al.*, 2008; Nayak *et al.*, 2007; Newaj-Fyzul *et al.*, 2007; Salinas *et al.*, 2005; Salinas *et al.*, 2008).

Aquaculture farming of the channel catfish has been one of the most successful animal production industries in North America in the past 30 years and currently represents the largest aquaculture industry in the United States. Over 90% of all catfish produced in the U.S. are raised in Alabama, Arkansas, Louisiana, and Mississippi and are

primarily grown in earthen ponds ranging in size from 2 to 10 ha (USDA, 2003a, 2003b).

Enteric Septicemia of Catfish (ESC), caused by the Gram negative bacterium *E. ictaluri* (Hawke, 1979), is the most important endemic infectious disease in the channel catfish aquaculture industry (Hawke and Khoo, 2004). Losses resulting from ESC were reported in over 78% of all operations with outbreaks being reported in 42% of catfish production ponds, with an economic loss between $20 and $30 million yearly (USDA, 2003a, 2003b; Wagner *et al.*, 2002).

Another important pathogen in channel catfish is *A. hydrophila,* which is the primary causative agent of motile aeromonad septicaemia (MAS) (Harikrishnan *et al.*, 2003) and can infect multiple fish species including tilapia, catfish, goldfish, common carp, and eel (Pridgeon *et al.*, 2011). In 2009 and 2010, *A. hydrophila* was identified as the etiologic agent of a disease epidemic in farmed channel catfish, resulting in higher mortality rates than typical for MAS with over five million pounds of catfish lost in the Alabama commercial catfish industry. The *A. hydrophila* strains (e.g., strain AL09-119) isolated from diseased fish during this epidemic are highly virulent in aquaria disease challenge trials compared to *A. hydrophila* reference strains (Pridgeon *et al.*, 2011).

Pangasianodon hypophthalmus Sauvage, commonly known as the striped catfish, is the native catfish in the Mekong Delta of Vietnam. The farming sector of *P. hypophthalmus* has recorded the highest growth rate in volume compared to any other aquaculture commodity globally over the last decade (Phan *et al.*, 2009; Phuong and Oanh, 2009). The sector accounted for 687,000 and 1,094,879 t production in 2007 and 2008, respectively, the latter amounting to 34% of the total aquaculture production in

Vietnam, the fifth-ranked nation in global aquaculture production (De Silva *et al.*, 2010). Furthermore, over 90% of the farmed catfish is processed and exported to more than 100 countries globally (Phuong and Oanh, 2009). Bacillary necrosis of *Pangasius* spp. (BNP), also caused by *E. ictaluri*, is an economically significant disease for the striped catfish aquaculture industry in the Mekong Delta, which can cause 50-90% mortality and occurs in 98% of farms (Phan *et al.*, 2009).

Few studies have been conducted to investigate probiotic bacteria for mitigating infectious diseases in channel catfish, and no studies have been reported using direct administration in feed. Queiroz and Boyd (1998) applied a commercial probiotic product, Biostart, which contained a few species of *Bacillus* spp., to channel catfish pond water and demonstrated that survival and net production of fish treated with *Bacillus* spp. were significantly greater than the control. However, the bacteria used in this previous study were not isolated specifically for use in channel catfish nor were their antimicrobial activities against important pathogens of channel catfish characterized.

Streptococcal infections in fish, particularly those caused by *S. iniae*, have increased markedly with intensification of aquaculture practices (Pier and Madin, 1976; Buchanan *et al.*, 2005). Although originally isolated from freshwater dolphins (Pier and Madin, 1976), *S. iniae* has emerged as an important etiological agent of streptococcosis in cultured finfish. It has gained recognition as the most important bacterial disease of cultured Nile tilapia, *O. niloticus* causing mass mortality and severe economic losses (Shoemaker *et al.*, 2001). According to Shoemaker *et al.* (2010), the estimated economic impact of *S. iniae* outbreaks on the US aquaculture industry is approximately US $10 million and greater than US $100 million globally. This bacterium has also been

discovered as a zoonotic pathogen with the confirmation of a number of cases involving the elderly or immuno-compromised humans (Weinstein, 1997; Koh *et al.*, 2004; Facklam *et al.*, 2005; Lau *et al.*, 2003; Agnew and Barnes, 2007). Thus, the need for an effective control method is not only limited to the economic loss in aquaculture, but also to protect the health of fish farmers and processers. Conventionally, antibiotics are used to control *S. iniae* infection in aquaculture; however, reported cases of lack of efficacy and resistance of bacteria to antibiotics (Stoffregen *et al.*, 1996; Shoemaker and Klesius, 1997; Locke *et al.*, 2008; Gaunt *et al.*, 2010) have heightened the need for alternative disease control methods. An alternative to prevent and control pathogenic bacteria is the use of probiotics. These are biologically active components of single or mixed cultures of live microorganisms, which when administered in adequate amounts are capable of improving the growth and health of the host (Salminen *et al.*, 1999; Lara-Flores *et al.*, 2010). Due to their reported benefits, probiotics have been commercialized and sold in the aquaculture industry as feed additives. Prevention of disease by inclusion of individual probiotic bacteria strains and/or their mixtures in the diet of fish have become preferential to antibiotic therapy (Boyd and Gross, 1998; Shelby *et al.*, 2006; Welker and Lim, 2011).

In Nile tilapia, the use of probiotics in feeds to improve growth and disease resistance has been investigated by many researchers with mixed results (Lara- Flores *et al.*, 2003; El-Haroun *et al.*, 2006; Shelby *et al.*, 2006, 2009; Taoka *et al.*, 2006b; Aly *et al.*, 2008b, 2008c; Marzouk *et al.*, 2008; El-Rhman *et al.*, 2009; Essa *et al.*, 2010; Ferguson *et al.*, 2010; Ghazala *et al.*, 2010; Zhou *et al.*, 2010a; Pirarat *et al.*, 2011). Because of these mixed results, it is imperative for more studies to be carried out to

ascertain specific probiotic strains and/or their combinations that can significantly control infections in *Nile tilapia*.

Tilapia production is of increasing importance in aquaculture globally and is second only to carp by production volume estimates (FAO, 2012). Among the several species of tilapia cultured commercially, Nile tilapia, *O. niloticus* (Linnaeus, 1758) is the most abundant and important species. Global aquaculture production of this species has increased from around 200,000 metric tons in 1990 to about 2.8 million metric tons in 2010 (FAO, 2012). The current trend in tilapia aquaculture development is towards increased intensification and commercialization (Goncalves *et al.*, 2011); however, disease is a primary constraint to the growth of the industry and severely impedes both economic and socio-economic development in many producer countries (Austin and Austin, 2007). Diseases caused by bacterial pathogens are responsible for heavy mortalities and annual losses. Among the major bacterial pathogens is *A. hydrophila*, which continues to plague the culture of this animal resulting in decreased survivability and profitability.

As mentioned above, tilapia are also susceptible to *A. hydrophila* infection (causative agent of motile aeromonad septicemia (MAS)), often being associated as a secondary pathogen with a number of other diseases affecting Nile tilapia, such as epizootic ulcerative septicemia (EUS). Primarily, however, *A. hydrophila* is usually an opportunistic pathogen, causing disease outbreaks in fish farms with high mortality rates, resulting in severe economic losses to the aquaculture industry (Fang *et al.*, 2004). In recent years in the western region of Alabama, U.S.A, a MAS disease outbreak caused by a highly virulent strain of *A. hydrophila* has resulted in the loss of millions of kg of food

size channel catfish (USDA, 2010; Pridgeon and Klesius, 2011b). This virulent *A. hydrophila* genotype corresponds to sequence-type 251, and is affecting farmed fish in China and the United States (Hossain *et al.*, 2014; Pang *et al.*, 2015).

Anabolic steroids, growth promoters and some antibiotics, such as oxytetracycline (OTC), sulfadimethozine and ormetoprim among others, are commonly administered in feed to improve growth performance and to control the outbreak of diseases in aquaculture (Defoirdt *et al.*, 2011). However, abuse of these chemicals, especially antibiotics, has led to the development of drug-resistant bacteria, which has reduced the efficacy of the drugs. Further, accumulation of antibiotics both in the environment and in fish can pose potential risk to consumers and the environment (Carrias *et al.*, 2012). Hence, to meet the increasing consumer demands for animal products that have not been treated with antibiotics whilst maintaining good health and growth, fish farmers are turning to cost-effective feed formulations that will decrease the negative effects of bacterial pathogens on farm profitability. Consequently, prebiotics, probiotics and their combinations are under extensive investigation for their potential beneficial effects on fish health and growth. Whilst a prebiotic is a non-viable food component that confers health benefit on the host associated with modulation of the microbiota (FAO, 2007), a probiotic has been defined as live microorganisms which, when administered in adequate amounts, confers health benefits on the host (FAO, 2001). Prebiotics are dietary carbohydrates that escape digestion in the upper gastrointestinal tract but alter the bacterial composition of the lower gut by changing the type of substrate provided to the existing gut microbiota (Gibson and Roberfroid, 1995; Mei *et al.*, 2011). The inclusion of common probiotic strains, such as *Bacillus* spp., in fish feed can also help promote

beneficial bacterial taxa on the skin and intestine to out-compete pathogenic bacteria (El-Rhman *et al.,* 2009). A mixture of prebiotics and probiotics, according to Gibson and Roberfroid (1995), can beneficially affect the host by improving the survival and implantation of live microbial dietary supplements in the gastro-intestinal tract by selectively stimulating the growth and/or activating the metabolism of one or a limited number of probiotic bacteria to improve host welfare. Thus, an effective pairing of pre- and probiotics would potentially allow alteration of the gut environment for optimal host growth and disease resistance. Several studies have shown that pre- and/or probiotics and their combinations can improve growth performance and feed utilization of various fish species including Nile tilapia (Mahious *et al.*, 2006; Staykov *et al.*, 2007; Torrecillas *et al.*, 2007; Burr *et al.*, 2008; Grisdale-Helland *et al.*, 2008). For instance, studies conducted by Li and Gatlin (2005) and Buentello *et al.* (2010) indicated that prebiotics can enhance the non-specific immune responses and resistance to bacterial infections in hybrid striped sea bass and red drum. Other studies have also associated prebiotics with improvement of gut function and health, improvement of the ultrastructure of the intestinal mucosa of cobia (Salze *et al.*, 2008) and also the activation of health promoting bacteria in the intestine of shellfish (Zhou *et al.*, 2007). Some researchers have, on the contrary, noted that both pre- and probiotics have not been effective in their application in fish culture (Shelby *et al.*, 2006; Merrifield *et al.*, 2010).

Three separate, but connected, studies were conducted in order to evaluate the efficacy of probiotic amended feed on the growth, immune parameters, and disease control of the infections mentioned above for channel catfish and tilapia. In addition, a prebiotic was also evaluated for tilapia.

In the first study, an extensive collection of *Bacillus* strains (n=160) isolated from soil and from the intestine of channel catfish (n=17) was tested for *in vitro* antimicrobial activity against *E. ictaluri, A. hydrophila*, and other bacterial and fungal pathogens of channel catfish. *Bacillus* strains that showed effective antibiosis were evaluated for their respective survival in the intestine of channel catfish. The biological control activity of the best performing *Bacillus* strains when amended onto feed was investigated using channel and striped catfish disease challenge studies in an aquarium system. The safety of selected *Bacillus* strains was also assessed in terms of the presence of plasmids and resistance to antibiotics.

Secondly, a probiotic feeding trial was conducted on tilapia. A 21-day feeding was carried out in order to determine the effects of four *B. subtilis* strains, SB3086, SB3295, SB3615, AP193 and a combination of SB3086 and SB3615 on growth performance, non-specific immune activity and *S. iniae* susceptibility in juvenile Nile tilapia.

And finally, another probiotic diet was given to tilapia, now with the addition of the prebiotic Previda®; and then subsequently challenged with *A. hydrophila*. This study was conducted to: (1) explore the individual and combined effects of feeding diets containing a prebiotic and two probiotic strains on growth performance of juvenile Nile tilapia, and (2) investigate the potential effects of pre-feeding of these diets on the survival of the Nile tilapia when challenged with *A. hydrophila*.

3. Materials and Methods

Study 1

Bacterial strains

E. ictaluri strain S97-773 was used for the primary screening for *Bacillus* antibiosis and for ESC challenge experiments since this strain is highly pathogenic for channel catfish, has previously been used in challenge studies and was obtained from the Southeastern Cooperative Fish Disease Laboratory (SCFDL), Auburn University. *E. ictaluri* strain R-4383, *E. ictaluri* strain Alg-08-200**,** *E. tarda* AL09-82, *S. iniae* USDA 2009, *Y. ruckeri* ATCC 29473, and *F. columnare* AL09-10 were from the collection of pathogenic isolates at the SCFDL, and *S. ferax* was obtained from Carolina Biological (catalog # 156271, Burlington, NC). *E. ictaluri* NLF33 were isolated from diseased striped catfish in Vietnam. *A. hydrophila* ML09-119 was isolated from a diseased channel catfish with MAS in 2009. The collection of soil-derived *Bacillus* strains (n=160) was provided by the laboratory of Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University). *B. subtilis* 1E17 was obtained from the *Bacillus* Genetic Stock Center (http://www.bgsc.org/).

Isolation of *Bacillus* spp. strains from the intestine of channel catfish and evaluation of antimicrobial activity

Healthy catfish (7-10 cm) were killed by administration of an overdose of MS-222 (tricaine mesylate, FDA approved for anesthesia in fin fish), and the digestive tracts were removed in their entirety. Approximately 1.0 g of the intestinal tissue with gut content was homogenized in 9.0 mL of sterile saline (0.9% w/v) using a sterile mortar and pestle. Ten-fold serial dilutions were prepared to 10^{-6} in fresh 1x phosphate-buffered

saline (PBS), and 0.1 mL was spread over the surface of triplicate plates of tryptone soy agar (TSA) with incubation at 28°C for 48 h (Irianto and Austin, 2002). *Bacillus*-like colonies were picked at random, purified by streaking for isolated colonies on fresh media, and examined for inhibition against the growth of *E. ictaluri* using the double-layer soft agar method (Weisburg *et al.*, 1991). For the soft agar overlay, the bacterial isolates were grown in 5 mL of tryptone soy broth (TSB) for 24 h at 30°C. A volume of 5 µl was then spotted onto triplicate plates of TSA and incubated for a further 24 h. Soft agar (0.7% w/v agar) prepared with TSB was melted, cooled and seeded with an inoculum of log-phase *E. ictaluri* strain S97-773 to achieve slight turbidity (i.e., ~ 10^7 cells/mL). The bacterial cell suspension in soft agar was immediately poured over the TSA plates and incubated for 24 h at 30°C whereupon the presence of zones of clearing in the growth of of *E. ictaluri* were recorded (in mm) as evidence of growth inhibition. Cultures that were regarded as inhibitory to *E. ictaluri* were characterized by Gram staining and 16S rRNA gene sequencing using the 'universal bacteria' primer set 27F and 1492R (Weisburg *et al.*, 1991). A consensus 16S rRNA sequence was produced using ChromasPro (Technelysium Pty Ltd., Queensland, Australia), and each sequence was compared to the GenBank non-redundant nucleotide database by BLASTn. *Bacillus* spp. strains were cryopreserved at -80°C. The collection of soil-derived *Bacillus* strains (n=160) was tested for antimicrobial activity against *E. ictaluri* using the same method.

Fifty *Bacillus* strains with antagonistic activity against *E. ictaluri* S97-773 were tested for their inhibitory activity against other *E. ictaluri* strains (*E. ictaluri* R-483, *E. ictaluri* Alg-08-200). *Bacillus* strains that showed antimicrobial activity against all three *E. ictaluri* strains were evaluated further for their activity to inhibit the growth of *A.*

hydrophila strain AL09-119. Twenty-one *Bacillus* strains that showed significant antimicrobial activity against both *E. ictaluri* and *A. hydrophila* were tested for their activity against several other channel catfish pathogens including *E. tarda, S. iniae, Y. ruckeri, S. ferax* with the soft agar overlay method described above.

The antimicrobial activity against *F. columnare* was tested by an agar well diffusion method. For the well diffusion assay, the *Bacillus* strains were grown in 5mL of TSB for 48h at 30°C. After centrifugation at 3,600 x g for 10 min, the culture supernatant was filtered through a 0.2 μm filter. Then 200 μl of the filter-sterilized supernatant was added to a round well (approx. 10 mm in diameter) made in a *F. columnare* growth medium (FCGM) agar plate (Farmer, 2004). After the supernatant was absorbed into the agar medium, a log-phase *F. columnare* culture grown in FCGM broth was spread thoroughly over the plate using a sterile cotton swab. The plates were incubated for 48 h at 30°C. The zones of clearing in the growth of the lawn of *F. columnare* were measured by the same method as in double-layer soft agar protocol.

Bacillus strains AB01, AP79, AP143, AP193, and AP254 were sent to Vietnam and their *in vitro* antimicrobial activity was tested against *E. ictaluri* NLF33, the causative agent of BNP in striped catfish. A broth culture of *E. ictaluri* was adjusted to 10^6 CFU/mL and evenly swabbed onto TSA plates. Three wells were punched from the agar plate and 50 μL of a 10^8 CFU/mL of a *Bacillus* cell-free supernatant (48 h culture in TSB) was added into each well. Zones of inhibition were measured after 24 hours incubation at 30°C.

Preparation of *Bacillus* spores and spore-amended feed

Bacillus spores were prepared by the method described by Kenny and Couch (1981) with some modifications. *Bacillus* strains were grown in TSB at 30°C overnight.

The cell suspension was spread onto spore preparation agar (peptone 3.3 g/l, beef extract powder 1.0 g/l, NaCl 5.0 g/l, K_2HPO_4 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) using a sterile cotton swab and incubated at 28°C for 5 to 7 days. To collect the spores, 5 mL of sterile distilled water was added to the plate and the spores were suspended in water using an inoculation loop. The spore suspension was then incubated at 85°C for 15 min to kill the vegetative cells. The concentration of the spore suspension was determined by 10-fold serial dilution in 1x PBS and spreading onto TSA. The final concentration of the spore suspension was manipulated with sterile water to 1.25 x 10^{10} CFU/mL for the intestinal survival assay and 10^9 CFU/mL for the challenge study. To prepare spore-amended feed, 80 mL of the spore suspension was sprayed onto 1000 g commercially available slow-sinking pelleted fish feed (2 mm, 40 % protein, Zeigler, Gardners, PA) using a bleach- and ethanol-sterilized pump sprayer to achieve approximately 8% (v/w) spore suspension application. The feed was then mixed thoroughly with 30 mL fish oil. The control feed was ammended solely with fish oil.

Inoculation and quantification of *Bacillus* spp. in the intestine of channel catfish

Fingerling channel catfish (7-10 cm) were distributed into twenty-three 60 L tanks each containing 15 L water and three fish. Fish were starved for one week prior to the experiment. Catfish feed was amended in separate batches with the 21 *Bacillus* strains that showed good antimicrobial activity against both *E. ictaluri* and *A. hydrophila* using the spore application method described above. Each unique *Bacillus* strain-amended feed (~10^9 CFU/g feed) was given to one aquarium tank. The fish were fed once daily with spore-amended feed or control feed for one week, and thereafter all fish received the

control feed for three days. One tank was used as the control and received untreated fish feed for the duration of the experiment. Daily feeding rate was 3% of total body weight.

At the end of the experiment, all of the fish were killed by administration of an overdose of MS-222. The intestine was removed, weighed, and then homogenized in sterile saline (0.9% w/v). The final volume of the homogenized intestine sample was adjusted to 2 mL by sterile saline. Homogenized samples were then serially diluted in sterile saline and spread on TSA and incubated at 28°C for 48h. Three representative colonies with the same morphology as the applied *Bacillus* strain were randomly picked from the plate, purified on new plates and identified by 16S rRNA gene sequencing as described previously and compared with the known 16S rRNA gene sequence from each respective *Bacillus* strain. For the control and treatment groups, only the unique colony morphology corresponding to that of the amended *Bacillus* strains was recorded. Cultured counts for each *Bacillus* strain recovered from the intestine were determined as CFU/g of intestine sample.

Aquarium challenge studies

In the first challenge, five *Bacillus* strains (AB01, AP143, AP193, AP254, and AP79) were selected for evaluation of their biological control of ESC in an aquarium challenge. Five *Bacillus* treatments and one control, each with four replicate aquaria, were included. Each replicate aquarium was stocked with 25 fingerling channel catfish weighing about 13 g. Fish were acclimated to commercial dry feed for one week. Fish from each treatment group were then fed with an experimental diet supplemented with spores of one *Bacillus* strain (8×10^7 CFU/g) at a daily feeding rate of 2.5 % fw/bw (feed

weight / body weight) for two weeks. Fish in the control group received normal feed only.

Fish were challenged by immersion for 45 minutes in 10 L of water containing 4.5×10^6 CFU/mL *E. ictaluri* S97-773. All fish from the same group were immersed in a single container. The challenge condition for the control group was the same as other treatments except that Brain Heart Infusion (BHI) medium was added instead of *E. ictaluri* culture. Mortalities were monitored over a 21-day period, and dead fish were dissected and the presence of *E. ictaluri* confirmed by microbiological examination of kidney and liver swabs on TSA. The final mortality was calculated when there was no more mortality for five consecutive days after challenge. The identity of the recovered *E. ictaluri* was confirmed by biochemical analyses that included indole and hydrogen sulfide production, catalase and oxidase activities, hydrolysis of esculin and gelatin, nitrate reduction, and acid formation from carbohydrates.

Fish were reared in a recirculating system during the acclimation period. Upon initiation of *Bacillus* feeding and during the challenge phase, a static system was incorporated with a 20-30 min daily water exchange that resulted in turnover of approximately half of the aquarium water. Sponge biofilters and daily removal of uneaten/waste materials were incorporated to improve water quality. Water temperature was kept at 26 ± 2°C. During the static phase, the central room heating system in conjunction with submersible aquarium water heaters was used to control the required water temperature, and a water heater system was used to control the temperature of the incoming water during water exchange.

In a second challenge trial using channel catfish, flow-through conditions were used to reduce catfish mortality. In this challenge experiment, four *Bacillus* treatments (AP79, AP143, AP193, AB01) and one control each with four replicate aquaria were included. Each aquarium was stocked with 20 fingerling channel catfish (~12 g). A lower dose of *E. ictaluri* S97-773 (8×10^5 CFU/mL) was used to challenge fish and starting immediately after challenge the aquaria were flushed for 5-8 hours a day. All other conditions in this challenge were the same as described above. Mortalities were monitored over a 21-day period after challenge, and presence of *E. ictaluri* in the dead fish was confirmed as previously described.

A third challenge trial was conducted to evaluate the protective effect of four *Bacillus* strains (AP79, AP193, AP254 and AB01) against *E. ictaluri* for striped catfish. Four *Bacillus* treatments and one control each with four replicate tanks were included in this study. Each tank was stocked with 18 striped catfish (~14g). Striped catfish were administered feed amended with *Bacillus* spores (~10^7 CFU/g feed) or control feed for two weeks and the fish were transferred to 80 L tanks for a bath challenge with *E. ictaluri* NLF33. Fish were immersed for 30 min in static, aerated aquaria at a dose of ~10^6 CFU/mL to target about 70% mortality in the control group. The control and test diets were offered throughout the trial. The recording of mortality and confirmation of *E. ictaluri* in dead fish were conducted as above.

Plasmid analysis

Plasmid DNA was extracted from *Bacillus* strains AP79, AP143, AP193, and AB01 by the alkaline lysis method (Birnboim and Doly, 1979). *B. subtilis* 1E17 containing plasmid pC194 was used as a positive control. The extracted DNA was

analyzed by a Chef-DR II pulsed field electrophoresis system (Bio-Rad, Hercules, CA). Pulse time ranged from 1 to 15 seconds for 15 h at 6 V/cm. The gel was stained with ethidium bromide and visualized using an AlphaImager HP gel documentation system (ProteinSimple, Santa Clara, CA).

Antibiotic resistance analysis

The susceptibility of *Bacillus* strains AP79, AP143, AP193, and AB01 to carbenicillin, ampicillin, spectinomycin, oxacillin, vancomycin, cephalothin, novobiocin, sulfadiazine, amikacin, erythromycin, neomycin, penicillin, chloramphenicol, sulfamethoxazole, norfloxacin, gentamicin and ciprofloxacin was determined by disc diffusion test following procedures outlined by National Committee for Clinical Laboratory Standards (2012). A log-phase culture of each strain was diluted to a concentration of approximately 1×10^8 to 2×10^8 CFU/mL (McFarland standard 0.5). The inoculum was then seeded onto a Mueller-Hinton agar plate using a cotton swab. Antibiotic-impregnated discs (BD Biosciences) were placed on seeded plates, and the diameter of the zone of growth inhibition was measured after 18 h of incubation at 37°C. The experiments were repeated three times and the average diameter of inhibition zones was calculated.

Statistics

A completely randomized design was used in this research. Data were presented as mean ± standard error (SE). Challenge data were subjected to analysis of variance in SAS 9.2. Differences between means were tested by Tukey's range test and were considered significant when probability (P) values < 0.05 were obtained.

Study 2

Diet Preparation

Three proprietary probiotic strains of *B. subtilis* and one strain identified from a collection at Auburn University, AL, USA (Ran *et al.*, 2012) were added as feed additives to a basal diet. The basal diet was formulated to meet the nutritional requirement of tilapia containing 32% protein and 6% lipid. All the diets contained 3.3% menhaden fish oil to ensure palatability. The basal/control diet had no probiotic additives and contained 0.2% corn starch. The probiotic strains were added to their respective diets at 0.2% inclusion levels but did not contain corn starch (Table 3). Three of the probiotic strains (SB3086, SB3295, SB3615 and a combination of SB3086 and SB3615) were dry concentrates containing the probiotic strains blended with calcium carbonate (provided for testing by Novus International, Saint Charles, MO, USA). The fourth strain was a bacterial spore suspension of *B. velezensis* AP193. AP193 was isolated from a soybean rhizosphere and was part of a collection of plant growth promoting rhizobacteria that were screened for activity against aquaculture pathogens (Hossain *et al.*, 2013; Hanson *et al.*, 2014). The test diets were prepared at the fish nutrition laboratory at the E. W. Shell Fisheries Center, Auburn University. Pre-ground dry ingredients and fish oil were mixed in a food mixer (Hobart Corporation, Troy, OH, USA) for 15 minutes. Hot water was blended into the mixture for consistency and pelleted through a 3-mm die using the same equipment. Pelleted diets were dried in an oven to a moisture content of 8-10%, bagged, labeled and stored at 4°C until feeding.

Bacteria Quantification in Experimental Diets

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

Growth

The 21-day feeding of fish with the experimental diets was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn University, AL, USA. Fish of average size 16.5 ± 0.2 g were kept in 60-L aquaria containing approximately 45 L of well water (28 fish/aquarium). Each aquarium was equipped with aeration maintained at dissolved oxygen (DO) levels of 5.00 ± 0.5 mg/L. Prior to administering the diets, fish were acclimated for one week at 28 ± 1°C. During the acclimation period, tilapia was maintained on a commercial diet at 3% of their body weight. At the commencement of the feeding phase, treatment and control diets were adjusted to 6% body weight. Fish were fed twice a day, in the morning and late afternoon. Any un-eaten feed and waste materials were siphoned out of each aquarium as needed. Each treatment had its own set of equipment, such as nets and siphoning hose, and disinfected in iodine solution after every use to avoid cross contamination. At the end of the feeding period, final mean body weight, percent weight gain [100 x (final weight-initial weight)/initial weight,

specific growth rate [100 x (ln final weight-ln initial weight)/days] and feed conversion ratio (feed intake as fed / weight gain) were determined.

Serum Bactericidal and Lysozyme Activity

Blood samples were collected from three fish per tank in each treatment (n=18 fish/treatment) after 21 days of feeding fish with the experimental diets and before the challenge. Fish were anaesthetized to loss of equilibrium with MS-222 and blood samples collected with sterile syringes from the caudal vein around the caudal peduncle into 1.7 mL microcentrifuge tubes without anticoagulants. Blood samples were allowed to clot for 30 min at room temperature and stored at 4°C overnight. Blood serum was pipetted into sterile 1.7-mL microcentrifuge tubes from the blood samples as supernatant after centrifuging at 3000 x *g* for 15 min at 4°C. Serum samples were stored at -80°C and after one week of storage, samples were taken out and thawed for serum bactericidal and lysozyme activities determination.

In the serum bactericidal activity procedure, bacterial cultures of *S. iniae* were subjected to centrifugation and the pellet was washed and re-suspended in PBS. The optical density of the suspension was adjusted to 0.5 at 546 nm. The bacterial suspension was serially diluted (1:10) with PBS 5 times. The serum bactericidal activity was determined by incubating 2 µL of the diluted bacterial suspension with 20 µL of the serum for 1 h. at 37°C. A control in which PBS replaced the serum was included. The numbers of viable bacteria was determined by counting the colonies after culturing on TSA plates for 24 h at 30 C. Lysozyme activity of serum was measured using the turbidity assay. On a flat-bottomed 96-well microtitre plate, 200 µL of 0.2 mg/mL suspension of *Micrococcus lysodeikticus* in sodium phosphate buffer (0.05 mol/L, pH

5.2) was added to 5 µL of serum. The reduction in the absorbance at 570 nm was determined at 0, 15, 30, 45 and 60 minutes. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of 0.001 units per minute. Chicken egg lysozyme (Sigma) was used as a standard (Kajita *et al.*, 1990; Rainger and Rowley, 1993; Lange *et al.*, 2001).

Preparation of *S. iniae* for the Challenge

The *S. iniae* challenge strain was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University. The bacteria isolate used was previously passed through tilapia to confirm virulence. A bacterial culture for *S. iniae* challenge was prepared by inoculating 5 mL tryptic soy broth (TSB) with 200 µL of a frozen stock (–80°C) of the bacterium. The 5 mL culture was incubated for 36 h at 30°C while shaking at 150 rpm, and then used to inoculate 100 mL of fresh TSB. The second inoculated culture was then incubated for an additional 15 h at 30°C while shaking at 150 rpm. Prior to its use for the challenge, the bacterial culture was subjected to centrifugation at 3600 x *g* for 30 min, re-suspended in 100 mL of fresh TSB, allowed to grow an additional 3 h, and then standardized to an OD_{600} of 1.0. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

The challenge was performed 21 days after initial treatment diet administration with 25 fish/tank after the removal of three fish per tank for use in the immunological analysis. All fish were removed from aquaria and anaesthetized in 100 mg/L of MS-222 and challenged by administering 200 µL of *S. iniae* suspension in PBS by intraperitoneal injection to obtain a final dosage of 8×10^6 CFU/fish. Fish were replaced into original aquaria after injection. For the negative control, fish were exposed to the same challenge

conditions as those groups receiving challenge bacteria except that buffer was administered instead of bacteria. During challenge, flow-through water supply (0.4 L/min) and temperature of 28 ± 1°C were maintained.

After challenge, fish were observed daily for behavioral changes and for gross signs of disease. Moribund and dead fish were removed and counted early morning and late afternoon each day. Samples of moribund or freshly dead fish were necropsied and samples from trunk kidney, liver and brain were streaked on TSA plates for bacterial isolation. Isolated colonies were identified using biochemical tests. At the end of the challenge experiment, all surviving fish were counted, euthanized with 300 mg/L MS-222, and properly disposed.

Statistical Analysis

Data collected on growth, immunology and fish mortality were analyzed using SAS (SAS Institute Inc., Cary, NC). The mixed procedure (Wolfinger *et al.*, 1991) was used to make treatment contrasts. A complete randomized block design was incorporated in this study to minimize variation due to location of aquarium units in three different banks of aquaria. Differences between means were considered significant when probability (P) values < 0.05 were obtained.

Study 3

Diet Preparation

Two proprietary probiotic *B. subtilis* group strains and Previda®, a commercial hemicellulose extract prebiotic product (Novus International Inc., St Charles, MO, USA), were added singly and in combination as additives to a basal diet. One of the probiotic strains, Aqua NZ Blend, is a dry concentrate containing *B. subtilis* (provided for testing

by Novus International Inc.) and the other strain is a bacterial suspension of *B. velezensis* AP193. *B.velezensis* is a part of the *B. amyloliqufaciens* clade and thus a part of the *B. subtilis* group (Priest *et al.*, 1987; Fan *et. al.*,2017). The basal/control diet was formulated to meet the nutritional requirements of tilapia containing 32% protein and 6% lipid (Table 7). The diet contained 3.3% menhaden fish oil to ensure palatability of the diets due to the addition of the pre- and/or probiotic. The probiotic was added to the diet at a weight of 1.7 g (0.028% of feed) by replacing corn starch to obtain a final concentration of approximately 4.2 x 10^7 CFU/g of feed. Diet 1 was the basal/control diet (no additives) while diet 2 was the prebiotic Previda® only, supplemented at 0.5% of the total diet (30.0 g). Diet 3 contained the probiotic Aqua NZ only, diet 4 was a mixture of Aqua NZ and Previda®, diet 5 contained AP193 only while diet 6 was a mixture of AP193 and Previda® (Table 7). Test diets were prepared at the fish nutrition laboratory of E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA. Briefly, pre-ground dry ingredients and fish oil were mixed in a 6.0 kg capacity food mixer (Hobart Corporation, Troy, OH, USA) for 15 min. Hot water was blended into the mixture for consistency and pelleted through a 3 mm die using the food mixer equipment. Pelleted diets were dried in an oven to a moisture content of 8-10%, bagged, labeled and stored at 4°C until feeding. In all, three batches of diets (6.0 kg/batch/diet) were prepared.

Bacteria Quantification in Experimental Diets

Samples of the diets were analyzed to quantify the number of *Bacillus* spp. probiotic bacteria present in a gram of feed. One gram of each diet was placed in a 15 mL tube containing 9 mL of sterile phosphate buffered saline (PBS). Samples were left undisturbed for 30 min and then homogenized. Dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and

10^{-8} were made from four replicate samples and 100 µL of each dilution was spread on tryptic soy agar (TSA) plates and incubated at 30°C overnight. After overnight incubation, colonies on plates with typical morphology characteristics of *B. subtilis* group bacteria, Aqua NZ and AP193 were counted.

Growth Trial

The growth trial was conducted at the E. W. Shell Fisheries Center, Auburn University, Auburn, AL, USA, with tilapia fed the formulated diets (Diets 1 to 6) over a period of 8 weeks. Fingerlings of average size 7.47 ± 0.11 g were acclimated and then stocked at 40 fish/tank into 36 aquaria (132 L volume containing 100 L of water) supplied with flow-through water from a reservoir at a flow rate of 1.3 L/min/aquarium. Water temperature ranged from 27°C to 28°C during the first five weeks; however, from week 6-8 when temperatures began to drop below 26°C, the aquaria were placed on partial flow-through and re-circulation with water heated to 28°C using a submersible heater. During this time, water was UV treated using Aqua Logic UV Sterilizer model ALUV-30, 0.6A (Aqua Logic, San Diego, CA, U.S.A). Dissolved oxygen (DO) levels in the aquaria were kept near saturation using air stones in each aquarium from a common airline connected to a regenerative air blower. Each diet treatment was randomly assigned to 6 replicate aquaria and fish fed throughout the experiment. Feeding was done twice a day, in the morning and late afternoon, at a percent body weight ranging from a total of 5 to 10%. Feeding rates were adjusted every two weeks. Fish were removed, counted and weighed bi-weekly on a digital scale (Ohaus Scout Pro 4000g Ohaus Corporation, Parsippany, NJ, U.S.A) during which time the aquaria were cleaned. Temperature and DO were measured twice a day (early morning and late afternoon) using YSI-85 digital

temperature/DO meter (YSI Corporation, Yellow Spring, OH, USA). Total ammonia nitrogen (TAN) and nitrite-nitrogen were determined twice a week from randomly selected aquaria and the photoperiod was set at 14 h light and 10 h dark. After 8 weeks, fish were counted, weighed and moved collectively by treatment approximately 6 km to the S-6 Fish Disease Challenge Laboratory at the E. W. Shell Fisheries Center.

Lysozyme and Respiratory Burst Activity

For the determinations of respiratory burst and lysozyme activities, the assays used were adapted from Kumar *et al.* (2005) and Lange *et al.*, (2001) respectively. Blood samples were collected from 3 fish per tank in each treatment (n=18 fish/treatment) a day before the disease challenge. Fish were anaesthetized with 100 mg/L Trycaine methanesulfonate (MS-222, Western Chemical Inc., Ferndale, WA, USA) and blood samples collected with sterile syringes from the caudal vein around the caudal peduncle into 1.7 mL microcentrifuge tubes. For the respiratory burst activity, 50 µL of blood was placed into the wells of 96-well microtitre plates and incubated for one hour at room temperature to assist cell adhesion. The supernatant was gently removed and the adhered cells were washed three times with PBS. After washing, 50 µL of 0.2% (w⁄v) nitroblue tetrazolium in PBS was added to the wells and incubated for one hour at room temperature. The supernatant was removed and the cells were fixed with 100% methanol for three min and then washed three times with 30% methanol. The plates were air dried before 60 µL of 2 mol/L potassium hydroxide and 70 µL dimethyl sulfoxide were added to each well to dissolve the formazan blue crystals. The optical density (OD) of the resulting solution was read in a spectrophotometer at 540 nm (Kumar *et al.*, 2005). The remaining blood samples after drawing sub-samples for the respiratory burst

determination was prepared for the lysozyme activity test in blood serum. Blood serum as supernatant was extracted into sterile 1.7 mL microcentrifuge tubes after subjecting the blood samples to centrifugation at 3,000 x *g* for 15 min, and the serum was stored at 4°C overnight. In a 96-well microtitre plate, 100 µL of 0.4 mg/mL suspension of *Micrococcus lysodeikticus* (Sigma Chemical Co., St. Louis, MO) in 0.05 mol/L sodium phosphate buffer (SPB, pH 6.2) was added to 100 µL of the serum in serial dilutions of 1:5 to 1:40. The OD_{590} reading was recorded at 0, 15, 30, 45 and 60 min. A unit of lysozyme activity was defined as the amount of serum causing a decrease in absorbance of 0.001 units per min (Lange *et al.*, 2001).

Preparation of *A. hydrophila* for the Challenge

A -80°C, frozen stock of *A. hydrophila* strain ML09-119 was obtained from the Southeastern Cooperative Fish Disease Laboratory at Auburn University (Tekedar *et al.*, 2013). The isolate was tested through LD50 assessment on tilapia prior to the challenge to confirm virulence at the dosage used. *A. hydrophila* used for the challenge was prepared by inoculating 5 mL TSA broth with 200 µl of a frozen cryostock of the bacteria. The 5 mL culture was incubated for 24 h at 30°C while shaking at 200 rpm and then used to inoculate 100 mL of fresh TSA. The second inoculated culture was then incubated for an additional 15 h at 30°C while shaking at 200 rpm. Prior to use in challenges, the bacterial culture was centrifuged at 3,600 x *g* for 30 min, re-suspended in 100 mL of fresh TSA, allowed to grow an additional 3 h, and then standardized to an OD_{600} of 1. Bacterial culture was quantified using standard plate count methodologies to verify challenge dose.

A. hydrophila Challenge System and Conditions

Disease challenge in tilapia (78 g mean weight) was carried out at the S6 Disease Laboratory, E. W. Shell Fisheries Center, Auburn, AL, U.S.A. under controlled temperature conditions. Fish were maintained in 60 L aquaria containing ~ 45 L of well water. Each aquarium was equipped with aeration and maintained at average DO of 5.00 ± 0.5 mg/L. Prior to the challenge, fish were acclimated for one week and fed the appropriate treatment diets. This experiment was conducted in a flow-through water supply system (0.4 L/min) with water temperature maintained at 30 ± 1°C during and after *A. hydrophilla* challenge. The laboratory was equipped with an efficient water heating system fitted with regulating valves and thermometers to mix the water temperature.

Experimental Design and *A. hydrophila* Challenge Protocol

The challenge experiment maintained the same experimental design used in the eight week feeding study. Briefly, the experiment included a control treatment (Treatment 1), which was a diet with no amendment, and the following five treatments composed of diets amended as follows: Treatment 2- prebiotic Previda® only, Treatment 3- *B. subtilis* Aqua NZ strain only, Treatment 4- Aqua NZ probiotic plus prebiotic Previda®, Treatment 5- *B. velezensis* strain AP 193 only and Treatment 6- AP 193 plus prebiotic Previda® Each treatment was composed of six replicate aquaria each stocked with 25 fish obtained from the remaining fish after the eight week feeding study. Fish were challenged by administering 200 µL of *A. hydrophila* ML09-119 strain (initial concentration of 1.95×10^8 CFU/mL) by intragastric gavage obtaining a final dosage of 3.9×10^7 CFU/fish.

Feeding and Husbandry Activities During Challenge

During the one week acclimation period prior to challenge, tilapia were maintained on their treatment diets as in the growth trial for the previous eight weeks. During the challenge, feed was offered to fish; however, fish stopped feeding a day after the challenge and did not feed while mortalities occurred. Un-eaten feed and fecal wastes were siphoned out of each aquarium as needed. Each treatment had its own set of equipment, such as nets and siphoning hose, and was disinfected after every use to avoid cross contamination. Fish were observed daily and, moribund and dead fish were removed twice daily and counted. Moribund/freshly dead fish (18 from each treatment) were necropsied and samples from trunk kidney, liver, skin and gills were streaked on TSA plates for bacterial isolation. Isolated colonies were identified using specialized M9 media containing *myo*-inositol (Hanson *et al.*, 2014). At the end of the experiment, all surviving fish were counted, euthanized with 300 mgL-1 MS-222, and properly disposed.

Statistical Analysis

Data collected were analyzed by one-way analysis of variance using the mixed linear model procedure in SAS (SAS Institute Inc. Cary, NC). The mixed procedure (Wolfinger *et al.*, 1991) was used to identify differences among treatment means and pairwise comparisons made using Tukey's post hoc test. For the disease challenge experiment, the experiment was set up in a block design to minimize variation due to location of aquarium units in three different banks. The model used in the analysis included a generalized complete block design to test for block x treatment interaction. Differences between means were considered significant when probability (P) values < 0.05 were obtained.

4. Results

Study 1

Characterization of *Bacillus* isolates

Each of the *Bacillus* strains isolated from soil or catfish intestine that exhibited inhibitory activity against both *E. ictaluri* and *A. hydrophila* was capable of endospore formation. Each pure *Bacillus* culture was ribotyped, indicating that most of the *Bacillus* strains were within the *B. subtilis* group (inclusive of *B. amyloliquefaciens and B. velezensis*) (Table 1). The 16S rRNA gene sequences for the 21 strains were submitted to GenBank (Accession Numbers JX094283 to JX094303). Two strains of *B. pumilus* (AP18 and AP280) and one strain of *B. methylotrophicus* (AP191) were also within the collection. Strain AP76 was identified as *Bacillus cereus* and thus eliminated for further evaluation due to the potential for foodborne illness. All of the *Bacillus* strains that were subsequently determined to have *in vivo* biological control activity were within the *B. subtilis* group. This phylogenetic affiliation was also confirmed by comparison of genome sequences to the GenBank nr/nt database for strains AB01, AP79, AP143, and AP193, indicating > 80% average nucleotide identity to a previously sequenced genome within the *B. subtilis* group (data not shown).

Antimicrobial activity of *Bacillus* strains

The *B. subtilis* strain AB01 isolated from the catfish intestine showed significant antimicrobial activity against *E. ictaluri*. From the collection of soil-derived *Bacillus* strains, 49 strains showed significant antagonism against *E. ictaluri.* All of the 50 *Bacillus* strains also showed inhibitory activity against *E. ictaluri* R-4383 and *E. ictaluri* Alg-08-200. A total of 21 *Bacilllus* strains showed potent antibiotic activity against both

E. ictaluri and *A. hydrophila* (Table 1). The 21 *Bacillus* strains selected were tested for their activity against multiple pathogens in aquaculture. All of the strains were antagonistic against multiple catfish pathogens, including Gram-negative and -positive bacteria, and the oomycete *Saprolegnia*. *Bacillus* strains AB01, AP219, and AP301 showed antimicrobial activity against all of the tested pathogens (Table 1). Also, all five of the *Bacillus* strains tested for biological control of BNP (AP79, AP143, AP193, AP254, AB01) showed significant antagonistic activity against *E. ictaluri* NLF33 (data not shown).

Survival and persistence of *Bacillus* strains in the intestine of channel catfish

Bacillus spores administered to channel catfish via feed for one week followed by three days of control feed were recovered from the catfish intestine. Over 10^7 CFU/g of introduced *Bacillus* was observed in the gut for strains AB01, AP76, AP77, AP79, AP143, and AP254 (Fig. 1). For strains AP18, AP280, and AP303, the counts of recovered bacteria were relatively low, and they were eliminated from further investigation. None of the 21 *Bacillus* strains were recovered from the control group. In all cases the 16S rRNA gene sequence determined from representative colonies matched the 16S rRNA gene sequence from the respective *Bacillus* strain that was added to catfish feed (data not shown).

Challenge study

In the first immersion challenge, the mean mortality of control group was 98.0%, a very high level of mortality that was likely a consequence of the persistence of *E. ictaluri* within the aquaria water under static conditions. Treatment groups that were fed with feed amended with spores of *Bacillus* strains AP143 or AB01 showed significantly reduced

mortality compared with the control ($P<0.05$), with 83.1% and 84.8% mortality, respectively. There was no significant difference in the mortality observed between the two strains. The treatment groups fed with *Bacillus* strains AP79, AP193, or AP254 (with mortality 89.0%, 95.0%, and 93.7%, respectively) did not show significant differences compared with the control (Fig. 2A, Table 2). In every challenge experiment a control group was included that was not challenged with *E. ictaluri*, and in every case this control group had no observed mortalities, demonstrating that mortalities were due to infection with *E. ictaluri*.

For the second immersion challenge, flow-through conditions were adopted post-challenge to reduce catfish mortality, and this was successful as only 41.3% mortality was observed in the control group. However, under the flow-through conditions of this challenge the mortality in the treatment groups ranged from 35.0% to 46.3% with no significant differences observed between any of the treatment groups and the control (Fig. 2B, Table 2).

In the striped catfish challenge experiment, the catfish were again challenged by immersion and then maintained under static conditions similar to those used in the first channel catfish challenge. Under these conditions the control group had 70.8% mortality which is consistent with the higher mortality observed under static conditions when re-infection can occur (Fig. 2C, Table 2). The treatment group fed with feed amended with spores of strain AP79 had the lowest (9.7%) cumulative mortality and this was significantly different from the control ($P<0.05$). Catfish fed with spores of strains AP193, AP254 or AB01 had 30.6%, 54.2% and 56.9% mortality, respectively.

Plasmid and antibiotic resistance study

An analysis of plasmid DNA extracted from *Bacillus* strains AP79, AP143, AP193, and AB01 was conducted by PFGE. We did not observe the presence of any plasmid within AP79, AP193 and AB01 but the positive control did show the presence of plasmid pC194 (data not shown). A plasmid was evident from strain AP143. High coverage contigs from the assembled genome sequence of AP143 (data not shown) were screened by BLASTn and one of the contigs showed high identity (99%) with plasmid pBSG3 from *B. amyloliquefaciens* B3 (Qiao *et al.*, 2011). BLASTx analysis of the predicted open reading frames from this plasmid did not show any similarity to genetic loci involved in antibiotic resistance or virulence. Evaluation of antibiotic susceptibility determined that all of these strains were susceptible to all of the tested antibiotics to varying degrees. They were all highly susceptible to carbenicillin, cephalothin, sulfamethoxazone and ciprofloxacin (> 25mm diameter inhibition zone). Ampicillin, penicillin, vancomycin, novobiocin, amikacin, erythromycin, neomycin, chloramphenicol, norfloxacin and gentamicin also inhibited their growth effectively (20 - 25 mm zones of inhibition), whereas spectinomycin, oxacillin, sulfadiazine showed moderate inhibition (15-20 mm inhibition zones). These strains showed very similar antibiograms, with the variation in the diameter of inhibition zones less than 10% of the average diameter for each of the antibiotics tested.

Study 2

Experimental Diets

The diets were prepared based on a standard basal diet to which the 0.2% corn starch was substituted with equal concentrations (0.2%) of the probiotic *B. subtilis* group

strains (Table 3). Bacterial concentrations and colony morphology of the diets are presented in Table 4 The dose of the probiotic strains present in the amended diets were determined to be statistically indifferent among all treatments and were within the targeted range of 10^7 CFU/g of feed.

Growth

During the 21-day feeding trial, fish increased in biomass from a mean value of 16.5 ± 0.2 g to 33.7 ± 1.5 g. The mean percent weight gain, specific growth rates and FCRs for the treatments were not significantly different from the control diet at the end of the experiment (Table 5). No fish mortalities or abnormal behavior were observed during this phase of the experiment. Additionally, no gross external effects were observed.

Serum Bactericidal and Lysozyme Activity

Results from immunological parameters are available (data not shown; Addo *et al.*, 2017a). Mean serum bactericidal activities were significantly higher in all treatment groups ($P = 0.0004$) as compared to the control except for strain SB3295 ($P = 0.9020$). Statistically, the combined probiotic treatment did not show any improved advantage over the individual groups. Mean lysozyme activities were significantly higher in fish fed the probiotic diets than that of the control diet ($P < 0.0001$).

***S. iniae* Challenge**

The mean percent cumulative mortality of Nile tilapia fingerlings challenged with *S. iniae* revealed a significant reduction in mortality associated with each of the probiotic strains, with differences in mortality being observed as early as day 3 post-challenge and most mortalities occurring by day 7 post-challenge. The experiment was concluded 16 days post-challenge when mortalities had ceased two days earlier in all the treatment

groups. The mortalities ranged from the lowest value of 44.0 ± 7.2% in treatment 4 to the highest value 77.3 ± 7.0% in the control treatment group (Fig. 3, Table 6). A significant difference by treatment was observed between the control treatment group and all other treatments. In specific comparisons among treatments differences were observed only between diets with strain SB3295 (mean mortality = 61.3%) and strain SB3086 (mean mortality = 47.3%), as well as strain SB3615 (mean mortality = 44.0%) and strain AP193 (mean mortality = 58.0%). The effect of the combination of strains SB3086 and SB3615 did not result in improved fish survival relative to the use of these strains individually.

Study 3

Bacteria Quantification in Diets

Bacterial concentrations present in the diets are presented in Table 8. The doses of both *Bacillus* spp. strains present in the amended feed were determined to be very similar, approximately 4.7 x 10^7 CFU/g of feed, and were not significantly different from each other (P = 0.40). The counts coincided with the theoretical targeted dose of 4.2 x 10^7 CFU/g of feed.

Growth Parameters

In the growth trial, overall mean water temperature ranged from 27.8 ± 1.2°C in the morning to 28.4 ± 0.9°C in the afternoon. Dissolved oxygen readings in the morning averaged 5.47 ± 0.58 mg/L while the average measurement in the afternoon was 5.38 ± 0.59 mg/L. Average total ammonia-nitrogen and nitrite-nitrogen were 0.27 ± 0.22 mg/L and 0.12 ± 0.04 mg/L, respectively (data now shown; Addo, 2017b). No fish mortalities, behavioral abnormalities, and external or internal abnormal gross signs were observed during the eight week growth trial suggesting the safety of the prebiotic and the probiotic

strain as feed additives. Table 9 shows an increase in biomass of juvenile Nile tilapia over the eight week period of growth trial for all treatments and control, although the overall % mean weight gain, thermal growth coefficient feed intake and feed conversion ratio for the treatments were not significantly different ($P > 0.05$) from the control diet at the end of the experiment.

Lysozyme and Respiratory Burst Activity

Pre- and probiotic treatments did not significantly influence mean serum lysozyme activity compared with the control ($P = 0.14$; data now shown; Addo, 2017b). The lowest mean activity of 590 ± 92.5/mL was recorded for Previda® while the highest mean activity 675 ± 92.9/mL occurred in the treatment group fed the Aqua NZ probiotic. Respiratory burst activity, which is an important innate defense mechanism of fish, also did not change significantly between treatments and control ($P = 0.32$).

***A. hydrophila* Challenge**

In the challenge experiment, mean water temperature was 30 ± 1°C while dissolved oxygen levels were maintained at 5.0 ± 0.5 mg/L across all treatments. The initial analysis showed a significant effect in both the treatment and block terms but no interaction effect (Treatment p-value = 0.0011; Block p-value = 0.0013; Treatment x Block p-value = 0.3397). Because the interaction effect was not significant, the analysis was re-run as a randomized complete block removing the interaction effect from the model. From the analysis of variance involving the randomized complete block design, both the block and treatment effects were significant ($F=15.62$, $p<0.0001$ and $F=11.64$, $p<0.0001$, respectively) at the 95% confidence level (Table 10). With the exception of the prebiotic diet, which did not differ significantly from the control diet ($P = 0.17$), all other

treatment groups showed significantly lower fish mortality compared to the control group (Fig. 4; $P < 0.05$). Overall, the diet formulated with *B. velezensis* strain AP193 and Previda® combined had the lowest mean percent mortality (25 ± 15%) with the highest mean percent mortality (71 ± 15%) occurring in fish fed the control diet. Pairwise comparison showed that the two probiotic *B. subtilis* group strains used in this study, Aqua NZ and AP193, were not significantly different ($P = 0.17$). Similarly, the combined prebiotic and probiotic treatments (AP193 and Previda®) and (Aqua NZ and Previda®) did not show any significant difference ($P = 0.97$) in their ability to reduce mortality due to *A. hydrophila* infection in Nile tilapia. Bacteria isolated from moribund fish showed growth in M9 minimal medium containing *myo*-inositol and this was indicative of the presence of the ML09-119 strain of *A. hydrophila* used for the challenge (Hossain *et al.*, 2013; Hanson *et al.*, 2014).

5. Discussion

In the first study, *Bacillus* spp. were evaluated for their potential candidacy as probiotics for use in aquaculture. Twenty one strains were found to be antagonistic against *E. ictaluri* and *A. hydrophila, in vitro*; these same strains were also tested for antagonistic activity against a panel of other aquatic pathogens including: *E. tarda*, *Streptococcus iniae*, *Yersinia ruckeri*, *Flavobacterium columnare*, and/or the oomycete *Saprolegnia ferax.* Furthermore these *Bacillus* strains were confirmed to survive within the intestine of channel catfish as *Bacillus* CFU/g of intestinal tissue after seven days of feeding spore amended feed followed by 3 days of normal feeding. The five best performers were selected for an aquarium channel catfish experiment, in which feed was amended with spores at 8×10^7 CFU/g, fed for 14 days, and subsequently challenged

with *E. ictaluri*. In addition another study using four of the five *Bacillus* strains conducted in Vietnam showed similar results. From this first study one bacteria in particular performed well in all categories, *B. velezensis* AP193, which was used in the following two studies.

The second study evaluated the efficacy of AP193 plus three additional *B. subtilis* group strains, SB3086, SB3295, and SB3615, as probiotics for use on Nile tilapia. Tilapia were fed with feed amended with spores at 4×10^7 CFU/g for 21 days, followed by a challenge with *S. iniae*. From this study SB3086 incurred the lowest percent mortality.

The third and final study evaluated the efficacy of the *Bacillus* spp. probiotics, AP193 and Aqua NZ (a commercially available probiotic), in addition to a prebiotic Previda® on nile tilapia. Nile tilapia were fed diets formulated with the probiotics and/or the prebiotic, or a control diet for 8 weeks and, subsequently, challenged with *A. hydrophila.* With the exception of the prebiotic only diet, all diets resulted in a higher survival rate as compared to the control, with the diets consisting of both prebiotic and probiotic providing the most protection.

Study 1

The results of the first study indicate that specific strains within the *B. subtilis* group showed promise for biological control of disease in catfish aquaculture. It provided another evidence of the efficacy of the *Bacillus* strains in boosting disease resistance of fish (Ai *et al.*, 2011; Aly *et al.*, 2008a; Kumar *et al.*, 2008; Nayak *et al.*, 2007; Newaj-Fyzul *et al.*, 2007; Salinas *et al.*, 2005; Panigrahi *et al.*, 2007). This study is the first to select probiotic bacteria for control of ESC and other pathogens in catfish and to evaluate their biocontrol efficacy via feed administration.

Gatesoupe (1999) concluded that probiotics for aquaculture should be antagonistic to pathogens, colonize intestines, and increase resistance of the host to pathogens. Ideally probiotic bacteria should be selected by considering all three criteria. However, it is difficult to evaluate potential probiotic bacterial strains for the second and third criterion on a large number of candidate bacteria. Therefore, *in vitro* antimicrobial activity was the primary criteria by which a large number of strains were evaluated, with candidate bacterial strains that did not show antagonistic activity eliminated from further study. The primary objective of this research project was to identify bacterial strains that can be applied for the control of *E. ictaluri, A. hydrophila,* as well as other bacterial and oomycete pathogens of catfish. Since the bacterial pathogens *E. ictaluri* and *A. hydrophila* are responsible for the majority of the mortality observed currently in catfish aquaculture, the ability of a *Bacillus* strain to inhibit the growth of these two pathogens was of paramount importance and only the strains capable of inhibiting both pathogens were selected for testing in aquarium disease challenges.

The ability of a probiotic bacterial strain to colonize and survive within or on its host is also an important criterion for strain selection. However, in many cases the probiotic bacteria may not permanently colonize the gastrointestinal tract but instead achieve a sustained transient state (Irianto and Austin, 2002; Robertson *et al.*, 2000). Even transient bacteria may be efficient at mediating biological control of disease if the cells are introduced artificially via food either continuously or semi-continuously (Gatesoupe, 1999; Gournier-Chateau *et al.*, 1994). High population levels of several *Bacillus* strains were recovered from catfish intestines three days post-feeding with *Bacillus*-spore amended feed. For *Bacillus* strains with high counts in the intestine,

colonies with the same morphology as the applied *Bacillus* strain dominated the TSA plates, and the ribotype of the representative colonies confirmed their identity as the applied *Bacillus* strain. Considering that bacterial population levels in the intestine should decline after cessation of feeding with the spore-containing diets, the maximal level of *Bacillus* strain CFU/g of intestinal tissue reached during the feeding regime may be higher. The bacterial population levels here (10^6-10^7 CFU/g for most of the strains) are in general agreement with previous studies involving fish (Irianto and Austin, 2002; Robertson *et al.*, 2000; Jöborn *et al.*, 1997; Gildberg and Mikkelsen, 1998). These results demonstrate that some of the *Bacillus* strains evaluated in this study can persist within the catfish gastrointestinal tract for at least three days. However, at this point the degree of persistence and ability to colonize the intestinal mucosa are unknown for each strain. A more detailed experiment evaluating the colonization and/or persistence of specific *Bacillus* strains within the catfish intestine will be conducted to help understand the biocontrol mechanism(s) of *Bacillus* strains and guide the duration and timing of *Bacillus* feeding. Future studies will also examine the impact of each *Bacillus* strain on the intestinal microbiota and the health and growth of the fish in the absence of aquaculture pathogens.

Three aquarium disease challenges were conducted in this study, two of which evaluated biological control of ESC in channel catfish. In the first channel catfish disease challenge, a very high mortality (98.0%) due to ESC was observed in the control group. Ideally, an aquarium disease challenge would result in a mortality of 60% -70%, which more accurately simulates the natural development of ESC in an aquaculture pond. The high mortality was probably the consequence of the incorporation of a static system after

the immersion challenge, wherein the *E. ictaluri* persisted in the aquaria for an extended period of time and bacterial cells shed in feces could potentially infect other fish within the same aquarium. Despite the higher mortality observed in this first challenge, two *Bacillus* strains (AP143, AB01) provided significant protection to channel catfish. However, the degree of mortality reduction for the *Bacillus* treatment groups compared with control was lower in the first challenge compared to a later challenge with striped catfish. This discrepancy might be due to the much higher mortality in the first challenge, which could have reduced the biological control capacity of the *Bacillus* strains. Presumably the degree of biological control would be greater in an ESC challenge if lower mortality (~70%) was obtained in the control group. In addition, at the lower doses of *E. ictaluri* that catfish are typically exposed to in an aquaculture pond the degree of biocontrol provided by *Bacillus* strains would presumably be of an even greater magnitude.

One solution to reduce channel catfish mortality during an aquarium disease challenge is to use a flow-through system, but we discovered that the use of a flow-through system also resulted in the loss of *Bacillus* biological control activity. The lack of a protective effect for *Bacillus* strains when catfish were maintained in flow-through conditions after immersion challenge is likely a consequence of the removal of the *Bacillus* cells from the aquarium water, thereby preventing the persistence of the *Bacillus* cells within the aquarium that would naturally occur within an aquaculture pond. This suggests that a more pond-like environment wherein the probiotic is maintained within the water, and potentially the skin and gills of the fish, may be more conducive for effective biological control of disease. Since a static aquarium system is a better model

for evaluating the biological control of disease that would occur in an aquaculture pond, we considered the results of the first disease challenge more relevant to the evaluation of *Bacillus* strains for their future adoption in pond-scale aquaculture.

Considering the importance of striped catfish for the Vietnamese aquaculture industry, the best performing *Bacillus* strains were sent to collaborators in Vietnam to evaluate their biological control activity for striped catfish against *E. ictaluri*. Significant reductions in mortality were obtained in this experiment, with an especially large reduction in mortality observed for fish fed with feed amended with *Bacillus* strain AP79. The biological control activity observed for *Bacillus* strains in this striped catfish disease challenge was comparable with previous studies in other fish species (Aly *et al.*, 2008a; Kumar *et al.*, 2008; Nayak *et al.*, 2007; Newaj-Fyzul *et al.*, 2007). However, in this study fish were fed with *Bacillus*-amended feed for two weeks before challenge, which is a relatively short period of time compared with the feeding duration time of one to two months in previous studies (Aly *et al.*, 2008a; Kumar *et al.*, 2008; Nayak *et al.*, 2007; Newaj-Fyzul *et al.*, 2007). The duration of time for probiotic feeding is a factor that can influence the degree of protection activity against infection in challenge (Aly *et al.*, 2008a). Thus, the degree of protection afforded by the *Bacillus* strains in this study might be even better if a longer feeding duration was adopted.

It is interesting that the *Bacillus* strains that showed a significant protective effect in the disease challenges were different for the two catfish species. This could reflect a biologically meaningful difference in the interactions between *Bacillus* strains and their respective host. Also, there could be unique tripartite interactions between host, pathogen, and probiotic bacteria that could be influenced by environmental factors.

Clearly more research is needed to understand the complex interplay between host, pathogen and probiotic *Bacillus* strains, and how to manipulate the environment to achieve the optimal biological control of disease. Further studies using an aquarium disease model with static conditions need to be conducted to optimize important parameters for challenge such as dosage and timing with the best performing *Bacillus* strains, with subsequent studies at a pond-scale to evaluate biological control efficacy within an aquaculture pond ecosystem.

One of the safety requirements for live bacteria directly consumed by humans is the absence of any acquired resistance to clinically important antibiotics (Sorokulova, 2008). Although the *Bacillus* strains used in this research were not for direct consumption by humans, they might be consumed inadvertently, as their hosts were cultured for food. Thus, it is important to analyze antibiotic resistance in probiotic strains and to distinguish the natural resistance, which is one of the phenotypic characteristics of a species, and acquired (i.e., transferable) resistance, which is associated with occurrence of plasmids. Also, pathogenicity and enterotoxin production are closely associated with plasmids (Pannucci *et al.*, 2002). Each of the four selected strains was susceptible to a broad spectrum of antibiotics tested with very similar susceptibility profile. Strains AP79, AP193 and AB01 did not contain any plasmids, ensuring their inability to conjugally transfer any plasmid that might confer antibiotic resistance or other traits. Although a plasmid was extracted from AP143, draft genome sequences from this strain were used to identify plasmid sequences and there were no predicted genes involved in antibiotic resistance or virulence.

Diffusible antimicrobial compounds were clearly involved in the *in vitro* antagonistic activity observed in soft agar overlay and in diffusion tests. The relative importance of secondary metabolites for *in vivo* biological control is unknown compared to other mechanisms such as enhancement of immune response, competitive exclusion of pathogens and modulation of intestinal microbiota. Future studies will investigate the relative contribution of antibiotic compound(s) production to the biological control activity of the *Bacillus* strains by comparing the mortality reduction activity of transposon mutagenized *Bacillus* strains with no antagonism activity with the wild type strains in aquarium challenge experiments.

In conclusion, a collection of *Bacillus* strains was identified that are antagonistic to the primary pathogens of catfish and are beneficial to both channel catfish and striped catfish when administered on feed for the control of ESC and BNP, respectively. These bacteria have potential application in aquaculture as a cost-effective alternative to the current use of antimicrobial compounds.

Study 2

Feeding trials with probiotics produced mixed results when growth is measured. In this study, growth of juvenile Nile tilapia fed the selected probiotic diets (individually and one combination) for 21 days did not significantly improve. These results are not unexpected given the short duration of this trial. Apun-Molina *et al.* (2009) published results indicating a trend towards improved growth in Nile tilapia fry (0.14 g) only after 75 days of feeding with diets composed of *Bacillus* or *Lactobacillus* probiotics. Honsheng (2010) attributed improved weight gain and feed efficiency to increased enzyme production due to the inclusion of *B. subtilis* in tilapia diets. According to Ridha

and Azad (2012), probiotics may improve digestion by stimulating production of digestive enzymes or through other alterations in the gut environment of fish. The lack of a significant result in growth performance from this short study nonetheless corroborates findings from other studies on probiotics. For instance, non-viable *S. cerevisiae* (Marzouk *et al.*, 2008), *Pseudomonas* spp. (El-Rhman *et al.*, 2009), *Pediococcus acidilactici* and *E. faecium* (Biomate SF-20®) (Ferguson *et al.*, 2010), *B. subtilis* + *B. licheniformis* (Bioplus 2B®), *P. acidilactici* (Bactocell PA10 MD®) and viable *S. cerevisiae* (Levucell SB 20®) (Shelby *et al.*, 2006) have all been reported as not having any significant effect on tilapia growth. Contrarily, other studies conducted by different researchers using the same or different strains of probiotic bacteria have produced significant improvement in growth of Nile tilapia. Aly *et al.* (2008c) noted statistically significant increases in weight gain of Nile tilapia after four or eight weeks of feeding two doses of *B. pumilus* and the commercial probiotic product Organic Green™ as compared to the control group. According to Lara-Flores *et al.* (2010), supplementation of combined *S. faecium* and *L. acidophilus* or *S. cerevisiae* singly in tilapia diets containing 27% or 40% crude protein produced significantly higher weight gain and feed utilization efficiency compared to the control diet. Improved growth performance of Nile tilapia fed diets with *B. subtilis, L. plantarum*, or a mixture of *B. subtilis, L. plantarum*, and *S. cerevisiae* have been reported by Essa *et al.* (2010). The contradicting reports on the effects of probiotics fed to tilapia after both short and longer periods may suggest that variability in probiotic strain efficacy, research conditions, handling practices, and stocking rates among other factors might have affected the results, which consequently influenced the success or failure of probiotics and their combinations to improve growth. The lack of detectable growth

enhancement in this study could be possibly due to the fact that the experimental conditions were ideal for optimal growth of the fish. The GI tract colony present might have influenced the result since intestinal microbiota has important and specific metabolic and trophic functions (Denev *et al.*, 2009). Gutowska *et al.* (2004) noted that the microbiota of the GI tract of fishes in general, represents a very important and diversified enzymatic potential having the capacity to produce proteolytic, amylolytic, cellulolytic, lipolytic, and chitinolytic enzymes, which is important for digestion of proteins, carbohydrates, cellulose, lipids and chitin to enhance growth.

Serum bactericidal activity was higher in fish fed probiotics relative to the control except in fish fed the probiotic strain SB3295. There was also higher lysozyme activity in fish fed the probiotic diets than those fed the control diet. A number of systemic, non-specific immune functions including serum bactericidal and lysozyme activities have been observed to be enhanced by dietary probiotic supplementation (Nayak, 2010; Pirarat *et al.*, 2011). Ferguson *et al.* (2010) found that blood leucocyte numbers and serum lysozyme activity were enhanced in Nile tilapia fed the probiotic *Pediococcus acidilactici*. In a study to evaluate the use of *L. acidophilus* as a biocontrol agent against some common fish pathogenic bacteria including *Streptococcus* sp. in the African catfish, *Clarias gariepinus*, Al-Dohail *et al.* (2011) observed a higher immunological response and concluded that *L. acidophilus* was useful as a probiotic in *C. gariepinus* against bacterial pathogens. Taoka *et al* (2006b) investigated the effect of live and dead probiotic cells on the non-specific immune system of *O. niloticus* and found that probiotic administration enhanced non-specific immune parameters such as lysozyme activity, migration of neutrophils and plasma bactericidal activity, resulting in improvement of

resistance to *E. tarda* infection. However, the viable cells might not be stimulating the immune system, but rather their cellular products or components or just the high concentration of the viable cells might be causing the increased response. Shelby *et al.* (2006) did not find any effect on lysozyme activity, alternative complement, or total serum immunoglobulin in tilapia fed commercial probiotics containing *B. subtilis, B. licheniformis, P. acidilactici* and *S. cerevisiae*. They concluded that feeding Nile tilapia for 94 days with these commercial probiotics did not prevent streptococcal disease infection.

Various mechanisms have been proposed to explain the effects of probiotics in fish disease resistance. These include competition for adhesion sites on the intestinal epithelium or other tissue surfaces, competition for nutrient and energy sources, release of secondary metabolites that have bactericidal effects on other microbial populations and enhancement of the host immune response. It has been observed that the ability to adhere to enteric mucus and intestinal wall surfaces was indispensable for probiotic bacteria to become established in fish intestines (Onarheim and Raa, 1990; Westerdahl *et al.*, 1991; Olsson *et al.*, 1992). Montes and Pugh (1993) proposed that competition for adhesion receptors with pathogens might be a critical probiotic phenotype since bacterial adhesion to tissue surface is important during the initial stages of pathogenic infection (Verschuere *et al.*, 2000b; La Ragione *et al.*, 2003, 2004). According to FAO (2001), probiotics confer health benefits on the host when administered in adequate amounts. In this study, survival of Nile tilapia to *S. iniae* challenge was significantly higher with the probiotic diets than the control diet, indicating increased health due to the probiotic diets given to the fish.

Several studies have attributed a probiotic effect to competition for energy sources (Rico-Mora *et al.* 1999; Verschuere *et al.* 1999, 2000a, 2000b) and the production and release of inhibitory substances such as antibiotics, bacteriocins, siderophores, lysozymes, proteases and hydrogen peroxide, which constitute a barrier against the proliferation of pathogens (Marden *et al.*, 2008; Chaucheyras-Durand *et al.*, 2008; Chaucheyras-Durand and Durand, 2010). El-Rhman *et al.* (2009) noted that probiotic inclusion in fish feed can stimulate the growth of beneficial bacterial taxa on skin and in the intestine which could aid in the competitive exclusion of pathogens. The effectiveness of probiotics in terms of protection against infection has also been demonstrated to be as a result of enhanced immunity (Delcenserie *et al.*, 2008; Johnson-Henry *et al.*, 2008; Welker and Lim, 2011). Merrifield *et al.* (2010) stated that probiotic use can enhance the immune response of tilapia and improve disease resistance. It is more likely that the positive results reported in the present work may be due to enhanced immune response since the IP injection administered bypasses the defense system of the GI. Merrifield *et al.* (2010) noted that the capacity of probiotics to prevent disease may be greater than the results observed in many studies due to the use of intraperitoneal (IP) injection as a method of disease challenge. The IP method bypasses competitive exclusion, which is one of the most important ways probiotics can prevent infection in the GI tract. These authors stated that IP challenges may not reflect the effect of probiotics on resistance to infection but rather demonstrate the effect of probiotics on infected fish. According to Shoemaker *et al.* (2006), the majority of challenges performed in tilapia research studies are done by IP injection, especially with *Streptococcus*, which is difficult to reproduce reliably by bath immersion. In the current study, the challenge

was done by IP, which does not reflect the mode of infection by *S. iniae* under culture conditions. This means that the potential reduction in mortality could be better than the results obtained. Due to this limitation, it would be difficult to conclude on which of the probiotic treatments was most effective

Study 3

In aquaculture, probiotics can be applied either as feed additives or as additives to the water (Moriarty, 1998; Taoka *et al.*, 2006b). The form and duration of prebiotic and probiotic administration can influence their effectiveness in affecting fish health (Welker and Lim, 2011). The supplementation of pre- and probiotics through feed has been documented as a better method of ensuring the efficiency of the probiotic bacterial colonization in the gastro-intestinal tract of fish (Rurangwa *et al.*, 2009; Merrifield *et al.*, 2010; Mei *et al.*, 2011). However, their use in commercial fish feed production is uncommon.

In the current study, a prebiotic was formulated separately and in combination with probiotic strains in feed and fed to Nile tilapia for eight weeks prior to the challenge with *A. hydrophila*. The concentration of the probiotic strain obtained in the amended feed (4.7×10^7 CFU/g of feed) was not significantly different from the theoretical targeted dose (4.2×10^7 CFU/g of feed) suggesting that the process of feed preparation and storage did not negatively affect the viability of the bacteria; also, the similar concentrations of the two probiotic strains in the bacteria-amended diets provided a controlled basis for comparison of their treatment effects. Also, the water quality parameters maintained during the study were in the range acceptable for the growth of *O. niloticus* (Mjoun *et al.*, 2010). Nonetheless, under the conditions of the growth trial, none

of the diets significantly improved nor negatively affected growth of the fish ($P = 0.69$) as compared to the control diet. Although prebiotics, probiotics, and/or their combinations have been demonstrated to positively modulate the intestinal microflora and promote fish growth and health, results from some studies on their efficiency have been conflicting (Gatesoupe, 2005; Shelby *et al.*, 2006; Song *et al.*, 2006; Grimoud *et al.*, 2010). Results from an 8-week feeding trial conducted by Zhou *et al.* (2010a) with juvenile red drum to evaluate four different prebiotics, fructooligosaccharides (FOS) in the form of inulin, galactooligosaccharides (GOS), Bio-MOS® containing mannanoligosaccharides (MOS) derived from yeast, and Previda® containing galacto-gluco-mannans from hemicellulose extract, showed that fish fed the diet containing Previda® had significantly higher weight gain than fish fed the basal diet or the one supplemented with Bio-MOS®. The feed efficiency and protein efficiency ratio of fish fed the various diets were not significantly different, although fish fed the basal diet had the lowest values. In a study conducted by Hui-Yuan *et al.* (2007) with hybrid tilapia, *O. niloticus* x *O. aureus* fed FOS, mean specific growth rates, daily feed intakes and feed conversion ratios were significantly improved with increasing levels of the prebiotic. Increasing the prebiotic concentration in the diets in this study from the 0.5% level may have improved growth performance. However, in a study that showed a similar lack of effect of prebiotic effects using different levels of the prebiotic Immunogen® (0, 0.5, 1, 1.5 and 2.5 g prebiotic/kg diet) fed to common carp fingerlings for eight weeks, Ebrahimi *et al.* (2011) did not observe any significant difference in growth among the groups fed different inclusion levels.

Various probiotic bacteria either singly or in combinations have been reported to improve growth and disease resistance in a few fish species, including Nile tilapia. Essa *et al.* (2010) reported improved growth performance of Nile tilapia fed diets with *B. subtilis*, *Lactobacillus plantarum*, a mixture of *B. subtillis* and *L. plantarum* and *Saccharomyces cerevisiae*. Aly *et al.* (2008a) compared the potential effects of two doses of *B. pumilus* and the commercial probiotic product Organic Green™ in improving immune response, survival, growth and resistance in Nile tilapia to *A. hydrophila* infection after feeding for four and eight weeks. Mean body weight and survival rates of all treatment groups showed statistically significant increases as compared to the control group. Other studies conducted to evaluate the effects of some probiotic strains on growth of Nile tilapia, however, did not show any remarkable effects on growth performance similar to this study (Shelby *et al.*, 2006; Marzouk *et al.*, 2008; El-Rhman *et al.*, 2009; Ferguson *et al.*, 2010). The differing impact of probiotics on growth performance in tilapia and other fish can relate to differences in the antibiosis activities of specific probiotic strains as well as the differing interactions between probiotics, gut microbiome, diet and the host in each study.

Although this study did not show any significant treatment effects with respect to growth performance, there was enough evidence to conclude that the probiotic strain and its combination with the prebiotic resulted in a significant reduction in mortality due to *A. hydrophila* infection. Results from the combined effect of the pre- and probiotic strain showed a significant reduction in mortality compared to the prebiotic only and the control diets, which indicates the importance of the probiotic strains in mediating disease resistance. Feeding a combined pre- and probiotic diet improved survival of rainbow

trout challenged with *Vibrio anguillarum* compared to trout fed the individual prebiotic or probiotic (Rodriguez-Estrada *et al.*, 2009). When the Japanese flounder was fed a diet containing *Bacillus clausii* or in combination with the prebiotics fructo- or mannanoligosaccharides, there was an improvement of the non-specific immune function (Ye *et al.*, 2011). Although the diet containing either of the prebiotics with *B. clausii* exhibited the highest immune function, this activity was not significantly different compared to flounder fed only *B. clausii*. Prebiotics are known to modify the microbial community within the gastrointestinal tract and to boost non-specific immune responses (Bailey *et al.*, 1991). The microbiota in the colon ferments the prebiotic and causes significant modification of the colonic microflora providing the substrate needed for growth and proliferation of probiotic bacteria, which may have the capacity to inhibit the growth of putrefactive and pathogenic bacteria present in the colon (Mussatto and Mancilha, 2007; Yousefian and Amiri, 2009; Mei *et al.*, 2011). Thus, while the prebiotic used in this study was not observed to have any synergistic effect with the probiotic strains, it is possible that under different environmental conditions there might be beneficial interactions between pre- and probiotics in enhancing host protection against infection.

According to Welker and Lim (2011), the effectiveness of probiotics in terms of protection against infection is often attributed to enhanced immunity; however, in this study, lysozyme and respiratory burst activities were not influenced significantly by treatment effects. This agrees with the assertion that findings of lysozyme and respiratory burst activities following probiotics treatment in fish are often contradictory. While some studies have indicated probiotics do not have significant impact on these non-specific

defense mechanisms of fish (Irianto and Austin, 2003; Nayak *et al.*, 2007; Sharifuzzaman and Austin, 2009), other researchers have identified specific probiotics like *B. subtilis* and some members of *Lactobacillus* group to significantly stimulate respiratory burst activity in fish (Nikoskelainen *et al.*, 2003; Salinas *et al.*, 2005; Salinas *et al.*, 2006; Zhou *et al.*, 2010b). Dietary supplementation of probiotics like *Lactobacillus sakei* in *Salmo trutta* (Balcazar *et al.*, 2007a), *L. sakei*, *L. lactis* ssp. lactis, *L. mesenteroides*, and *L. rhamnosus* in *Oncorhynchus mykiss* (Panigrahi *et al.*, 2004; Panigrahi *et al.*, 2005; Balcazar *et al.*, 2007b); *A. sobria* in *O. mykiss* (Brunt *et al.*, 2007) as well as water supplementation of *B. coagulans*, *B. subtilis* and *Rhodopseudomonas palustris* and *E. faecium* in *O. niloticus* (Pieters *et al.*, 2008; Wang *et al.*, 2008b; Zhou *et al.*, 2010b) failed to elevate lysozyme level. It has also been suggested that variations in environmental conditions could be responsible for the conflicting results obtained in studies with pre- and probiotics due to differences in the choice of prebiotics, probiotics, pairing of pre- and probiotics, dietary concentrations, species strains, age/size of fish, feeding management and duration, dosage and virulence of challenge pathogens, and methods of challenge (Welker and Lim, 2011). Merrifield *et al.* (2010) noted that the potential of probiotics to prevent disease may be greater than the results obtained under experimental conditions. This is because the use of intragastric and/or intraperitoneal (IP) method of disease challenge and the challenge dose may be harsher than what pertains in the natural environment of the fish. Other factors, such as environmental conditions, handling practices, and stocking densities, may also affect results. All these factors can influence the success or failure of prebiotics, probiotics and their combination in the enhancement of growth, immunity and disease resistance in fish.

The three studies can be summarized by the following: *B. velezensis* AP193 administered as a probiotic can help to increase growth of channel catfish while also providing protection against MAS. In addition, *B. velezensis* AP193 and *B. subtilis SB3086* provide protection from MAS in nile tilapia, and to an even greater degree when combined with the prebiotic Previda®.

Table 1. Antimicrobial activity of 22 *Bacillus* strains against multiple aquatic pathogens. Note that (+) indicates a zone of inhibition up to 5 mm, (++) indicates a zone of inhibition from 5 mm to 1cm, (+++) indicates a zone of inhibition greater than 1cm, and (-) indicates no observable zone of inhibition.

Phylogeny	Strain	*Aeromonas hydrophila*	*Edwardsiella ictaluri*	*Edwardsiella tarda*	*Flavobacterium columnare*	*Saprolegnia ferax*	*Streptococcus iniae*	*Yersinia ruckeri*
B. subtilis group	AB01	+	+	+	+++	+	++	++
B. pumilus	AP18	+	+	-	-	+	+	-
B. subtilis group	AP71	+	++	+	-	-	++	+
B. cereus	AP76	+	++	++	-	+	++	++
B. subtilis group	AP77	+	+++	++	-	+	+	++
B. subtilis group	AP79	++	++	+	-	-	++	++
B. subtilis group	AP102	+	++	++	++	+	-	++
B. subtilis group, *B. amyloliquefaciens*‡	AP143	++	++	++	-	+	+	++
B. subtilis group	AP183	+	++	++	-	-	++	+
B. subtilis group	AP189	++	+++	++	-	-	++	+
Bacillus methylotrophicus	AP191	++	+++	+	+	+	++	-
B. subtilis group, *B. amyloliquefaciens*‡	AP193	++	++	++	++	+	+	-
B. subtilis group	AP215	+	+++	+	-	+	+	++
B. subtilis group	AP218	+	++	+	-	+	+	+
B. subtilis group	AP219	++	++	+	+	+	++	+
B. subtilis	AP254	+	++	+	++	-	-	-
B. pumilus	AP280	+	++	+	-	+	+	+
B. subtilis group	AP295	+	++	+	-	+	+	++
B. subtilis group	AP301	+	++	+	+++	+	++	+
B. subtilis group	AP303	++	++	++	-	+	+	++
B. subtilis group	AP305	++	++	++	-	-	+	++

‡Draft genome sequences are available for these *Bacillus* strains, so the phylogenetic affiliation is inferred from a comparison of these *Bacillus* strain genome sequences with previously sequenced *Bacillus* genomes.

Table 2. Mortality (%) (± SE) of groups of fish that received feed amended with different *Bacillus* strains or control feed and were challenged with *E. ictaluri* (n=4). Means in the same column sharing a common superscript letter were not significantly different (P > 0.05) as determined by Tukey's test.

Treatment	Channel catfish challenge (Fig 2A)	Channel catfish challenge (Fig 2B)	Striped catfish challenge (Fig 2C)
Control	98.0 ± 1.2^{a}	41.3 ± 5.9^{a}	70.8 ± 7.3^{a}
AB01	84.8 ± 2.0^{bc}	37.5 ± 9.5^{a}	56.9 ± 6.6^{ab}
AP143	83.1 ± 2.9^{bc}	43.3 ± 14.8^{a}	Not determined
AP193	95.0 ± 3.0^{ab}	35.0 ± 5.4^{a}	30.6 ± 23.7^{ab}
AP254	93.7 ± 2.8^{ab}	Not determined	54.2 ± 11.4^{ab}
AP79	89.0 ± 2.7^{abc}	46.3 ± 5.2^{a}	9.7 ± 6.6^{b}

Table 3. Composition (g/100g as is) of experimental diets, with or without probiotics, formulated to contain 32% protein and 6% lipid and fed to Nile tilapia.

Ingredients	1 Basal	2 SB3086	3 SB3295	4 SB3615	5 SB3086 +SB3615	6 AP193
Fishmeal [1]	4.00	4.00	4.00	4.00	4.00	4.00
Soybean meal solvent extracted [2]	46.50	46.50	46.50	46.50	46.50	46.50
Menhaden fish oil [1]	3.31	3.31	3.31	3.31	3.31	3.31
Yellow corn [2]	36.74	36.74	36.74	36.74	36.74	36.74
Corn starch [3]	0.20	0.00	0.00	0.00	0.00	0.00
Trace mineral premix[4]	0.50	0.50	0.50	0.50	0.50	0.50
Vitamin premix[5]	1.80	1.80	1.80	1.80	1.80	1.80
Choline chloride [3]	0.20	0.20	0.20	0.20	0.20	0.20
Stay C 250 mg kg [6]	0.10	0.10	0.10	0.10	0.10	0.10
CaP-dibasic[7]	2.00	2.00	2.00	2.00	2.00	2.00
Corn gluten meal [7]	4.65	4.65	4.65	4.65	4.65	4.65
Probiotic	0.00	0.20	0.20	0.20	0.20	0.20
Total %	100	100	100	100	100	100

[1] Omega Protein Inc., Reedville, VA, USA

[2] Faithway Feed Co., Guntersville, AL, USA.

[3] MP Biochemicals Inc., Solon, OH, USA.

[4]Trace mineral (g/100g Premix): Cobalt chloride 0.004, Cupric sulfate pentahydrate 0.25, Furrous sulfate 4.0, Magnesium sulfate anhydrous 13.862, Manganous sulfate monohydrate 0.65, Potassium iodide 0.067, Sodium selenite 0.01, Zinc sulfate hepahydrate 13.193, cellulose 67.964.

[5]Vitamin (g/kg Premix): Thiamin HCl 0.44, Riboflavin 0.63, Pyridoxine HCl 0.91, D pantothenic acid 1.72, Nicotinic acid 4.58, Biotin 0.21, Folic acid 0.55, Inositol 21.05, Menadione sodium bisulfite 0.89, Vitamin A acetate (500,000 IU/g) 0.68, Vitamin D_3 (400,000 IU/g) 0.12, DL-alpha-tocoperol acetate (250 IU/g) 12.63, cellulose 955.59.

[6] Stay-C® (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.

[7] Grain Processing Corporation, Muscatine, IA, USA.

Table 4. Concentrations of *Bacillus*-like colonies recovered from probiotic-supplemented diets.

Diet	Mean ± SD (CFU/g)
Control	0
SB3086	$8.5 \pm 9.3 \times 10^7$
SB3295	$7.3 \pm 4.2 \times 10^7$
SB3615	$8.2 \pm 3.9 \times 10^7$
SB3086 + SB3615	$7.0 \pm 4.2 \times 10^7$
AP193	$7.7 \pm 5.1 \times 10^7$

Table 5. Effects of experimental diets on the growth of juvenile *O. niloticus* grown for 21 days in flow-through aquaria.

Growth					Diets		*P*-value
Parameter	Control	SB3086	SB3295	SB3615	SB3086 + SB3615	AP193	
IBW	16.2 ± 0.5	16.6 ± 0.8	16.6 ± 0.8	16.6 ± 0.8	16.7 ± 1.1	16.7 ± 0.8	0.6205
FBW	33.7 ± 1.6	30.6 ± 1.5	35.6 ± 1.6	34.1 ± 2.6	34.6 ± 2.2	33.3 ± 1.3	0.1390
%WG	107.5 ± 7.1	101.6 ± 10.7	115.0 ± 9.5	106 ± 14.3	107.6 ± 9.7	100.2 ± 6.7	0.3910
SGR	3.5 ± 0.05	3.0 ± 0.07	3.6 ± 0.06	3.4 ± 0.08	3.5 ± 0.08	3.3 ± 0.05	0.0955
FCR	1.42 ± 0.11	1.41 ± 0.09	1.54 ± 0.12	1.42 ± 0.12	1.45 ± 0.05	1.35 ± 0.18	0.1172

IBW (g/fish) = initial mean body weight;

FBW (g/fish) = final mean body weight.

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

SGR (specific growth rate) (%/day) = 100 x [(ln final weight-ln initial weight)/days]

FCR (feed conversion ratio) = feed intake / (FBW-IBW).

Table 6. Percent mortality of juvenile *O. niloticus* fed probiotic diets and challenged with *S. iniae*.

Treatment	Mortality (%)	*P*-value
Control	77.3 ± 7.0	0.0001
SB3086	47.3 ± 4.7	0.9768
SB3295	61.3 ± 8.6	0.0170
SB3615	44.0 ± 7.2	0.0001
SB3086 + SB3615	46.7 ± 9.7	1.0000
AP193	58.0 ± 10.0	0.2097

Table 7. Composition (g/100g) of test diets designed to contain 32% protein and 6% lipid for Nile tilapia.

Ingredients	Diets					
	Control	Previda	Aqua NZ	AquaNZ+Previda	AP193	AP193+Previda
Fishmeal[1]	3.97	3.97	3.97	3.97	3.97	3.97
Soybean meal solvent extracted[2]	46.5	46.5	46.5	46.5	46.5	46.5
Corn gluten meal[3]	4.65	4.65	4.65	4.65	4.65	4.65
Menhaden fish oil[1]	3.31	3.31	3.31	3.31	3.31	3.31
Yellow corn[2]	36.0	36.0	36.0	36.0	36.0	36.0
Corn starch[4]	0.97	0.47	0.94	0.44	0.97	0.47
Trace mineral premix[5]	0.5	0.5	0.5	0.5	0.5	0.5
Vitamin premix[6]	1.8	1.8	1.8	1.8	1.8	1.8
Choline chloride[4]	0.2	0.2	0.2	0.2	0.2	0.2
Stay C 250 mg kg[7]	0.1	0.1	0.1	0.1	0.1	0.1
CaP-dibasic[3]	2.0	2.0	2.0	2.0	2.0	2.0
Prebiotic[8]	0.00	0.50	0.00	0.50	0.00	0.50
Probiotic[9]	0.00	0.00	0.028	0.028	0.028	0.028
Total %	100	100	100	100	100	100

[1] Omega Protein Inc., Reedville, VA, USA
[2] Faithway Feed Co., Guntersville, AL, USA.
[3] Grain Processing Corporation, Muscatine, IA, USA.
[4] MP Biochemicals Inc., Solon, OH, USA.
[5]Trace mineral (g/100g Premix): Cobalt chloride 0.004, Cupric sulfate pentahydrate 0.25, Furrous sulfate 4.0, Magnesium sulfate anhydrous 13.862, Manganous sulfate monohydrate 0.65, Potassium iodide 0.067, Sodium selenite 0.01, Zinc sulfate hepahydrate 13.193, cellulose 67.964.
[6]Vitamin (g/kg Premix): Thiamin HCl 0.44, Riboflavin 0.63, Pyridoxine HCl 0.91, D pantothenic acid 1.72, Nicotinic acid 4.58, Biotin 0.21, Folic acid 0.55, Inositol 21.05, Menadione sodium bisulfite 0.89, Vitamin A acetate (500,000 IU/g) 0.68, Vitamin D_3 (400,000 IU/g) 0.12, DL-alpha-tocopherol acetate (250 IU/g) 12.63, cellulose 955.59.
[7] Stay-C® (L-ascorbyl-2-polyphosphate), Roche Vitamins Inc., Parsippany, NJ, USA.
[8]Prebiotic, Previda® (hemicellulose extract), Novus International Inc., St Charles, MO, USA.
[9]Probiotics (Aqua NZ and AP193), *B. subtilis* strains provided by Novus International Inc. and Auburn University, Auburn, AL USA respectively.

Table 8. Mean concentrations of *B. subtilis*-like colonies recovered from prebiotic and probiotic-supplemented diets.

Treatment	Mean (CFU/g)
Control	0
Previda	0
Aqua NZ	4.75×10^7
Aqua NZ+Previda	4.75×10^7
AP193	5.5×10^7
AP193+Previda	4.5×10^7

Table 9. Prebiotic, probiotic and combined effects of formulated diets on the growth of juvenile *O. niloticus* L. under laboratory conditions.

Growth	Diet						
Parameter	Control	Previda	Aqua NZ	AquaNZ+Previda	AP193	AP193+Previda	p-value
IBW	7.49 ± 0.07	7.39 ± 0.17	7.45 ± 0.14	7.49 ± 0.06	7.50 ± 0.06	7.48 ± 0.07	0.94
FBW	60.1 ± 4.6	60.5 ± 2.5	60.2 ± 4.3	59.4 ± 2.6	57.8 ± 3.1	59.1 ± 3.8	0.68
%WG	602.0 ± 57.4	628.9 ± 41.5	603.8 ± 50.4	592.9 ± 32.5	570.9 ± 38.2	590.0 ± 46.3	0.70
TGC	1.26±0.02	1.28±0.01	1.26±0.03	1.25±0.02	1.23±0.01	1.25±0.02	0.88
FI	65.3 ± 2.04	65.3 ± 2.14	65.4 ± 5.30	65.00 ± 1.99	62.4 ± 2.06	64.4 ± 1.85	0.77
FCR	1.25 ± 0.08	1.22 ± 0.02	1.25 ± 0.09	1.26 ± 0.06	1.24 ± 0.06	1.25 ± 0.07	0.87

Values are means ± s. d. of six replicates

IBW (g/fish), initial mean body weight; FBW (g/fish), final mean body weight.

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

TGC (Thermal Growth Coefficient) = $[(\sqrt[3]{W_t} - \sqrt[3]{W_0}) / (T \times t)] \times 1000$; where W_t=final weight, W_0=initial weight, T=mean temperature, t=duration of growth

FI (feed intake) = (g $fish^{-1}$ in 56 days)

FCR (feed conversion ratio) = feed intake/(FBW-IBW).

Table 10. Percent mortality of treatment groups that received feed amended with probiotics, prebiotic, or a combination of both, and were challenged with *A. hydrophila*. Statistical analysis was performed using an ANOVA with a randomized complete block design. Analysis was followed by a Tukey's Test to determine differences between treatments. Equal numbers of replicate treatment aquaria (6 replicate aquaria total; 2 replicate aquaria per block; 3 blocks).

Treatment			% Mortality
Control			71 ± 15^{a}
Previda			54 ± 18^{ab}
Aqua NZ			46 ± 16^{bc}
Aqua NZ + Previda			29 ± 18^{c}
AP193			27 ± 9^{c}
AP193 + Previda			25 ± 15^{c}
Treatment Effect	n=6	F value = 11.64	p<0.0001
Block Effect	n=3	F value = 15.62	p<0.0001

Values are mean percentages ± s.d. Significance between treatments ($p < 0.05$) is indicated by different letters within the same column.

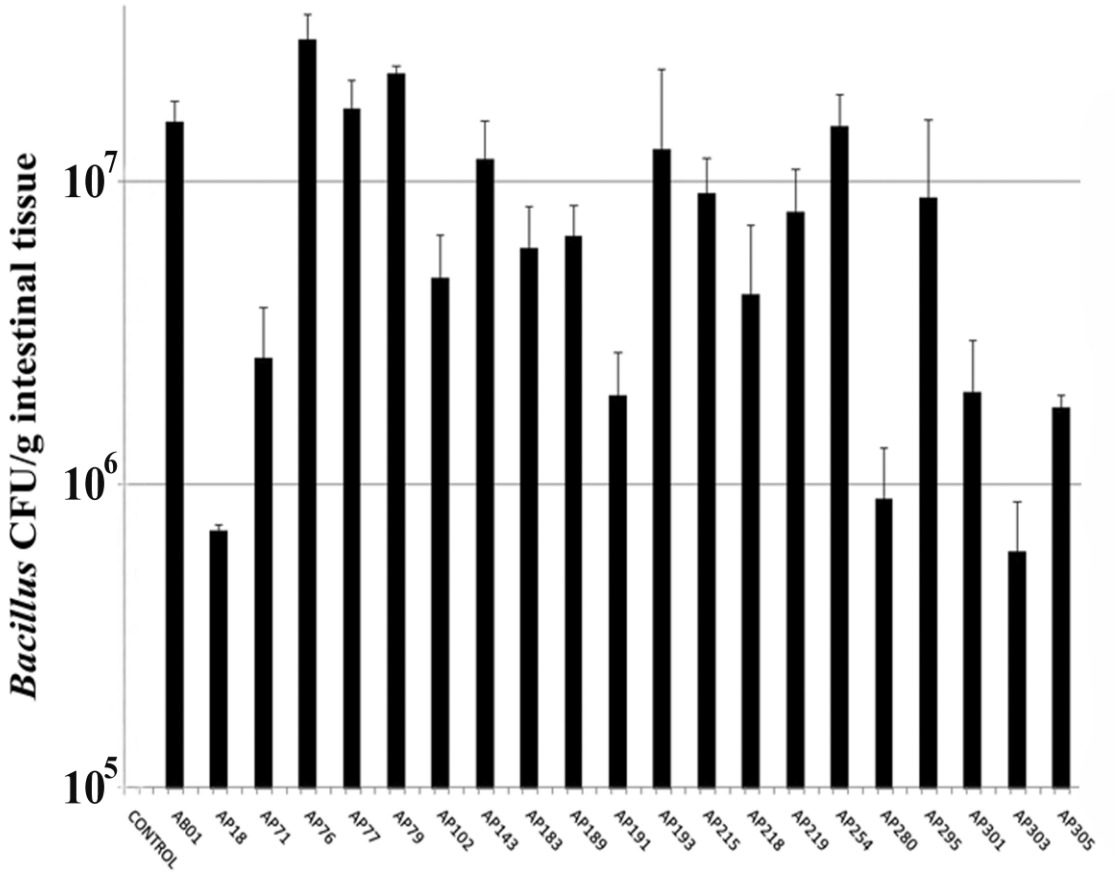

Figure 1. *Bacillus* strain CFUs/g of catfish intestine, after feeding with *Bacillus*-amended or non-amended feed (n=3 animals per *Bacillus* strain).

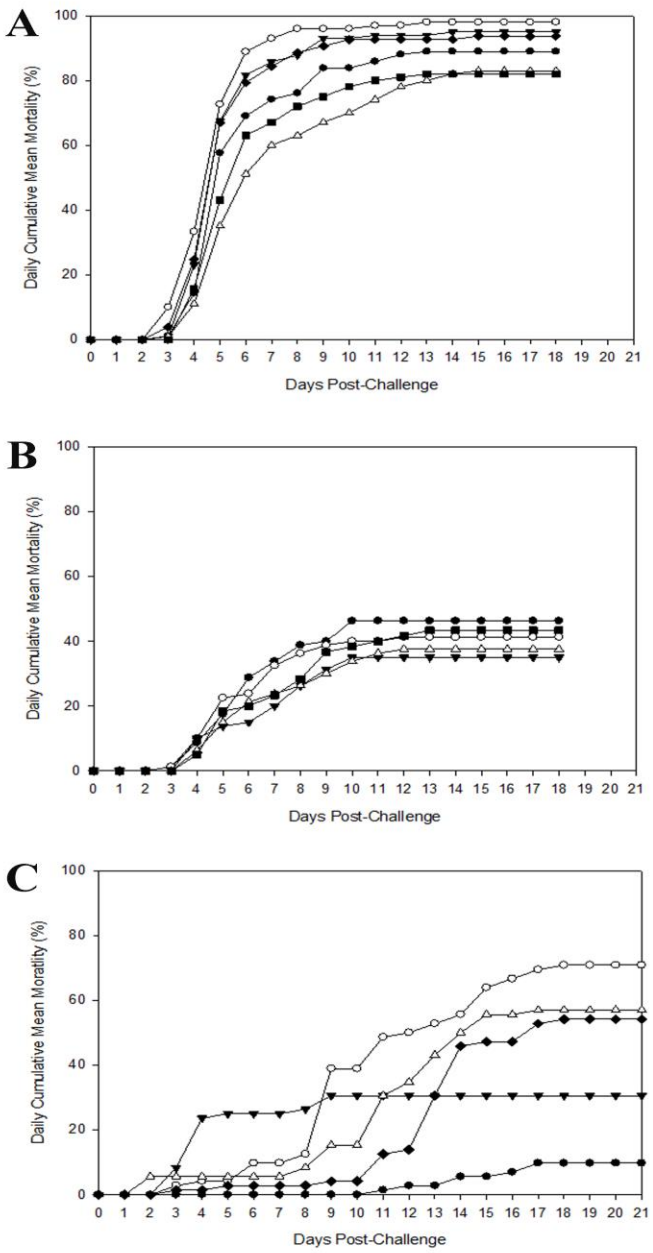

Figure 2. Daily mean cumulative mortality of **(A)** channel catfish in static system with 20-30 min daily water exchange and **(B)** channel catfish with 5-7 h flow through water daily , or **(C)** striped catfish in static system with 20-30 min daily water exchange, fed with and without addition of *Bacillus* strains and challenged with *E. ictaluri*. All values are means of four replicates per treatment. Treatments: (○) Control, (●) AP79, (▼) AP193, (△) AB01, (■) AP143, and (◆) AP254.

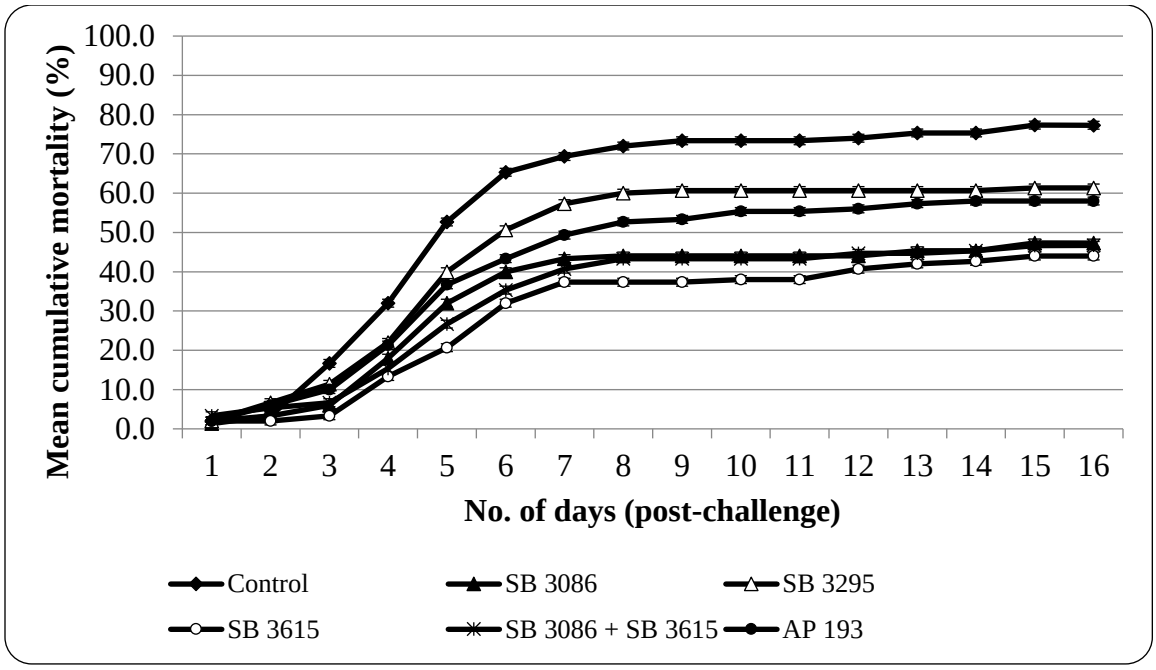

Figure 3. Mean percent cumulative mortality of Nile tilapia fed probiotic diets for 21 days and challenged with *Streptococcus iniae* using IP injection.

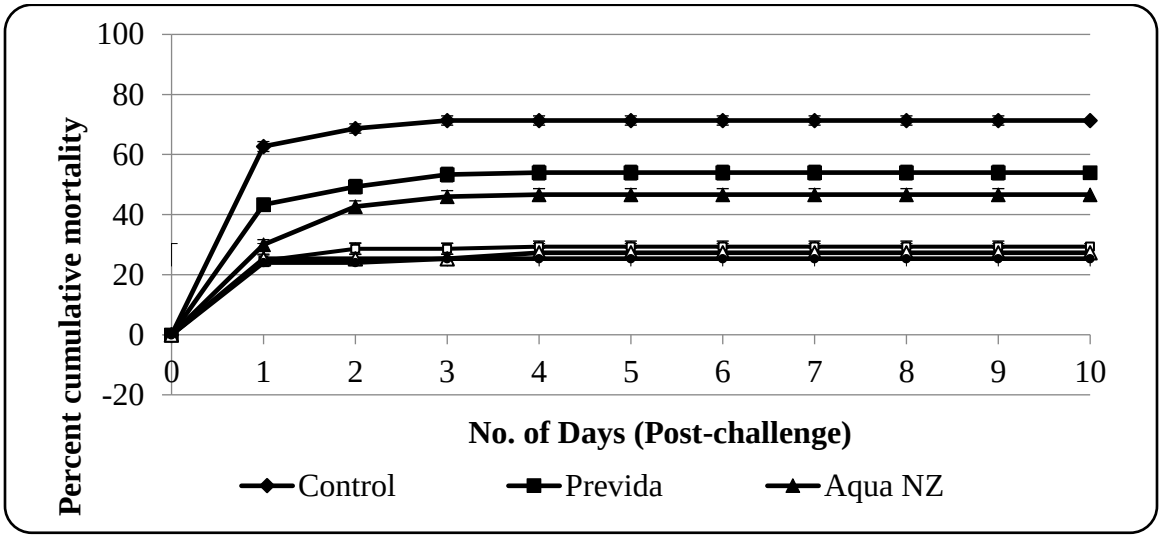

Figure 4. Percent cumulative mortality of Nile tilapia fed probiotics, prebiotic, and a mixture of both, and challenged with *A. hydrophila* by intragastric gavage.

Chapter V

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, pond water quality, and on the microbiomes in fish intestines and pond water. Firstly, four *B. velezensis* strains were evaluated for their probiotic effects and disease reduction due to *Edwardsiella ictaluri* in an aquarium study. 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. No significant

differences were observed in the catfish intestinal microbiota or the pond microbiota due to probiotic-amended feed. 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%). These data suggest that *B. velezensis* AP193 can promote catfish growth and improve pond water quality when used as a feed amendment.

2. Introduction

Due to its rapid growth rate, low cost, and proficient reproduction capabilities, the channel catfish is an economically important aquaculture species, particularly in the southeastern United States (Agriculture, 2003). 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). Phosphorus found in plant protein sources are unusable by fish, and phytate, a common storage component of phosphorus, 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 catalyses 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. 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).

Another factor responsible for significant economic losses in aquaculture is disease (Stentiford *et al.*, 2017). One traditional treatment for disease is the use of antibiotics, and there are currently only three FDA-approved treatments for use in aquaculture production facilities (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.*, 2006a; Macfarlane and Cummings, 1999; Wang *et al.*, 2008a). *Bacillus* spp. have good potential as probiotics 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; 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.

Previous research evaluated a collection of 160 *Bacillus* spp. strains for their antimicrobial activity against bacterial and fungal fish pathogens and evaluating the impact on mortality after challenge (Ran *et al.*, 2012). The 21 *Bacillus* spp. strains that showed production of secondary metabolites that inhibited the growth of fish pathogens were then tested for their survival and persistence in the catfish intestine and protection against infection by *E. ictaluri* (Ran *et al.*, 2012). In Nile tilapia (*Oreochromis niloticus*), *B. velezensis* AP193 showed protection against infection by *Aeromonas hydrophila* (Addo *et al.*, 2017b) or *Streptococcus iniae* (Addo *et al.*, 2017a). Out of the 21 strains tested, the five strains that indicated the greatest enhancement of growth and best in vitro antagonistic activity against *E. icatluri*, *S. iniae*, and *A. hydrophila* were selected for further testing. This study presents the evaluation of these probiotic strains when used as a feed additive in a 10-week aquaria trial testing their growth promoting capabilities and protective effects against E. ictaluri. Further, this study also presents the effects that feeding *B. velenzensis* AP193 has in 1) promoting the growth of channel catfish, 2) determining its impact on the microbiome in fish intestines and ponds and 3) assessing its effects on pond water quality when delivered as a feed amendment 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

accordance with U.S. welfare guidelines for the humane care and use of laboratory animals.

Bacterial 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 (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 poured 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 used.

Aquaria study conditions. Channel Catfish were obtained from Auburn University North Fisheries Unit. Each fish was roughly 4 months old and weight approximately 20 g and had no immediate history of infection and no previous history of ESC and *E. ictaluri*. Fish were fed once a day at approximately 3% of the total biomass of the fish for ten weeks. A ten-week trial was deemed necessary based on previously performed challenges. Channel catfish fingerlings were batched-weighed and placed into their respective units. 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. Fish were randomly assigned to their designated feed treatment with five replicate tanks per treatment and 30 fish per replicate tank.

Following ten weeks of probiotic feeding, the fish were evaluated for their levels of protection against *E. ictaluri* infection. Each treatment group consisted of five replicates of 60 L aquaria containing 16 fish, with the exception of the control group that only contained 4 replicate aquaria. Fish were challenged by immersion for 1 hour in 10 L of water containing 5.2×10^6 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. All remaining fish were euthanized 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.

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 pond 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 feeding behavior was observed to subside. To determine fish growth performance, 100 fish were randomly pulled from the overall population at 10 weeks and weighed. The remainder of the fish were euthanized with an overdose of MS-222 (>250 mg/l) and incinerated.

Fish intestine and pond sampling and DNA isolation. For the aquarium study, intestine samples were obtained just before the start of probiotic feeding or after ten weeks of probiotic-amended feeding. Intestinal tissue samples were obtained aseptically from the end of the stomach to the anus. Three fish were sacrificed through an overdose of MS-22 (>250 mg/l) and the intestine samples were combined to give one sample. The intestine samples were then homogenized in sterile water and frozen at -80°C until the DNA extraction was performed. For the initial time point, which consisted only of control samples, there were 23 replicates of fish intestinal samples, each containing pooled samples from three fish, used for DNA isolation. For the ten-week time point, replicate pooled intestine samples were taken from each treatment group (n=5) in each aquarium.

For the pond study, intestine samples were obtained just before the start of amended feeding and again after ten weeks of probiotic-amended feeding. Fish were sacrificed, then the intestine from the end of the stomach to the anus was aseptically removed. The intestine samples were then homogenized in sterile water, and DNA was immediately extracted. For the initial time point consisting only of control samples there were 16 independent replicates. For the ten week-post feeding initiation time point, there were eight independent replicates for both the control and AP193 groups and each sample was used for DNA isolation.

Pond water samples were collected in 50 mL sterile tubes at times zero, four and ten weeks post-feeding initiation. The samples were then filtered through a 0.2 μm filter, and DNA was extracted. For the initial time point, which consisted only of control samples there were eight replicates. For the four and ten-week post-feeding time points there were four replicates for both the control and AP193 groups. All samples were used for DNA isolation.

For both aquarium and pond studies, fish intestine DNA was isolated using a stool extraction kit (E.Z.N.A.® Stool DNA Kit, Omega Bio-Tek, Inc., Norcross, GA) according to manufacturer instructions. Pond water DNA was isolated using a water extraction kit (PowerWater® DNA Isolation Kit, MO BIO Laboratories Inc., Carlsbad, CA) according to manufacturer instructions.

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 (n=24). 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; Le and Boyd, 2012); nitrite-nitrogen by the diazotization method (Boyd, 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).

16S rRNA gene sequencing and analyses. 16S rRNA gene sequencing was conducted using an Illumina MiSeq next-generation sequencer (San Diego, CA). The V4 variable region (515bp-806bp) was PCR amplified with a unique barcode identifier included in the forward primer. The amplicons were then pooled to an equimolar concentration and the Illumina TruSeq DNA library preparation protocol was followed according to manufacturer instructions. The sequences were analyzed using the software program Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). For the aquarium study, sequences were trimmed using CLC genomics workbench (CLC bio, Boston, MA), ambiguous base calls were removed, and all sequences less than 200 bp were omitted. Sequences were then analyzed for taxa relative abundance using QIIME. Libraries were separated according to barcode, and only sequences with a minimum quality score of 10 were used. QIIME was used to identify operational taxonomic units (OTUs) by the "uclust" method with a divergence of 3% (Edgar, 2010). OTUs were aligned by to the Greengenes Core reference alignment by PyNAST (Caporaso *et al.*, 2010; DeSantis *et al.*, 2006). QIIME was then used to summarize OTUs according to treatment and generate a table of phylum relative abundance data. The relative abundance data from QIIME was used to construct graphs with GraphPad Prism (GraphPad Software, La Jolla, CA). For the pond study, sequencing was performed by Molecular Research LP (Shallowater, TX). Sequences were trimmed, ambiguous base calls were removed, and sequences less than 150bp was omitted. OTUs were generated by clustering at 3% divergence, and taxonomically classified by alignment to the Greengenes Core, RDPII, and NCBI (Agarwala *et al.*, 2016; Benson *et al.*, 2017; Cole *et al.*, 2003; DeSantis *et al.*, 2006). Additional ordination analysis of beta diversity,

specifically Principal Coordinates Analysis (PCoA) using the Bray-Curtis distance, was conducted via the Phyoseq package in R (McMurdie and Holmes, 2013) and visualized using the R packages ggplot2 (v2.2.1.9000), scales (v0.4.1) and grid (Ginestet, 2011). 16S rRNA gene sequence reads were submitted to the short read archive database at the National Center for Biotechnical Information (NCBI) within the BioProject accession # PRJNA418129, with catfish intestinal microbiome samples having accession numbers SAMN08105202 to SAMN08105264, and the pond water microbiome samples having accession numbers SAMN08016039 to SAMN08016062.

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 samples were removed from each pond sample, with one sample heat-treated at 80°C for ten minutes 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 hours at 30°C. After 24 h the CFUs/mL of pond water were determined 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 sec at 94°C, 30 sec 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. 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 (Table 2). Fish fed with a diet amended with the other three *Bacillus* strains did show an improved weight gain and FCR compared to the control (Table 2). 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 (Fig. 1A). 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% ($P = 0.07$) (Table 3). Albeit the marginal insignificance, *B. velezensis* AP193 was selected for evaluation in a replicated pond study based on the observed increase potential in promoting fish growth and disease resistance

Analysis of catfish intestinal microbiota from fish grown in aquaria

Successful PCR amplification of the V4 region of the 16S rRNA gene was achieved for all samples of AP193-, AP79-, and AP143- amended feed, while there were two successful PCR amplifications for the control, and four successful PCR amplifications for AB01-amended feed. The analysis of the intestinal microbiota from

catfish raised in replicate aquaria indicated the predominance of the bacterial phyla *Bacteroidetes*, *Firmicutes*, *Fusobacteria*, and *Proteobacteria* (Fig. 2). Similar to previously published results based on 16S rRNA gene sequences, a high relative abundance of *Fusobacteria* taxa was observed within the catfish intestinal microbiota (Larsen *et. al.*, 2014). While *Fusobacteria* taxa were dominant within fish in the control group at time zero, a significant 36% decrease in their relative abundance was observed by the ten-week time point ($P < 0.05$). In contrast, in all of the treatment groups fed with probiotic-amended feed, the relative abundance of the *Fusobacteria* taxa did not decrease to the same extent as in control fish. For all treatment groups, the relative abundance of *Proteobacteria* taxa was observed to increase over time, and this was especially evident for control fish and fish fed with strain AP143 (relative abundance increased 22%; $P < 0.05$). All significant changes in the relative abundance of bacterial taxa at the genus level are available (data not shown; Thurlow *et al.*, 2019).

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 (Fig. 1B; $P = 0.04$). 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 ($P = 0.04$) (Fig. 1B).

Analysis of catfish intestinal microbiota from fish grown in ponds

The analysis of the intestinal microbiota from catfish raised in ponds indicated the predominance of the bacterial phyla *Bacteroidetes, Firmicutes, Fusobacteria*, and *Proteobacteria* (Fig. 3A). A significant 14.9% decrease in *Bacteroidetes* taxa relative abundance was observed for fish with the *B. velezensis* AP193-amended diet relative to the initial time point ($P < 0.05$). This decrease in *Bacteroidetes* taxa included reductions in the relative abundance of the genera *Paludibacter* (decreased by 2.8%), *Parabacteroides* (decreased by 4.4%), *Barnesiella* (decreased by 5.3%) and *Dysgonomonas* (decreased by 2.7%). Additionally, *Paludibacter* and *Parabacteroides* spp. also showed significant decreases (1.4% and 1.0%, respectively) at ten weeks for fish with the AP193-amended diet as compared to the ten-week control group. All other significant changes in the relative abundance of bacterial taxa are available (data not shown; Thurlow *et al.*, 2019). There was a 20.3% increase in the relative abundance of *Proteobacteria* taxa in these probiotic-fed fish ($P < 0.05$) at ten weeks as compared to the initial time. However, while many *Proteobacteria* taxa increased in their relative abundance over time with probiotic feeding, *Pseudomonas* spp. were observed to decrease in their relative abundance over time by 2.2% in fish fed with a control diet and by 6.6% in fish fed with an AP193-amended diet (data not shown; Thurlow *et al.*, 2019).

Furthermore, there was a significant decrease of *Pseudomonas* spp. relative abundance of 4.4% when comparing the ten-week control diet with the ten-week AP193-amended diet. In both control and probiotic-fed fish, a significant decrease in the relative abundance of *Firmicutes* taxa was observed from time zero to week ten, with the control group having decreased by 16.8% and the AP193-amended diet group decreasing by 16.6%.

Additionally, the PCoA plot indicated clustering of the intestinal microbiota based on time, as can be seen when comparing initial time versus the ten week time point for the control and AP193-amended diets (Fig. 3B). However, no separation by treatment group was observed, as can be seen by the close clustering of the ten week control samples together with samples from the AP193-amended diet.

Analysis of pond water microbiota

The analysis of pond water microbiota indicated the predominance of the bacterial phyla *Fusobacteria*, *Chloroflexi*, *Firmicutes*, *Verrucomicrobia*, *Cyanobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Proteobacteria* (Fig. 4A). No significant change in the relative abundance of bacterial taxa affiliated with the above phyla was observed across treatment groups or over time. Additionally, the PCoA plot indicated clustering of the different treatments, but with no clear difference based on either time or treatment (Fig. 4B).

Detection of strain AP193 from pond water

While no AP193-like colonies were detected in pond water samples at earlier time points, at the eighth-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

There were differences between the control and probiotic fed ponds for total phosphorus, total nitrogen, and nitrate-nitrogen (data not shown; Thurlow, 2019). 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, than 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

The four best-performing *Bacillus* spp. strains (AB01, AP79, AP143 and AP193) 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.*, 2015), without any predicted virulence factors (data not shown). Each of these *B. velezensis* strains was evaluated separately for their relative degree of promoting channel catfish growth and reducing mortality due to *E. ictaluri*. While two of the treatments 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, there was a marginal insignificance observed in the relative protection

between any of the treatment groups when compared to the control. 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.*, 2015). The previous study 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.

While 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. However, it is important to note that enhanced performance observed in fish delivered *B. velenzensis* AP193 was insignificantly different from the enhanced performance observed in all the Bacillus strains tested. Additionally, none of the strains tested indicated any adverse side effects in the fish during the duration of these challenges. Based on these aquaria results, it was apparent that each of the *B. velezensis* strains may have the potential to enhance channel catfish growth performance. Even though all the *Bacillus* isolates tested during the aquaria trial indicated relative growth enhancement and disease resistance in catfish when compared to the control, the pond trials could only be carried out using one of the Bacillus isolates. Albeit the marginal insignificance, *B. velenzensis* strain AP193 was chosen as the most appropriate candidate moving forward based on an observed increase in growth performance and disease resistances compared to control. Interestingly, there

was a much more evident 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. The two-way ANOVA and Tukey Test indicated that there was a significant difference in weight gain between the fish that were fed a control diet versus fish that were fed a diet amended with AP193 spores ($P = 0.04$). 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, we believe that this difference in weight gain was due to the differences in starting weights of the two different population of fish, as the two-way ANOVA's could not confirm that the enhanced growth observed in the raceway derived fish was due to feeding *B. velenzensis* AP193 (P=0.79). Future research will explore the mechanism(s) by which *B. velezensis* AP193 and other 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 did result in improved 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). In addition, genomic analysis of *B. velezensis* AP193 indicates that this strain contains a phytase gene (Hossain *et al.*, 2015), 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*.

The addition of *B. velezensis* AP193 as a probiotic resulted in no significant changes in the intestinal microbiota, as compared to a 36% decrease in *Fusobacteria* over

time for control samples, as well as an increase of *Proteobacteria* by 38% and 22% over the ten-week study for control and AP143 treatments, respectively. This indicated that despite the addition of high levels (4×10^7 CFU/g feed) of AP193 amended to channel catfish feed, the fish intestinal microbiota remained stable throughout the ten-week study, in contrast to that observed with the control diet or even with the AP143 treatment. The absence of a decrease in the relative abundance of *Fusobacteria* taxa or an increase in *Proteobacteria* taxa in response to AP193 dietary amendment 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. *Fusobacteria* are gram-negative anaerobic, rod-shaped bacteria that produce butyrate and are known to be commensal microbiota in the channel catfish intestine. 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 in this study and in an earlier report (Larsen *et al.*, 2014). However, this study did not show as high of a relative abundance of *Fusobacteria* as was observed by this previous study. Interestingly, while the *Fusobacteria* abundance dropped precipitously within the control fish intestinal microbiota over the course of ten weeks, 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 in pond water, indicating that *Fusobacteria* taxa are natural inhabitants of the catfish gastrointestinal tract.

It is interesting to note the decrease in *Firmicutes* spp. over time in the pond study, particularly for the AP193 treatment, especially considering that *Firmicutes* spp. were added to the diet of the fish. Perhaps AP193 outcompeted its closely related bacteria for resources and/or attachment within the intestine. However, due to the low resolution of 16S rDNA analysis as it pertains to species identification, we cannot conclude based on these sequence data that AP193 was specifically detected from this ribotype analysis. The use of higher resolution phylogenetic analyses and/or strain-specific primer sets in future studies could track the relative abundance of this or other strains within the dynamic intestinal microbiota.

No significant differences were observed in the pond water microbiota based on a culture-independent based analysis of 16S rRNA gene amplicon relative abundance. Due to lower resolution of this genetic marker, we could not determine any changes in the relative abundance of *B. velezensis* or strain AP193. We therefore used a culture-dependent approach to determine the levels of *B. velenzensis* 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 *B. velezensis* AP193 to channel catfish feed resulted in an observed increase in fish growth rate compared to control feed, and these increases were particularly pronounced in a pond study. Additionally, the ponds in which feed was amended with the probiotic were observed to have improved water quality parameters. These results suggest that *B. velezensis* 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. Channel catfish growth and FCR in aquaria (N = 5).

Treatment	**Weight Gain per Fish (g) in Aquaria (Mean ± SE)**	**FCR in Aquaria**
Control	6.25 ± 0.17	1.09 ± 0.01
AB01	6.64 ± 0.36*	1.07 ± 0.04
AP79	6.58 ± 0.18*	1.04 ± 0.01
AP143	6.32 ± 0.15*	1.09 ± 0.02
AP193	6.78 ± 0.15*	1.04 ± 0.03

* $P > 0.05$ compared to control

Table 3. Mean percent mortality of channel catfish fingerlings challenged with *E. ictaluri* S97-773 after being fed *B. velezensis* probiotic strains for 10 weeks (N = 5).

Treatment	**Percent Mortality (Mean ± SE**
Control	62.1 ± 8.07
AB01	65.6 ± 3.63
AP79	54.6 ± 5.29
AP143	69.8 ± 1.61
AP193	47.8 ± 3.00*

*P = 0.07 compared to control

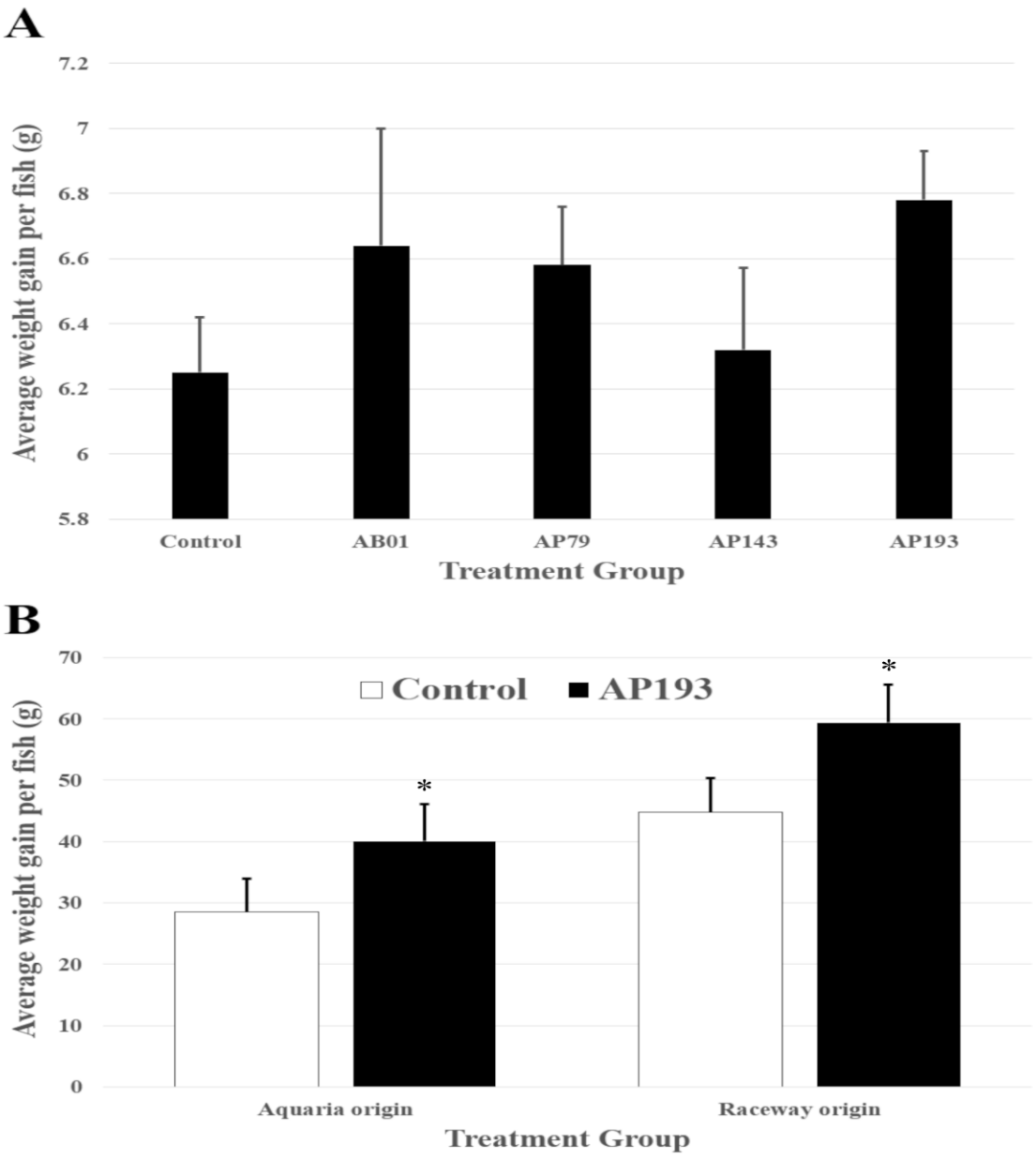

Figure 1. Growth performance of channel catfish after 10 weeks of probiotic feeding in (**Panel A**) aquaria or (**Panel B**) ponds. For the pond study fish were obtained from either aquaria or from a raceway, and fins were clipped to distinguish between the two origins.

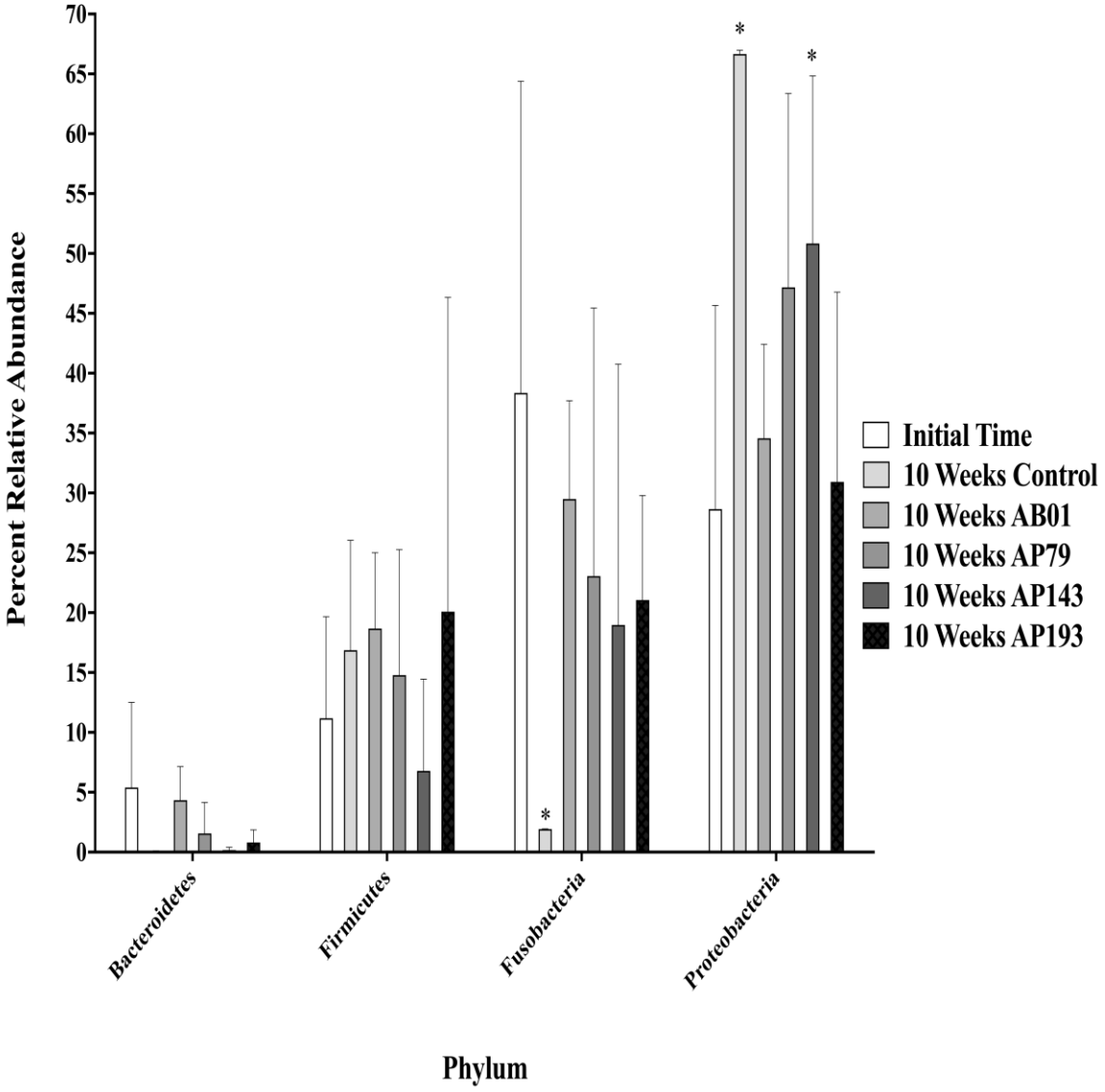

Figure 2. Phylum relative abundance based on 16S rRNA gene sequences amplified from channel catfish intestinal microbiota for the initial time and after 10 weeks in the aquarium study.

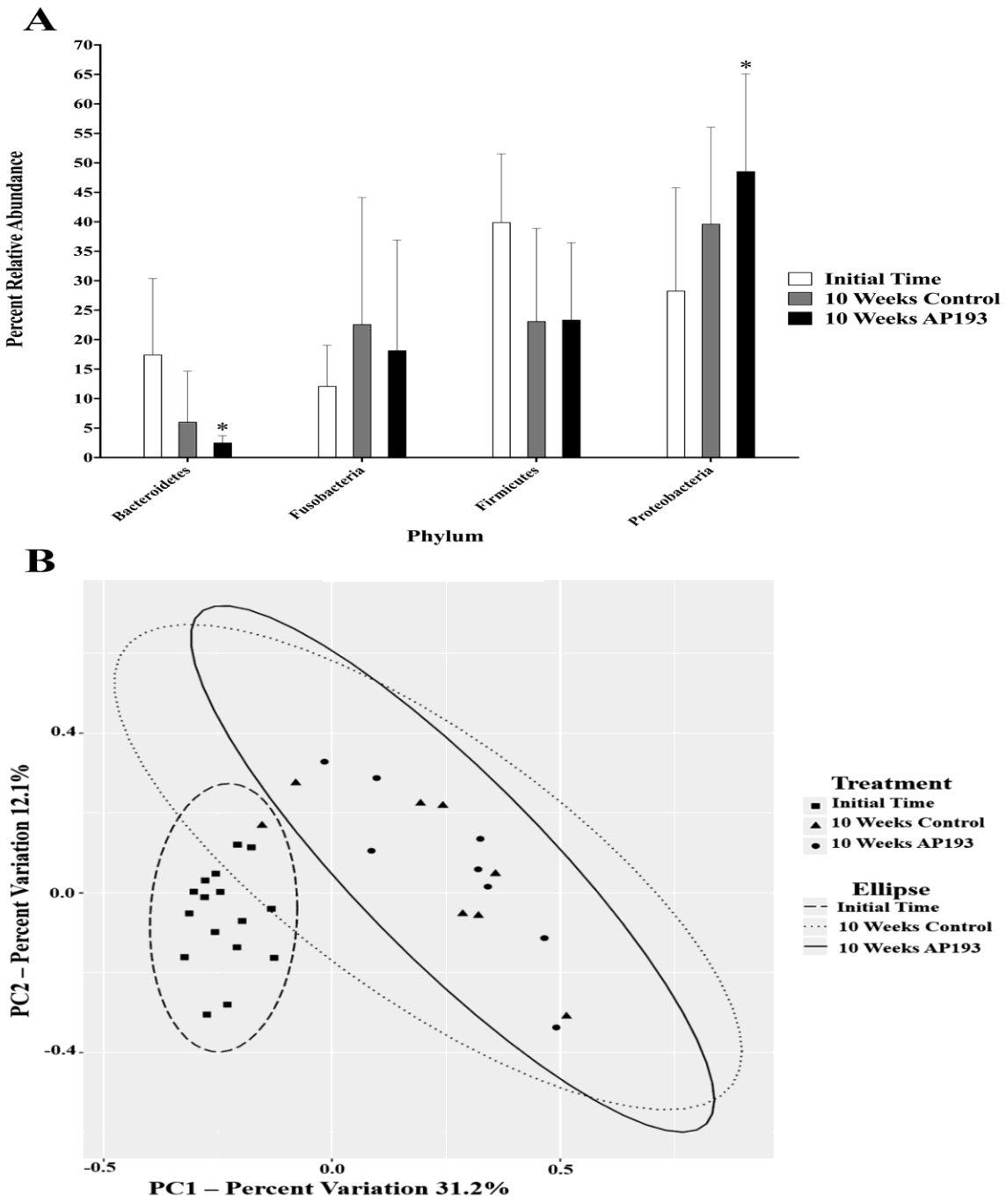

Figure 3. (**Panel A**) Phylum relative abundance based on 16S rRNA gene sequences amplified from channel catfish intestinal microbiota for the initial time and after 10 weeks of feeding in the pond study; (**Panel B**) PCoA plot with 95% confidence interval ellipses, based on Bray-Curtis distance.

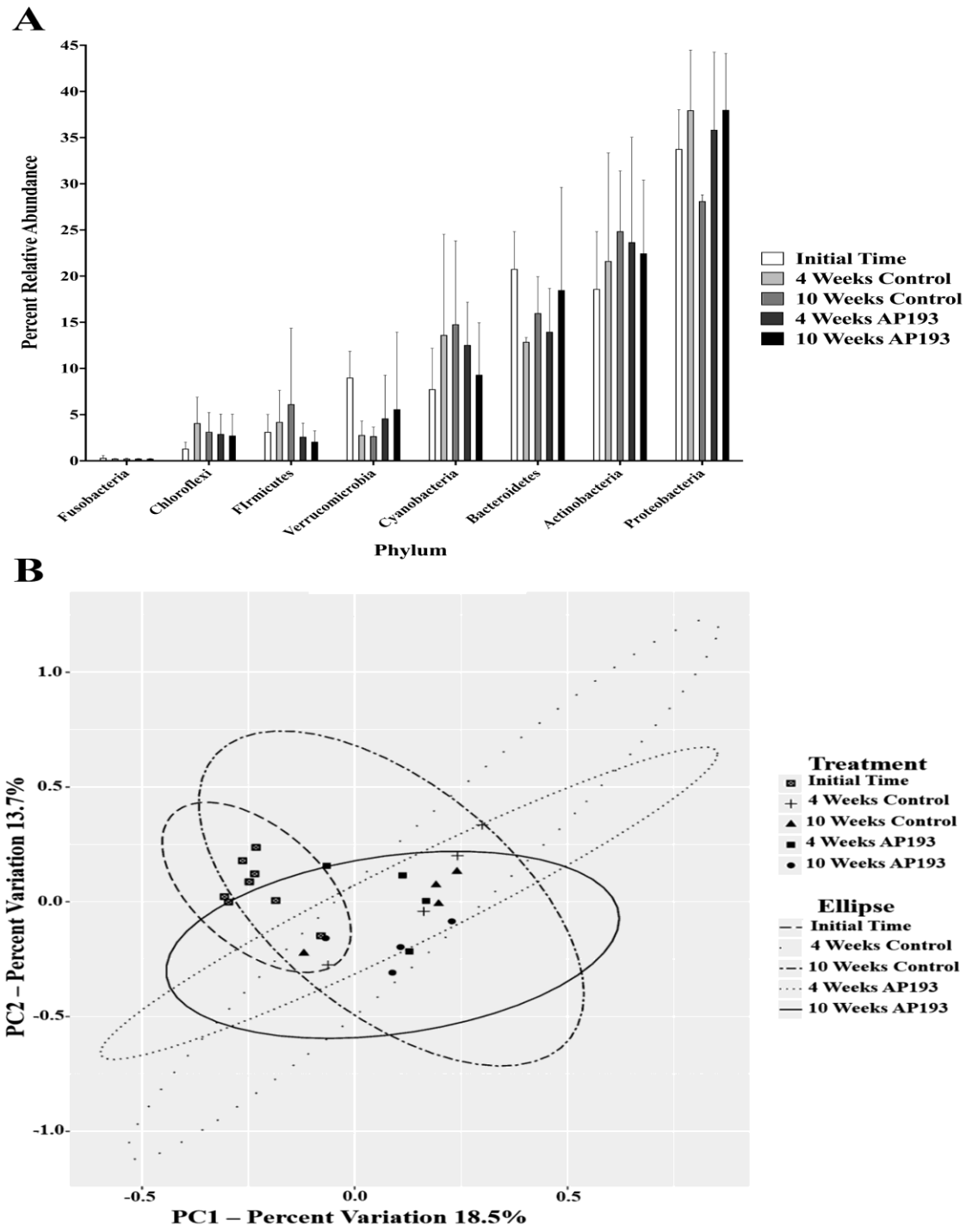

Figure 4. (**Panel A**) Phylum relative abundance based on 16S rRNA gene sequences amplified from pond water microbiota for the initial time, 4, and 10 weeks after the start of feeding; (**Panel B**) PCoA plot with 95% confidence interval ellipses, based on Bray-Curtis distance.

Chapter VI

Conclusions

This dissertation evaluates the use of bacteria as biological control agents for growth promotion and disease control in the agriculture and aquaculture industries. Several soil isolated bacteria were evaluated for their efficacy as probiotics for channel catfish, Nile tilapia, and stripped catfish; as well as PGPR for tomato plants.

PGPR

In a genetic analysis of *B. subtilis* group bacteria, it was determined that *B. subtilis* strains have high genetic diversity while *Bacillus amyloliquefaciens* strains are conserved. Further, the *B. amyloliquefaciens* clade is a part of the *B. subtilis* group and *B. velezensis* is within the *B. amyloliquefaciens* clade. Through further genetic analysis of 53 complete *B. subtilis* genomes from the GenBank database and 12 newly sequenced PGPR genomes, a core genome of *B. subtilis* was created. Further, core genomes for the *B. amyloliquefaciens* group (generated through comparative analysis of 32 complete genomes) and a core genome for *B. velezensis* (generated through comparative analysis of 28 complete genomes) were obtained. Because *B. velezensis* is well established as a PGPR species, conserved genes shared across strains can be identified that are involved in plant growth promotion and disease control. Greenhouse experiments with *B.*

velezensis AP193 and a difficidin (type of polyketide) deletion mutant correlated with the genetic data and showed that AP193 protected against bacterial spot caused by *Xanthomonas axonopodis* pv. vesicatoria in tomato plants, while the mutant was deficit. In fact, difficidin production is critical for AP193's protective effects.

Given the success seen with soil isolates as PGPR, a search for new isolates was conducted using novel techniques. A media was created using M9 minimal media plus finely homogenized soybean root tissue. The complex root carbohydrates from the root tissue to serve as a carbon source, in addition to long incubation times, allowed for the cultivation of new bacterial isolates from soybean rhizosphere samples. Those isolated were tested *in vitro* for antibiosis against a panel of plant pathogens including *Xanthomonas*. One strain stood out from the isolates, *Burkholderia gladioli* C101, which produces potent and heat stable secondary metabolites. A cell-free formulation of *B. gladioli* C101 was created to use as a foliar spray. When tested *in planta*, this spray provided protection against bacterial spot on tomato plants caused by *Xanthomonas perforans.* A cell-free formulation of *B. velezensis* AP193 was also created, and though it was effective in reducing the severity of bacterial spot, it was not as effective as the *B. gladioli* C101. The *Xanthomonas* used to challenge in this study is resistant to copper-based biocide pathogen control, so an alternative method of disease control is important.

Probiotics in aquaculture

A panel of soil isolated bacteria provided by the laboratory of Dr. Joseph Kloepper (Department of Entomology and Plant Pathology, Auburn University) was evaluated for probiotic use in the aquaculture industry. Initially, the panel was tested *in vitro* for antibiosis against the aquaculturally relevant pathogens: *Edwardsiella ictalurid,*

Aeromonas hydrophila, and *Streptococcus iniae*. *E. ictaluri* is the causative agent of enteric septicemia of catfish, an economically important disease affecting catfish farming in the Southeastern United States. *A. hydrophila* is the causative agent of motile aeromonad septicemia, a disease affecting both catfish and tilapia, among others. And finally, *S. iniae* is the causative agent of streptococcal infections in tilapia.

The top 21 strains were antagonistic to the three bacteria listed above in addition to *Yersinia ruckeri*, *Flavobacterium columnare*, and the oomycete *Saprolegnia ferax*. Additionally, survival of the 21 strains in channel catfish intestinal tissue was determined by CFU/g of tissue when fed *Bacillus* spp. spore-amended feed for seven days plus threedays of normal feed. Five top performers from the antibiosis and intestinal survival experiments were selected for further study: 1) *Bacillus velezensis* AB01, 2) *B. velezensis* AP79, 3) *B. velezensis* AP143, 4) *B. velezensis* AP193, and 5) *Bacillus subtilis AP254*. Two of these strains, *B. velezensis* AB01 and *B. velezensis* AP143, significantly reduced mortality in channel catfish when challenged with *E. ictaluri* following 14 days of feeding with *Bacillus* spore-amended feed at a dose of 8×10^7 CFU/g. In addition, *B. velezensis* AP79 conferred protection against *E. ictaluri* in a similar experiment carried out in Vietnam on stripped catfish.

Another study was carried out on Nile tilapia using *B.* velezensis AP193 and other *B. subtilis* group bacteria as probiotics amended to feed in spore form. When fed spore-amended feed of *Bacillus licheniformis* SB3086, *B. subtilis* SB3395, *B. subtilis* SB3615, or *B. velezensis* AP193, for 21 days at a dosage of 4×10^7 CFU/g of feed there was no significant change in growth as compared to control diets. However, despite no significant increase in growth, these *Bacillus* spp. probiotics did significantly ($P <=$

0.0001 vs. control) improve mortality of Nile tilapia when challenged with *S. iniae* by intraperitoneal injection at a dosage of 8 x 10^6 CFU/fish.

Furthermore *B. velezensis* AP193 was found to significantly decrease mortality in Nile tilapia when challenged with *A. hydrophila* by intragastric gavage at a dosage of 3.9 x 10^7 CFU/fish following eight weeks of feeding with amended feed. The effect was even greater when the probiotic stain was combined with Previda®, a commercial prebiotic hemicellulose extract.

Additionally, there were improvements to growth of channel catfish and pond water quality when channel catfish were fed *B. velezensis* AP193-amended feed at a dosage of 4x10^7 CFU/g of feed for 10 weeks in a pond study; while there was no significant impact on catfish intestinal microbiota or pond microbiota. There was a 40.4% and 32.6% increase in growth relative to control feed in fingerling catfish that originated from aquaria and raceways, respectively. 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%).

As a final note, it is interesting that *B. velezensis* AP193 had success as both a probiotic for fish and a PGPR. Fish feed is plant-based, often soybean-based, and contains a number of antinutrients including: phytates, glucosinolates, saponins tannins, lectins, oligosaccharides and non-starch polysaccharides, phytoestrogens, alkaloids, antigenic compounds, gossypols, cyanogens, mimosine, cyclopropenoid fatty acids, canavanine, antivitamins, and phorbol esters (Francis *et al.*, 2001). Thus, AP193's phytase activity and ability to degrade complex plant carbohydrates allow it to function

as both, an aquaculture probiotic and a PGPR; this is in addition to its production of secondary metabolites and any competitive exclusion of harmful microbes.

References

Acosta-Martinez, V., Dowd, S.E., Sun, Y., and Allen, V. (2008). Tag-encoded pyrosequencing analysis of bacterial diversity in a single soil type as affected by management and land use. Soil Biology and Biochemistry. 40(11): 2762-2770.

Addo, S., Carrias, A.A., Williams, M.A., Liles, M.R., Terhune, J.S., and Davis, D.A. (2017a). Effects of *Bacillus subtilis* Strains on Growth, Immune Parameters, and *Streptococcus iniae* Susceptibility in Nile Tilapia, *Oreochromis niloticus*. J World Aquacult Soc. 48: 257-267.

Addo, S., Carrias, A.A., Williams, M.A., Liles, M.R., Terhune, J.S., and Davis, D.A. (2017b). Effects of *Bacillus subtilis* strains and the prebiotic Previda® on growth, immune parameters and susceptibility to *Aeromonas hydrophila* infection in Nile tilapia, *Oreochromis niloticus*. Aquac Res. 48: 4798-4810.

Agarwala, R., Barrett, T., Beck, J., Benson, D.A., Bollin, C., Bolton, E., Bourexis, D., Brister, J.R., Bryant, S.H., Lanese, K., Charowhas, C., Clark, K., DiCuccio, M., Dondoshansky, I., Federhen, S., Feolo, M., Funk, K., Geer, L.Y., Gorelenkov, V., Hoeppner, M., Holmes, B., Johnson, M., Khotomlianski, V., Kimchi, A., Kimelman, M., Kitts, P., Klimke, W., Krasnov, S., Kuznetsov, A., Landrum, M.J., Landsman, D., Lee, J.M., Lipman, D.J., Lu, Z.Y., Madden, T.L., Madcj, T., Marchler-Bauer, A., Karsch-Mizrachi, I., Murphy, T., Orris, R., Ostell, J., O'Sullivan, C., Panchenko, A., Phan, L., Preuss, D.,

Pruitt, K.D., Rodarmer, K., Rubinstein, W., Sayers, E.W., Schneider, V., Schuler, G.D., Sherry, S.T., Sirotkin, K., Siyan, K., Slotta, D., Soboleva, A., Soussov, V., Starchenko, G., Tatusova, T.A., Todorov, K., Trawick, B.W., Vakatov, D., Wang, Y.L., Ward, M., Wilbur, W.J., Yaschenko, E., and Zbicz, K., Coordinators, N.R. (2016). Database resources of the National Center for Biotechnology Information. Nucleic acids research. 44: D7-D19.

Agnew, W. and Barnes, A.C. (2007). *Streptococcus iniae*: an aquatic pathogen of global veterinary significance and a challenging candidate for reliable vaccination. Veterinary Microbiology. 122: 1–15.

Agriculture, U.D.O. (2003). Part I: Reference of Fingerling Catfish Health and Production Practices in the United States. USDA, Fort Collins, CO, USA.

Ai, Q.H., Xu, H.G., Mai, K.S., Xu, W., Wang, J., and Zhang, W.B. (2011). Effects of dietary supplementation of *Bacillus subtilis* and fructooligosaccharide on growth performance, survival, non-specific immune response and disease resistance of juvenile large yellow croaker, *Larimichthys crocea.* Aquaculture. 317: 155-161.

Al-Dohail, M.A., Hashim, R., and Aliyu-Paiko, M. (2011). Evaluating the use of *Lactobacillus acidophilus* as a biocontrol agent against common pathogenic bacteria and the effects on the haematology parameters and histopathology in African catfish *Clarias gariepinus* juveniles. Aquaculture Research. 42: 196-209.

Alegre, M.T., Rodriguez, M.C., and Mesas, J.M. (2004). Transformation of Lactobacillus plantarum by electroporation with in vitro modified plasmid DNA. FEMS Microbiol Lett. 241: 73-77. doi: 10.1016/j.femsle.2004.10.006.

Allareddy, V., Martinez-Schlurmann, N., Rampa, S., Romesh, N., Allareddy, V., and Rotta, A. (2015). Incidence, Predictors, and Impact of C. Difficile and Mrsa Infections in Patients Undergoing Ecaohs. Crit Care Med. 43(12).

Aly, S.M., Ahmed, Y.A.G., Ghareeb, A.A.A., and Mohamed, M.F. (2008a). Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica*, *Oreochromis niloticus* to challenge infections. Fish and Shellfish Immunology. 25: 128-136.

Aly, S.M., Azza, M., John, G., Rahman, A-E., and Mohamed, M.F. (2008b). Characterization of some bacteria isolated from *Oreochromis niloticus* and their potential use as probiotics. Aquaculture. 277: 1–6. Open access journal.

Aly, S.M., Mohamed, M.F., and John, G. (2008c). Effect of probiotics on the survival, growth and challenge infection in Tilapia nilotica (*Oreochromis niloticus*). Aquaculture Research. 39: 647–656. Open access journal.

Anderson, D.M., Glibert, P.M., and Burkholder, J.M. (2002). Harmful algal blooms and eutrophication: Nutrient sources, composition, and consequences. Estuaries. 25: 704-726.

Apun-Molina, J.P., Santamaria-Miranda, A., Luna-Gonzalez, A., Martinez-Diaz, S.F., and Rojas-Contreras, M. (2009). Effect of potential probiotic bacteria on growth and survival of tilapia *Oreochromis niloticus L.*, cultured in the laboratory under high density and suboptimum temperature. Aquaculture Research. 40: 887-894.

Arp, J., Gotze, S., Mukherji, R., Mattern, D.J., Garcia-Altares, M., Klapper, M., Brock, D.A., Brakhage, A.A., Strassmann, J.E., Queller, D.C., Bardl, B.,

Willing, K., Peschel, G., and Stallforth, P. (2018). Synergistic activity of cosecreted natural products from amoebae-associated bacteria. Proc Natl Acad Sci USA. 115(15): 3758-3763.

Askelson, T.E., Campasino, A., Lee, J.T., and Duong, T. (2014). Evaluation of Phytate-Degrading Lactobacillus Culture Administration to Broiler Chickens. Applied and Environmental Microbiology. 80: 943-950.

Austin, B. and Austin, D.A. (2007). Bacterial fish pathogens: Diseases of farmed and wild fish, 4th edn. Chichester, UK: Springer-Praxis. 580p.

Austin, B., Stuckey, L.F., Robertson, P., Effendi, I., and Griffith, D. (1995). A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida,Vibrio anguillarum* and *Vibrio ordalii*. J Fish Dis. 18: 93-96.

Aziz, R.K., Bartels, D., Best, A.A., Dejongh, M., Disz, T., Edwards, R.A., Formsma, K., Gerdes, S., Glass, E.M., Kubal, M., Meyer, F., Olsen, G.J., Olson, R., Osterman, A.L., Overbeek, R.A., Mcneil, L.K., Paarmann, D., Paczian, T., Parrello, B., Pusch, G.D., Reich, C., Stevens, R., Vassieva, O., Vonstein, V., Wilke, A., and Zagnitko, O. (2008). The RAST server: Rapid annotations using subsystems technology. BMC Genomics. 9: 75. doi: Artn 75

Bailey, J., Blankenship, L., and Cox, N. (1991). Effect of fructooligosaccharide on *Salmonella* colonization of the chicken intestine. Poultry Science. 70: 2433-2438.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Cunningham, D., Vendrell, D., and Muzquiz, J.L. (2006a). The role of probiotics in aquaculture. Veterinary microbiology. 114: 173-186.

Balcazar, J.L., de Blas, I., Ruiz-Zarzuela, I., Vendrell, D., Calvo, A.C., and Marquez, I. (2007a). Changes in intestinal microbiota and humoral immune response following probiotic administration in brown trout, *Salmo trutta*. British Journal of Nutrition. 97: 522-527.

Balcazar, J.L., de Blas, I., Ruiz-Zazuela, I., Vandrell, D., Girones, O., and Muzquiz, J.L. (2007b). Enhancement of the immune response and protection induced by probiotic lactic acid bacteria against furunculosis in rainbow trout, *Oncorhynchus mykiss*. FEMS Immunology and Medical Microbiology. 51: 185-193.

Balcazar, J.L., Decamp, O., Vendrell, D., de Blas, I., Ruiz-Zarzuela, I. (2006b). Health and nutritional properties of probiotics in fish and shellfish. Microb Ecol Health Dis. 18: 65-70.

Benson, D.A., Cavanaugh, M., Clark, K., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Sayers, E.W. (2017). GenBank. Nucleic acids research. 45: D37-D42.

Birnboim, H.C. and Doly, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. 7: 1513-1523.

Bledsoe, J.W., Peterson, B.C., Swanson, K.S., and Small, B.C. (2016). Ontogenetic Characterization of the Intestinal Microbiota of Channel Catfish through 16S rRNA Gene Sequencing Reveals Insights on Temporal Shifts and the Influence of Environmental Microbes. PLOS ONE. 11(11): e0166379. doi:10.1371/journal.pone.0166379

Blin, K., Medema, M.H., Kazempour, D., Fischbach, M.A., Breitling, R., Takano, E., and Weber, T. (2013). antiSMASH 2.0—a versatile platform for genome mining

of secondary metabolite producers. Nucleic Acids Res. 4: 1, W204-W212. doi: 10.1093/nar/gkt449.

Blin, K., Wolf, T., Chevrette, M.G., Lu, X.W., Schwalen, C.J., Kautsar, S.A., Duran, H.G.S., Santos, E.L.C.D.L., Kim, H.U., Nave, M., Dickschat, J.S., Mitchell, D.A., Shelest, E., Breitling, R., Takano, E., Lee, S.Y., Weber, T., and Medema, M.H. (2017). antiSMASH 4.0-improvements in chemistry prediction and gene cluster boundary identification. Nucleic Acids Res. 45(W1): W36-W41.

Borriss, R., Chen, X.H., Rueckert, C., Blom, J., Becker, A., Baumgarth, B., Fan, B., Pukall, R., Schumann, P., Sproer, C., Junge, H., Vater, J., Puhler, A., and Klenk, H.P. (2011). Relationship of Bacillus amyloliquefaciens clades associated with strains DSM 7T and FZB42T: a proposal for Bacillus amyloliquefaciens subsp. amyloliquefaciens subsp. nov. and Bacillus amyloliquefaciens subsp. plantarum subsp. nov. based on complete genome sequence comparisons. Int J Syst Evol Microbiol. 61: 1786-1801. doi: 10.1099/ijs.0.023267-0.

Bower, C.E. and Holmhansen, T. (1980). A Salicylate-Hypochlorite Method for Determining Ammonia in Seawater. Can J Fish Aquat Sci. 37: 794-798.

Boyd, C.E. (1982). Managing Water-Quality in Channel Catfish Ponds. J Soil Water Conserv. 37: 207-209.

Boyd, C.E. and Gross, A. (1998). "Use of probiotics for improving soil and water quality in aquaculture ponds," in Advances in shrimp biotechnology, ed. T.W. Flegel. (National Center for Genetic Engineering and Biotechnology, Bangkok).

Boyd, C.E. and Tucker, C.S. (1992). Water Quality and Pond Soil Analyses for Aquaculture. Alabama Agricultural Experiment Station, Auburn, AL.

Brunt, J., Newaj-Fyzul, A., and Austin, B. (2007). The development of probiotics for the control of multiple bacterial diseases of rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases. 30: 573-579.

Buchanan, J.T., Stannard, J.A., Lauth, X., Ostland, V.E., Powell, H.C., Westerman, M.E., and Nizet, V. (2005). *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. Infection and Immunity. 73: 6935-6944.

Buentello, J.A., Neill, W.H., and Gatlin, D.M. III. (2010). Effects of dietary prebiotics on growth, feed efficiency and non-specific immunity of juvenile red drum, *Sciaenops ocellatus* fed soybean-based diets. Aquaculture Research. 14: 411–418.

Burkett-Cadena, M., Kokalis-Burelle, N., Lawrence, K.S., van Santen, E., and Kloepper, J.W. (2008). Suppressiveness of root-knot nematodes mediated by rhizobacteria. Biological Control. 47(1): 55-59.

Burr, G., Hume, M., Neill, W.H., and Gatlin, D. M. III. (2008). Effects of prebiotics on nutrient digestibility of soybean-meal-based diets by red drum, *Sciaenops ocellatus*. Aquaculture Research. 39: 1680–1686.

Cao, L., Wang, W.M., Yang, C.T., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z., and Li, D.P. (2007). Application of microbial phytase in fish feed. Enzyme and microbial technology. 40: 497-507.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R.,

Tumbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., and Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. Nature methods. 7: 335-336.

Carrias, A., Ran, C., Terhune, J.S., and Liles, M.R. (2012). "Bacteria and bacteriophages as biological agents for disease control in aquaculture," in Infectious disease in aquaculture: prevention and control, ed. B. Austin. (Woodhead Publishing Series in food science, technology and nutrition, No. 231). 353-393.

Case, R.J., Boucher, Y., Dahllof, I., Holstrom, C., Doolittle, W.F., and Kjelleberg, S. (2007). Use of 16S rRNA and *rpoB* genes as molecular markers for microbial ecology studies. Applied and Environmental Microbiology. 73: 278-288.

Casula, G. and Cutting, S.M. (2002). *Bacillus* probiotics: Spore germination in the gastrointestinal tract. Applied and Environmental Microbiology. 68: 2344-2352.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., and Hanage, W.P. (2015). Antibiotics in agriculture and the risk to human health: how worried should we be? Evol Appl. 8(3): 240–247. doi:10.1111/eva.12185

Chang, Z., Flatt, P., Gerwick, W.H., Nguyen, V.A., Willis, C.L., and Sherman, D.H. (2002). The barbamide biosynthetic gene cluster: a novel marine cyanobacterial system of mixed polyketide synthase (PKS)-non-ribosomal peptide synthetase (NRPS) origin involving an unusual trichloroleucyl starter unit. Gene. 296(1-2): 235-47.

Chaucheyras-Durand, F. and Durand, H. (2010). Probiotics in animal nutrition and health. Beneficial Microbes. 1: 3-9.

Chaucheyras-Durand, F., Walker, N.D., and Bach, A. (2008). Effects of active dry yeasts on the rumen microbial ecosystem: past, present and future. Animal Feed Science and Technology. 145: 5-26.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I., Morgenstern, B., Voss, B., Hess, W.R., Reva, O., Junge, H., Voigt, B., Jungblut, P.R., Vater, J., Sussmuth, R., Liesegang, H., Strittmatter, A., Gottschalk, G., and Borriss, R. (2007). Comparative analysis of the complete genome sequence of the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol. 25: 1007-1014. doi: 10.1038/nbt1325.

Chen, X.H., Scholz, R., Borriss, M., Junge, H., Mogel, G., Kunz, S., and Borriss, R. (2009). Difficidin and bacilysin produced by plant-associated Bacillus amyloliquefaciens are efficient in controlling fire blight disease. J Biotechnol. 140: 38-44. doi: 10.1016/j.jbiotec.2008.10.015.

Chen, X.H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., Koumoutsi, A., Hitzeroth, G., Grammel, N., Strittmatter, A.W., Gottschalk, G., Sussmuth, R.D., and Borriss, R. (2006). Structural and functional characterization of three polyketide synthase gene clusters in Bacillus amyloliquefaciens FZB 42. J Bacteriol. 188: 4024-4036. doi: 10.1128/JB.00052-06.

Chin, A., F., W.T., Bloemberg, G.V., Mulders, I.H., Dekkers, L.C., and Lugtenberg, B.J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. Mol Plant Microbe Interact. 13: 1340-1345.

Cho, C.Y. and Bureau, D.P. (2001). A review of diet formulation strategies and feeding systems to reduce excretory and feed wastes in aquaculture. Aquac Res. 32: 349-360.

Choi, S.K., Jeong, H., Kloepper, J.W., and Ryu, C.M. (2014). Genome Sequence of Bacillus amyloliquefaciens GB03, an Active Ingredient of the First Commercial Biological Control Product. Genome Announc. 2: 01092-01014.

Chowdhury, S.P., Hartmann, A., Gao, X.W., and Borriss, R. (2015). Biocontrol mechanism by root-associated Bacillus amyloliquefaciens FZB42-a review. Front Microbiol. 6.

Cole, J.R., Chai, B., Marsh, T.L., Farris, R.J., Wang, Q., Kulam, S.A., Chandra, S., McGarrell, D.M., Schmidt, T.M., Garrity, G.M., and Tiedje, J.M. (2003). The Ribosomal Database Project (RDP-II): previewing a new autoaligner that allows regular updates and the new prokaryotic taxonomy. Nucleic acids research. 31: 442-443.

Coppolecchia, R., DeGrazia, H., and Moran, C.P. (1991). Deletion of spoIIAB Blocks endospore formation in *Bacillus subtilis* at an early stage. Journal of Bacteriology. 173(21): 6678-6685.

Cowan, A.E., Koppel, D.E., Setlow, B. and Setlow, P. (2003). A soluble protein is immobile in dormant spores of Bacillus subtilis but is mobile in germinated spores: implications for spore dormancy. Proc Natl Acad Sci USA. 100: 4209–4214.

Da, C.T., Lundh, T., and Lindberg, J.E. (2013). Digestibility of dietary components and amino acids in animal and plant protein feed ingredients in striped catfish (Pangasianodon hypophthalmus) fingerlings. Aquaculture Nutrition. 19: 741-750.

Darling, A.C., Mau, B., Blattner, F.R., and Perna, N.T. (2004). Mauve: multiple alignment of conserved genomic sequence with rearrangements. Genome Res. 14: 1394-1403. doi: 10.1101/gr.2289704.

de Los Santos-Villalobos, S., Kremer, J.M., Parra-Cota, F.I., Hayano-Kanashiro, A.C., Garcia-Ortega, L.F., Gunturu, S.K., Tiedje, J.M., He, S.Y., and Pena-Cabriales, J.J. (2018). Draft genome of the fungicidal biological control agent Burkholderia anthina strain XXVI. Arch Microbiol. 200(5): 803-810.

De Silva, S.S., Ingram, B.A., Nguyen, P.T., Bui, T.M., Gooley, G.J., and Turchini, G.M. (2010). Estimation of nitrogen and phosphorus in effluent from the striped catfish farming sector in the Mekong Delta, Vietnam. Ambio. 39: 504-514.

Defoirdt, T., Sorgeloos, P., and Bossier, P. (2011). Alternatives to antibiotics for the control of bacterial disease in aquaculture. Current Opinion in Microbiology. 14: 251–258.

Dekkers, L.C., Mulders, I.H., Phoelich, C.C., Chin, A.W.T.F., Wijfjes, A.H., and Lugtenberg, B.J. (2000). The sss colonization gene of the tomato-Fusarium oxysporum f. sp. radicis-lycopersici biocontrol strain Pseudomonas fluorescens WCS365 can improve root colonization of other wild-type pseudomonas spp.bacteria. Mol Plant Microbe Interact. 13: 1177-1183. doi: 10.1094/MPMI.2000.13.11.1177.

Delcenserie, V., Martel, D., Lamoureux, M., Amiot, J., Boutin, Y., and Roy, D. (2008). Immunomodulatory effects of probiotics in the intestinal tract. Current Issues in Molecular Biology. 10: 37-54.

DeLorenze, G.N., Horberg, M.A., Silverberg, M.J., Tsai, A., Quesenberry, C.P., and Baxter, R. (2013). Trends in annual incidence of methicillin-resistant Staphylococcus aureus (MRSA) infection in HIV-infected and HIV-uninfected patients. Epidemiol Infect. 141(11): 2392-2402.

Denev, S., Staykov, Y., Moutafchieva, R., and Beev, G. (2009). Microbial ecology of the gastrointestinal tract of fish and the potential application of probiotics and prebiotics in finfish aquaculture. International Aquatic Research. 1: 1-29.

Derek, S.L., Lebeis, S.L., Paredes, S.H., Yourstone, S., Gehring, J., Malfatti, S., Tremblay, J., Engelbrektson, A., Kunin, V., Glavina del Rio, T., Edgar, R.C., Eickhorst, T., Ley, R.E., Hugenholtz, P., Tringe, S.G., Dangl, J.L. (2012). Defining the core *Arabidopsis thaliana* root microbiome. Nature. 488(7409): 86-90.

DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G.L. (2006). Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Applied and Environmental Microbiology. 72: 5069-5072.

Dietel, K., Beator, B., Budiharjo, A., Fan, B., and Borriss, R. (2013). Bacterial Traits Involved in Colonization of Arabidopsis thaliana Roots by *Bacillus amyloliquefaciens* FZB42. Plant Pathol J. 29: 59-66.

Driks, A. (2002). Overview: development in bacteria: spore formation in Bacillus subtilis. Cell Mol Life Sci. 59: 389–391.

Dunlap, C.A., Kim, S.J., Kwon, S.W., and Rooney, A.P. (2016). *Bacillus velezensis* is not a later heterotypic synonym of Bacillus amyloliquefaciens; Bacillus methylotrophicus, Bacillus amyloliquefaciens subsp plantarum and 'Bacillus oryzicola' are later heterotypic synonyms of Bacillus velezensis based on phylogenomics. International journal of systematic and evolutionary microbiology. 66: 1212-1217.

Ebrahimi, Gh., Ouraji, H., Khalesi, M.K., Sudagar, M., Barari, A., Zarei Dangesaraki, M., and JaniKhalili, K.H. (2011). Effects of a prebiotic Immunogen®, on feed utilization, body composition, immunity and resistance to *Aeromonas hydrophila* infection in the common carp, *Cyprinus carpio* (Linnaeus) fingerlings. Journal of Animal Physiology and Animal Nutrition. 96: 591–599.

Edgar, R.C. (2010). Search and clustering orders of magnitude faster than BLAST. Bioinformatics. 26: 2460-2461.

El-Haroun, E.R., Goda, A.M. A-S., and Kabir Chowdhury, M.A. (2006). Effect of dietary probiotic biogens supplementation as a growth promoter on growth performance and feed utilization of Nile tilapia *Oreochromis niloticus* (L.). Aquaculture Research. 37: 1473–1480.

El-Rhman, A.M.A., Khattab, Y.A., and Shalaby, A.M. (2009). *Micrococcus luteus* and *Pseudomonas* species as probiotics for promoting the growth performance and health of Nile tilapia, *Oreochromis niloticus*. Fish and Shellfish Immunology. 27: 175-180.

Emmert, E.a.B., Klimowicz, A.K., Thomas, M.G., and Handelsman, J. (2004). Genetics of Zwittermicin A Production by *Bacillus* cereus. Appl Environ Microbiol. 70: 104-113. doi: 10.1128/aem.70.1.104-113.2004.

Essa, M.A., El-Serafy, S.S., El-Ezabi, M.M., Daboor, S.M., and Esmael, N.A. (2010). Effect of different dietary probiotics on growth, feed utilization and digestive enzymes activities of Nile tilapia, Oreochromis niloticus. Journal of Arabian Aquaculture Society. 5: 143-161.

Fabret, C., Dusko Ehrlich, S., and Noirot, P. (2002). A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol Microbiol. 46: 25-36. doi: 10.1046/j.1365-2958.2002.03140.x.

Facklam, R., Elliott, J., Shewmaker, L., and Reingold, A. (2005). Identification and characterization of sporadic isolates of *Streptococcus iniae* isolated from humans. Journal of Clinical Microbiology. 43: 933–937.

Fan, B., Blom, J., Klenk, H.P., and Borriss, R. (2017). *Bacillus amyloliquefaciens, Bacillus velezensis*, and *Bacillus siamensis* Form an "Operational Group *B. amyloliquefaciens*" within the *B. subtilis* Species Complex. Frontiers in Microbiology. 8: 22. doi:10.3389/fmicb.2017.00022

Fan, B., Chen, X.H., Budiharjo, A., Bleiss, W., Vater, J., and Borriss, R. (2011). Efficient colonization of plant roots by the plant growth promoting bacterium *Bacillus amyloliquefaciens* FZB42, engineered to express green fluorescent protein. J Biotechnol. 151: 303-311. doi: 10.1016/j.jbiotec.2010.12.022.

Fang, H.M., Ge, R., and Sin, Y.M. (2004). Cloning, characterization and expression of *Aeromonas hydrophila* major adhesion. Fish and Shellfish Immunology. 16: 645–658.

FAO (Food and Agriculture Organization). (2001). Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Online: http://ftp.fao.org/es/esn/food/probio_report_en.pdf.

FAO (Food and Agriculture Organization). (2007). FAO Technical meeting on prebiotics. FAO: Rome. September 15-16, 2007. http://www.fao.org-/ag/agn/agns/files/Prebiotics_Tech_ Meeting_ Report.pdf.

FAO (Food and Agriculture Organization). (2012). The state of world fisheries and aquaculture 2012. Food and Agriculture Organization FAO, Rome, 209.

Farmer, B. (2004). Improved methods for the isolation and characterization of *Flavobacterium columnare*. Thesis. Baton Rouge, LA: Department of Pathobiological Sciences, Louisiana State University.

Fellas, A., Lindenlaub, P., Tammer, I., and Gollnick, H. (2011). Comparison of the Incidence of MRSA and MRSA Resistance Profiles of Primary Isolates of Inpatiens at the University Skin Clinic of Magdeburg in the Years 2000 to 2009. Journal Der Deutschen Dermatologischen Gesellschaft. 9: 223-223.

Felsenstein, J. (1981). Evolutionary trees from DNA sequences: a maximum likelihood approach. J Mol Evol. 17: 368-376.

Ferguson, R.M.W., Merrifield, D.L., Harper, G.M., Rawling, M.D., and Mustafa, S. (2010). The effect of *Pediococcus acidilactici* on the gut microbiota and immune

status of on-growing red tilapia, *Oreochromis niloticus*. Journal of Applied Microbiology. 109: 851-862.

Ficke, A., Cowger, C., Bergstrom, G., and Brodal, G. (2018). Understanding Yield Loss and Pathogen Biology to Improve Disease Management: Septoria Nodorum Blotch - A Case Study in Wheat. Plant Disease. 102(4): 696-707

Fowler, J.R., Greenhill, D., Schaffer, A.A., Thoder, J.J., and Ilyas, A.M. (2013). Evolving Incidence of MRSA in Urban Hand Infections. Orthopedics. 36(6): 796-800.

Francis, G., Makkar, H.P.S., and Becker, K. (2001). Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. Aquaculture. 199(3-4): 197-227.

Friis, C., Wassenaar, T.M., Javed, M.A., Snipen, L., Lagesen, K., Hallin, P.F., Newell, D.G., Toszeghy, M., Ridley, A., Manning, G., and Ussery, D.W. (2010). Genomic characterization of *Campylobacter jejuni* strain M1. PLOS ONE. 5: e12253. doi: 10.1371/journal.pone.0012253.

Fritze, D. (2004). Taxonomy of the Genus *Bacillus* and Related Genera: The Aerobic Endospore-Forming Bacteria. Phytopathology. 94: 1245-1248.

Gatesoupe, F. J. (2005). Probiotics and prebiotics for fish culture, at the parting of the ways. Aqua Feeds: Formulation and Beyond. 2: 3-5.

Gatesoupe, F.J. (1999). The use of probiotics in aquaculture. Aquaculture. 180: 147-165.

Gaunt, P.S., Endris, R., McGinnis, A., Baumgartner, W., Camus, A., Steadman, J., Sweeney, D., and Sun, F. (2010). Determination of florfenicol dose rate in feed

for control of mortality in Nile tilapia Infected with *Streptococcus iniae*, Journal of Aquatic Animal Health. 22: 158-166.

Gerardo, N.A., Sanuth, B., and Khudeira, Z. (2014). Incidence of methicillin-resistant Staphylococcus aureus (MRSA) pneumonia infection in patients with nares-positive cultures compared to nares-negative cultures for MRSA in a medical intensive care unit. Pharmacotherapy. 34(6): E79-E79.

Gessmann, J., Kammler, J., Schildhauer, T.A., and Kaminski, A. (2012). MRSA colonisation in patients with proximal femur fractures in a German trauma centre: incidence, infection rates and outcomes. Langenbecks Archives of Surgery. 397(1): 117-123.

Ghazalah, A.A., Ali, H.M., Gehad, E.A., Hammouda, Y.A., and Abo-State, H.A. (2010). Effect of probiotics on performance and nutrients digestibility of Nile tilapia (*Oreochromis niloticus*) fed low protein diets. Nature and Science. 8: 46-53.

Ghosh, S., Sinha, A., and Sahu, C. (2007). Effect of probiotic on reproductive performance in female livebearing ornamental fish. Aquaculture Research. 38(5): 518–526.

Gibson, G.R. and Roberfroid, M.B. (1995). Dietary modulation of the human colon microbiota: introducing the concept of prebiotics. Journal of Nutrition. 125: 1401-1412.

Gildberg, A. and Mikkelsen, H. (1998). Effects of supplementing the feed to Atlantic cod (*Gadus morhua*) fry with lactic acid bacteria and immuno-stimulating

peptides during a challenge trial with *Vibrio anguillarum*. Aquaculture. 167: 103-113.

Ginestet, C. (2011). ggplot2: Elegant Graphics for Data Analysis. J R Stat Soc a Stat. 174: 245-245.

Giovannoni, S.J., Britschgi, T.B, Moyer, C.L., and Field, K.G. (1990). Genetic diversity in Sargasso Sea bacterioplankton. Nature. 345: 60–63.

Goncalves, A.T., Maita, M., Futami, K., Endo, M., and Katagiri, T. (2011). Effects of a probiotic bacterial *Lactobacillus rhamnosus* dietary supplement on the crowding stress response of juvenile Nile tilapia, *Oreochromis niloticus*. Fisheries Science. 77: 633–642.

Goris, J., Konstantinidis, K.T., Klappenbach, J.A., Coenye, T., Vandamme, P., and Tiedje, J.M. (2007). DNA-DNA hybridization values and their relationship to whole-genome sequence similarities. Int J Syst Evol Microbiol. 57: 81-91.

Gournier-Chateau, N., Larpent, J.P., Castellanos, I., and Larpent, J.L. (1994). Les Probiotiques en Alimentation Animale et Humaine. Paris: Technique et Documentation Lavoisier. 192.

Grimoud, J., Durand, H., Courtin, C., Monsan, P., Ouarné, d F., Theodoroue, V., and Roques, C. (2010). In vitro screening of probiotic lactic acid bacteria and prebiotic glucooligosaccharides to select effective synbiotics. Anaerobe. 16: 493-500.

Grisdale-Helland, B., Helland, S.J., and Gatlin, D.M. (2008). The effects of dietary supplementation with mannanoligosaccharide, fructooligosaccharide or

galactooligosaccharide on the growth and feed utilization of Atlantic salmon, *Salmo salar*. Aquaculture. 283: 163-167.

Gross, A., Boyd, C.E., and Seo, J.W. (1999). Evaluation of the ultraviolet spectrophotometric method for the measurement of total nitrogen in water. J World Aquacult Soc. 30: 388-393.

Gutowska, M.A., Drazen, J.C., and Robison, B.H. (2004). Digestive chitinolytic activity in marine fishes of Monterey Bay, California. Comp Biochem and Physiol, Part A. 139: 351-358.

Handelsman, J. (2005). Metagenomics: Application of genomics to uncultured microorganisms (vol 68, pg 669, 2004). Microbiology and Molecular Biology Reviews. 69(1): 195-195.

Hanson, L.A., Liles, M.R., Hossain, M.J., Griffin, M.J., and Hemstreet, W.G. (2014). 1.2.9 Motile Aeromonas Septicemia. http://afs-fhs.org/perch/resources/1.2.9-motile-aeromonas-septicemia-2014.pdf.

Hao, K., He, P., Blom, J., Rueckert, C., Mao, Z., Wu, Y., He, Y., and Borriss, R. (2012). The genome of plant growth promoting *Bacillus amyloliquefaciens* subsp. *plantarum* strain YAU B9601-Y2 contains a gene cluster for mersacidin synthesis. J Bacteriol. 194: 3264-3265. doi: 10.1128/JB.00545-12.

Harikrishnan, R., Nisha Rani, M., and Balasundaram, C. (2003). Hematological and biochemical parameters in common carp, *Cyprinus carpio*, following herbal treatment for *Aeromonas hydrophila* infection. Aquaculture. 221: 41-50.

Hawke, J.P. (1979). A bacterium associated with disease of pond cultured channel catfish, *Ictalurus punctatus*. J Fish Res Board Can. 36: 1508–1512.

Hawke, J.P. and Khoo, L.H. (2004). "Infectious diseases," in Biology and Culture of Channel Catfish, ed. C.S. Tucker and J.A. Hargreaves. (Amsterdam, The Netherlands: Elsevier). 387-443.

He, P., Hao, K., Blom, J., Ruckert, C., Vater, J., Mao, Z., Wu, Y., Hou, M., He, P., He, Y., and Borriss, R. (2012). Genome sequence of the plant growth promoting strain Bacillus amyloliquefaciens subsp. plantarum B9601-Y2 and expression of mersacidin and other secondary metabolites. J Biotechnol. 164: 281-291.

Heil, M. and Bostock, R. M. (2002). Induced systemic resistance (ISR) against pathogens in the context of induced plant defenses. Annals of Botany. 89: 503-512.

Hiltner, L. (1904). Ueber neuere Erfahrungen und Probleme auf dem Gebiete der Bodenbakteriologie und unter besonderer BerUcksichtigung der Grundungung und Brache. Arb. Deut. Landw. Gesell. 98: 59-78.

Hoffmann, T., Muller, S., Nadmid, S., Garcia, R., and Muller, R. (2013). Microsclerodermins from terrestrial myxobacteria: an intriguing biosynthesis likely connected to a sponge symbiont. J Am Chem Soc. 135(45): 16904-11.

Hong, H.A., Duc, L.H., and Cutting, S.M. (2005). The use of bacterial spore formers as probiotics. FEMS Microbiology Reviews. 29: 813-835.

Honsheng, M. (2010). Effects of probiotics on activities of digestive enzyme for tilapia. Journal of South China Normal Univ. 2: 10-15.

Horton, R.M., Hunt, H.D., Ho, S.N., Pullen, J.K., and Pease, L.R. (1989). Engineering hybrid genes without the use of restriction enzymes: gene splicing by overlap extension. Gene. 77: 61-68.

Hossain M.J., Sun, D., McGarey, D.J., Wrenn, S., Alexander, L.M., Martino, M.E., Xing, Y., Terhune, J.S., and Liles, M.R. (2014). An Asian Origin of Virulent *Aeromonas hydrophila* Responsible for Disease Epidemics in United States-Farmed Catfish. http://mbio.asm.org/content/5/3/e00848-14.short.

Hossain, M.J., Ran, C., Liu, K., Ryu, C.M., Rasmussen-Ivey, C.R., Williams, M.A., Hassan, M.K., Choi, S.K., Jeong, H., Newman, M., Kloepper, J.W., and Liles, M.R. (2015). Deciphering the conserved genetic loci implicated in plant disease control through comparative genomics of Bacillus amyloliquefaciens subsp plantarum. Frontiers in plant science. 6: 631.

Hossain, M.J., Waldbieser, G.C., Sun, D., Capps, N.K., Hemstreet, W.B., Carlisle, K., Griffin, M.J., Khoo, L., Goodwin, A.E., Sonstegard, T.S., Schroeder, S., Hayden, K., Newton, J.C., Terhune, J.S., and Liles, M.R. (2013). Implication of lateral genetic transfer in the emergence of Aeromonas hydrophila isolates of epidemic outbreaks in channel catfish. PLOS ONE. 8: e80943. doi: 10.1371/journal.pone.0080943.

Howe, C.G. (1921). Pectic Material in Root Hairs. JSTOR. 72: 313-320.

Hugouvieux-Cotte-Pattat, N. and Robert-Baudouy, J. (1987). Hexuronate Catabolism in *Erwinia chrysanthemi*. Journal of Bacteriology. 169: 1223-1231.

Hui-Yuan, L., Zhigang, Z., Rudeaux, F., and Respondek, F. (2007). Effects of dietary short chain fructo-oligosaccharides on intestinal microflora, mortality and growth performance of *Oreochromis aureus* x *O. niloticus*. Chinese Journal of Animal Nutrition. 19: 1–6.

Hyronimus, B., Le Marrec, C., Hadji Sassi, A., and Deschamps, A. (2000). Acid and bile tolerance of spore-forming lactic acid bacteria. Int J Food Microbiol. 61: 193–197.

Ibal, J.C., Pham, H.Q., Park, C.E., and Shin, J.H. (2019). Information about variations in multiple copies of bacterial 16S rRNA genes may aid in species identification. PLOS ONE. 14(2): e0212090.

Idriss, E.E., Makarewicz, O., Farouk, A., Rosner, K., Greiner, R., Bochow, H., Richter, T., and Borriss, R. (2002). Extracellular phytase activity of Bacillus amyloliquefaciens FZB45 contributes to its plant-growth promoting effect. Microbiology. 148: 2097-2109.

Intrakamhaeng, M., Komutarin, T., Pimpukdee, K., and Aengwanich, W. (2012). Incidence of Enterotoxin-Producing MRSA in Bovine Mastitis Cases, Bulk Milk Tanks and Processing Plants in Thailand. Journal of Animal and Veterinary Advances. 11(5): 87-93.

Irianto, A. and Austin, B. (2002). Use of probiotics to control furunculosis in rainbow trout, *Oncorhynchus mykiss* (Walbaum). J Fish Dis. 25: 333-342.

Irianto, A. and Austin, B. (2003). Use of dead probiotic cells to control furunculosis in rainbow trout, *Onchorhynchus mykiss* (Walbaum). Journal of Fish Diseases. 26: 59-62.

Jacoby, R.P., Martyn, A., and Kopriva, S. (2018). Exometabolomic Profiling of Bacterial Strains as Cultivated Using Arabidopsis Root Extract as the Sole Carbon Source. Molecular Plant-Microbe Interactions. 31(8): 803-813.

Jeong, H., Choi, S.K., Kloepper, J.W., and Ryu, C.M. (2014). Genome Sequence of the Plant Endophyte Bacillus pumilus INR7, Triggering Induced Systemic Resistance in Field Crops. Genome Announc. 2: 01093-01014.

Jeong, H., Jeong, D.-E., Kim, S.H., Song, G.C., Park, S.-Y., Ryu, C.-M., Park, S.-H., and Choi, S.-K. (2012). Draft Genome Sequence of the Plant Growth Promoting Bacterium *Bacillus siamensis* KCTC 13613T. J Bacteriol. 194: 4148-4149. doi: 10.1128/jb.00805-12.

Johnson-Henry, K.C., Donato, K.A., Dhen-Tu, G., Gordanpour, M., and Sherman, P.M. (2008). *Lactobacillus rhamnosus* strain GG prevents entero-hemorrhagic *Escherichia coli* O157:H7-induced changes in epithelial barrier function. Infection and Immunity. 76: 1340-1348.

Joseph, S.J., Hugenholtz, P., Sangwan, P., Osborne, C.A., and Janssen, P.H. (2003). Laboratory cultivation of widespread and previously uncultured soil bacteria. Applied and Environmental Microbiology. 69(12): 7210-7215.

Ju, J., Lim, S.K., Jiang, H., and Shen, B. (2005). Migrastatin and dorrigocins are shunt metabolites of iso-migrastatin. J Am Chem Soc. 127(6): 1622-3.

JÖborn, A., Olsson, J.C., Westerdah, M.A., Conway, P.L., and Kjelleberg, S. (1997). Colonization in the fish intestinal tract and production of inhibitory substances in intestinal mucus and faecal extracts by *Carnobacterium* sp. strain K1. J Fish Dis. 20: 383-392.

Kajita, Y., Sakai, M., Atsuta, S., and Kobayash, M. (1990). The immunostimulatory effects of levamisole on rainbow trout, *Oncorhnchus mykiss*. Fish Pathology. 25: 93–98.

Kamilova, F., Validov, S., Azarova, T., Mulders, I., and Lugtenberg, B. (2005). Enrichment for enhanced competitive plant root tip colonizers selects for a new class of biocontrol bacteria. Environ Microbiol. 7: 1809-1817.

Kenney, D.S. and Couch, T.L. (1981). "Mass production of biological agents for plant disease, weed and insect control," in Biological Control in Crop Production BARC Symposium No. 5, ed. G.C. Papavizas. (Totowa, NJ: Allenheld and Osmum). 143-150.

Kim, B.K., Chung, J.H., Kim, S.Y., Jeong, H., Kang, S.G., Kwon, S.K., Lee, C.H., Song, J.Y., Yu, D.S., Ryu, C.M., and Kim, J.F. (2012). Genome sequence of the leaf-colonizing Bacterium *Bacillus* sp. strain 5B6, isolated from a cherry tree. J Bacteriol. 194: 3758-3759. doi: 10.1128/JB.00682-12.

Kloepper, J.W. and Schroth, M.N. (1978). Plant growth promoting rhizobacteria on radishes. Proceedings of the 4th International Conference on Plant Pathogenic Bacteria. Station de Pathologie Végétale et Phytobactériologie, INRA, Angers, France. 2: 879–882.

Kloepper, J.W., Leong, J., Teintze, M., and Schroth, M.N. (1981). Enhanced Plant-Growth by Siderophores Produced by Plant Growth Promoting Rhizobacteria. Phytopathology. 71(2): 231-231.

Kloepper, J.W., Lifshtz, R., and Zablotowicz, R.M. (1989). Free-living bacterial inocula for enhancing crop productivity. Trends in Biotechnology 7(2): 39-44.

Kloepper, J.W., Reddy, M.S., Kenney, D.S., Vavrina, C., Kokalis-Burelle, N., and Martinez-Ochoa, N. (2004). "Theory and applications of rhizobacteria for transplant production and yield enhancement. Proc. XXVI IHC – Transplant

Production and Stand Establishment," in Acta Hort., eds. S. Nicola, J. Nowak, and C.S. Vavrina. 631:217-229.

Kloepper, J.W., Ryu, C.-M., Zhang, S. (2004). Induced systemic resistance and promotion of plant growth by *Bacillus* spp. Phytopathology. 94: 1259-1266.

Knief, C. (2014). Analysis of plant microbe interactions in the era of next generation sequencing technologies. Frontiers in Plant Science. 5: article216.

Koh, T.H., Kurup, A., and Chen, J. (2004). *Streptococcus iniae* discitis in Singapore. Emerging Infectious Diseases. 10: 1694–1696.

Kokalis-Burelle, N., Vavrina, C.S., Reddy, M.S., and Kloepper, J.W. (2003). Amendment of muskmelon transplant media with plant growth promoting rhizobacteria: effects on seedling quality, disease, and nematode resistance. Hortechnology. 13: 476-482.

Kokalis–Burelle, N., Vavrina, C.S., Rosskopf, E.N., and Shelby, R.A. (2002). Field evaluation of plant growth promoting Rhizobacteria amended transplant mixes and soil solarization for tomato and pepper production in Florida. Plant and Soil. 238: 257-266.

Koumoutsi, A., Chen, X.-H., Henne, A., Liesegang, H., Hitzeroth, G., Franke, P., Vater, J., and Borriss, R. (2004). Structural and Functional Characterization of Gene Clusters Directing Nonribosomal Synthesis of Bioactive Cyclic Lipopeptides in *Bacillus amyloliquefaciens* Strain FZB42. J Bacteriol. 186: 1084-1096. doi: 10.1128/jb.186.4.1084-1096.2004.

Kumar, R., Mukherjee, S.C., Ranjan, R., and Nayak, S.K. (2008). Enhanced innate immune parameters in *Labeo rohita* (Ham.) following oral administration of *Bacillus subtilis*. Fish Shellfish. Immunol. 24: 168–172.

Kumar, S., Sahu, N.P., Pal, A.K., Choudhury, D., Yengkokpam, S., and Mukherjee, S.C. (2005). Effect of dietary carbohydrate on haematology, respiratory burst activity and histological changes in *L. rohita* juveniles. Fish Shellfish Immunology. 19: 331–344.

Kumar, V., Sinha, A.K., Makkar, H.P.S., De Boeck, G., and Becker, K. (2012). Phytate and phytase in fish nutrition. Journal of animal physiology and animal nutrition. 96: 335-364.

Kunakom, S. and Eustaquio, A.S. (2019). Burkholderia as a Source of Natural Products. Journal of Natural Products.

La Ragione, R.M. and Woodward, M.J. (2003). Competitive exclusion by *Bacillus subtilis* spores of *Salmonella enterica* serotype *enteritidis* and *Clostridium perfringens* in young chickens. Veterinary Microbiology. 94: 245-256.

La Ragione, R.M., Narbad, A., Gasson, M.J., and Woodward, M.J. (2004). In vivo characterization of *Lactobacillus johnsonii* FI9785 for use as a defined competitive exclusion agent against bacterial pathogens in poultry. Letters in Applied Microbiology. 38: 197-205.

Lacroix, C. and Yildirim, S. (2007). Fermentation technologies for the production of probiotics with high viability and functionality. Current Opinion in Biotechnology. 18(2): 176–183.

Lange, S., Gudmundsdottir, B.K., and Magnadottir, B. (2001). Humoral immune parameters of cultured Atlantic halibut, *Hippoglossus hippoglossus* L. Fish Shellfish Immunology. 11: 523–535.

Lara-Flores, M., Olivera-Castillo, L., and Olvera-Novoa, M.A. (2010). Effect of the inclusion of a bacterial mix (*Streptococcus faecium* and *Lactobacillus acidophilus*), and the yeast (*Saccharomyces cerevisiae*) on growth, feed utilization and intestinal enzymatic activity of Nile tilapia (*Oreochromis niloticus*). International Journal of Fish Aquaculture. 2: 93-101.

Lara-Flores, M., Olvera-Novoa, M.A., Guzmán-Méndez, B.E., and López-Madrid, W. (2003). Use of the bacteria *Streptococcus faecium* and *Lactobacillus acidophilus*, and the yeast *Saccharomyces cerevisiae* as growth promoters in Nile tilapia (*Oreochromis niloticus*). Aquaculture. 216: 193-201.

Larsen, A.M., Mohammed, H.H., and Arias, C.R. (2014). Characterization of the gut microbiota of three commercially valuable warmwater fish species. Journal of Applied Microbiology. 116(6): 1396-1404.

Lau, S.K.P., Woo, P.C.Y., Tse, H., Leung, K.-W., Wong, S.S.Y., and Yuen, K.-Y. (2003). Invasive *Streptococcus iniae* infections outside North America. Journal of Clinical Microbiology. 41: 1004–1009.

Le, P.T.T. and Boyd, C.E. (2012). Comparison of Phenate and Salicylate Methods for Determination of Total Ammonia Nitrogen in Freshwater and Saline Water. J World Aquacult Soc. 43: 885-889.

Lee, Y.T., Tsao, S.M., Lin, H.C., Huang, H.J., Lee, M.C., and Hsueh, P.R. (2010). Decline in the incidence of healthcare-associated methicillin-resistant

Staphylococcus aureus (HA-MRSA) correlates with deceased antimicrobial consumption at a tertiary care hospital in Taiwan, 2001-2009. Int J Antimicrob Agents. 36(6): 523-530.

Letunic, I. and Bork, P. (2011). Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. Nucleic Acids Res. 39: W475-W478. doi: 10.1093/nar/gkr201.

Lewis, W.J., van Lenteren, J.C., Sharad, C., Phatak, C.,and Tumlinson III, J.H. (1997). A total system approach to sustainable pest management. Proceedings of the National Academy of Science USA. 94: 12243–12248.

Li, P. and Gatlin, D.M. (2005). Evaluation of the prebiotic Grobiotic®-A and brewers yeast as dietary supplements for sub-adult hybrid striped sea bass, *Morone chrysops* x *M. saxatilis* challenged in situ with. Aquaculture. 248: 197-205.

Li, Q., Song, Y., Qin, X., Zhang, X., Sun, A., and Ju, J. (2015). Identification of the Biosynthetic Gene Cluster for the Anti-infective Desotamides and Production of a New Analogue in a Heterologous Host. J Nat Prod. 78(4): 944-948.

Li, Y.L. and Boyd, C.E. (2016). Influence of a Bacterial Amendment on Water Quality in Small Research Ponds for Channel Catfish, Ictalurus punctatus, Production. J World Aquacult Soc. 47: 464-469.

Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A., Schaberle, T.F., Hughes, D.E., Epstein, S., Jones, M., Lazarides, L., Steadman, V.A., Cohen, D.R., Felix, C.R., Fetterman, K.A., Millett, W.P., Nitti, A.G., Zullo, A.M., Chen, C., and Lewis, K. (2015). A new antibiotic kills

pathogens without detectable resistance (vol 517, pg 455, 2015). Nature. 520(7547).

Liu, S., Endo, K., Ara, K., Ozaki, K., and Ogasawara, N. (2008). Introduction of marker-free deletions in Bacillus subtilis using the AraR repressor and the ara promoter. Microbiology. 154: 2562-2570. doi: 10.1099/mic.0.2008/016881-0.

Liu, W.-T., Mirzabekov A. D., and Stahl, D. A. (2001). Optimization of an oligonucleotide microchip for microbial identification studies: a non-equilibrium dissociation approach. Environmental Microbiology. 3: 619-629.

Locke, J.B., Aziz, R.K., Vicknair, M.R., Nizet, V., and Buchanan, J.T. (2008). *Streptococcus iniae* m-like protein contributes to virulence in fish and is a target for live attenuated vaccine development. PLOS ONE. Open access journal.

Lugtenberg, B., and Kamilova, F. (2009). Plant Growth Promoting Rhizobacteria. Annu Rev Microbiol. 63: 541-556. doi: doi:10.1146/annurev.micro.62.081307.162918.

Lukashin, A.V. and Borodovsky, M. (1998). GeneMark.hmm: new solutions for gene finding. Nucleic Acids Res. 26: 1107-1115.

Lynch, J.M. and Whipps, J.M. (1990). Substrate flow in the rhizosphere. Plant Soil. 129: 1–10.

Lynch, M.D.J. and Neufeld, J.D. (2015). Ecology and exploration of the rare biosphere. Nature Reviews Microbiology. 13(4): 217-229.

Macfarlane, G.T. and Cummings, J.H. (1999). Probiotics and prebiotics: can regulating the activities of intestinal bacteria benefit health? British medical journal. 318: 999-1003.

Mahenthiralingam, E., Song, L.J., Sass, A., White, J., Wilmot, C., Marchbank, A., Boaisha, O., Paine, J., Knight, D., and Challis, G.L. (2011). Enacyloxins are products of an unusual hybrid modular polyketide synthase encoded by a cryptic Burkholderia ambifaria Genomic Island. Chem Biol. 18(5): 665-77.

Mahious, A.S., Gatesoupe, F.J., Hervi, M., Metailler, R., and Ollevier, F. (2006). Effect of dietary inulin and oligosaccharides as prebiotics for weaning turbot, *Psetta maxima* (Linnaeus, C. 1758). Aquaculture International. 14: 219–229.

Marden, J.P., Julien, C., Monteils, V., Auclair, E., Moncoulon, R., and Bayourthe, C. (2008). How does live yeast differ from sodium bicarbonate to stabilize ruminal pH in high-yielding dairy cows? Journal of Dairy Science. 91: 3528-3535.

Marilley, L. and Aragno, M. (1999). Phylogenetic diversity of bacterial communities differing in degree of proximity of *Lolium perenne* and *Trifolium repens* roots. Appl Soil Ecol. 13: 127–136.

Marilley, L., Vogt, G., Blanc, M., and Aragno, M. (1998). Bacterial diversity in the bulk soil and rhizosphere fractions of *Lolium perenne* and *Trifolium repens* as revealed by PCR restriction analysis of 16S rDNA. Plant Soil. 198: 219–224.

Martinez Cruz, P., Ibanez, A.L., Monroy Hermosillo, O.A., and Ramirez Saad, H.C. (2012). Use of probiotics in aquaculture. ISRN Microbiology. 2012: 916845.

Marzouk, M.S., Moustafa, M.M., and Mohamed, N.M. (2008). The influence of some probiotics on the growth performance and intestinal microbial flora of *Oreochromis niloticus*. Proceedings of 8th International Symposium on Tilapia in Aquaculture, Cairo, Egypt. 1059-1071.

Matsumoto, K., Shigemi, A., Yaji, K., Shimodozono, Y., Takeda, Y., Ikawa, K., Morikawa, N., Miyanohara, H., Kawamura, H., Orita, M., Tokuda, K., Nishi, J., and Yamada, K. (2012). Reduction in the incidence of MRSA with use of alcohol-based hand rub solutions and gloves. Journal of Infection and Chemotherapy. 18(2): 269-271.

McCullough, A.C., Seifried, M., Zhao, X.C., Haase, J., Kabat, W.J., Yogev, R., Blumenthal, R.M., and Mukundan, D. (2011). Higher incidence of perineal community acquired MRSA infections among toddlers. BMC Pediatr. 11.

McMurdie, P.J. and Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLOS ONE. 8.

McNear Jr., D.H. (2013). The Rhizosphere - Roots, Soil and Everything In Between. Nature Education Knowledge. 4(3): 1.

Medini, D., Serruto, D., Parkhill, J., Relman, D.A., Donati, C., Moxon, R., Falkow, S., and Rappuoli, R. (2008). Microbiology in the post-genomic era. Nat Rev Microbiol. 6: 419-430. doi: 10.1038/nrmicro1901.

Mei, G-Y., Carey, C.M., Tosh, S., and Kostrzynska, M. (2011). Utilization of different types of dietary fibers by potential probiotics. Canadian Journal of Microbiology. 57: 857–865.

Meisner, J., Wang, X., Serrano, M., Henriques, A.O., and Moran, C.P. Jr. (2008). A channel connecting the mother cell and forespore during bacterial endospore formation. PNAS. 105(39): 15100-15105. doi:10.1073/pnas.0806301105

Merrifield, D.L., Dimitroglou, A., Foey, A., Davies, S.J., and Baker, R.T.M. (2010). The current status and future focus of probiotic and prebiotic applications for salmonids. Aquaculture. 302: 1-18.

Migita, A., Watanabe, M., Hirose, Y., Watanabe, K., Tokiwano, T., Kinashi, H., and Oikawa, H. (2009). Identification of a gene cluster of polyether antibiotic lasalocid from Streptomyces lasaliensis. Biosci Biotechnol Biochem. 73(1): 169-176.

Mjoun, K., Rosentrater, K.A., and Brown, M.L. (2010). Tilapia: Environmental biology and nutritional requirements. South Dakota Cooperative Extension Service, SDSU, USDA. Accessed at http://pubstorage.sdstate.edu/agBio_publications/articles/Fs963-02.pdf.

Montes, A.J. and Pugh, D.G. (1993). The use of probiotics in food-animal practice. Veterinary Medicine. 88: 282–288.

Moreira, S.D., Medeiros, S.S., Pereira, L.L., Romano, L.A., Wasielesky, W., and Cupertino, B.E. (2011). The use of probiotics during the nursery rearing or the pink shrimp *Farfantepenaeus brasiliensis* (Latreille, 1817) in a zero exchange system. Aquaculture Research. 1–10.

Moriarty, D.J.W. (1998). Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture. 164(1–4): 351–358.

Mukhtar, A., Hasanin, A., Farouk, S., Mostafa, H., Lofty, A., Elazizi, H., Zaghlol, A., Gado, A., Botros, M., and Ghaith, D. (2013). *V*entilator Care Bundle Reduced the Incidence of Ventilator Associated Pneumonia Induced by Mrsa but

Not Acinetobacter Baumannii: Epidemiological Study and Antimicrobial Resistance Pattern. Intensive Care Med. 39: S312-S312.

Murillo, J., Heitzer, V., Ruiz, M., and Ordieres, F. (2010). Initiatives to decrease the incidence of methicillin resistant Staphylococcus aureus (MRSA). International Journal of Infectious Diseases. 14: E252-E252.

Mussatto, S.I. and Mancilha, I.M. (2007). Non-digestible oligosaccharides: A review, Carbohydrate Polymers. 68: 587-597.

Nakkeeran, S., Fernando, W.G.D., and Siddiqui, Z.A. (2005). "Plant growth promoting rhizobacteria formulations and its scope in commercialization for the management of pests and diseases," in PGPR: Biocontrol and Biofertilization, ed. Z.A. Siddiqui. (Dordrecht, The Netherlands: Springer). 257-296.

National Committee for Clinical Laboratory Standards. (2012). Performance Standards for Antimicrobial Disk Susceptibility Test; Approved Standard-Ninth Edition. Wayne, PA: Clinical and Laboratory Standards Institute.

Nayak, S.K. (2010). Role of gastrointestinal microbiota in fish. Aquaculture Research. 41: 1553-1573.

Nayak, S.K., Swain, P., and Mukherjee, S.C. (2007). Effect of dietary supplementation of probiotic and vitamin C on the immune response of Indian major carp, *Labeo rohita* (Ham). Fish and Shellfish Immunology. 23: 892-896

Naylor, R.L., Hardy, R.W., Bureau, D.P., Chiu, A., Elliott, M., Farrell, A.P., Forster, I., Gatlin, D.M., Goldburg, R.J., Hua, K., and Nichols, P.D. (2009). Feeding aquaculture in an era of finite resources (vol 106, 15103, 2009).

Proceedings of the National Academy of Sciences of the United States of America. 106: 18040-18040.

Nemoz, G., Robert-Baudouy, J., and Stoeber, F. (1976). Physiological and genetic regulation of the aldohexuronate transport system in *Escherichia coli*. J Bacteriol. 127: 706-718.

Newaj-Fyzul, A., Adesiyun, A.A., Mutani, A., Ramsubhag, A., Brunt, J., and Austin, B. (2007). *Bacillus subtilis* AB1 controls *Aeromonas* infection in rainbow trout (*Oncorhynchus mykiss*, Walbaum). J Appl Microbiol. 103 (5), 1699–1706.

Newton, A.C., Fitt, B.D.L., Atkins, S.D., Walters, D.R., and Daniell, T.J. (2010). Pathogenesis, parasitism and mutualism in the trophic space of microbe-plant interactions. Trends Microbiol. 18: 365–373.

Nichols, D., Cahoon, N., Trakhtenberg, E.M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K., and Epstein, S.S. (2010). Use of Ichip for High-Throughput In Situ Cultivation of "Uncultivable" Microbial Species. Applied and Environmental Microbiology. 76(8): 2445-2450.

Nicholson, W.L., Munakata, N., Horneck, G., Melosh, H.J., and Setlow, P. (2000). Resistance of bacillus endospores to extreme terrestrial and extraterrestrial environments. Microbiol Mol Biol Rev. 64: 548–572.

Nikoskelainen, S., Ouwehand, A.C., Bylund, G., Salminen, S., and Lilius, E. (2003). Immune enhancement in rainbow trout (*Oncorhynchus mykiss*) by potential probiotic bacteria, *Lactobacillus rhamnosus*. Fish and Shellfish Immunology. 15: 443-452.

Nuez-Ortin, W.G., Prado, S., and Toranzo, A.E. (2012). Antimicrobial properties of butyric acid and other organic acids against pathogenic bacteria affecting the main aquatic species. Conference proceedings aqua conference.

Olsson, J.C., Westerdahl, A., Conway, P., and Kjelleberg, S. (1992). Intestinal colonization potential of turbot (*Scophthalmus maximus*) and dab (*Limanda limanda*)- associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology. 58: 551–556.

Onarheim, A.M. and Raa, J. (1990). "Characteristics and possible biological significance of an autochthonous flora in the intestinal mucosa in sea-water fish," in Microbiology in poecilotherms. Proceedings of the International Symposium on Microbiology in Poecilotherms, ed. R. Lésel. (Elsevier Science Publishers B.V., Paris, France). 197– 201.

Ong, C., Ooi, C.H., Wang, D., Chong, H., Ng, K.C., Rodrigues, F., Lee, M.A., and Tan, P. (2004). Patterns of large-scale genomic variation in virulent and avirulent Burkholderia species. Genome Res. 14(11): 2295-307.

Ongena, M., Jourdan, E., Adam, A., Paquot, M., Brans, A., Joris, B., Arpigny, J.L., and Thonart, P. (2007). Surfactin and fengycin lipopeptides of Bacillus subtilis as elicitors of induced systemic resistance in plants. Environ Microbiol. 9: 1084-1090. doi: 10.1111/j.1462-2920.2006.01202.x.

Pang, M., Jiang, J., Xie, X., Wu, Y., Dong, Y., Kwok, A.H., Zhang, W., Yao, H., Lu, C., Leung, F.C., and Liu, Y. (2015). Novel insights into the pathogenicity of epidemic *Aeromonas hydrophila* ST251 clones from comparative genomics. http://www.ncbi.nlm.nih.gov/pubmed/26014286

Panigrahi, A., Kiron, V., Kobayashi, T., Puangkaew, J., Satoh, S., and Sugita, H. (2004). Immune responses in rainbow trout, *Oncorhynchus mykiss* induced by a potential probiotic bacteria, *Lactobacillus rhamnosus* JCM 1136. Veterinary Immunology and Immunopathology. 102: 379-388.

Panigrahi, A., Kiron, V., Puangkaew, J., Kobayashi, T., Satoh, S., and Sugita, H. (2005). The viability of probiotic bacteria as a factor influencing the immune response in rainbow trout, *Oncorhynchus mykiss*. Aquaculture. 243: 241-254.

Panigrahi, A., Kiron, V., Satoh, S., Hirono, I., Kobayashi, T., Sugita, H., Puangkaew, J., and Aoki, T. (2007). Immune modulation and expression of cytokine genes in rainbow trout *Oncorhynchus mykiss* upon probiotic feeding. Dev Comp Immunol. 31: 372–382.

Pannucci, J., Okinaka, R.T., Sabin, R., and Kuske, C.R. (2002). *Bacillus anthracis* pXO1 plasmid sequence conservation among closely related bacterial species. J Bacteriol. 184: 134-141.

Patil, H.J., Benet-Perelberg, A., Naor, A., Smirnov, M., Ofek, T., Nasser, A., Minz, D., and Cytryn, E. (2016). Evidence of Increased Antibiotic Resistance in Phylogenetically-Diverse Aeromonas Isolates from Semi-Intensive Fish Ponds Treated with Antibiotics. Frontiers in microbiology. 7.

Pfeiffer, W. and Stamatakis, A. (2010). "Hybrid MPI/Pthreads Parallelization of the RAxML Phylogenetics Code," in Ninth IEEE International Workshop on High Performance Computational Biology (HiCOMB 2010). (Atlanta, USA).

Phan, L.T., Bui, T.M., Nguyen, T.T.T., Gooley, G.J., Ingram, B.A., Nguyen, H.V., Nguyen, P.T., and De Silva, S.S. (2009). Current status of farming practices of

striped catifish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. Aquaculture. 296: 227-236

Phuong, N.T. and Oanh, D.T.H. (2009). "Striped catfish (*Pangasianodon hypophthalmus*) aquaculture in Viet Nam: an unprecedented development within a decade," in Success Stories in Asian Aquaculture, ed. S.S. De Silva and F.B. Davy. (Dordrecht, Bangkok and Ottawa: Springer, NACA and IDRC). 133-149.

Pier, G.B. and Madin, S.H. (1976). *Streptococcus iniae* sp. nov., a beta-hemolytic *Streptococcus* isolated from an Amazon freshwater dolphin, *Inia geoffrensis*. International Journal of Systematic Bacteriology. 26: 545–553.

Pieters, N., Brunt, J., Austin, B., and Lyndon, A.R. (2008). Efficacy of in-feed probiotics against *Aeromonas bestiarum* and *Ichthyophthirius multifiliis* skin infections in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Applied Microbiology. 105: 723-732

Piggot, P.J. and Hilbert, D.W. (2004). Sporulation of Bacillus subtilis. Curr Opin Microbiol. 7: 579–586.

Pirarat, N., Pinpimai, K., Endo, M., Katagiri, T., and Ponpornpisit, A. (2011). Modulation of intestinal morphology and immunity in Nile tilapia (*Oreochromis niloticus*) by *Lactobacillus rhamnosus* GG. Research Veterinary Science. 91: 92-97.

Podile, A.R., and Kishore, G.K. (2006). "Plant growth promoting rhizobacteria," in Plant-Associated Bacteria, ed. S.S. Gnanamanickam. (Netherlands: Springer). 195–230.

Popovici, O. (2016). The Incidence of Invasive Mrsa Infections in Germany May Be Declining. Medical-Surgical Journal-Revista Medico-Chirurgicala. 120(1): 89-89.

Pridgeon, J.W. and Klesius, P.H. (2011a). Development and efficacy of a novobiocin-resistant *Streptococcus iniae* as a novel vaccine in Nile tilapia (*Oreochromis niloticus*). Vaccine. 29: 5986– 5993.

Pridgeon, J.W. and Klesius, P.H. (2011b). Molecular identification and virulence of three *Aeromonas hydrophila* isolates cultured from infected channel catfish during a disease outbreak in West Alabama in 2009. Diseases of Aquatic Organisms. Open access journal.

Pridgeon, J.W., Klesius, P.H., Mu, X., and Song, L. (2011). An *in vitro* screening method to evaluate chemicals as potential chemotherapeutants to control *Aeromonas hydrophila* infection in channel catfish. J Appl Microbiol. 111: 114-124.

Priest, F.G., Goodfellow, M., Shute, L.A., and Berkeley, R.C.W. (1987). *Bacillus amyloliquefaciens* sp. nov. norn. rev. International Journal of Systematic Bacteriology. 69-71.

Pu, W.X., Su, Y., Li, J.X., Li, C.H., Yang, Z.Q., Deng, H.P., and Ni, C.X. (2014). High Incidence of Oxacillin-Susceptible mecA-Positive Staphylococcus aureus (OS-MRSA) Associated with Bovine Mastitis in China. PLOS ONE. 9(2).

Qiao, J.Q., Tian, D.W., Huo, R., Wu, H.J., and Gao, X.W. (2011). Functional analysis and application of the cryptic plasmid Pbsg3 harboring the RapQ-PhrQ system in *Bacillus amyloliquefaciens* B3. Plamid. 65: 141-149.

Queiroz, J.F. and Boyd, C.E. (1998). Effects of a bacterial inoculum in channel catfish ponds. Journal of the World Aquaculture Society. 29(1): 67–73.

Rahiman, M., Yousuf, J., Ambat, T., and Hatha, M. (2010). Probiotic effect of *Bacillus* NL110 and *Vibrio* NE17 on the survival, growth performance and immune response of *Macrobrachium rosenbergii* (de Man). Aquaculture Research. 41(9): e120–e134.

Rainger, G.E. and Rowley, A.F. (1993). Antibacterial activity in the serum and mucus of rainbow trout, *Oncorhynchus mykiss,* following immunization with *Aeromonas salmonicida*. Fish Shellfish Immunology. 3: 475-482.

Ran, C., Carrias, A., Williams, M.A., Capps, N., Dan, B.C., Newton, J.C., Kloepper, J.W., Ooi, E.L., Browdy, C.L., Terhune, J.S., and Liles, M.R. (2012). Identification of *Bacillus* strains for biological control of catfish pathogens. PLOS ONE. 7(9): e45793. doi: 10.1371/journal.pone.0045793.

Raupach, G.S. and Kloepper, J.W. (1998). Mixtures of plant growth promoting rhizobacteria enhance biological control of multiple cucumber pathogens. Phytopathology. 88(11): 1158-1164.

Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., and Menasveta, P. (1998). Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. Aquaculture. 167(3-4): 301–313.

Reva, O.N., Dixelius, C., Meijer, J., and Priest, F.G. (2004). Taxonomic characterization and plant colonizing abilities of some bacteria related to Bacillus amyloliquefaciens and Bacillus subtilis. FEMS Microbiol Ecol. 48: 249-259. doi: 10.1016/j.femsec.2004.02.003.

Richard, D., Ravigne, V., Rieux, A., Facon, B., Boyer, C., Boyer, K., Grygiel, P., Javegny, S., Terville, M., Canteros, B.I., Robene, I., Verniere, C., Chabirand, A., Pruvost, O., and Lefeuvre, P. (2017). Adaptation of genetically monomorphic bacteria: evolution of copper resistance through multiple horizontal gene transfers of complex and versatile mobile genetic elements. Mol Ecol. 26(7): 2131-2149.

Richter, M. and Rosselló-Móra, R. (2009). Shifting the genomic gold standard for the prokaryotic species definition. Proc Natl Acad Sci USA. 106: 19126-19131. doi: 10.1073/pnas.0906412106.

Rico-Mora, R., Voltolina, D., and Villaescusa-Celaya, J.A. (1998). Biological control of *Vibrio alginilyticus* in *Skeletonema costatum* (Bacilliophyceae) cultures. Agricultural Engineering. 19: 1-6.

Ridha, M.T. and Azad, I.S. (2012). Preliminary evaluation of growth performance and immune response of Nile tilapia *Oreochromis niloticus* supplemented with two putative probiotic bacteria. Aquaculture Research. 43: 843–852.

Robertson, P.A.W., O'Dowd, C., Burrells, C., Williams, P., and Austin, B. (2000). Use of *Carnobacterium* sp. as a probiotic for Atlantic salmon (*Salmo salar* L.) and rainbow trout (*Oncorhynchus mykiss*, Walbaum). Aquaculture. 185: 235-243

Rodriguez-Estrada, U., Satoh, S., Haga, Y., Fushimi, H., and Sweetman, J. (2009). Effects of single and combined supplementation of *Enterococcus faecalis*, mannanoligosaccharide and polyhydrobutyric acid on growth performance and immune response of rainbow trout, *Oncorhynchus mykiss*. Aquaculture Science. 57: 609–617.

Roesch, L.F., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K.M., Kent, A.D., Daroub, S.H., Camargo, F.A.O., Farmerie, W.G., and Triplett, E.W. (2007). Pyrosequencing enumerates and contrasts soil microbial diversity. Isme Journal. 1(4): 283-290.

Rojas-Rojas, F.U., Salazar-Gomez, A., Vargas-Diaz, M.E., Vasquez-Murrieta, M.S., Hirsch, A.M., De Mot, R., Ghequire, M.G.K., Ibarra, J.A., and Estrada-de los Santos, P. (2018). Broad-spectrum antimicrobial activity by Burkholderia cenocepacia TAtl-371, a strain isolated from the tomato rhizosphere. Microbiology-Sgm. 164(9): 1072-1086.

Ruckert, C., Blom, J., Chen, X., Reva, O., and Borriss, R. (2011). Genome sequence of *B. amyloliquefaciens* type strain DSM7(T) reveals differences to plant-associated *B. amyloliquefaciens* FZB42. J Biotechnol. 155: 78-85. doi: 10.1016/j.jbiotec.2011.01.006.

Rurangwa, E., Laranja, J.L., Van Houdt, R., Delaedt, Y., Geraylou, Z., Van de Wiele, T., Van Loo, J., Van Craeyveld, V., Courtin, C.M., Delcour, J.A., and Ollevier, F. (2009). Selected nondigestible carbohydrates and prebiotics support the growth of probiotic fish bacteria mono-cultures in vitro. Journal of Applied Microbiology. 106: 932–940

Ryu, C.-M., Farag, M.A., Hu, C.-H., Reddy, M.S., Kloepper, J.W., and Paré, P.W. (2004). Bacterial Volatiles Induce Systemic Resistance in *Arabidopsis*. Plant Physiol. 134: 1017-1026. doi: 10.1104/pp.103.026583.

Saitou, N. and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evo. 4: 406-425.

Sales, J. (2009). The effect of fish meal replacement by soyabean products on fish growth: a meta-analysis. Brit J Nutr. 102: 1709-1722.

Salinas, I., Abelli, L., Bertoni, F., Picchietti, S., Roque, A., Furones, D., Cuesta, A., Meseguer, J., and Esteban, M.Á. (2008). Monospecies and multispecies probiotic formulations produce different systemic and local immunostimulatory effects in the gilthead seabream (*Sparus aurata* L.). Fish Shellfish Immunol. 25: 114–123.

Salinas, I., Cuesta, A., Esteban, M.A., and Meseguer, J. (2005). Dietary administration of *Lactobacillus delbrueckii* and *Bacillus subtilis*, single or combined, on gilthead seabream cellular innate immune responses. Fish and Shellfish Immunology. 19: 67-77.

Salinas, I., Diaz-Rosales, P., Cuesta, A., Meseguer, J., Chabrillon, M., and Morinigo, M.A. (2006). Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). Veterinary Immunology and Immunopathology. 111: 279-286.

Salminen, S., Ouwehand, A., Benno, Y., and Lee, Y.K. (1999). Probiotics: how should they be defined? Trends in Food Science and Technology. 10: 107–110.

Salze, G., McLean, E., Schwarz, M.H., and Craig, S. R. (2008). Dietary mannanoligosaccharide enhances salinity tolerance and gut development of larval cobia. *Aquaculture* **274**:148-152.

Schnick, R. (2007). Major aquaculture drug approval. Fisheries. 32: 162-+.

Schwartz, D., Grammel, N., Heinzelmann, E., Keller, U., and Wohlleben, W. (2005). Phosphinothricin tripeptide synthetases in Streptomyces viridochromogenes Tu494. Antimicrob Agents Chemother. 49(11): 4598-4607.

Schwartz, D., Recktenwald, J., Pelzer, S., and Wohlleben, W. (1998). Isolation and characterization of the PEP-phosphomutase and the phosphonopyruvate decarboxylase genes from the phosphinothricin tripeptide producer Streptomyces viridochromogenes Tu494. FEMS Microbiol Lett. 163(2): 149-157.

Sekirov, I., Russell, S.L., Antunes, L.C., and Finlay, B.B. (2010). Gut microbiota in health and disease. Physiol Rev. 90: 859-904.

Semenov, A.M., van Bruggen, A.H.C., and Zelenev, V.V. (1999). Moving waves of bacterial populations and total organic carbon along roots of wheat. Microb Ecol. 37: 116–128.

Setlow, P. (1983). "Germination and outgrowth," in The Bacterial Spore. Vol. II, ed. A. Hurst and G.W. Gould. (London: Academic Press). 211–254.

Setlow, P. (1994). Mechanisms which contribute to the longterm survival of spores of Bacillus species. J Appl Bacteriol. 76: 49S–60S.

Setlow, P. (2005). Spores of *Bacillus subtilis:* their resistance to and killing by radiation, heat and chemicals. J Applied Microbiology. 1364-5072.

Sevrinreyssac, J. and Pletikosic, M. (1990). Cyanobacteria in Fish Ponds. Aquaculture. 88: 1-20.

Shantharaj, D., Williams, M.A., Potnis, N.S., Liles, M.R. (2019). *Burkholderia gladioli* C101 metabolites protect tomato plants against *Xanthomonas perforans* infection. Auburn University Auburn, Alabama.

Sharifuzzaman, S.M. and Austin, B. (2009). Influence of probiotic feeding duration on disease resistance and immune parameters in rainbow trout. Fish and Shellfish Immunology. 27: 440-445.

Shelby, R.A., Lim, C., Yildirim-Aksoy, M., Welker, T.L., and Klesius, P.H. (2009). Effects of yeast oligosaccharide diet supplements on growth and disease resistance in juvenile Nile tilapia, *Oreochromis niloticus*. Journal of Applied Aquaculture. 21: 61-71.

Shelby, R.A., Lim, C.E., Aksoy, M., and Klesius, P.H. (2006). Effect of probiotic diet supplements on disease resistance and immune response of young Nile tilapia, *Oreochromis niloticus*. Journal of Applied Aquaculture. 18: 23-34.

Shishehchian, F., Yusoff, F.M., and Shariff, M. (2001). The effects of commercial bacterial products on macrobenthos community in shrimp culture ponds. Aquaculture International. 9(5): 429–436.

Shoemaker, C. and Klesius, P. (1997). "Streptococcal disease problems and control, a review," in Tilapia aquaculture, volume 2," ed. K. Fitzsimmons. (Northeast Regional Agricultural Engineering Service, Ithaca, New York). 671–682.

Shoemaker, C.A., Evans, J.J., and Klesius, P.H. (2001). Prevalence of *Streptococcus iniae* in tilapia, hybrid striped bass, and channel catfish on commercial farms in the US. American Journal of Veterinary Research. 62: 174–177.

Shoemaker, C.A., LaFrentz, B.R., Klesius, P. H., and Evans, J.J. (2010). Protection against heterologous *Streptococcus iniae* isolates using a modified bacterin

vaccine in Nile tilapia, *Oreochromis niloticus* (L.). Journal of Fish Diseases. 33: 537–544.

Shoemaker, C.A., Xu, D.-H., Evans, J.J., and Klesius, P.H. (2006). "Parasites and diseases," in Tilapia biology, culture and nutrition, ed. C. E. Lim and C. D. Webster. (The Haworth Press, Binghamton, NY, USA). 561–582.

Simon, H.M., Jahn, C.E., Bergerud, L.T., Sliwinski, M.K., Weimer, P.J., Willis, D.K., and Goodman, R.M. (2005). Cultivation of mesophilic soil crenarchaeotes in enrichment cultures from plant roots. Applied and Environmental Microbiology. 71(8): 4751-4760.

Song, Z., Wu, T., Cai, L., Zhang, L., and Zheng, X. (2006). Effects of dietary supplementation with *Clostridium butyricum* on the growth performance and humoral immune response in *Miichthys miiuy*. Journal of Zhejiang University of Science and Biotechnology. 7: 596-602.

Sorokulova, I. (2008). Preclinical testing in the development of probiotics: a regulatory perspective with *Bacillus* strains as an example. Clin Infect Dis. 46: SS92-95.

Staykov, Y., Spring, P., Denev, S., and Sweetman, J. (2007). Effect of a mannanoligosaccharide on the growth performance and immune status of rainbow trout, *Oncorhynchus mykiss*. Aquaculture International. 15: 153-161.

Steichen, J., Roelliker, J., and Grosh, D. (1986). Kansas farmstead well survey for contamination by pesticides and volatile organics: National Water Well Association. Proceedings of the Agricultural Impacts on Ground Water, August 11-13, 1986, p. 530-541.

Stentiford, G.D., Sritunyalucksana, K., Flegel, T.W., Williams, B.A.P., Withyachumnarnkul, B., Itsathitphaisarn, O., and Bass, D. (2017). New Paradigms to Help Solve the Global Aquaculture Disease Crisis. PLoS pathogens. 13.

Stoffregen, D., Backman, S., Perham, R., Bowser, P., and Babish, J. (1996). Initial disease report of *Streptococcus iniae* infection in hybrid striped (sunshine) bass and successful therapeutic intervention with the fluoroquinolone antibacterial enrofloxacin. Journal of the World Aquaculture Society. 27: 420–434.

Storebakken, T., Shearer, K.D., and Roem, A.J. (1998). Availability of protein, phosphorus and other elements in fish meal, soy-protein concentrate and phytase-treated soy-protein-concentrate-based diets to Atlantic salmon, Salmo salar. Aquaculture. 161: 365-379.

Sumpavapol, P., Tongyonk, L., Tanasupawat, S., Chokesajjawatee, N., Luxananil, P., and Visessanguan, W. (2010). Bacillus siamensis sp. nov., isolated from salted crab (poo-khem) in Thailand. Int J Syst Evol Microbiol. 60: 2364-2370. doi: 10.1099/ijs.0.018879-0.

Svoboda, M., Farkas, J., and Sicher, J. (1954). Studies in the Chloramphenicol Series .8. Some Unsaturated Derivatives. Collection of Czechoslovak Chemical Communications. 19(3): 545-550.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., and Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evo. 28: 2731-2739.

Taoka, Y., Maeda, H., Jo, J.-Y., Jeon, M.-J., Bai, S.C., Lee, W.-J., Yuge, K., and Koshio, S. (2006a). Growth, stress tolerance and non-specific immune response of Japanese flounder *Paralichthys olivaceus* to probiotics in a closed recirculating system. Fisheries Science. 72(2): 310–321.

Taoka, Y., Maeda, H., Jo, J.-Y., Kim, S.M., Park, S., and Yoshikawa, T. (2006b). Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. Fishery Science. 72: 755- 766.

Taylor, E.P. (1953). A Tasteless Derivative of Chloramphenicol. Journal of Pharmacy and Pharmacology. 5(4): 254-256.

Tekedar, H.C., Waldbieser, G.C., Karsi, A., Liles, M.R., Griffin, M.J., Vamenta, S., Sonstegard, T., Hossain, M., Schroeder, S.G., Khoo, L., and Lawrence, M.L. (2013). Complete Genome Sequence of a Channel Catfish Epidemic Isolate, *Aeromonas hydrophila* Strain ML09-119. Genome Announcements. 1(5): e00755-13. doi: 10.1128/genomeA.00755-13.

Thurlow, C.M., Williams, M.A., Carrias, A., Ran, C., Newman, M., Tweedie, J., Allison, E., Jescovitch, L.N., Wilson, A.E., Terhune, J.S., and Liles, M.R. (2019). Bacillus velezensisAP193 exerts probiotic effects in channel catfish (Ictaluruspunctatus) and reduces aquaculture pond eutrophication. Aquaculture. 503: 347-356.

Torrecillas, S., Makol, A., Caballero, M.J., Montero, D., Robaina, L., Real, F., Sweetman, J., Tort, L., and Izquierdo, M.S. (2007). Immune stimulation and improved infection resistance in European sea bass, *Dicentrarchus labrax* fed mannanoligosaccharides. Fish and Shellfish Immunology. 23: 969-981.

US Department of Agriculture, USDA. (2003a). Part I: Reference of Fingerling Catfish Health and Production Practices in the United States. Fort Collins, CO, USA: National Health Monitoring System.

US Department of Agriculture, USDA. (2003b). Part II: Reference of Foodsize Catfish Health and Production Practices in the United States. Fort Collins, CO, USA: National Health Monitoring System.

US Department of Agriculture, USDA. (2010). Catfish 2010 part I: reference of catfish health and production practices in the United States, 2009. USDA-APHIS-VS, CEAH, Ft. Collins, CO.

Van Rijn, J. (1993). Methods to Evaluate Water Quality in Aquaculture. The Hebrew University of Jerusalem, Rehovot, Israel.

Vanderploeg, M., Tucker, C.S., and Boyd, C.E. (1992). Geosmin and 2-Methylisoborneol Production by Cyanobacteria in Fish Ponds in the Southeastern United-States. Water Science and Technology. 25: 283-290.

Vardakas, K.Z., Matthaiou, D.K., and Falagas, M.E. (2009). Incidence, characteristics and outcomes of patients with severe community acquired-MRSA pneumonia. European Respiratory Journal. 34(5): 1148-1158.

Vercauteren, I., Engler, J.A., Groodt, R. and Gheysen, G. (2002). An *Arabidopsis thaliana* Pectin Acetylesterase Gene Is Upregulated in Nematode Feeding Sites Induced by Root-knot and Cyst Nematodes. The American Phytopathological Society. 15: 404-407.

Verschuere, L., Rombaut, G., Heang, H., Criel, G., Sorgeloos, P., and Verstraete, W. (2000a). Selected bacterial strains protect *Artemia* spp. from the pathogenic

effects of *Vibrio proteolyticus* CW8T2. Applied and Environmental Microbiology. 66: 1139- 1146.

Verschuere, L., Rombaut, G., Huys, G., Dhont, J., Sorgeloos, P., and Verstraete, W. (1999). Microbial control of the culture of Artemia juveniles through pre-emptive colonization by selected bacterial strains. Applied and Environmental Microbiology. 65: 2527-2533.

Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000b). Probiotic bacteria as biological control agents in aquaculture. Microbial and Molecular Biology Review. 64: 655-671.

Wagner, B.A., Wise, D.J., Khoo, L.H., and Terhune, J.S. (2002). The epidemiology of bacterial diseases in food-size channel catfish. J Aquat Anim Health. 14: 263-272.

Walakira, J.K., Carrias, A.A., Hossain, M.J., Jones, E., Terhune, J.S., and Liles, M.R. (2008). Identification and characterization of bacteriophages specific to the catfish pathogen, Edwardsiella ictaluri. Journal of applied microbiology. 105: 2133-2142.

Wang, L.-T., Lee, F.-L., Tai, C.-J., and Kasai, H. (2007). Comparison of *gyr*B gene sequences, 16S rRNA gene sequences and DNA-DNA hybridization in the *Bacillus subtilis* group. Internaltional Journal of Systematic and Evolutionary Microbiology. 57(8): 1846-1850.

Wang, Y.B., Li, J.R., and Lin, J. (2008a). Probiotics in aquaculture: Challenges and outlook. Aquaculture. 281(1–4): 1–4.

Wang, Y.B., Tian, Z.Q., Yao, J.T., and Li, W.F. (2008b). Effect of probiotics, *Enteroccus faecium*, on tilapia, *Oreochromis niloticus* growth performance and immune response. Aquaculture. 277: 203-207.

Weinstein, M.R., Litt, M., Kertesz, D.A., Wyper, P., Rose, D., and Coulter, M. (1997). Invasive infections due to a fish pathogen, *Streptococcus iniae*. *S. iniae* Study Group. New England Journal of Medicine. 337: 589–94.

Weisburg, W.G., Barns, S.M., Pelletier, D.A., and Lane, D.J. (1991). 16S Ribosomal DNA Amplification for Phylogenetic Study. J Bacteriol. 173: 697-703.

Welker, T.L. and Lim, C. (2011). Use of probiotics in diets of tilapia. Journal of Aquaculture Research and Development. 8. Open access journal.

Westerdahl, A., Olsson, C., Kjelleberg, S., and Conway, P. (1991). Isolation and characterization of turbot (*Scophthalmus maximus*)-associated bacteria with inhibitory effects against *Vibrio anguillarum*. Applied and Environmental Microbiology. 57: 2223–2228.

Wilson, K.E., Flor, J.E., Schwartz, R.E., Joshua, H., Smith, J.L., Pelak, B.A., Liesch, J.M., and Hensens, O.D. (1987). Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by Bacillus subtilis. II. Isolation and physico-chemical characterization. J Antibiot (Tokyo). 40: 1682-1691.

Woese, C.R., Kandler, O., Wheelis, M.L. (1990). Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria, Eucarya. Proc. Natl. Acad. Sci. USA. 87: 4576-4579.

Wolfinger, R.D., Tobias, R.D., and Sall, J. (1991). Mixed models: A future direction. Proceedings of the sixteenth annual SAS users group conference. 1380–1388.

Xu, Z., Zhang, R., Wang, D., Qiu, M., Feng, H., Zhang, N., and Shen, Q. (2014). Enhanced control of cucumber wilt disease by Bacillus amyloliquefaciens SQR9 by altering the regulation of Its DegU phosphorylation. Appl Environ Microbiol. 80: 2941-2950. doi: 10.1128/AEM.03943-13.

Yan, X., Yu, H.-J., Hong, Q., and Li, S.-P. (2008). Cre/lox System and PCR-Based Genome Engineering in *Bacillus subtilis*. Appl Environ Microbiol. 74: 5556-5562. doi: 10.1128/aem.01156-08.

Yang, J., Kloepper, J.W., and Ryu, C.-M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. Trends in Plant Science. 14(1): 1-4.

Ye, J-D., Wang, K., Li, F-D., and Sun, Y-Z. (2011). Single or combined effects of fructo- and mannan oligosaccharide supplements and *Bacillus clausii* on the growth, feed utilization, body composition, digestive enzyme activity, innate immune response and lipid metabolism of the Japanese flounder, *Paralychthys olivaceus*. Aquaculture Nutrition. 17: 902-911.

Yousefian, M. and Amiri, M.S. (2009). A review of the use of prebiotic in aquaculture for fish and shrimp. African Journal of Biotechnology. 8: 7313-7318.

Yuan, J., Raza, W., Shen, Q., and Huang, Q. (2012). Antifungal Activity of *Bacillus amyloliquefaciens* NJN-6 Volatile Compounds against *Fusarium oxysporum* f. sp. *cubense*. Appl Environ Microbiol. 78: 5942-5944. doi: 10.1128/aem.01357-12.

Zakataeva, N.P., Nikitina, O.V., Gronskiy, S.V., Romanenkov, D.V., and Livshits, V.A. (2010). A simple method to introduce marker-free genetic modifications into the chromosome of naturally nontransformable *Bacillus amyloliquefaciens* strains. Appl Microbiol Biotechnol. 85: 1201-1209. doi: 10.1007/s00253-009-2276-1.

Zehnder, G.W., Murphy, J.F., Sikora, E.J. and Kloepper, J.W. (2001). Application of rhizobacteria for induced resistance. European Journal of Plant Pathology. 107: 39-50.

Zhang, G-Q., Bao, P., Zhang, Y., Deng, A-H., Chen, N., Wen, T-Y. (2011). Enhancing electro-transformation competency of recalcitrant *Bacillus amyloliquefaciens* by combining cell-wall weakening and cell-membrane fluidity disturbing. Analytical Biochemistry. 409: 130-137.

Zhou, Q-C., Alejandro Buentello, J., and Gatlin, D. M. III. (2010a). Effects of dietary prebiotics on growth performance, immune response and intestinal morphology of red drum, Sciaenops ocellatus. Aquaculture. 309: 253–257.

Zhou, X., Tian, Z., Wang, Y., and Li, W. (2010b). Effect of treatment with probiotics as water additives on tilapia, *Oreochromis niloticus* growth performance and immune response. Fish Physiology and Biochemistry. 36: 501-509.

Zhou, Z.G., Ding, Z.K., and Huiyuan, L.V. (2007). Effects of dietary short-chain fructooligosaccharides on intestinal microflora, survival, and growth performance of juvenile white shrimp, *Litopenaeus vannamei*. Journal of the World Aquaculture Society. 38: 296–301.

Zhu, Y., Qiu, X., Ding, Q.L., Duan, M.M., and Wang, C.F. (2014). Combined effects of dietary phytase and organic acid on growth and phosphorus utilization of juvenile yellow catfish Pelteobagrus fulvidraco. Aquaculture. 430: 1-8.

Zimmerman, S.B., Schwartz, C.D., Monaghan, R.L., Pelak, B.A., Weissberger, B., Gilfillan, E.C., Mochales, S., Hernandez, S., Currie, S.A., Tejera, E., and *et al.* (1987). Difficidin and oxydifficidin: novel broad spectrum antibacterial

antibiotics produced by Bacillus subtilis. I. Production, taxonomy and antibacterial activity. J Antibiot (Tokyo). 40: 1677-1681.