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CHEMICAL INVESTIGATION OF CANDIDATE PROBIOTICS IN AQUACULTURE AND FORMULATION OF A PROBIOTIC AGENT FOR OYSTER LARVICULURE

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CHEMICAL INVESTIGATION OF CANDIDATE PROBIOTICS IN AQUACULTURE AND FORMULATION OF A PROBIOTIC AGENT FOR OYSTER LARVICULURE

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ABSTRACT

Aquaculture is a multi-billion dollar industry worldwide. The United States is a significant consumer to both fresh and marine aquaculture products. Aquaculture sales in Rhode Island have dramatically increased in the last 20 years. In Rhode Island nearly 4.3 million oysters were produced via aquaculture in 2012. Currently, hatcheries and nurseries in the United States produce large amounts of a variety of species of oysters, clams and scallops. Oysters are filter feeders and are exposed to many microbes in the hatchery. Infectious diseases from bacterial pathogens in the hatcheries can have serious impacts on production. *Vibrio* species are often responsible for vibriosis disease outbreaks in bivalve larviculture hatcheries worldwide. Another prevalent disease observed in oyster nurseries in the Northeastern US is Juvenile Oyster Disease (JOD).

Probiotic agents are promising tools to reduce the risks of disease outbreaks in aquaculture facilities. Two marine bacteria, *Bacillus pumilus* RI0695 and *Phaeobacter gallaeciensis* S4, were previously reported to provide significant protection of the Eastern oyster larvae *Crassostreae virginica* when challenged with the shellfish pathogen *Vibrio tubiashii.* The goals of my dissertation research were to isolate and identify the antibiotic(s) secreted by *Phaeobacter gallaeciensis* S4, to chemically examine their mechanisms of action for probiotic activity, and to create probiotic formulations of *Bacillus pumilus* RI0695 and of *Phaeobacter gallaeciensis* S4 for delivery in shellfish larviculture facilities.

Chapter 2 describes the isolation and identification of the antibiotic tropodithietic acid (TDA) from *Phaeobacter gallaeciensis* S4. Genes *tdaA*, *tdaB*, *clpX* and *rpoE*

were previously found to be necessary for the biosynthesis of TDA in *Silicibacter* sp. TM104 (Geng, Bruhn et al. 2008, Karim, Zhao et al. 2013). Gene *exoP* is responsible for the exopolysaccharide biosynthesis (Zhao 2014). Collaborative work suggests that TDA contributes to the probiotic activity of *P. gallaeciensis* S4 but that antibiotic production is not the sole mechanism of action. The basis for this finding was biological and chemical analysis of S4 wild-type and genetic mutant strains producing different levels of TDA (*tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, *clpX*, *rpoE*$^-$, *exoP*$^-$, and complement strains *rpoE*$^-$, *exoP*$^-$) by high pressure liquid chromatography (HPLC) and ultra high pressure liquid chromatography (UHPLC). HPLC analysis of culture extracts from the *tdaA*$^-$, *tdaB*$^-$ and *tdbD*$^-$ mutants confirmed loss of TDA production as compared to S4 wild type. Additional genetic mutant strains, *clpX*, *rpoE*$^-$, *exoP*$^-$, and complement strains *rpoE*$^-$ and *exoP*$^-$ were created by insertional mutagenesis to further explore the role of TDA and mechanisms regulating its production. UHPLC analysis of *clpX* stationary phase culture extracts confirmed the lost production of TDA when compared to a TDA standard. UHPLC analysis of complement strains *clpX* demonstrated that TDA was present compared to a TDA standard. The *exoP*$^-$ mutant produced TDA similar to the wild-type strain. Mutant strains that lack the production of TDA had less protection of Eastern oyster larvae *C. virginica* to bacterial challenge than the wild type or the genetic mutant strain that produced similar TDA concentrations as the wild type. This research determined that TDA was necessary for optimal probiotic activity.

Chapter 3 describes efforts to create a stable formulation of *B. pumilus* RI0695 for delivery at shellfish hatcheries. Currently there are no commercially available

probiotics for shellfish aquaculture. Granulation is robust, cost effective, and simple proven method of formulation. A granular probiotic formulation of *B. pumilus* RI0695 was created by extruding dried *B. pumilus* RI0695 cells through three particle size sieves (40s, 80s, and 325s). Three granule sizes of 420 µ, 177 µ and 43 µ were successfully created. Granular (177 µ and 43 µ) formulations stored for 29 weeks and 22 weeks at room temperature (RT) were able to reduce mortality in *C. virginica* larvae and seed, respectively, when challenged with *V. tubiashii*. This study suggests the 43 µ granule formulation of *B. pumilus* RI0695 is a good candidate for commercial use in shellfish hatcheries.

In Chapter 4, a study is presented showing an effort to create a lyophilized probiotic formulation of *P. gallaeciensis* S4 that provides reduced mortality of *C. virginica* larvae when exposed to the shellfish pathogen *V. tubiashii* RE22. Several lyophilized formulations were prepared using varying amounts of two cryoprotectants at two growth stage phases of the bacterium. The two best formulations used log phase cells lyophilized with either 30% or and 40% mannitol as a cryoprotectant. The cell viabilities of the two formulations were measured under various storage conditions (27 °C, 4 °C, 30 °C, and 30 °C with 75% humidity) over a 5-week period. S4 formulations (30-M and 40-M) were tested at 1 week for probiotic protection of *C. virginica* seed against *V. tubiashii* infection. Unfortunately, the lyophilization process and storage significantly decreased the cell viability of both formulations (30-M and 40-M). Further, there was no protection of the larvae when pre-exposed to either formulation. Based on the *in vivo* results, a liquid *P. gallaeciensis* S4 formulation under starvation conditions in NSS medium was prepared. The liquid formulation maintained a cell

viability of 10^8 CFU/mL over 8 weeks. Further research should be done to evaluate these formulations in a hatchery study. More research must be done to refine the formulation processes for commercial scale up.

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Lastly I would like to dedicate my PhD to my mom, thank you so much for your never ending love, encouragement, support and the innumerable sacrifices you have made for me.

PREFACE

This dissertation was in accordance with the manuscript format guidelines established by the Graduate School of the University of Rhode Island. The dissertation includes an introduction and the following three manuscripts:

1. Literature review: Probiotics for disease management in commercial aquaculture systems.
2. Isolation, Purification and Quantification of Tropodithietic Acid a Major Contributor to the Probiotic Activity of *Phaeobacter gallaeciensis* S4 in Eastern Oyster *Crassostrea virginica*
3. Preparation of a Granular Formulation of *Bacillus pumilus* RI0695 for Disease Management in Eastern Oyster *Crassostrea virginica* hatcheries.
4. Preparation of a Lyophilized Formulation of *Phaeobacter gallaeciensis* S4 for Disease Management in Eastern Oyster *Crassostrea virginica* hatcheries.

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ANOVA	Analysis of variance
CFU	Colony forming unit
EA	Ethyl acetate
ESIMS	Electrospray ionization mass spectrometer
FA	Formic acid
FAO	Food and Agriculture Organization
FSSW	Fresh sterilized seawater
HPLC	High performance liquid chromatography
JOD	Juvenile oyster disease
LGP	Log growth phase
MEOH	Methanol
MIC	Minimum inhibitory concentration
NOAA	National Oceanic and Atmospheric Administration
OD	Optical density
RO	Reverse osmosis
RPS	Relative percent survival
RT	Room temperature
SGP	Stationary growth phase
TDA	Tropodithietic acid
UHPLC	Ultra high performance liquid chromatography
wt	wild type
20-M	20 (w/v) % mannitol

30-M	30 (w/v) % mannitol
40-M	40 (w/v) % mannitol
20-S	20 (w/v) % sucrose
30-S	30 (w/v) % sucrose
40-S	40 (w/v) % sucrose

CHAPTER 1

LITERATURE REVIEW:

PROBIOTICS FOR DISEASE MANAGEMENT IN COMMERCIAL AQUACULTURE SYSTEMS

Aquaculture value

Global aquaculture is a multi-billion dollar industry. Aquaculture is defined by NOAA as the production of freshwater fish, marine fish, shellfish, and marine plants (Elston, Hasegawa et al. 2008). In 2014, the Fisheries and Aquaculture Organization (FAO) released an overview of major trends and issues which estimated aquaculture production to be worth 137.7 billion USD worldwide (FAO 2014). The United States is a significant contributor to both fresh and marine aquaculture sales, adding approximately one billion dollars to the worldwide total (Elston, Hasegawa et al. 2008). Aquaculture sales in Rhode Island have dramatically increased from $83,518 in 1995 to $4.2 million in 2013 (Beutel 2013). In addition to this production boom, the number of oyster farms in Rhode Island has grown from six to fifty-two (Beutel 2013). In 2013, nearly 6.8 million oysters were produced *via* aquaculture in Rhode Island (Beutel 2013).

Aquaculture production growth

The growth of worldwide aquaculture production revenue increased average 6.1 percent annually between 2001-2012 (FAO 2014). The total production of fish, crustaceans, mollusks and other aquatic animals around the world increased continuously to 158 million tonnes in 2010 (FAO 2014). In comparison, the capture production has remained around 90 million tonnes over the last 15 years (FAO 2014). Approximately 86% of seafood consumed by the U.S. is imported, around half of which is produced via aquaculture (FAO 2014). According to The 2010 State of World Fisheries and Aquaculture by the FAO, sixty-two percent of all farmed seafood in the world is produced in China. An additional 26% of all farmed seafood is

produced in Asia outside of China. Europe and the Americas produced 4.5 percent each of all farmed seafood (NOAA 2012).

United States Aquaculture Production

United States' aquaculture industry, both freshwater and marine, supplies five to seven percent of the national demand for seafood. Wild catch and aquaculture, both imported and domestic, account for the sources of seafood production in the United States. Many of the existing farms are located on territorial waters in Maine, Washington, Hawaii RI and other states , and on land in ponds and tanks in several states (NOAA 2012).

The U.S freshwater aquaculture industry produces primarily catfish, trout, and tilapia. Two-thirds of U.S. marine aquaculture production is comprised of molluscan shellfish such as oysters, clams, and mussels. The other third consists mainly of shrimp, Atlantic salmon, steelhead trout, coho salmon, cod, sturgeon, red drum, Pacific threadfin (moi), Hawaiian yellowtail, and cobia, with lesser amounts of barramundi, seabass, seabream, and other species (NOAA 2012).

Diseases in aquaculture

Approximately 430 aquatic species have been domesticated for aquaculture (Duarte, Marba et al. 2007). Rigorous culture of bivalve shellfish on a production scale developed in the 1970's. Currently, hatcheries and nurseries in the United States produce large numbers of a variety of species of oysters, clams and scallops (Elston 1998). However, infectious diseases in the hatcheries have serious impacts on

production, with 45.5% of losses in aquaculture being due to diseases (Elston 1998, FDA 2012).

Aquaculture hatchery operations are especially prone to bacterial diseases that rapidly kill larvae. The hatchery environment has many factors that may impact the growth and survival of cultured species. There are a combination of controllable and chance factors that determine the composition of the resulting microflora in aquatic animals (Vine, Leukes et al. 2006). The controllable factors include: salinity; temperature; and feed quality (Vine, Leukes et al. 2006) . The gastrointestinal flora of aquatic animals reared in hatchery settings usually resemble the microflora initially present in the rearing water, microalgae, and livefood (Gatesoupe 1999, Riquelme, Jorquera et al. 2001, Vine, Leukes et al. 2006). Diseases in bivalve shellfish hatcheries are caused by infectious organisms entering the hatchery from the same three sources as other microbes: brood stock; seawater source; and algal food source (Elston 1998). Once a pathogen has been introduced into the system, it can rapidly cause disease and death in the aquatic animal. Major causative factors for opportunistic infections in shellfish hatcheries are bacterial infections, which can enter the system through the seawater source and/or the algal food source (Elston 1998). Another source of opportunistic infections is viral. Viruses are generally transferred from infected brood stock (Elston 1998). Aside from bacteria and viruses, other pathogens include fungi and eukaryotic parasites.

Prevalent diseases observed in Eastern oysters (*Crassostrea virginica)* in the Northeastern US and Atlantic Canada are multi-nucleated sphere X (MSX) disease, dermo disease, juvenile oyster disease (JOD) and bonamiasis (shown in Table 1). A

major player in the development of infectious disease in Pacific oyster larvae (*Crassostrea gigas)* hatcheries is *Vibrio tubiashii*.

Table 1. Diseases associated with the Eastern oyster *Crassostrea virginica* and the Pacific oyster *Crassostrea gigas.*

Disease	Pathogenic Agent	Effects on Host	Occurrence of Disease	Mechanism of Virulence	Reference
Multi-nucleated Sphere X (MSX) disease	*Haplosporidium nelson*	Decreased meat quality, reduced reproductive capacity and mortality	Observed from Maine to Florida in the late summer and early fall	Unknown	Elston 1990, Barber 1999
Dermo Disease	*Perkinsus marinus*	Decreased growth, typically between 60% and 80% after two years of exposure, and mortality	Observed in Northeast US and Atlantic Canada, in increased temperatures of seawater (>20 °C) and salinity (> 15 parts per trillion)	Infection through ingestion, because it is often observed in gut tissue	Elston 1990, Barber 1999, Ford 2011
Juvenile (*Roseovarious*) Oyster Disease	*Roseovarious crassostreae*	Reduced growth of juveniles along with uneven shell growth. Mortalities of over 90% in infected areas.	Observed in New York and New England, in mid- to late summer when water temperature is elevated (>25 °C)	Unknown	Gomez-Leon, Villamil et al. 2008Romalde and Barja 2010
Bonamiasis	*Bonamia ostreae*	Inability to close shell completely. Decimation of infected hatchery.	Washington State and Maine, outbreaks occur year round when seawater temperatures range from 12-20 °C.	Infects the host blood cells, it can multiply within blood cells and spreads to all tissues	Elston 1990, Barber 1999

Disease	Pathogenic Agent	Effects on Host	Occurrence of Disease	Mechanism of action	Reference
Vibriosis	*Vibrio tubiashii*	Larvae exhibit mantle and feeding disruption, and visceral damage infection, resulting in death within 24 hours. Infection have been attributed to 59% decline in production	Pacific oyster hatcheries, when seawater temperature is elevated (>20 ℃)	Two virulence factors associated with bacterium: Metalloprotease and hemolysin	Decamp, Moriarty et al. 2008, Elston 2008
Vibriosis	*Vibrio parahaemolyticus*	Human consumption of infected oysters can cause gastroenteritis and septicemia..	Abundant in marine environments, when seawater temperature is elevated (>20 ℃)	Clinical illness is associated with strains is hemolysin	DePaola, McLeroy et al. 1997
Vibriosis	*V. vulnificus*	Distributed throughout the tissues of the oyster. Causative agent of gastroenteritis and septicemia through human consumption.	Abundant in mollucs in the Gulf coast.	Virulence factor associated with bacterium is hemolysin	DePaola, McLeroy et al. 1997

MSX disease is caused by the eukaryotic parasite *Haplosporidium nelsoni*. *H. nelsoni* cells usually appear first in gill tissue, indicating that the infective stage is waterborne, which can only be confirmed by microscopic examination of oyster tissue (Elston 1990, Barber 1999). Effects of the disease include decreased meat quality, reduced reproductive capacity, and mortality (Elston 1990, Barber 1999). These outbreaks mainly occur in the late summer and fall and have been observed from Maine to Florida (Elston 1990, Barber 1999). The mode of transmission of this pathogen is unknown.

Dermo disease of Eastern oysters, *C. virginica*, is caused by a highly infectious eukaryotic parasite *Perkinsus marinus.* The presence of *P. marinus* is determined by microscopic examination of oyster gut tissue cultured in thioglycollate medium (Elston 1990, Barber 1999, Ford 2011). It has been reported that the primary route of infection is through ingestion, because it is often observed in gut tissue (Elston 1990, Barber 1999, Ford 2011). The primary effect of Dermo disease is a decrease in growth, typically between 60% and 80% after two years of exposure (Ford 2011). The disease can be directly passed from infected to uninfected oysters. It has been reported that *P. marinus* causes the greatest mortality at temperatures greater than 20 °C and at a salinity above 15 parts per trillion (ppt) (Ford 2011).

Roseovarious crassostreae is another bacterium that causes serious infections in juvenile oysters. This pathogen, the causative agent of Juvenile or *Roseovarious* Oyster Disease (JOD), is endemic to New York and the New England area (NOAA 2009, Romalde and Barja 2010). JOD has been responsible for population mortalities of over 90% in highly impacted areas. Outbreaks of *R. crassostreae* may be associated

with elevated water temperature (>25 °C) (Gomez-Leon, Villamil et al. 2008, NOAA 2009) (NOAA 2009, Romalde and Barja 2010). Indications of the disease are reduced growth of the oyster along with uneven shell growth. The disease is characterized by the presence of conchiolin deposits, or the presence of brown rings, on the inner shell surfaces. JOD follows a seasonal pattern, with most mortality occurring in mid- to late-summer, coinciding with warm water temperatures. The disease can be transmitted from oyster to oyster, but the toxins and virulence factors are still unknown for this pathogen (Romalde and Barja 2010).

Bonamiasis, a disease that originated in the European oyster *Ostrea edulis*, is caused by the eukaryotic parasite *Bonamia ostreae* (Elston 1990, Barber 1999). It has been reported to occur in Washington State and in Maine (Elston 1990, Barber 1999). *B. ostreae* has been reported to multiply within blood cells and spreads to all tissues, interfering with physiological processes (Elston 1990, Barber 1999). A sign of early stage bonamiasis is the inability of the shell to close completely (Elston 1990, Barber 1999). Diagnosis of *B. ostreae* requires microscopic examination of oyster blood or tissue (Elston 1990, Barber 1999). Outbreaks of bonamiasis can be devastating to a hatchery as it is highly infectious and is transmitted directly from oyster to oyster. It has been reported that mortality can approach 100% (Elston 1990, Barber 1999). These outbreaks can occur year round, with ideal temperature conditions for infections ranging from 12 °C to 20 °C. *B. ostreae* is reportedly found consistently in Maine with a low prevalence in some populations of *O. edulis* (Elston 1990, Barber 1999).

A variety of *Vibrio* species are often responsible for disease outbreaks in bivalve larviculture hatcheries, including *Vibrio coralliilyticus* which has previously

been mistaken for *V. tubiashii* RE22 which cause bacillary necrosis in oyster larvae. Vibriosis outbreaks in larvae are characterized by mantle and feeding disruption, loss of motility, and visceral damage usually resulting in larval death within 24 hours (Porsby, Nielsen et al. 2008). This disease is particularly problematic in hatcheries that culture bivalves (Decamp, Moriarty et al. 2008, NOAA 2009, Romalde and Barja 2010). From 2006 to 2007, increased water temperature, and bad water quality maybe the causative factors for several outbreaks of *V. tubiashii* in shellfish hatcheries and nurseries in North America (Elston, Hasegawa et al. 2008). These pathogenic outbreaks caused a 59% decline in oyster larvae production , thus affecting the overall production of cultured oysters for human consumption. In addition, although they don't affect the shellfish hosts, *V. parahaemolyticus and V. vulnificus* have been linked to foodborne illnesses in humans such as gasterenteritis or in severe cases septicemia through consumption of raw oysters (DePaola, McLeroy et al. 1997, CDC 2015). Both bacterial species are prevalent in seawater with elevated temperatures (>20 °C) (DePaola, McLeroy et al. 1997, CDC 2015).

Studies by Hasegawa and coworkers determined two potential virulence factors in *Vibrio tubiashii*, metalloprotease and hemolysin, which they termed "extracellular products" (Hasegawa, Lind et al. 2008, Porsby, Nielsen et al. 2008). Both extracellular products are regulated by quorum sensing (Hasegawa, Lind et al. 2008). Similar to *Vibrio anguillarum*, at high cell density in *V. tubiashii* the metalloprotease gene is expressed while the hemolysin gene is down regulated (Li, Rock et al. 2008). Toxicity in oyster larvae was diminished when *V. tubiashii* supernatants were treated with metalloprotease inhibitors. It was reported that

inhibition of hemolysin also caused a decrease in larval toxicity (Hasegawa, Lind et al. 2008). The study identified the structural genes encoding for metalloprotease (*vtpA*) and hemolysin (*vthA)* for *V. tubiashii* (Hasegawa, Lind et al. 2008). The *vtpA* gene encoded for a zinc metalloprotease, and had a high sequence similarity to genes found in other *Vibrio* species (Hasegawa, Lind et al. 2008). It is still unknown how *vtpA* contributes the bacillary necrosis. However, it has been suggested that *vtpA* contributes to the degradation of oyster larvae tissues. The *vthA* gene from *V. tubiashii* has significant homology with the *vvhA* gene found in *V. vulnificus* (Hasegawa, Lind et al. 2008). The virulence factor associated with the gastroenteritis in *V. parahaemolyticus* is thermostable direct hymolysin (TDH). It has been studied that both trh and tdh are significant in the virulence of *V. parahaemolyticus* (Shirai, Ito et al. 1990).

Disease Management in Bivalve Aquaculture Facilities

In the last two decades there has been an increased incidence of disease outbreaks due to bacterial pathogens in shellfish hatcheries (Boettcher, Geaghan et al. 2005, Gomez-Leon, Villamil et al. 2008, USDA 2008, NOAA 2009). In particular, pathogen outbreaks of *Vibrio* spp. can have devastating effects on oyster production in shellfish hatcheries (Romalde and Barja 2010). *Vibrio* pathogenic outbreaks can cause a 59% decline in oyster larvae production (Balcazar, de Blas et al. 2006).

The treatment of pathogenic disease in aquaculture is problematic due to limited resources and treatment options (Decamp, Moriarty et al. 2008). Aquaculturists have explored using filtration systems, ozonolysis, UV and electrolytic

treatments of seawater, adjusting salinity, and selective breeding (Boettcher, Barber et al. 1999, Ford and Borrero 2001, Jorquera, Valencia et al. 2002, Wijesekara, Nomura et al. 2006, Park, Kim et al. 2011, FAO 2014). Additionally, investigations into the use of probiotics as a new disease management tool are being explored (Westerdahl, Olsson et al. 1991, Elston 1998, Rengpipat, Phianphak et al. 1998, Verschuere, Rombaut et al. 2000, Spanggaard, Huber et al. 2001, Farzanfar 2006, Vine, Leukes et al. 2006, Decamp, Moriarty et al. 2008, Kesarcodi-Watson, Kaspar et al. 2008). Vaccines are not feasible in bivalves because they lack an antibiody immune response (Gomez-Chiarri 1999). The use of antibiotics in aquaculture can have potentially detrimental effects for increasing the development of drug-resistant bacteria involved in human and other animal diseases (Vaseeharan and Ramasamy 2003, Farzanfar 2006).

Disease reduction strategies can be matched to the pathogens of interest. Management of MSX disease is aided by the fact that *H. nelsoni* cannot tolerate salinity below 10 ppt and only causes severe mortality above 20 ppt. Sites that regularly experience salinity below 20 ppt are be less impacted by MSX than other areas. *H. nelsoni* can be eliminated from oysters by moving them into an environment of a salinity of 10 ppt or less. Research has also shown that MSX mortality can be reduced through selective breeding (Burreson and Ford 2004). Ford and Borrero (2001) reported that filtration of the seawater entering the hatchery with a 1µm filter cartridge and followed by exposure to UV irradiation (30,000 $\mu Ws^{-1}cm^{-2}$) prevented infection by both eukaryotic parasites *H. nelsoni*, the causative agent for MSX disease, and *P. marinus,* the causative agent for Dermo disease (Ford and Borrero 2001).

Filtered sterilized seawater also yielded good results in preventing (82%) JOD in the eastern oyster *C. virginica* (Boettcher, Barber et al. 1999).

Ozonation in freshwater recirculating hatchery systems has been widely used for almost 30 years to form oxidized organic matter to control bacterial diseases. There have been some conflicting reports that ozonation in seawater can have toxic effects due to a reaction with bromine. Ozonation by-products are known to oxidize organic material forming total oxidized residual compounds (TRO). A TRO concentration of 0.06-0.1 mg/L is needed to inactivate microorganisms. Ozonation in seawater recirculation systems for black seabream, *Acanthopargus schlegelii,* fed at 20g O_3/kg feed day^{-1} significantly reduced heterotrophic bacteria in the inlet water measured after six consecutive days of treatment (Park, Kim et al. 2011). Electrolytic methods have been successful in reducing microalgae, bacteria, and viruses (Jorquera, Valencia et al. 2002, Wijesekara, Nomura et al. 2006, FAO 2014). This process uses a nonmembrane electrolytic cell to dissociate sodium chloride molecules in seawater to form hypochloride ion and sodium ion. (Jorquera, Valencia et al. 2002). A study using electrolytically treated seawater in aquaculture systems reported that an electrolytic current intensity of 1.3 A was able to reduce *V. anguillarum* to undetectable concentrations and produced minimal free chloride ions (Jorquera, Valencia et al. 2002). Electrolysis of seawater at 4.0 A followed by neutralization with sodium thiosulfate was able to stimulate the growth of cultured *Isochrysis galbana* as compared to both the control (sterilized seawater) and UV treatments of seawater (Jorquera, Valencia et al. 2002).

The methods for treating the hatchery water supply, including treatments with ozone, filtration, heat, UV irradiation, and electrolysis (Jorquera, Valencia et al. 2002), are effective for minimizing pathogenic diseases. However, they can have a high cost due to the need for expensive equipment and high energy input (Park, Kim et al. 2011). Thus, they lack practicality for many hatchery facilities.

Probiotics have shown promise as a disease management tool for aquaculture (Kesarcodi-Watson, Kaspar et al. 2008). Unlike chemical antibiotics, probiotics are provided to larvae prior to infection outbreaks in order to control pathogens in the hatchery. They are defined by the World Health Organization as "live microbes which have a beneficial effect on the host" (Liang 2003). They are a "green" alternative to the use of antibiotics since they are unlikely to contribute to the rising problem of antimicrobial resistance (Verschuere, Rombaut et al. 2000).

Probiotics in shellfish aquaculture

A full definition of a marine probiotic suggested by Vershuere et al. (2000) is:

> A live microbial adjunct which has a beneficial effect on the host by modifying the host-associated or ambient microbial community, by ensuring improved use of the feed or enhancing its nutritional value, by enhancing the host response towards disease, or by improving the quality of its ambient environment including water quality and interaction with phytoplankton (Verschuere, Rombaut et al. 2000).

More widely stated, "a probiotic is an entire or component(s) of a microorganism that is beneficial to the health of the host" (Salminen, von Wright et al. 1998, Irianto and Austin 2002). Additionally, immunostimulants or bacterial by-products, such as peptidoglycan and lipopolysaccharides that have been shown to promote an enhanced immune response from the host can be considered to be prebiotics (Itami, Asano et al. 1998, Smith, Brown et al. 2003, Kesarcodi-Watson, Kaspar et al. 2008).

Investigations of probiotics in aquaculture have been aimed at improving survival rate and/or growth of various aquacultured animals (Table 1). Probiotics that are chosen for aquaculture must be able to proliferate in an aquatic environment and there must be a consideration of the relationship of the aquatic animal and its external environment. Potential pathogens have the ability to proliferate in the seawater that may enter the hatcheries (Hansen and Olafsen 1999, Verschuere, Rombaut et al. 2000, Kesarcodi-Watson, Kaspar et al. 2008).

Marine probiotics have several proposed modes of action, including the production of antimicrobial compounds, competition for adhesion sites and nutrients, stimulation of the host immune system, and providing enhanced nutrition to the host (Queiroz and Boyd 1998, Murthy and TJ 1999, Ziaei-Nejad, Rezaei et al. 2006, Cude, Mooney et al. 2012). The most common examined mechanism of action in probiotic candidate bacteria has been investigating the production of inhibitory substances. Such studies seek to identify bacteria that secrete molecule(s) that limit the growth of pathogens *in vitro* as candidate probiotics that then are tested *in vivo* (Westerdahl, Olsson et al. 1991, Sugita, Shibuya et al. 1996, Bly, Quiniou et al. 1997, Sugita, Matsuo et al. 1997, Spanggaard, Huber et al. 2001, Chythanya, Karunasagar et al. 2002, Sugita, Okano et al. 2002, Hjelm, Riaza et al. 2004).

There are four methods that are commonly employed to screen for growth inhibitory substances *in vitro*: the double layer method, the well diffusion method, the cross-streak method, and the disc diffusion method. All methods are based on the principle that a bacterium (the producer) secretes an extracellular substance which is inhibitory to itself or another bacterial strain (the indicator). The inhibitory activity is

displayed by growth inhibition of the indicator in agar medium. In some cases, initial *in vitro* screening was followed by small scale testing of short-listed candidates *in vivo* for either pathogenicity to the host (Makridis, Jon Fjellheim et al. 2000, Chythanya, Karunasagar et al. 2002, Hjelm, Riaza et al. 2004) or host protection when challenged with a pathogen (Rengpipat, Phianphak et al. 1998, Gram, Melchiorsen et al. 1999, Robertson, O'Dowd et al. 2000, Irianto and Austin 2002, Vaseeharan and Ramasamy 2003, Lategan, Torpy et al. 2004, Lategan, Torpy et al. 2004). For example, Irianto et al. (2002) demonstrated that several candidate probiotics identified as *Aeromonas hydrophila A3-51, V. fluvialis, Carnobacterium* sp. displayed antagonistic properties towards the pathogen *Aeromonas salmonicida* (Irianto and Austin 2002). Many investigations have observed a positive protective effect in *in vivo* studies following positive antagonism assays *in vitro* (see Table 2) (Gibson, Woodworth et al. 1998, Rengpipat, Phianphak et al. 1998, Makridis, Jon Fjellheim et al. 2000, Robertson, O'Dowd et al. 2000, Chythanya, Karunasagar et al. 2002, Irianto and Austin 2002, Vaseeharan and Ramasamy 2003, Hjelm, Riaza et al. 2004, Lategan, Torpy et al. 2004, Lategan, Torpy et al. 2004).

Table 2. Some examples of promising probiotics in shellfish larviculture and their effect on the host.

Microbe	Host Species	Effect on Host	Reference
Arthrobacter sp. strain 77	*Argopecten purpuratus*	Production of inhibitory compounds; Probiotic replaced resident microflora within 24 hours.	Riquelme et al. (2000)
Vibrio sp. strains 11 and C33	*Argopecten purpuratus*	Colonize the digestive tract when administered with microalgae	Avendano & Riquelme (1999)
Vibrio sp. C33, *Pseudomonas* sp. 11 and *Bacillus* sp. strain B2	*Argopecten purpuratus*	Increased the number of larvae when compared to antibiotic treatment	Riquelme et al. (2001)
Aeromonas media strain A199	*Crassostrea gigas*	Increased survival rate when challenged with *V. tubiashii;*	Gibson et al. (1998)
Aeromonas media strain A199	*Anguilla australis* Richardson	Production of inhibitory substance inhibiting growth of *Saprolegnia* sp.	Lategan et al. (2003)
Bacillus S11	*Penaeus monodon* PL-10	Post larvae survival was increased when challenged with *V. harveyi*; Probiont provided cellular and humoral immune defense responses	Rengpipat et al. (2000)

Microbe	Host Species	Effect on Host	Reference
Scophthalmus maximus L. gut strains 4:44 PB52	*Brachionus plicatilis*	Colonization of the gut of larvae	Makridis et al. (2000)
Bacillus S11	*Penaeus monodon*	Improvement in survival when coupled with ozonation treatments	Meunpol et al. (2003)
Lactobacillus sporogenes	*Macrobrachium rosebergii*	Improved growth rate and feed efficiency of post larvae when fed as a bio-encapsulated probiotic.	Venkat et al. (2004)
Bifidobacterium thermophilum	*Penaeus japonicus*	Improved survival by peptidoglycan against *Vibrio penaecida*	Itami et al. (2002)
Bacillus subtilis BT23	*Penaeus* sp.	Reduced mortality by 90% when challenged with *V. harveyi.*	Vaseeharan et al. (2003)
Bacillus spp.	*Fenneropenaus indicus*	Increase in survival and wet weight, increases in specific activities of amylase, total protease	Ziaei-Nejad (2006)
Pseudomonas strain I-2	*Penaeus monodon*	Production of inhibitory compounds against *V. harveyi, V. fluvailis, Vibrio parahaemolyticus, Vibrio damsela and Vibrio vulnificus*	Chythanya et al. (2002)

Microbe	*Host Species*	Effect on Host	Reference
Pseudomonas sp. PM11 and *Vibrio fluvalis* sp.	*Penaeus monodon*	Increased survival of *P. monodon* because of its antagonistic effect towards *V. harveyi*	Meunpol et al. (2003) Vaseeharan et al. (2003)
Vibrio P62, and *Bacillus* P64	*Penaeus vannamei*	Both showed inhibitory effects against *Vibrio harveyi*. However P64 showed immunostimulatory features as well	Gullian et al. (2004)
Carnobacterium sp.	*Salmo salar* L. and *Oncorhynchus mykiss*	Reduction of disease by *A. salmonicida* in *Oncorhynchus mykiss, V. ordalii* in *Salmo salar* L., and *Yersinia ruckeri* in *Salmo salar* L. and *Oncorhynchus mykiss*	
Bacillus sp. RI0695	*Crassostrea virginica*	Increased survival of larvae when challenged with *Vibrio tubiashii,* through production of inhibitory compounds and colonization.	Karim et al. (2013)
Claulobacter sp. PK654		Demonstrated against two phytoplankton, *Skeletonema costatum* and *Heterosigma akashiwo*	Kawano, Asada et al. 1998
Phaeobacter gallaeciensis S4	*Crassostrea virginica*	Increased survival of larvae when challenged with *Vibrio tubiashii.* Through inhibitory compounds, colonization and biofilm formation.	Karim et al. (2013)

Various microorganisms produce antimicrobial compounds that limit the growth of aquaculture pathogens. While not a probiotic organism, *Skeletonema costatum*, a common phytoplankton used in larviculture of mollusks and crustaceans, produces an organic extract capable of inhibiting the growth of *V. anguillarum* and three *Vibrio* species (Kesarcodi-Watson, Kaspar et al. 2008). Additionally, the antibiotic thiotropocin produced by *Claulobacter* sp. PK654 demonstrated inhibitory activity towards the fish pathogen *Lactococcus garvieae*, but also had activity against two phytoplankton, *Skeletonema costatum* and *Heterosigma akashiwo* (Kawano, Asada et al. 1998, Naviner, Bergé et al. 1999, Kesarcodi-Watson, Kaspar et al. 2008). A study by Meunpol et al. (2003) suggested that the probiotic *Bacillus* S11 was a contributing factor to the survival of black tiger shrimp (*Penaeus monodon*) in the presence of the bacterial pathogen *V. harveyi* (Meunpol, Lopinyosiri et al. 2003). Certain *Bacillus* sp. have been shown to antagonize the growth of pathogenic *Vibrio* spp. (Vaseeharan and Ramasamy 2003), thus suggesting a possible mechanism of action for *Bacillus* S11.

There are two major restrictions to the approach of only screening for the production of antimicrobial substances by potential probiotic microbes (Kesarcodi-Watson, Kaspar et al. 2008). One is that other modes of probiotic activity, such as immunostimulation, digestive enzymes production, competition for attachment sites, and nutrient enhancement, are not revealed by these types of experiments. These could be major contributing mechanisms of action and would be otherwise overlooked. Another drawback is that positive results *in vitro* may not translate into the anticipated *in vivo* effects (Kesarcodi-Watson, Kaspar et al. 2008). For example, *P. fluorescens*

strain AH2 demonstrated inhibitory effects against the salmon pathogen *A. salmonicida in vitro*, but no protective effect was found when the probiotic was tested *in vivo* (Gram, Melchiorsen et al. 1999, Gram, Løvold et al. 2001). The same result was seen by Ruiz-Ponte et al. (1999), when *in vitro* antagonism of a putative probiotic bacterium did not translate to protection of scallop larvae once challenged by a pathogen. Similarly, a bacterium that is not inhibitory in the laboratory might actually be antagonistic *in vivo* (Ruiz-Ponte, Samain et al. 1999). This suggests other traits that might be required, such as successful colonization of the host by the putative probiotic organism. In other studies, probiotics have been tested further for properties such as bile resistance (Chabrillón, Arijo et al. 2006), attachment capacity (Olsson, Westerdahl et al. 1992, Hjelm, Riaza et al. 2004), immunostimulation (Rengpipat, Phianphak et al. 1998, Irianto and Austin 2002, Gullian, Thompson et al. 2004), competition for adhesion sites (Vine, Leukes et al. 2004, Chabrillón, Arijo et al. 2006) and competition for nutrients (siderophore production) (Gram, Melchiorsen et al. 1999). In practice, these latter studies test whether or not a probiotic that produces diffusible inhibitory substances also possesses other modes of probiotic action. Although competition for adhesion sites has been widely suggested as a mode of action, this has been demonstrated only *in vitro*. There are studies reporting an adhesion of certain bacteria to intestinal mucus *in vitro* (Krovacek, Faris et al. 1987, Olsson, Westerdahl et al. 1992, Garcia, Otto et al. 1997, Jöborn, Olsson et al. 1997, Hansen and Olafsen 1999, Gullian, Thompson et al. 2004, Vine, Leukes et al. 2004). The attachment properties of potential probiotics measured *in vitro* cannot be presumed to exert the same effect *in vivo* (Vine, Leukes et al. 2004). This study

examined the competitive exclusion effect of five probiotics isolated from the gut of the common clownfish, *Amphirion percula*, versus the pathogens *A. hydrophila* and *V. alginolyticus* on fish intestinal mucus. Only one of the probiotic strains inhibited the attachment of one of the pathogens on the intestinal mucus of *A. percula* (Vine, Leukes et al. 2004).

Another probiotic mechanism of action is the ability to outcompete a pathogen for attachment sites. Probiotics that use this particular mechanism of action would have a distinct advantage if the addition of probiotic bacterium was introduced during the initial egg fertilization steps of larviculture (Irianto and Austin 2002). Several studies have suggested that a probiotic effect may be seen due to competition for energy sources with pathogenic bacteria (Rico-Mora, Voltolina et al. 1998, Verschuere, Rombaut et al. 1999, Verschuere, Rombaut et al. 2000). Enhanced growth and survival were observed in *Artemia* sp. pre-exposed to nine strains (LVS_1–LVS_9) of bacteria before challenge with *V. proteolyticus*. The protective effect was attributed to the competition for energy sources and for adhesion sites between the probiotic bacteria and pathogen *V. proteolyticus* (Verschuere, Rombaut et al. 1999).

Competition for iron has been reported as an important factor in marine bacterial systems. Iron is necessary for the growth of most bacteria. However, the ferric Fe^{3+} form is found in limited amounts in the tissues and body fluids of the host animals (Verschuere, Rombaut et al. 2000). Both probiotic and pathogenic bacteria may produce siderophores that bind iron, increasing microbial growth (Gram, Melchiorsen et al. 1999). It has been hypothesized that probiotics producing siderophores could compete with potential pathogens for iron (Kesarcodi-Watson,

Kaspar et al. 2008, Watson-Kesarcodi 2008). This may deprive pathogens of iron and limit their propagation (Gram, Melchiorsen et al. 1999, Kesarcodi-Watson, Kaspar et al. 2008). This was observed by Gram et al. (1999) who found that a culture supernatant of *P. fluorescens* inhibited the growth of *V. anguillarum* when incubated in low iron conditions (Gram, Melchiorsen et al. 1999, Kesarcodi-Watson, Kaspar et al. 2008). A similar result was observed *in vivo.* Constant exposure of *P. fluorescens* either in feed or in the water reduced mortality in *Calta calta* when challenged with pathogenic *Vibrio* (Mohideen, Mohanb et al. 2010*).*

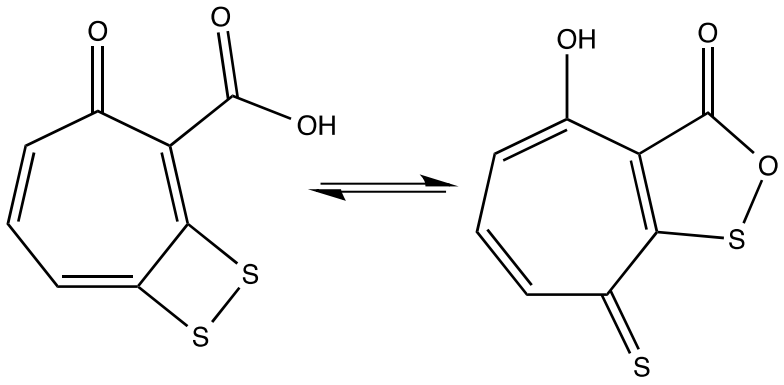
Another mode of action that probiotics may provide protection to the host is an immunostimulant effect (Kesarcodi-Watson, Kaspar et al. 2008). Itami et al. (1998) found increased phagocytic activity of shrimp granulocytes compared to controls of *Marsupenaeus japonicas,* the Japanese tiger prawn, when treated with the peptidoglycan of *Bifidobacterium thermophilum* and then challenged with *V. penaeicida* (Itami, Asano et al. 1998). A study by Gullian et al. (2004) tested the immunostimulation of healthy wild shrimp by a live *Vibrio* sp. (P62 and P63) and *Bacillus* sp. (P64) using the pathogen *V. alginolyticus* (IIi) as a positive control (Gullian, Thompson et al. 2004). They concluded that *Bacillus* sp. P64 showed both probiotic and immunostimulatory effects (Gullian, Thompson et al. 2004, Kesarcodi-Watson, Kaspar et al. 2008).

There are many probiotic strains of *Bacillus* bacteria cited as useful for the treatment of bacterial disease in aquaculture (Rengpipat, Phianphak et al. 1998, Gatesoupe 1999, Murthy and TJ 1999, Riquelme, Jorquera et al. 2001, Meunpol, Lopinyosiri et al. 2003, Farzanfar 2006, Vine, Leukes et al. 2006, Ziaei-Nejad, Rezaei

et al. 2006, Decamp, Moriarty et al. 2008, Romalde and Barja 2010). In particular, *Bacillus* spp. have shown promise in crustacean and mollusk aquaculture (Queiroz and Boyd 1998). Several species of *Bacillus* have been used to increase the survival of crustaceans (Queiroz and Boyd 1998, Rengpipat, Phianphak et al. 1998, Meunpol, Lopinyosiri et al. 2003, Vaseeharan and Ramasamy 2003, Cude, Mooney et al. 2012). These probiotic candidates were initially screened *in vitro* for inhibitory substances and many were further tested *in vivo* for their probiotic activity. For example, a study by Rengpipat et al. examined the growth of *P. monodon* and its resistance to *Vibrio* when fed with *Bacillus* (BS11) (Rengpipat, Phianphak et al. 1998). Their results suggested that the growth and survival rates of *P. monodon* were significantly higher than the controls due to probiotic stimulation of cellular and humoral immunity (Rengpipat, Phianphak et al. 1998).

Bacteria belonging to the genus *Phaeobacter* have also shown promise as probiotic agents for aquaculture. *Phaeobacter* are α-proteobacteria belonging to the *Roseobacter* clade, which comprises the majority of oceanic bacterioplankton (Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011). Previous studies have reported several *Roseobacter* isolates that exhibit antagonistic effects against pathogenic *Vibrio* species that are problematic in cod and turbot larviculture (Brinkhoff, Bach et al. 2004, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011). *Roseobacter* clade isolates have been shown to produce tropodithetic acid (TDA), an antibiotic with potent effects against marine and human pathogens.

The structure of TDA was reported by Liang to be a tropolone fused to a four membered ring incorporating a disulfide (Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Webber et al. 2011, D'Alvise, Lillebo et al. 2012). TDA has been proposed to be a tautomer of thiotropocin, another broad-spectrum antibiotic (Bentley 2008, Greer, Aebisher et al. 2008, Porsby, Webber et al. 2011, Seyedsayamdost, Carr et al. 2011). The structure of thiotropocin is a tropolone attached to a five membered thiolactone ring (Figure 1), and was supported by ^{13}C labeling studies by Cane and coworkers (Cane, Wu et al. 1992). Tropodithietic acid (TDA) was first described by Liang from bacteria belonging to the *Roseobacter* clade (Liang 2003). The structure elucidation data included mass spectrometry,UV, ^{1}H, ^{13}C, HSQC, HMBC NMR, IR, and X-ray data. Many papers that work with *Roseobacter* clade bacteria attribute the antibiotic activity to TDA and subsequently cite this dissertation (Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011, Seyedsayamdost, Carr et al. 2011). Thiotropocin had broad spectrum antibiotic activity against human pathogens was previously isolated from *Pseudomonas* (Kintaka, Ono et al. 1984). In 2006 Laatsch cited that the unpublished data suggesting the structural assignments of TDA were incorrect (Laatsch 2006). Computational studies carried out by Greer et al. provided insight that tropodithietic acid and thiotropocin exist as isomers rather than a single structure with a symmetric hydrogen bond (Greer, Aebisher et al. 2008).



Tropodithietic Acid Thiotropocin

Figure 1. Antibiotic produced by certain bacteria belonging to the genera *Phaeobacter*, *Ruegeria*, and *Roseobacter* that exhibit probiotic activity against *V. anguillarum.*

Several *in vivo* studies have shown promise toward the development of probiotic agents in shellfish, particularly in crustaceans. For example, *Lactobacillus* sp. was examined for activity against gram negative bacteria in freshwater prawns (Venkat, Sahu et al. 2004). *Bifidobacterium thermophilum* derived peptidoglycan proved to be successful *in vivo* against *V. penaeicida* in shrimp (Itami, Asano et al. 1998). Several *Bacillus* sp. are active against *V. harveyi* in shrimp aquaculture (Vaseeharan and Ramasamy 2003) (Moriarty 1998, Meunpol, Lopinyosiri et al. 2003, Gullian, Thompson et al. 2004). Alavandi et al. (2004) examined two candidate probiotics, *Pseudomonas* sp. PM11 and *Vibrio fluvalis* sp. PM 17, for their ability to cause immunostimulation in shrimp (Alavandi, Vijayan et al. 2004). Gibson et al. demonstrated that probiotic candidate *Aeromonas media* sp. 199 produced an inhibitory substance that contributed to the antagonistic effects *in vitro* as well *in vivo* (Gibson, Woodworth et al. 1998). *Aeromonas media* sp. 199 increased the survival

rate of 2-6 day old Pacific oyster larvae *Crassostrea gigas* over five days in a *V. tubiashii* challenge (Gibson, Woodworth et al. 1998). Karim et al. (2013) determined both *Bacillus pumilus* RI0695 and *Phaeobacter gallaeciensis* S4 exhibited antagonistic effects towards *V. tubiashii in vitro* (Karim, Zhao et al. 2013). Additionally the probionts were able to protect *Crassostrea virginica* larvae when challenged with *V. tubiashii in vivo* (Karim, Zhao et al. 2013). Karim et al. (2012) postulated that the potential mechanisms of action for *B. pumilus* RI0695 and *P. gallaeciensis* S4 could be the excretion of an antibiotic molecule, biofilm formation by the bacterium, immune modulation of the oyster larvae, and colonization of marine surfaces (Karim, Zhao et al. 2013).

Use of probiotics in bivalve shellfish hatcheries

Understanding the potential mechanisms of action of probiotics would be useful for determining the optimal modes of delivery in order to achieve disease management in commercial settings. It is necessary to ensure that probiotic microbes are non-toxic to both the intended host and the microalgal feed. Microalgae are generally used as a food source in larviculture and probiotic epiphytes could promote phytoplankton growth (Haines and Guillard 1974, Ukeles and Bishop 1975, Fukami, Nishijima et al. 1992, Fukami, Nishijima et al. 1997, Kesarcodi-Watson, Kaspar et al. 2008). Probionts that attach to microalgae may also be delivered to larvae as algal epiphytes. Gomez-Gil et al. (2002) co-cultured shrimp probiotic, *V. alginolyticus* strain C7b, with *Chaetoceros muelleri*, a feed for shrimp larvae, without having any adverse effect on the microalgae (Gomez-Gil, Roque et al. 2002). Avendaño and Riquelme (1999) investigated the growth of seven bacterial strains co-cultured with

Isochrysis galbana (Avendaño and Riquelme 1999). The co-culture of bacterium *Vibrio* sp. C33 significantly enhanced the ingestion of the candidate probiotic bacterium in larval scallop, *Argopecten purpuratus* (Avendaño and Riquelme 1999). Bairagi et al. (2004) examined the benefit of adding two probiotic candidates, *B. subtilis* and *B. circulans*, derived from adult carp *Cyprinus carpio*, to the diet of *Labeo rohita* (rohu) (Bairagi, Sarkar Ghosh et al. 2004). They found that the addition of both *Bacillus* bacteria increased rohu's growth, feed conversion ratio, and protein efficiency ratio (Bairagi, Sarkar Ghosh et al. 2004). It was hypothesized that this increase was due to the extracellular cellulolytic and amylolytic enzyme production by the bacteria (Bairagi, Sarkar Ghosh et al. 2004, Kesarcodi-Watson, Kaspar et al. 2008).

Commercially available probiotics for shellfish aquaculture

Although a number of potential probiotics have been studied in shellfish aquaculture, no commercial products are currently available. This is despite the fact that probiotic agents have been developed for other aquacultured species, including penaeid shrimps, catfish, rotifers, and tilapia (Hirata, Murata et al. 1998, Queiroz and Boyd 1998, Murthy and TJ 1999, Irianto and Austin 2002, Cutting 2011, Parthasarathy, Ramasubramanian et al. 2012). All of these commercial probiotics are derived from gram-positive bacteria, such as *Bacillus* spp.

It has been suggested that various gram positive and gram negative marine bacteria are possible candidate probiotics for commercial hatchery settings. However, more research, including the evaluation and development of various probiotic formulation techniques, must be performed in order to overcome the challenges of

creating a product that meets the criteria of a dry, stable, and viable formulation over time while inducing a safe and protective probiotic effect.

Conclusion

Aquaculture production is estimated to be worth 130.2 billion USD worldwide according to FAO overview of major trends and issues (FAO 2014) . Hatcheries and nurseries in the United States produce large numbers of a variety of species of oysters, clams and scallops (Elston 1998). These hatcheries can be infected with bacterial diseases and such infection can have serious impacts on production, with 45.5% of losses in hatchery aquaculture attributed to infectious diseases (FDA 2012). Losses in production due to disease may thus result in tremendous economic loss. Aquaculturists have explored using filtration systems, electrolytic, ozonolysis and UV treatments of seawater, adjusting salinity, and selective breeding as disease management tools. Vaccines are not feasible in bivalves. The use of antibiotics in aquaculture can have potentially detrimental effects, including the development of antibiotic-resistant organisms, including the development of drug- resistant bacteria that are causative factors for human and animal diseases (Vaseeharan and Ramasamy 2003, Farzanfar 2006). Additionally, investigations into the use of probiotics as new disease management tools are being conducted.

Karim et al. (2013) demonstrated that probiotic candidates *Bacillus pumilus* RI0695 and *Phaeobacter gallaeciensis* S4 were antagonistic against growth of *V. tubiashii in vitro* and were able to protect *C. virginica* larvae when challenged with *V. tubiashii in vivo.* Chapter Two presents a chemical investigation into the mechanism of action of candidate probiotic *P. gallaeciensis* S4. Although there has been much

promise in the study of potential probiotics in shellfish aquaculture, no commercial products are currently available, a situation which affords ample opportunities for the investigation and development of probiotics for this application. Chapters Three and Four summarize experiments based upon classic pharmaceutical techniques, including granulation and lyophilization, in order to formulate two promising probiotic bacteria, *B. pumilus* RI0695 and *P. gallaeciensis* S4, respectively, for eventual use in commercial aquaculture operations.

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CHAPTER 2

CHEMICAL INVESTIGATION OF THE PROBIOTIC ACTIVITY OF *PHAEOBACTER GALLACIESIS* S4 IN THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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Abstract

Previous studies have shown that the marine bacterium *Phaeobacter gallaeciensis* S4 can reduce mortality in larvae of the Eastern oyster *Crassostreae virginica* challenged with the pathogens *Vibrio tubiashii* and *Roseovarious crassostreae*. However, the mechanisms involved in this probiotic protection remain to be elucidated. In this study, secondary metabolites produced by *P. gallaeciensis* S4 were investigated for their contributions to larval protection. Using a bioassay-guided fractionation approach, it was found that the S4 strain produces tropodithietic acid (TDA) and that this compound has potent antibiotic properties against the marine pathogens *V. tubiashii, R. crassostreae,* and *V. anguillarum*. A series of mutants of S4 (*tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, *clpX*$^-$, *rpoE*$^-$, *exoP*) were tested for their production of TDA. The *tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, and *clpX*$^-$ mutants lost the ability to produce TDA, while the *rpoE*$^-$ and *exoP*$^-$ strains produced TDA at levels similar to the wild-type strain. The *tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$ strains were deficient in TDA production and had 70-80% reduction in biofilm formation. The *clpX*$^-$ strain maintained its ability to form a normal biofilm, while the *exoP*$^-$ strain was biofilm deficient. In further collaboration with the laboratory of Dr. Marta Gomez-Chiarri at URI, challenge assays were conducted using oyster larvae, *V. tubiashii*, and the *clpX*$^-$, *exoP*$^-$, and wild-type strains. These *in vivo* experiments revealed that both TDA production and biofilm formation contribute to the probiotic activity of *P. gallaeciensis* S4.

Introduction

Bacteria belonging to the genus *Phaeobacter* have shown promise as probiotic agents for aquaculture. *Phaeobacter* are α-proteobacteria belonging to the *Roseobacter* clade, which comprises the majority of oceanic bacterioplankton (Liang 2003, Brinkhoff, Bach et al. 2004, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011). Previous studies have reported several *Roseobacter* isolates that exhibit antagonistic effects against pathogenic *Vibrio* species that are problematic in cod and turbot larviculture (Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011, D'Alvise, Lillebo et al. 2012).

In the last two decades, there has been an increased incidence of disease outbreaks by oyster pathogens (Boettcher, Geaghan et al. 2005, Gomez-Leon, Villamil et al. 2008, USDA 2008, NOAA 2012). Some of these diseases are detrimental to hatchery production of larvae and can subsequently impact shellfish production by farmers. In particular, pathogen outbreaks of *Vibrio* spp. can have devastating effects on oyster larviculture (Romalde and Barja 2010). In 2006, *Vibrio* outbreaks were reported to have caused a 59% decline in oyster larvae production (Balcazar, de Blas et al. 2006). Other infectious diseases also affect the shellfish industry. Outbreaks of the bacterial pathogen *Roseovarious crassostreae* can cause up to 90% losses of total production. New disease management tools are required to combat pathogen outbreaks in shellfish aquaculture facilities.

The treatment of pathogenic disease in aquaculture is problematic due to limited resources and treatment options (Decamp, Moriarty et al. 2008). Filtration, UV

irradiation, ozonolysis, and electrolysis of incoming seawater have all been explored to reduce the risk of introducing pathogens to hatcheries. However, all of these methods have drawbacks, mainly high costs (Meunpol, Lopinyosiri et al. 2003, Park, Kim et al. 2011). Seawater filtration using a 1µm filter cartridge and followed by exposure to UV irradiation (30,000 $\mu Ws^{-1}cm^{-2}$) prevented infection of oyster juveniles by both eukaryotic parasites *H. nelsoni* and *Perkinsus marinus,* the causative agent for Dermo disease (Ford, Xu et al. 2001). Filter sterilized seawater has yielded good results in preventing Juvenile Oyster Disease JOD in cultures of the Eastern oyster *C. virginica* (Boettcher, Barber et al. 1999). Ozonation in freshwater recirculating hatchery systems has been used for almost 30 years to control bacterial disease (Park, Kim et al. 2011). There have been some conflicting reports that ozonation in seawater can have toxic effects due to a reaction with bromine (Park, Kim et al. 2011). Electrolytic methods have been successful in reducing microalgae, bacteria, and viruses (Jorquera, Valencia et al. 2002, Wijesekara, Nomura et al. 2006, FAO 2014). Despite some success in water treatment, shellfish hatcheries are still impacted by disease outbreaks. Vaccines are not feasible in bivalves and overuse of antibiotics can promote drug resistance. In order to minimize this threat, new alternatives must be sought out for disease management.

Probiotics have shown promise as a disease management tool for aquaculture, particularly in shrimp (Rengpipat, Phianphak et al. 1998, Farzanfar 2006, Decamp, Moriarty et al. 2008). They are defined by the World Health Organization to be 'Live microbes which have a beneficial effect on the host'(Liang 2003). They are a "green" alternative to the use of antibiotics since they are unlikely to contribute to the rising

problem of antimicrobial resistance. Marine probiotics have several proposed modes of action, including the production of antimicrobial compounds, competition for adhesion sites and nutrients, stimulation of the host immune system, and enhancement of nutrition for the host (Queiroz and Boyd 1998, Murthy and TJ 1999, Ziaei-Nejad, Rezaei et al. 2006, Kesarcodi-Watson, Kaspar et al. 2008, Cude, Mooney et al. 2012). Unlike chemical antibiotics, probiotics are provided to larvae prior to infection outbreaks in order to control pathogens in the aquaculture setting.

Previously, *Phaeobacter gallaeciensis* S4 was isolated from the inner shell surface of an apparently healthy oyster. *Phaeobacter* spp. can form rosettes, are excellent biofilm formers, and are considered to be dominant colonizers of surfaces in marine environments. When S4 was used as a potential probiotic treatment of oyster larvae, it showed strong antagonistic properties against pathogens and increased host survival in the presence of bacterial pathogens. S4 can be used to mitigate the effects of several important diseases, but little is known about the probiotic mechanisms of action (Karim, Zhao et al. 2013). In this study, we examined the roles of antibiotic production and biofilm formation in the probiotic activity of *P. gallaeciensis* S4. Bioassay-guided fractionation was used to isolate tropodithietic acid (TDA), a potent antibiotic against important marine pathogens. Using a series of gene-knockout experiments and *in vivo* challenges with oyster larvae, the contributions of TDA and biofilm production by S4 were individually interrogated for their contributions to the probiotic activity of S4.

Methods and Materials

General Experimental Procedures

^{1}H NMR spectra were recorded in benzene-d_6 at 20 °C on a Varian 500 MHz VNMRS spectrometer (Agilent Technologies, Wilmington, DE USA). Chemical shifts were referenced with to the solvent signal at δ_H 7.16. Data processing was performed using VNMRJ software (Agilent Technologies, Wilmington, DE USA). ESIMS of the purified compound was measured using an AB Sciex QSTAR Elite quadrupole time-of-flight (qTOF) mass spectrometer (m/z 5-40,000) equipped with a Turbo Ion Spray source (AB Sciex, Framingham, MA USA). Preparative high-pressure liquid chromatography HPLC was conducted on a Hitachi Elite LaChrom system consisting of a L2130 pump, L-2200 autosampler and an L-2455 diode array detector. Data processing was performed on EZ Chrom Elite software (Hitachi Santa Clara, CA USA). The HPLC analysis was carried out on a Waters Xterra 5µm C18 100 x 3.0 mm column (Milford, MA USA). UHPLC experiments were conducted using a Hitachi La Chrom Ultra® equipped with a diode-array detector L2445U, a column oven L-2300, an auto sampler L-2200U, two L2160U pumps, and a Fortis® (Cheshire, CH64 3UG) 1.7µ 2.1x50 mm C18 column connected to a 3 µ C18 guard column.

Bacteria strains and cultivation

Phaeobacter gallaeciensis S4, *R. crassostreae* Cv 919-312^{T} and *V. tubiashii* RE22 were provided by Dr. Marta Gómez-Chiarri (University of Rhode Island), and *Vibrio harveyi* BB120 was provided by Dr. David Nelson (University of Rhode Island). *P. gallaeciensis* S4 mutant and complement strains (*tdaA*$^{-}$, *tdaB*$^{-}$, *tdbD*$^{-}$, *clpX* $^{-}$ and *exoP*$^{-}$) were provided by Dr. Wenjing Zhao and Dr. David Nelson (University of Rhode Island). All bacteria were cultured in a seawater-based yeast extract-peptone

culture medium [YP; 5 g/L of peptone (Sigma Aldrich, St. Louis, MO USA), 1 g/L of yeast extract (Becton, Dickinson and Co, Franklin Lakes, NJ USA), and 30 g/L of Instant Ocean (United Pet Group Inc., Cincinnati, OH USA) in pure, reverse osmosis (RO) water] at 28 °C with shaking at 175 rpm. Bacterial stocks were stored at −80 °C in YP broth with 25% glycerol until use.

Purification and identification of bioactive molecule from *Phaeobacter gallaeciensis* S4

P. gallaeciensis S4 was cultured in 7 x 1 L volumes of YP liquid culture medium at 28 °C with shaking at 175 rpm. After 96 h, the cells were pelleted by centrifugation at 10,000 rpm for 10 min. The resulting culture supernatants were acidified to pH 3 with formic acid (FA) (Sigma Aldrich, St. Louis, MO USA) and extracted with acidified (0.1% FA) ethyl acetate. The ethyl acetate extract was concentrated *in vacuo* to yield 0.673 g of crude extract. The extract was fractionated using C18 flash chromatography (Redi sep Rf high performance gold 30g hp combiflash column; linear gradient elution 5% - 100% CH_3OH in H_2O, 0.1% FA, 45 min). This led to fractions containing pure TDA, which was identified based on comparison of ^1H NMR and ESIMS data with literature values. To construct a standard curve for determining concentrations of TDA, solutions of pure TDA in methanol (MeOH) at various concentrations were analyzed by reversed-phased HPLC. Eluent A was Milli-Q water acidified with 0.1% FA and eluent B was HPLC grade methanol acidified with 0.1% FA. The method program was a linear gradient of 5% to 100% eluent B over 9 min. The injection volume was 3 µL and the flow rate was 0.25 mL/min. All chromatograms were analyzed at a wavelength of 302 nm for detection of

TDA (t_R = 7.35 min). Each concentration was analyzed in triplicate, and averages were calculated for the peak areas. A standard curve was constructed by plotting peak area versus concentration.

Disc Diffusion assay

Extracts were dissolved in MeOH at 25 mg/mL and 10 µL of the sample was pipetted onto a paper disc and allowed to dry (250 µg/disc). Meanwhile, overnight culture of *V. harveyi* BB120 was swabbed onto YP plates. Discs were laid onto the agar surface and the plates were incubated for 24 h at 28 °C. Zones of growth inhibition were measured in mm.

Minimum inhibitory concentration (MIC) assay

The MIC of TDA was determined against *V. tubiashii* and *V. anguillarum* following standard protocols (Andrew 2001). Briefly, 5 µL of a two-fold serial dilution of TDA in methanol was added to wells of a 96 well microtiter plate. The final concentration of TDA in the well ranged from 25 µg/mL to 0.196 µg/mL. *V. tubiashii* RE22 or *V. anguillarum* (195 µL, 10^5 CFU/ mL) was added to the wells containing TDA. Control wells received 5 µL MeOH, tetracycline (antibiotic control, concentrations from 20 µg/mL to 0.391 µg/mL) or negative control (YP media only). All treatments or controls were tested in triplicate. The plate was incubated at 30 °C for 24 h. The presence or absence of *V. tubiashii* RE22, *V. anguillarum* growth was determined visually after 24 h. The MIC was defined as the lowest concentration of antibiotic resulting in no visible bacterial growth.

Construction of mutant strains of *Phaeobacter gallaeciensis* S4

S4 mutant strains were created to investigate the role of TDA in the probiotic activity of *P. gallaeciensis* S4. The mutant S4 strains (*tdaA*$^{-}$, *tdaB*$^{-}$, *tdbD*$^{-}$, *clpX*$^{-}$, *rpoE*$^{-}$, *exoP*$^{-}$*)* and complement strains (*clpX*$^{+}$ and *exoP*$^{+}$*)* were constructed and provided by the Nelson laboratory (Cell and Molecular Biology Department, University of Rhode Island). Briefly described below are the methods for the construction of the mutant strains and the complement mutation strains by Dr. Wenjing Zhao and Dr. David Nelson.

Insertional mutagenesis

Insertional mutagenesis by homologous recombination was used to create interruptions within specific genes using a modification of the procedure described by Milton and Wolf-Watz (Milton, O'Toole et al. 1996, Li, Mou et al. 2011). Briefly, primers (Table 1) were designed to amplify specific *Phaeobacter* genes based on homologous sequences from *P. gallaeciensis* 2.10 (GenBank accession No.CP002972.1). A fragment of the selected gene was PCR amplified, then digested with SacI and XbaI restriction enzymes, and the DNA fragments separated on a 1% agarose gel. Subsequently, the gel-purified PCR fragment was ligated into the suicide vector pNQ705 after digestion with SacI and XbaI and the ligation mixture was introduced into *E. coli* Sm10 (λ pir) by electroporation with Bio-Rad Gene Pulser II. The resulting recombinant plasmids were confirmed by both PCR amplification and sequencing. The mobilizable suicide vector was transferred from *E. coli* Sm10 (λ pir) into S4Sm by conjugation. Transconjugants were selected by utilizing the chloramphenicol resistance gene located on the suicide plasmid. The incorporation of the suicide vector into the gene of interest was confirmed by PCR analysis and DNA

sequencing.

Complementation of mutants

P. gallaeciensis mutants were complemented by cloning the appropriate gene fragment into the shuttle vector pBBR1MCS4 (GenBank accession No. U25060), using a modification of the method described previously by Rock and Nelson (Rock and Nelson 2006). Briefly, primers (Table 1) were designed with a SacI or XbaI site added to the 5' end of the appropriate primer. The primer pair was then used to amplify the entire gene plus ~500 bp of the 5′ and 3′ flanking regions from genomic DNA sequences of *P. gallaeciensis* 2.10 (GenBank accession No.CP002972.1). The resulting amplicon was ligated into the pBBR1MCS4 plasmid after digestion with SacI and XbaI and the ligation mixture introduced into *E. coli* Sm10 (λ pir) by electroporation with Bio-Rad Gene Pulser II. Transformants were selected on LB10-Amp100 agar plates and the recombinant plasmids confirmed by both PCR amplification and sequencing. The complementing plasmid, pBBR1MCS4-*clpX* or pBBR1MCS4-*exo*P, was transferred from *E. coli* Sm10 into *clpX* or *exoP* mutants by conjugation using the procedures described previously. The transconjugants were confirmed by PCR amplification.

Table 1. Primers used to construct S4 genetic knock out mutants

Primer	Sequence (5' to 3', underlined sequences are engineered restriction sites)	Description
pw108	GAAGAGCTCGGACGACTATGTGATTGGTCAGGC	For *clpX* insertional mutation, forward, with *Sac*I site
pw109	GGGTCTAGACGACGTTATATTCCGACGCCTGCA	For *clpX* insertional mutation, reverse, with *Xba*I site
pw153	GTATTAGAGCTCGAGCATAACCGCTTTGCCCGCCGCCCA	For *exoP* insertional mutation, forward, with *Sac*I site
pw154	CGACTATCTAGACCATGCTGAGTGCAAGGTTGACGGCGG	For *exoP* insertional mutation, reverse, with *Xba*I site
pw127	GCATTAGAGCTCGTCAGATTGGCCGAAGCCCCTTTT	For *clpX in trans* complement, forward, with *Sac*I site
pw128	CGGCTATCTAGACGAACTCACCACCTGAGGAGATACGT	For *clpX in trans* complement, reverse, with *Xba*I site
pw166	GTATTAGAGCTCCCCGTCCGATGTGTCAAAATAGGT	For e*xoP in trans* complement, forward, with *Sac*I site
pw165	CGTCTTTCTAGAGGTGCCTGCGGTCATCACCATGAC	For *exoP in trans* complement, reverse, with *Xba*I site
pwGFP-F	GCGGTACATATGTAAGGAGGAAAAACATATG	For amplification of *gfp* ORF, forward, with *Nde*I site
pwGFP-R	CTATATGGATCCCAGATCTATTTGTATAGTTCATCCA	For amplification of *gfp* ORF, reverse, with *BamH*I site
Pm113	GGTACCTGTCTGTCGCCTCTTGT	For amplification of PflaB, forward, with *Kpn*I site
Pm114	GGTACCATATCATTCCTCCATGAT	For amplification of PflaB, forward, with *Kpn*I site
pwmO-F	GCGGTACATATGATGGTGAGCAAGGGCGAGGAGAAT	For amplification of *ofp* ORF, forward, with *Nde*I site
pwmO-R	CTATATGGATCCCTTGTACAGCTCGTCCATGCCGCC	For amplification of *ofp* ORF, reverse, with *BamH*I site

Chemical Extraction of wild type strain and mutant strains

S4 wild type and S4 mutant strains (*tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, *clpX*$^-$, *rpoE*$^-$, *exoP*$^-$ and complement strains *clpX*$^+$ and *exoP*$^+$*)* were cultured in triplicate in 50 mL cultures (YP medium, 27 °C, 175 rpm) until stationary growth phase was reached as indicated by an OD_{600} absorbance of 0.7-0.8 (10^8 CFU). The cells were pelleted by centrifugation (4,000 rpm, 10 min). The supernatants were passed through a 0.2 micron filter, acidified to pH 3 with FA, and extracted twice with 50 mL of acidified (0.1% FA) ethyl acetate. The two organic layers for each culture were combined and dried *in vacuo* at 27 °C, yielding 27 crude extracts. The procedure was additionally used to prepare and analyze culture extracts of the S4 wild type and *tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, *clpX*$^-$, *rpoE*$^-$, and *exoP*$^-$ mutants at 6 h and 24 h time points, yielding an additional 42 extracts.

HPLC Detection of TDA in S4 wild type and *tdaA-, tdaB*$^-$*, tdbD*$^-$ extracts

S4 culture extracts (wild type, *tdaA*$^-$, *tdaB*$^-$, and *tdbD*$^-$) were prepared in 10 mg/mL solutions in HPLC grade MeOH. The extracts were analyzed by reversed-phase HPLC system equipped with a diode array detector (DAD) and Xterra 5µm C18 100 x 3.0 mm column. Eluent A was Milli-Q water acidified with 0.1% FA and eluent B was HPLC grade methanol acidified with 0.1% FA. The program was a linear gradient of 5% to 100% eluent B over 24 min. The injection volume was 10 µL and the flow rate was 0.5 mL/min. Spectra were analyzed at a wavelength of 302 nm for optimal detection of TDA.

UHPLC Detection and Quantification of TDA in *P. gallaeciensis* S4 culture extracts

The analysis of the crude extracts was also conducted using reversed-phase UHPLC. S4 culture extracts (wild type, *clpX*$^{-}$, *rpoE*$^{-}$, and *exoP*$^{-}$) were prepared in 10 mg/mL solutions in HPLC grade MeOH. Eluent A was Milli-Q water acidified with 0.1% FA and eluent B was HPLC grade MeOH acidified with 0.1% FA. The program was a linear gradient of 5% to 100% eluent B over 9 min. The injection volume was 3 µL and the flow rate was 0.25 mL/min. All chromatograms were analyzed at a wavelength of 302 nm for detection of TDA. Each strain was analyzed in triplicate. TDA concentrations were determined based on a standard curve.

Oyster larvae bacterial challenges

Oyster larvae challenges were performed by Sae Bom Sohn and Dr. Marta Gomez-Chiarri (URI) as previously described (Karim, Zhao et al. 2013). Briefly, oyster larvae (25-30) were placed in each well of a 6 well plate containing 5 mL of fresh sterile seawater (FSSW) at 28 psu. Each treatment was run in triplicate. The wild type S4 or mutants were added to wells at a final concentration of 10^5 CFU/mL. Oyster larvae were fed with commercial algal paste (Reed Mariculture Inc., San Jose, CA, USA) in order to promote ingestion of probiotics by the larvae while feeding. Plates were incubated at 22-23 °C for 24 h with gentle rocking. Water was changed after 24 h of incubation with the probiotic and *V. tubiashii* RE22 was added to 5 mL of FSSW to achieve a final concentration of 10^5 CFU/mL. Larvae and pathogen were incubated for an additional 24 h. In order to determine the survival of larval oysters, 200 µL of neutral red (stock concentration 13.3 mg/L) was added to each well (final concentration of 0.53 mg//L in each well) and incubated for 2 h. The neutral red staining technique helps to distinguish the live (stained) and dead (unstained) oysters

(Gomez-Leon, Villamil et al. 2008). Both the survival rate (SR) and relative percent survival (RPS) were calculated using the following formulas:

SR= (number of survivors/total number of oysters) x100

RPS = [1-(%survival challenge control oysters/% survival challenged treatment oysters)] x100

Statistical analysis

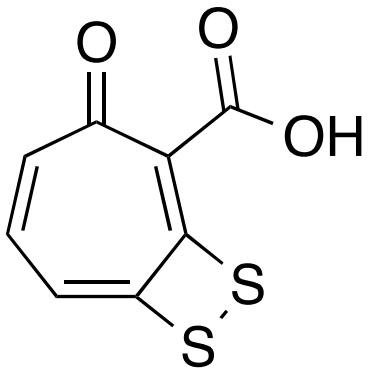
All experiments were performed in triplicate, and a q-test was performed on the area underneath the curve for each data set for all concentrations of the TDA analysis. As previously described (Karim, Zhao et al. 2013), oyster challenge assays were analyzed using One Way Analysis of Variance (ANOVA) and multiple comparison tests (Tukey Test) was used to determine significant level between groups. Statistics were analyzed using Sigmastat 3.1 software (Systat) (Karim, Zhao et al. 2013).

Results

***P. gallaeciensis* S4 wild-type produces the antibiotic tropodithietic acid**

P. gallaeciensis S4 was previously shown to inhibit the growth of several oyster pathogens (Karim, Zhao et al. 2013). However, the identity of the putative antibiotic was unknown. *P. gallaeciensis* S4 was cultured in 7 x 1 L scale for 96 h and the cells were removed by centrifugation to prevent emulsion formation during liquid-liquid partitioning. The resulting cell-free broths were extracted with acidified ethyl acetate to yield 1.26 g of a crude extract that contained the antibiotic activity. Antibiotic activity was assessed using a disc diffusion assay and found to reside in the organic fraction. Further bioassay-guided fractionation was pursued by C18 medium

pressure chromatography yielding 6 fractions. The disc diffusion tests indicated only one active fraction (F5), which was further analyzed by analytical HPLC. This fraction appeared to contain a single compound with a retention time of (t_R= 15.1 min) and a characteristic UV absorbance at 302 nm. Thus, this fraction afforded compound 1 shown in Figure 1 with a yield of 0.71 mg/L. The molecule was also analyzed by UHPLC monitored at 302 nm (t_R =7.35 min) as shown in Figure 2. Compound 1 was obtained as a copper orange solid. ESIMS data showed an $[M+H]^+$ ion at *m/z* 212.9, $[M+Na]^+$ ion at *m/z* 234.9, $[M-COOH]^+$ ion at *m/z* 164.0 and $[M-H_2O]^+$ ion at 194.0 (Figure 3). The ^1H NMR spectra (Figure 4) had three aromatic protons [δ_H 5.36, 5.89, and 6.67] and one proton downfield at δ_H 16.77 (Table 1). The ^1H NMR data and the ESIMS data were consistent with literature values for tropodithietic acid (TDA) which has a molecular formula of $C_8H_4O_3S_2$ (Figure 1) (Liang 2003). Pure TDA had a MIC of 6.25 µg/mL (29.5 µM) against *V. tubiashii* and 2.38 $x10^{-3}$ µg/mL (0.011 µM) against *V. anguillarum*.



(1)

Figure 1. Structure of Tropodithietic Acid (1)

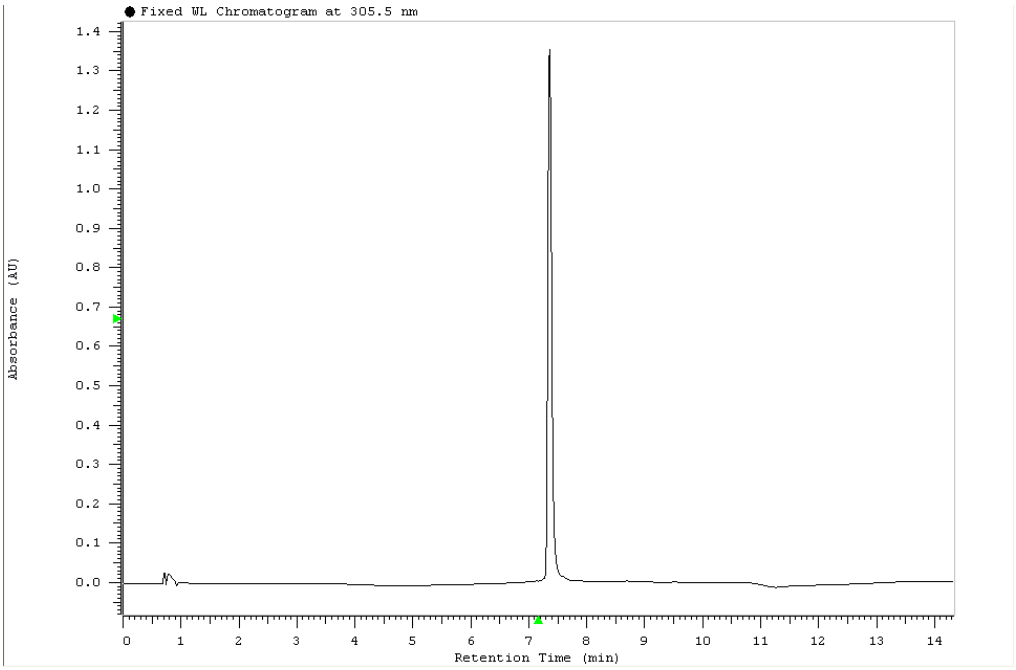


Figure 2. UHPLC chromatogram of pure TDA monitored at 302 nm.

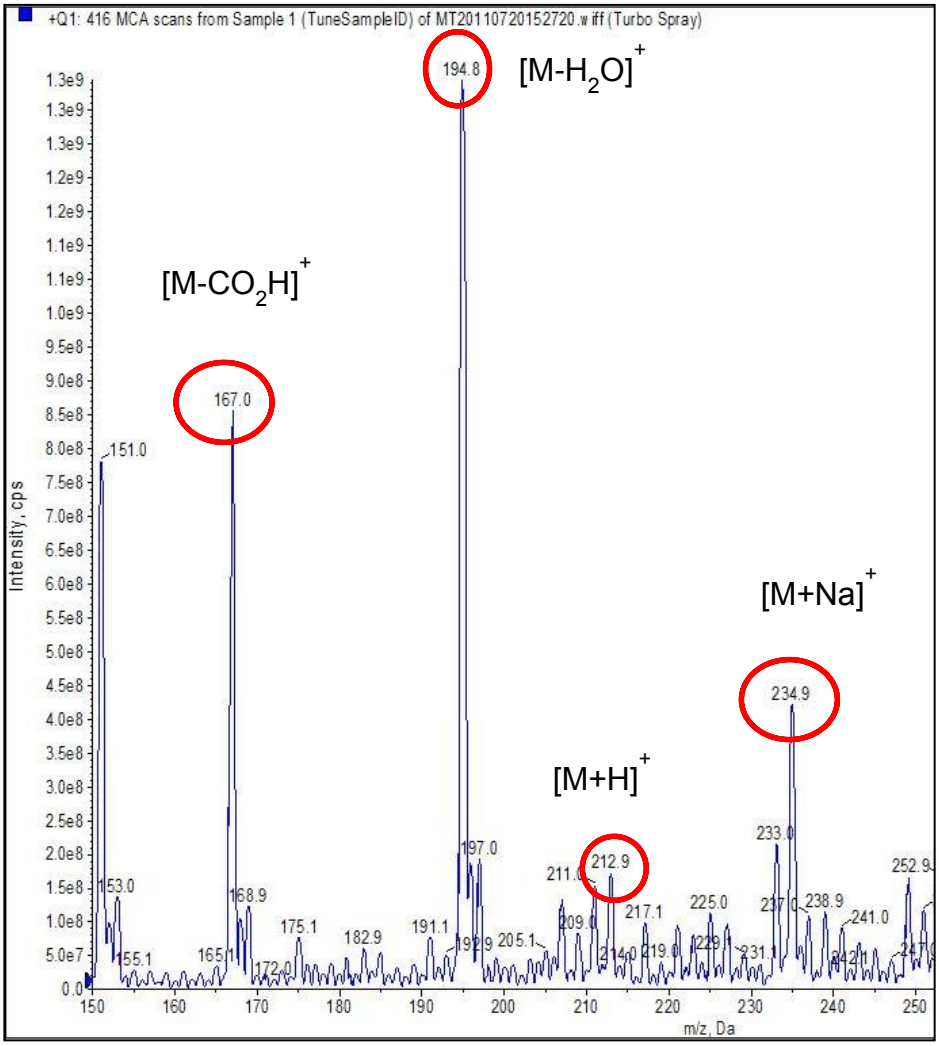
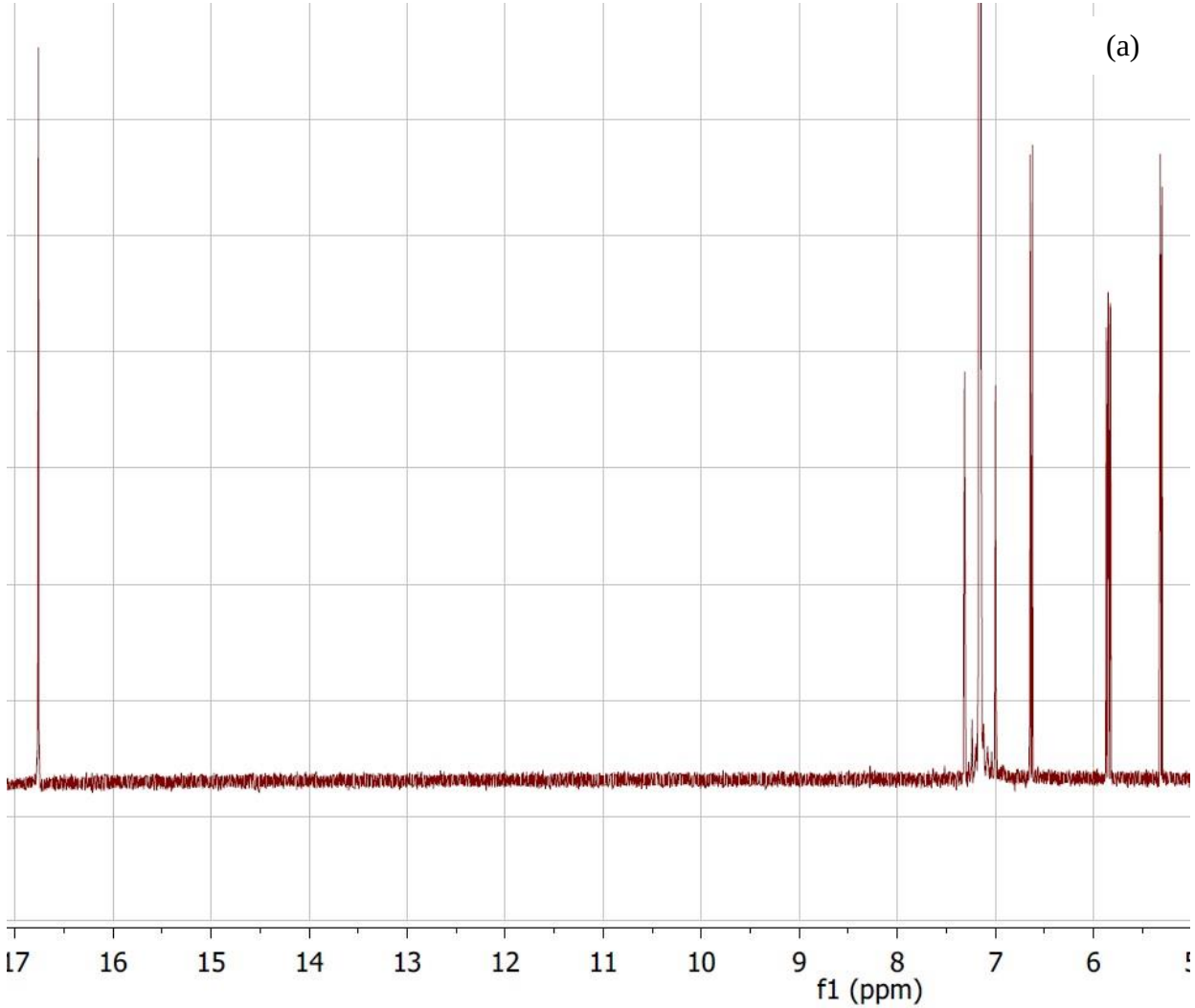


Figure 3. ESIMS of purified TDA in the positive ion mode.


(a)
17
16
15
14
13
12
11
10
9
8
7
6
f1 (ppm)

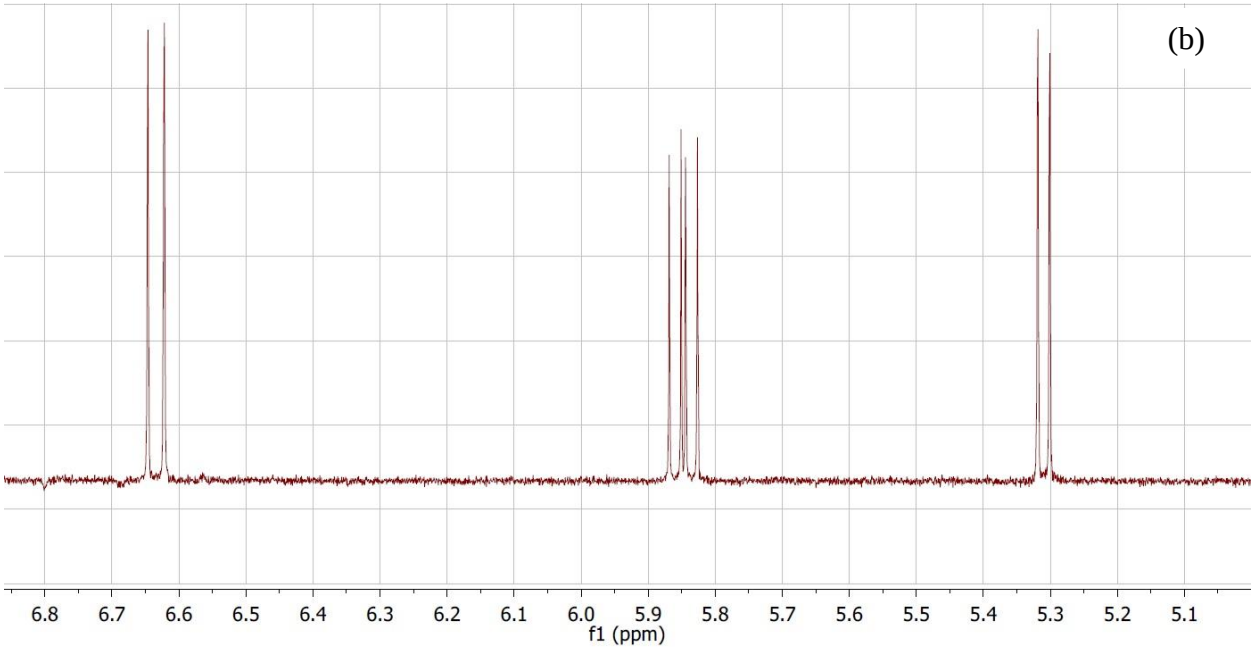


Figure 4. (a) ^{1}H NMR of spectrum for purified TDA in C_6D_6. Region of spectrum showing resonances shown. (b) Expanded ^{1}H NMR of spectrum for purified TDA in C_6D_6 from 5-7 ppm.

Table 2. ^{1}H NMR spectroscopic data of TDA (**1**)

H	δ_H, *m, J=Hz literature* (Liang 2003)	δ_H *m, J=Hz experimental*
5	1H, 5.38, d, J=8.9	1H, 5.36, d J=9.0
6	1H, 5.90, dd, J=12.2, 8.9	1H, 5.89, dd, J=12.1, 8.8
7	1H, 6.63, d J=12.2	1H, 6.67, d, J=12.5
8	1H, 16.80 s	1H, 16.77 s

Construction and chemical extraction of mutant strains of *P. gallaeciensis* S4

Mutant strains were constructed to understand the contribution of biofilm formation and antibiotic production toward probiotic activity. Target genes were those associated with biofilm production and antibiotic production. Six mutant strains of *P. gallaeciensis* S4 (*tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$, *clpX*$^-$, *rpoE*$^-$, and *exoP*$^-$) were created and provided by Dr. Wenjing Zhao and Dr. David Nelson. Organic extracts were generated from 50 mL cultures and analyzed by analytical HPLC and/or UHPLC for the presence of TDA. HPLC analysis of *tdaA*$^-$, *tdaB*$^-$ and *tdbD*$^-$ extracts confirmed the lost production of TDA when compared to S4 wild type crude extract (Figure 5). UHPLC analysis of *clpX*$^-$ extract also confirmed the lost production of TDA (Figure 9). Thus, it was concluded that the *tdaA, tdaB, tdbD* and *clpX* genes are all required for the biosynthesis of TDA. UHPLC analysis of the *rpoE*$^-$ and *exoP*$^-$ mutants confirmed that these genes are not required for TDA production. TDA was produced at all-time

points (6h, 24h and stationary phase) with a retention time of T_R = 7.35 min (Figure 6).

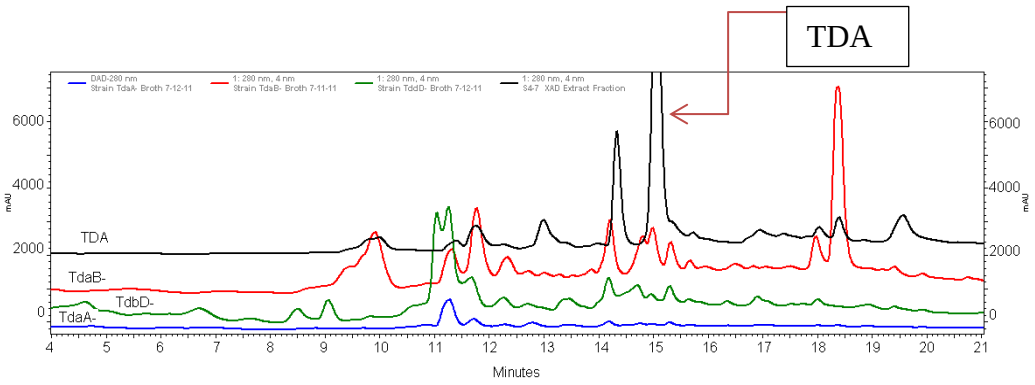
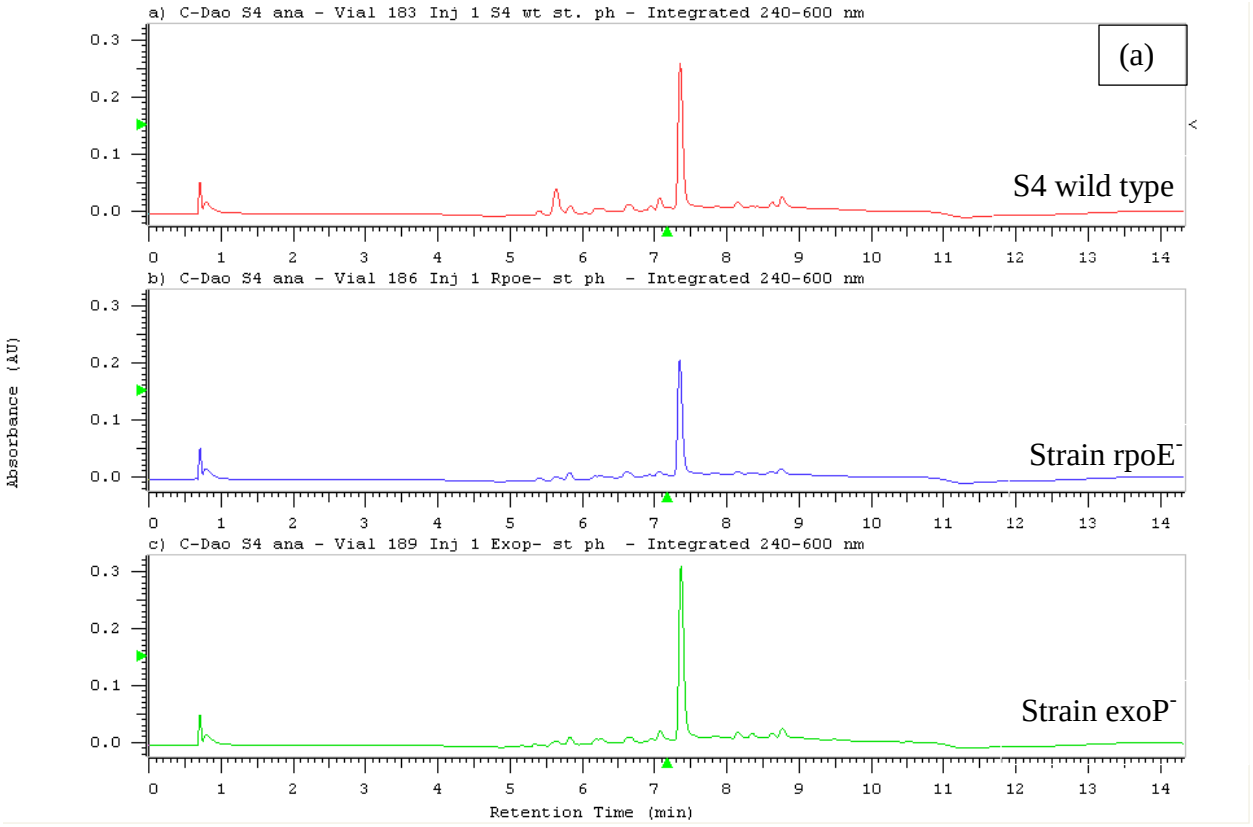


Figure 5. HPLC analysis of *P. gallaeciensis* S4 wild type and mutant strains *tdaA*$^-$, *tdaB*$^-$, *tdbD*$^-$ monitored at 290 nm.


a) C-Dao S4 ana - Vial 183 Inj 1 S4 wt st. ph - Integrated 240-600 nm
(a)
S4 wild type
b) C-Dao S4 ana - Vial 186 Inj 1 Rpoe- st ph - Integrated 240-600 nm
Strain rpoE-
c) C-Dao S4 ana - Vial 189 Inj 1 Exop- st ph - Integrated 240-600 nm
Strain exoP-
Absorbance (AU)
0.3
0.2
0.1
0.0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Retention Time (min)

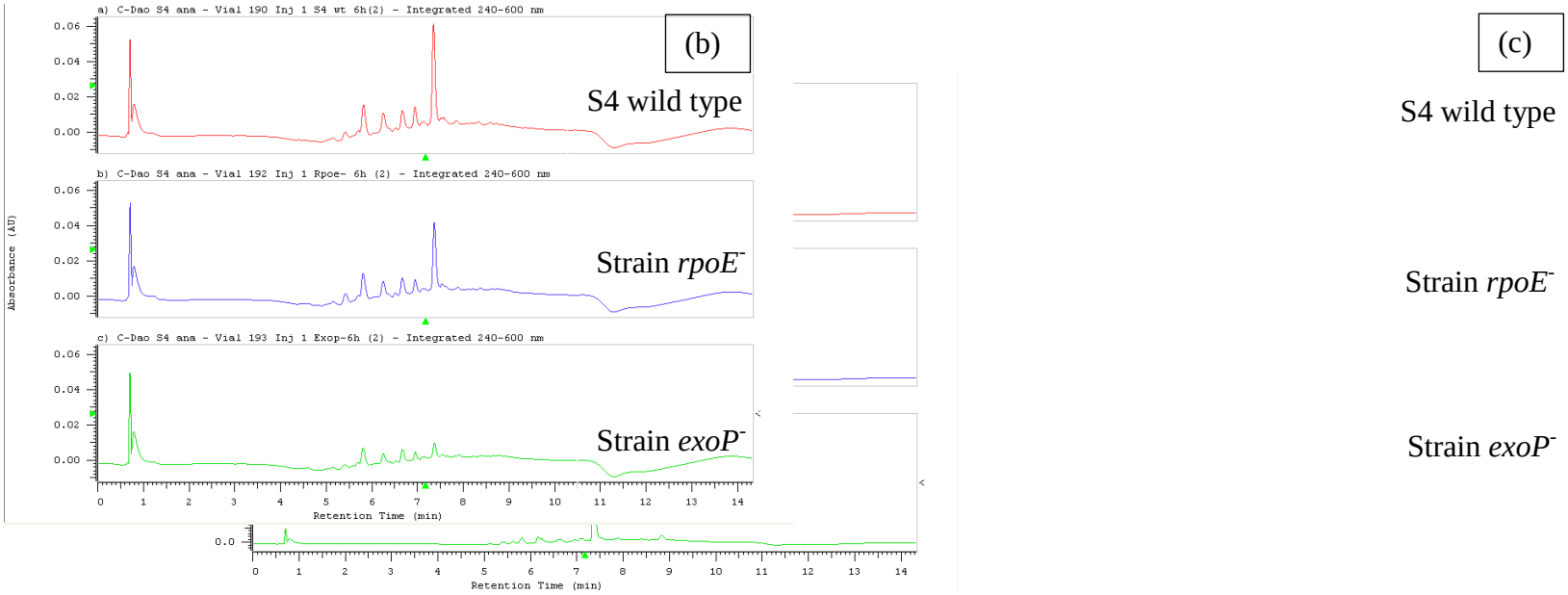


Figure 6. (a). UHPLC of S4 wild type and mutant strains *rpoE*$^-$ and *exoP*$^-$ at stationary phase monitored at 302 nm (b) UHPLC of *P. gallaeciensis* S4 wild type and *rpoE*$^-$ and *exoP*$^-$ mutant strains at 6 hours. TDA production (peak at T_R= 7.35 minutes) was monitored at 302nm. (c) UHPLC of *P. gallaeciensis* S4 wild type, *rpoE*$^-$ and *exoP*$^-$ strains at 24 hours.

P. gallaeciensis S4 wild-type is an excellent biofilm former

The crystal violet staining assay was used to demonstrate that S4 wild-type produced thick biofilms. The absorbance (OD_{580} value) for S4 wild type strain was measured to be 3.89±0.06 at 27 °C under static conditions after 60 h (Table 3). As expected, the absorbance of (OD_{580} value) of *rpoE*$^{-}$ and *clpX*$^{-}$ strains were similar to the S4 wild-type. The exopolysaccharide *exoP* gene was predicted to be involved in biofilm formation. This was confirmed by a low absorbance (OD_{580} value = 1.60± 0.09) of the mutant *exoP*$^{-}$ strain when compared to the wild type shown in Table 3. The complement mutant *exoP*$^{+}$ should have re-inserted the gene *exoP,* thus restoring biofilm formation. The complement mutant *exoP*$^{+}$ demonstrated that gene *exoP* was successfully re-inserted, due to biofilm formation similar to the wild type, as shown in Table 3. In contrast, all three pathogens (*V. anguillarum, V. tubiashii,* and *R. crassostreae*) used in this study had biofilms that were between 13.4-14.9% of the S4 wild type (Table 3). These data suggested S4 has the ability to form thick and dense biofilm matrix on glass coverslips.

Creation of a standard curve of pure TDA

A standard curve was constructed using pure TDA to quantify concentrations of the antibiotic in bacterial culture extracts. A total of 11 concentrations of TDA ranging from 1000 µg/mL to 0.97 µg/mL were prepared in 2-fold serial dilution. The analysis of all TDA concentrations was carried out by reversed-phase UHPLC in triplicate. Before the standard curve was constructed a q-test was performed on the

area underneath the peak for each data set for all concentrations (1000 µg/mL – 0.97 µg/mL). All concentrations were determined to have a 95% confidence interval. The variation between each sample was <10% difference. A graph was constructed of average peak area versus concentration and a linear regression trend line was added (Figure 7). All of the concentration data points fit well with the linear regression trend line, shown by the r^2 value = 0.998.

Table 3. Quantification of biofilm formation by measuring OD580 of crystal violet dye assay.

Strains	**OD_{580}***
P. gallaeciensis S4Sm	3.89±0.06
P. gallaeciensis WZ10 (*clpX*-)	3.90±0.12
P. gallaeciensis WZ11 (*clpX*+)	4.0±0.06
P. gallaeciensis WZ20 (*exoP*-)	1.60±0.09[a]
P. gallaeciensis WZ21 (*exoP*+)	3.90±0.10
V. anguillarum NB10Sm	0.58±0.02[a]
V. tubiashii RE22Sm	0.54±0.02[a]
R. crassostreae CV919Sm	0.52±0.08[a]

*Biofilm formation quantified by crystal violet dye assay as described in the Materials and Methods. The data presented are the average of two independent experiments and each independent experiment has three replicates.

[a]Statistically significant difference compared to S4 wild-type.

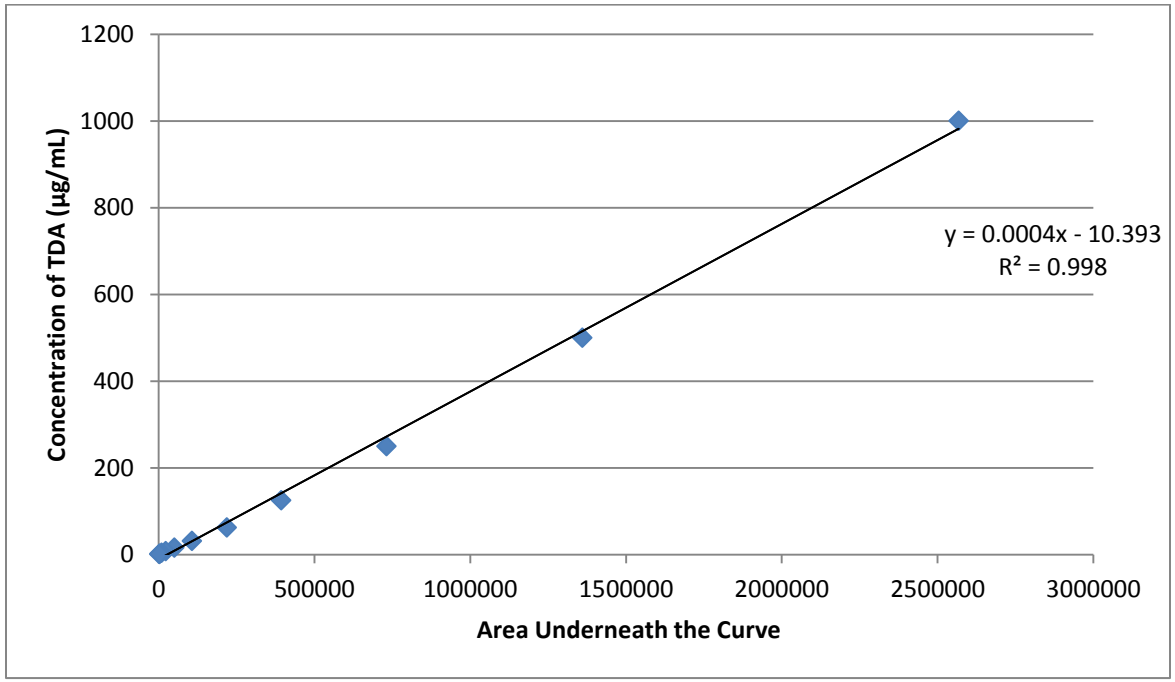


Figure 7. Standard curve of TDA based on UHPLC analysis of pure TDA in concentrations ranging from 1000 µg/mL to 0.97µg/mL.

Quantification of TDA in S4 wild-type and mutant strains of *rpoE*$^-$ and *exoP*$^-$

UHPLC analysis of S4 wild type, *rpoE*$^-$, and *exoP*$^-$ extracts at all-time points confirmed the production of TDA (Table 4). At the first time point (6 hours), the S4 wild type had produced the highest amount of TDA at all time points (Table 4 and Figure 8). The concentration of TDA at both 24 h and the stationary phase is 4-5 times the MIC against *V. tubiashii.*

Table 4. Quantification of TDA in strains of *P. gallaeciensis* S4 in 50 mL cultures at three time points.

Bacterial Strain	**Quantity of TDA produced (µg/mL)**		
	6h	24h	27h
Wild type S4	4.16±0.07	32.60±0.73	21.80±2.35
exoP$^-$	0.45±0.12	30.70±2.61	18.70±0.08
rpoE$^-$	2.47±0.11	24.80±1.61	14.20±0.52

TDA production detected using UHPLC analysis and compared to a standard curve described in the Materials and Methods. The data presented are the average of two independent experiments and each independent experiments has three replicates.

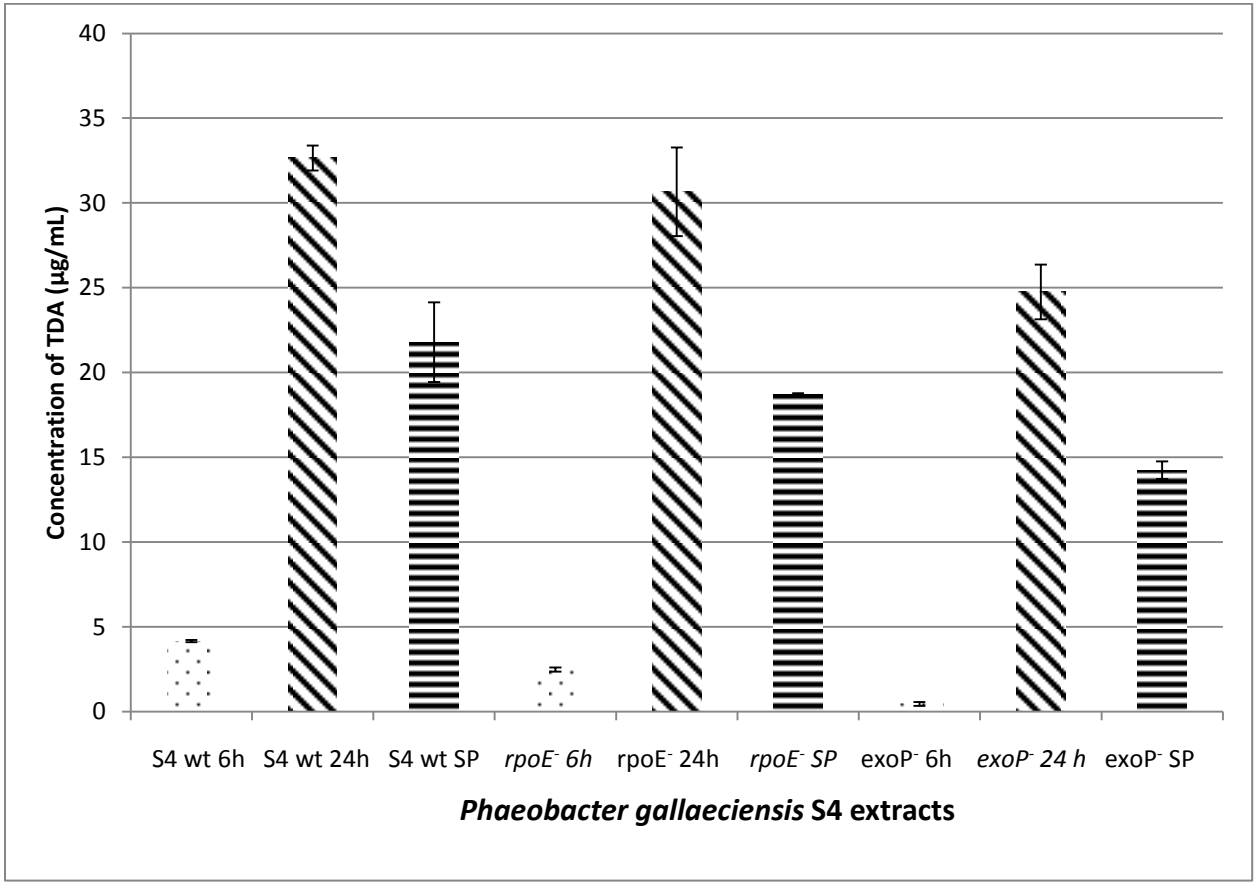


Figure 8. TDA production by S4 wild type, *rpoE*$^-$ and *exoP*$^-$ strains cultured in 50 mL scale, YP medium, at 6h, 24 h, and stationary phase (~27 h).

Detection of TDA in culture supernatants of *clpX*$^-$ and *exoP*$^-$ strains and complement *clpX*$^+$ and *exoP*$^+$ strains

UHPLC analysis of culture extracts from the *clpX*$^-$ mutant confirmed that this gene is required for optimal TDA production. Analysis of the *clpX*$^+$ strain confirmed that reinsertion of this gene restored TDA biosynthesis. TDA production was observed for both the *exoP*$^-$ and *exoP*$^+$ strains (Figure 9). Together, these results confirm that genes *tdaA*, *tdaB*, *tdbD* and *clpX*, but not *exoP*, are necessary in the biosynthesis of TDA by *P. gallaeciensis* S4. These results were in agreement with Geng et al (Geng, Bruhn et al. 2008).

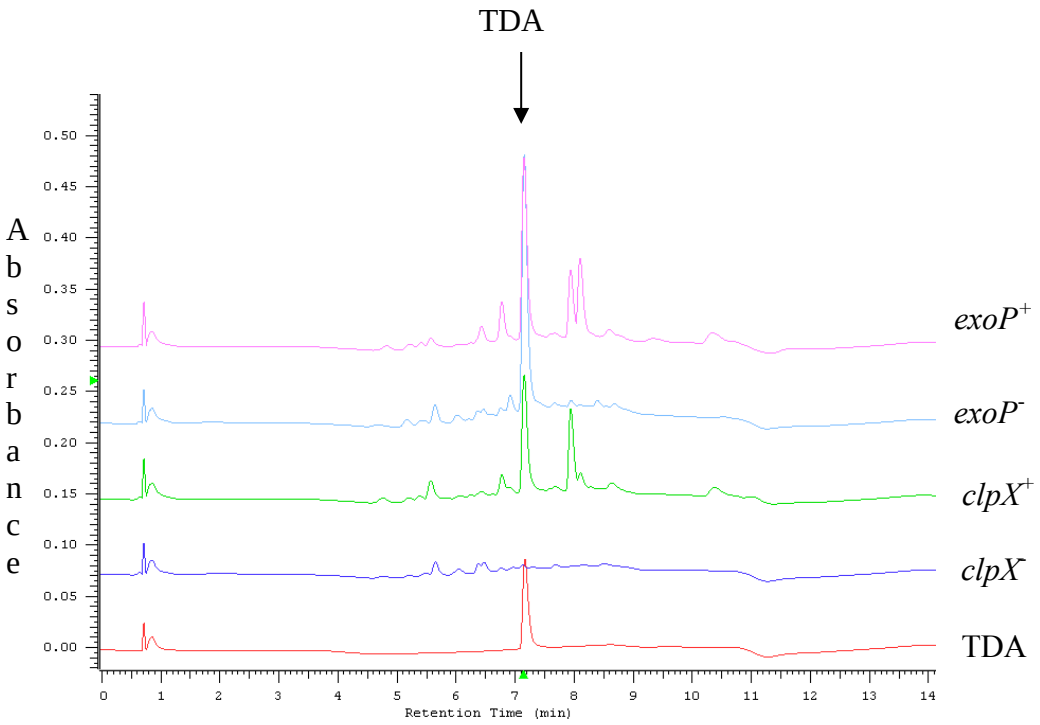


Figure 9. Detection of TDA in culture supernatants of *clpX*$^-$ and *exoP*$^-$ strains and complement strains *clpX*$^+$ and *exoP*$^+$.

Mutations in *tdaA, tdaB* and *tdbD* affect probiotic activity of *P. gallaeciensis* against *V. tubiashii* in oyster larvae.

Karim et al. (2013) demonstrated that S4 wt provides protection to oyster larvae against infections by *V. tubiashii* RE22 (Karim, Zhao et al. 2013). Oyster challenge assays were performed with both wt and mutant strains to determine if TDA production and biofilm formation are necessary for probiotic activity against *V. tubiashii*. *P. gallaeciensis* S4 mutants *tdaA*$^-$, *tdaB*$^-$ *and tdbD*$^-$ provided partial protection to the oysters against *V. tubiashii* challenge compared to wild-type S4. The *tdaA*$^-$, *tdaB*$^-$ *and tdbD*$^-$ mutants showed a >50% decline in oyster larvae survival compared to S4 wild-type as shown in Table 4. S4 mutants *tdaA*$^-$, *tdaB*$^-$ *and tdbD*$^-$ provided slight protection of the oyster larvae compared to the survival of larvae treated only with the RE22 pathogen.

Mutations in *clpX* and *exoP* affect probiotic activity of *P. gallaeciensis* against *V. tubiashii* in oyster larvae.

To further assess if mutations in TDA production or biofilm formation would affect the probiotic activity of S4 against *V. tubiashii in vivo*, oyster challenge assays were performed with the *exoP* (biofilm deficient) and *clpX* (TDA deficient) mutants. Both *P. gallaeciensis* mutants showed a partial protection of the oysters against *V. tubiashii* challenge compared to wild type. The *clpX* mutant exhibited a >50% decline in oyster larvae survival compared to S4 wild type (RPS, S4 wild type: 72% ± 1% vs. *clpX*$^-$: 35% ± 3%), while the *exoP* mutant provided almost 70% of the protection as wild type (wild type: 72% ± 1%; e*xoP*$^-$: 50% ± 8 %) (Table 5). The RPS increases in

larval survival provided by *clpX*$^{-}$ and *exoP*$^{-}$ mutants were equal to 35% ± 3% and 50% ± 8%, respectively.

Table 5. Effect of a 24 h preincubation with *Phaeobacter gallaeciensis* S4 wild-type or mutant strains on oyster larval survival 24 h after challenge with the bacterial pathogen *Vibrio tubiashii* RE22.

***P. gallaeciensis* S4 strains**	**Quantity of TDA produced at 24 h (µg/mL)**	**Relative Percent Survival (%RPS)***
Wild type	32.60± 0.73	72±1
tdaA^{-}	NP	24±2
tdaB^{-}	NP	24±4
tdbD^{-}	NP	23±2
clpX^{-}	NP	35±3
exoP^{-}	30.70± 2.61	50±8

*Larval oysters were challenged using the protocol described above in the Methods and Materials. These experiments are representative of at least 3 independent replicates.

Discussion

Probiotics have been hypothesized to possess several modes of action, including colonization, biofilm formation, production of antibiotic molecules, immunostimulation of the host, and enhanced water quality (Verschuere, Rombaut et al. 2000, Vine, Leukes et al. 2006, Kesarcodi-Watson, Kaspar et al. 2008). In this study, mutant strains of *P. gallaeciensis* S4 that were deficient in TDA production or biofilm formation were constructed and analyzed. After the confirmation of these either loss in TDA production or variations in biofilm formation these strains were tested for the ability to provide protection of *C. virginica* larvae when challenged with *V. tubiashii.* This study confirmed that antibiotic production and biofilm formation were found to be critical factors in probiotic protection of oyster larvae afforded by *P. gallaeciensis* S4..

Our research confirms the role of TDA in the probiotic activity for *Phaeobacter* sp. strain (S4). *Roseobacter* isolates have been shown to produce tropodithetic acid (TDA), an antibiotic with potent effects against marine and human pathogens (Kintaka, Ono et al. 1984, Kawano, Nakagomi Kazuya. et al. 1998, Gram, Melchiorsen et al. 1999, Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Geng, Bruhn et al. 2008, Porsby, Nielsen et al. 2008). Liang (2003) originally isolated and described TDA from *Roseobacter gallaeciensis.* The structure of TDA was reported to be a tropolone fused to a four-membered ring incorporating a disulfide (Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Webber et al. 2011, D'Alvise, Lillebo et al. 2012). TDA may convert to thiotropocin, another broad-spectrum antibiotic. The structure of thiotropocin is a tropolone attached to a five-membered

thiolactone ring (Kintaka, Ono et al. 1984, Cane, Wu et al. 1992, Bentley 2008). TDA and thiotropocin have been proposed as tautomers of each other (Bentley 2008, Greer, Aebisher et al. 2008, Porsby, Webber et al. 2011, Seyedsayamdost, Carr et al. 2011).

In addition to producing TDA, *Phaeobacter* species are typically excellent biofilm formers, colonizing a variety of surfaces including microalgae and shells (Belas, Horikawa et al. 2009, Prado, Montes et al. 2009, D'Alvise, Melchiorsen et al. 2010). Biofilms can be formed from compact bacteria communities that adhere to surfaces. Bacterial biofilms are thought to be linked to chronic or persistent diseases found in both marine and human environments (Hancock, Dahl et al. 2010). In this study, mutation in the *exoP* gene was found to reduce biofilm formation by ~60%, but production of TDA was unaffected at (24 h). Loss of optimal biofilm production resulted in reduction of probiotic protection of larval oysters from *V. tubiashii* infections when tested *in vivo*. These results demonstrate the requirement for biofilm formation to provide maximum probiotic effects.

Pure TDA exhibited antibiotic effects toward *V. tubiashii* and *V. anguillarum in vitro*, suggesting that it plays a role in animal protection. To determine the role of TDA production and biofilm formation in the probiotic activity of S4, mutant strains (*tdaA*$^{-}$, *tdaB*$^{-}$, *tdbD*$^{-}$, *clpX*, *rpoE*,$^{-}$ and *exoP*$^{-}$) were created and subjected to chemical analysis. Genes *tdaA*, *tdaB*, *clpX* and *rpoE* were previously found to be necessary for the biosynthesis of TDA in *Silicibacter* sp. TM104 (Geng, Bruhn et al. 2008, Karim, Zhao et al. 2013), so these were an initial focus for this study. These genes have roles in ring precursors, oxidation and expansion and regulatory mechanisms for synthesis of TDA.

Gene *rpoE* is an alternative sigma factor for RNA polymerase, it has a possible role in regulation of gene expression (Geng, Bruhn et al. 2008, Rattanama, Thompson et al. 2012). This study confirmed that the genes *tdaA, tdaB* and *tdbD* are necessary for TDA biosynthesis, but mutations in these genes resulted in strains that also displayed a 70-80% reduction in biofilm formation. Thus, these mutations were not suitable for dissecting the independent contributions of biofilm capability and antibiotic production. However, mutation in *clpX* (an ATP-binding protein regulatory subunit) resulted in lost production of TDA but had no effect on biofilm formation. *In vivo* testing of this mutant also resulted in only partial protection of oyster larvae, further suggesting that TDA production plays a role in the probiotic activity of *P. gallaeciensis* S4. Treatment of oyster larvae with sub-MIC levels of TDA (3.25 µg/mL and 1.56 µg/mL) also provides partial protection following challenge with *V. tubiashii* (Karim, Zhao et al. 2013). It is possible that there are other contributing factors besides antibiotic production and biofilm formation leading to probiotic activity for *P. gallaeciensis* S4.

TDA was present in all of the growth phases of S4 wild type, *rpoE*$^{-}$ and *exoP*$^{-}$. It appears that the TDA is produced in the highest concentration at 24 h in all strains, which was considered the late exponential phase. A decline in TDA concentration appears during the stationary phase, and this may correspond to degradation of the molecule, or a down regulation of the genes responsible for TDA biosynthesis. While mutations in *tdaA, tdaB, tdbD, clpX, exoP* and *rpoE* all resulted in reduced protection of larval oysters when challenged with *V. tubiashii*, each still provided a higher relative percent survival than oysters treated with *V. tubiashii* only. It is possible that

these mutants may act by modulating the immune response of the larvae, or that other yet unidentified mechanisms of probiotic activity maybe also involved. This study strongly supports that the production of TDA and the ability to form biofilms contribute to the probiotic effects of *P. gallaeciensis* S4 in oyster larviculture.

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CHAPTER 3

PREPARATION OF A GRANULAR FORMULATION OF *BACILLUS PUMILUS* RI0695 FOR POTENTIAL USE IN DISEASE MANAGEMENT IN *CRASSOSTREA VIRGINICA* HATCHERIES

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Abstract

Probiotic agents are promising tools to reduce the risks of disease outbreaks in aquaculture facilities. However, there are currently no commercially available probiotics for shellfish aquaculture. The marine bacterium *Bacillus pumilus* RI0695 was previously reported to provide significant protection of Eastern oyster, *Crassostreae virginica,* larvae when challenged with the shellfish pathogen *Vibrio tubiashii.* This investigation aimed to create a stable formulation of *B. pumilus* RI0695 for delivery to larval tanks at shellfish hatcheries. Granulation is a proven and cost effective method of formulation. A granular probiotic formulation of *B. pumilus* RI0695 was created by extruding dried *B. pumilus* RI0695 cells through three particle size sieves (40s, 80s, and 325s) resulting in 420 µ, 177 µ and 43 µ granule sizes. The 43 µ and 177 µ granular formulations stored at 27° C and sampled at 22 weeks and 29 weeks reduced mortality in *C. virginica* larvae and seed when challenged with *V. tubiashii*. This study suggests that the 43 µ and 177 µ granular formulations of *B. pumilus* RI0695 are good candidates for commercial use in shellfish hatcheries.

Introduction

The world's aquaculture production is a multi-billion dollar industry. In 2010, the Fisheries and Aquaculture Organization (FAO) released an overview of major trends and issues which estimated aquaculture production to be worth 130.2 billion USD worldwide (FAO 2014). The United States is a significant contributor to both fresh and marine aquaculture sales by adding approximately one billion dollars to the

worldwide total (Elston, Hasegawa et al. 2008). Aquaculture sales in Rhode Island have dramatically increased from $83,518 in 1995 to $2.8 million in 2012. In 2012, nearly 4.3 million oysters were produced via aquaculture in Rhode Island (Beutel 2013).

Rigorous culture of bivalve shellfish on a production scale was developed in the 1970's. Currently, hatcheries and nurseries in the United States produce large numbers of a variety of oysters, clams and scallops species (Elston 1998). However, hatchery operations are especially prone to bacterial diseases that rapidly kill larvae. Infectious diseases in the hatcheries have serious impacts on production, with 45.5% of losses in aquaculture being due to diseases (Elston 1998, FDA 2012).

Vibriosis and Juvenile Oyster Disease (JOD) are two prevalent diseases observed in hatcheries and nurseries (respectively) culturing the Eastern oyster *Crassostrea virginica* in the Northeastern US. *Vibrio* species are responsible for disease outbreaks in bivalve larviculture hatcheries. Vibriosis, or bacillary necrosis, in larvae yields massive mortality rates in contaminated hatcheries. Vibriosis in larvae is characterized by mantle and feeding disruption, loss of motility, and visceral damage, usually resulting in larval death within 24 hours (Porsby, Nielsen et al. 2008). In 2006-2007, vibriosis outbreaks caused by *Vibrio tubiashii* spread through hatcheries and nurseries in North America causing a 59% decline in oyster larvae production (Elston, Hasegawa et al. 2008). Metalloprotease and hemolysin are two virulence factors associated with the pathogenicity of *V. tubiashii* (Hasegawa, Lind et al. 2008, Porsby, Nielsen et al. 2008).

The bacterium *Roseovarious crassostreae* is the causative agent of Juvenile

Oyster Disease (JOD). JOD is endemic to New York and the New England area (NOAA 2009, Romalde and Barja 2010). JOD has been responsible for mortalities of over 90% in areas that have been infected with the disease. Indications of the disease are reduced growth of the oyster along with uneven shell growth. JOD follows a seasonal pattern, with most mortality occurring in middle to late summer, coinciding with warm water temperatures. The disease can be transmitted from oyster to oyster, but the toxins and virulence factors are still unknown for this invasive pathogen (NOAA 2009, Romalde and Barja 2010). Although not reported to be problematic in oyster hatcheries, *R. crassostreae* is able to cause mortality of oyster larvae in experimental challenges (Gomez-Leon et al. 2008).

The use of marine probiotics is being explored as a new disease management tools. Several *in vivo* studies have shown promise toward the development of probiotic agents in shellfish, particularly in crustaceans. Moriarty and others determined *Bacillus* sp. were active against *V. harveyi* in shrimp aquaculture (Moriarty 1998, Meunpol, Lopinyosiri et al. 2003, Vaseeharan and Ramasamy 2003, Gullian, Thompson et al. 2004). Alavandi et al. examined two candidate probiotics, *Pseudomonas* sp. PM11 and *Vibrio fluvalis* sp. PM 17, for their ability to cause immunostimulation in shrimp (Alavandi, Vijayan et al. 2004). Gibson et al. demonstrated that probiotic candidate *Aeromonas media* sp. 199 produced an inhibitory substance that contributed to the antagonistic effects *in vitro* as well *in vivo* (Gibson, Woodworth et al. 1998). *Bacillus* spp. bacteria have shown promise as probiotic agents in crustacean, and mollusk aquaculture (Queiroz and Boyd 1998). Several species of *Bacillus* have also been used to increase the survival of crustaceans

(Queiroz and Boyd 1998, Rengpipat, Phianphak et al. 1998, Meunpol, Lopinyosiri et al. 2003, Vaseeharan and Ramasamy 2003, Cude, Mooney et al. 2012). When probiotic bacteria pre-mixed in algae feed was used to treat shrimp ponds in a high density, there were antagonistic properties exhibited against pathogenic *Vibrio* spp. in the gut of the crustaceans to improve host survival (Decamp, Moriarty et al. 2008). There are many probiotic bacteria that have been cited to be useful for the treatment of bacterial disease in aquaculture (Rengpipat 1998, Rengpipat, Phianphak et al. 1998, Gatesoupe 1999, Naik A.T.R. 1999, Riquelme, Jorquera et al. 2001, Meunpol, Lopinyosiri et al. 2003, Farzanfar 2006, Vine, Leukes et al. 2006, Ziaei-Nejad, Rezaei et al. 2006, Decamp, Moriarty et al. 2008, Romalde and Barja 2010). A study by Meunpol et al. suggested that the probiotic Bacillus S11 was a contributing factor to the survival of black tiger shrimp (Penaeus monodon) against bacterial pathogen *V. harveryi* (Meunpol, Lopinyosiri et al. 2003). One of *Bacillus'* mechanism of action is the antagonistic effect of *Bacillus* against the pathogenic *Vibrios* sp. (Vaseeharan and Ramasamy 2003). A study by Rengpipat et al. examined the growth of *P. monodon* and its resistance to *Vibrio* when fed with *Bacillus* (BS11) (Rengpipat, Phianphak et al. 1998). Their results suggest that the growth and survival rates of *P. monodon* fed on the probiotic supplement were significantly higher than the controls (Rengpipat, Phianphak et al. 1998).

Although there has been much promise in the study of potential probiotics in shellfish aquaculture, no commercial products are currently available for bivalve larviculture. This is despite the fact that probiotic agents have been created for other aquacultured species, including penaeids, catfish, water rotifers and tilapia (Hirata,

Murata et al. 1998, Queiroz and Boyd 1998, Murthy and TJ 1999, Irianto and Austin 2002, Cutting 2011, Parthasarathy, Ramasubramanian et al. 2012). All of these commercial probiotics are gram-positive bacteria, such as *Bacillus* sp.

Previous results by Karim et al. determined both *Bacillus pumilus* RI0695 and *Phaeobacter gallaeciensis* S4 exhibited antagonistic effects towards *Vibrio tubiashii in vitro* (Karim, Zhao et al. 2013). Additionally they were able to protect *C. virginica* larvae when challenged with *V. tubiashii in vivo* (Karim, Zhao et al. 2013). Karim et al. proposed the potential mechanisms of action for *B. pumilus* RI0695 and *P. gallaeciensis* S4 could include excretion of an antibiotic molecule, biofilm formation by the bacterium, immune modulation of the oyster larvae, and colonization of marine surfaces play a role in its probiotic activity (Karim, Zhao et al. 2013). *B. pumilus* RI0695 was previously found to produce the antibiotic amicoumacin (Socha 2008). In this study, we explore creating a granular formulation of *B. pumilus* RI0695 for delivery as a disease management tool in commercial shellfish larviculture facilities. Granular formulations were tested for the viability of the bacteria over time and at different storage temperatures, and were evaluated for their protection of oyster larvae against bacterial pathogens.

Materials and methods

Bacterial Cultivation

B. pumilus RI0695 was isolated by the Rowley laboratory at URI (Socha 2008). *R. crassostreae* Cv *919-312*^{T} and *V. tubiashii* RE22 were provided by Dr. Marta Gómez-Chiarri (University of Rhode Island), and *Vibrio harveyi* BB120 was provided by Dr. David Nelson (University of Rhode Island). All bacteria were

cultured in a seawater-based yeast extract-peptone culture medium (YP; 5 g/L of peptone, 1 g/L of yeast extract, and 30 g/L of Instant Ocean (Blacksburg, VA) in pure, reverse osmosis (RO) water) at 28 °C and shaking at 175 rpm. *V. harveyi* and *R. crassostreae* were used for *in vitro* antibiotic susceptibility testing, while *V. tubiashii* was used for larval oyster challenges. Bacterial stocks were stored at −80 °C in YP broth with 25% glycerol until use.

Formulation of Bacterial Cells

Four 1L cultures of RI0695 were incubated at 28 °C and shaking for 96 hours. The cells in the cultures were harvested by centrifugation at 18,600 x *g* for 10 min at 20 °C. The supernatant was decanted, and the cell pellet was then resuspended in 150 mL of sterile artificial seawater and re-pelleted. The resulting bacterial cell pellet was then either (1) dried to a 'damp mass' at 30 °C for 12 h in a convection oven with continuous airflow, or (2) dried at 22 °C for 48 h. The dried cell pellets were extruded through three different-sized USA standard sieve stainless steel screens (Cole Palmer, Illinois, USA): 40 mesh, 80 mesh, and 325 mesh. The 40 mesh, 80 mesh, and 325 mesh screens yield average size particle sizes of 420 µ, 177 µ and 43 µ, respectively. Each formulated product was stored in glass vials at either room temperature (approximately 25 °C) or 4 °C.

Colony-Forming Unit (CFU) Viability Assay

The cell viability of all formulated probiotics was determined at 1, 2, 5, 8 and 12 weeks after formulation. Formulated products were prepared at a 5 mg/mL concentration in sterile artificial seawater, allowed to dissolve standing for 10 min,

and vortexed for 1 min. Dilutions of 1:10 and 1:100 of the stock (5 mg/mL) in sterile artificial seawater were prepared in triplicate. 10 µL of each dilution was spread onto YP agar plates in triplicate. The YP plates were incubated for 48 h at 27 °C and then colonies were counted. Each assay was performed in duplicate.

Antibiotic activity of the formulations against bacterial pathogens

Bacteria from each formulation were cultivated on agar plates and assessed for their ability to inhibit the growth of *R. crassostreae* and *V. harveyi* BB120 (Karim, Zhao et al. 2013). Overnight cultures of either *R. crassostreae* or *V. harveyi* BB120 were spread onto YP agar plates. Each of the formulated products (5 mg/mL in sterile artificial seawater) and an overnight culture of RI0695 (control) were pipetted (10 µL) in triplicate onto each YP plate. Each plate was incubated for two days at 27 °C and then assessed for zones of growth inhibition surrounding the colonies of RI0695. Assays were performed in triplicate.

Characterization of cell morphology of granular products

The cell morphology of the granular formulations (420 µ, 177 µ and 43 µ) was examined by phase contrast microscopy (Zeiss Axio Imager 2 microscope using phase-contrast optics, 100x magnification). Three aliquots of 10^4 CFU/mL dilutions in sterile artificial seawater (28psu) were prepared for each formulation from their rehydrated 5 mg/mL stock solutions. A 48 h, 10 mL fresh culture of the probiotic candidate RI0695 bacteria was examined as the control.

Oyster challenge assays

Oyster Larvae

Eastern oyster (*C. virginica)* larvae (10 – 15 days old) were obtained from the Blount Shellfish Hatchery at Roger Williams University, Bristol, RI. The larvae were divided into six wells containing 5 mL of aerated filtered sterile seawater (FSSW, 28 psu) and allowed to acclimate at room temperature (approx. 20 °C) for 24 hours before treatment. The larvae were fed commercial algal paste (Reed Mariculture Inc., San Jose, CA. USA) daily during the experiments. The water in each well was changed with FSSW every 48 hours.

Probiotic treatments of oyster larvae and challenge experiments

The ability of the RI06-95 formulations to protect larvae to challenge with the pathogen *V. tubiashii* was tested following established protocols (Karim et al. 2013). These experiments were run in triplicate. Treatments (each performed in duplicate) included: no probiotic treatment and no challenge; no probiotic treatment and challenge; probiotic treatments (either the 43 µ or the 177 µ formulation) and no challenge; and each of the probiotic treatments and challenge. Probiotics (fresh or formulations) were added to the larvae at a concentration of 10^4 CFU/mL with the algal food and incubated at room temperature for 24 h. Larvae were then removed from each well and filtered through a nylon mesh (75 µ). Larvae retained on the mesh were resuspended in 5 ml of FSSW, and placed back in the original wells. Larvae were then challenged with *V. tubiashii* (10^5 CFU/mL). Larval survival was monitored at 24 h after challenge by staining with neutral red (200 µL). The relative percent

survival compared to the challenged control (no probiotic treatment) was calculated using the following formula:

$$\text{Relative Percent Survival} = [1- (\frac{\%\text{survival challenged control oysters}}{\%\text{survival challenged treatment oysters}})] \text{ x100}$$

Probiotic treatments of oyster juveniles and challenge experiments

A total of 261 all ages Eastern oyster triploid juveniles (1.36+0.32 mm x1, 32+0.40 mm) were obtained from Blount Shellfish Hatchery at Roger Williams University, Bristol, RI.. The oyster juveniles were divided into six 1 L containers containing 700 mL of FSSW as follows: one control (no probiotic, no challenge), one challenge control (no probiotic, challenged), two containers treated with the 43 µ RI0695 granules before challenge, and two containers treated with the 177 µ RI0695 granules before challenge. Juvenile oysters were maintained in aerated FSSW at room temperature for 24 h prior to any treatment. Oysters were fed commercial algal paste (Reed Mariculture) throughout the experiment and the water was exchanged changed with fresh FSSW every 48 h. Probiotics (10^4 CFU/mL) were added to the larvae daily with food or 7 days. On day 2 after the start of the treatment, oysters were challenged with *V. tubiashii* (10^5 CFU/mL) after a water exchange. Juvenile oysters were assessed for viability using a dissecting microscope over the 9 d period. Percent survival and relative percent survival relative to the non-treated challenge control was calculated as described above. These experiments were run in duplicate.

Statistical analysis

The challenge data set survival counts data was analyzed by one way

(ANOVA) followed by Tukey's Test was used to determine significant level between groups. Data colleged as a percentage were arsine of the square root-transformed before analysis. Results were considered significant at the 95% level of confidence ($p<0.05$). Statistics were run using Sigmastat 3.1 software (Systat).The cell viability data granular size and biological replicates were analyzed by two way (ANOVA) followed by Tukey's Test for each temperature and each time point. Cell viability data biological replicates were analyzed for both temperature conditions at each time point using a one-way analysis of variance (ANOVA) followed by Dunn's method. All statistical analysis were performed using Sigma stat 3.1 software (Systat). Differences were considered to be significant at values of $P < 0.05$.

Results

Effect of granulation on cell viability

Liquid cultures of *B. pumilus* RI0695 cells were successfully formulated into granules. In a representative experiment, 1L of a 96 h culture of RI06-95 provided a total of 450.3 mg of 420 µ granules, 245.5 mg of 177 µ granules, and 155.1 mg of 43 µ granules.

The effect of the granulation processes on cell viability was assessed by measuring live cells both prior to and after formulation (Table 1). Prior to formulation, RI0695 cultures yielded at least 10^8 CFU/mL. The cell viability of the formulated granules was assessed over 8 weeks using by measuring CFU per mg over time (Tables 1-3). In general, the granulation process consistently delivered products that contained between 5×10^5 and 5×10^6 CFU/mg, and the viability of the formulated products remained relatively stable over an 8 week period, regardless of storage

temperature ($p>0.05$). Interestingly, granules stored at room temperature were significantly different. There were often demonstrated increases in CFU/mg over time, perhaps due to some humidity in the particles following the formulation step causing inaccurate weight of samples and inaccurate cell viability (Figures 1-6).

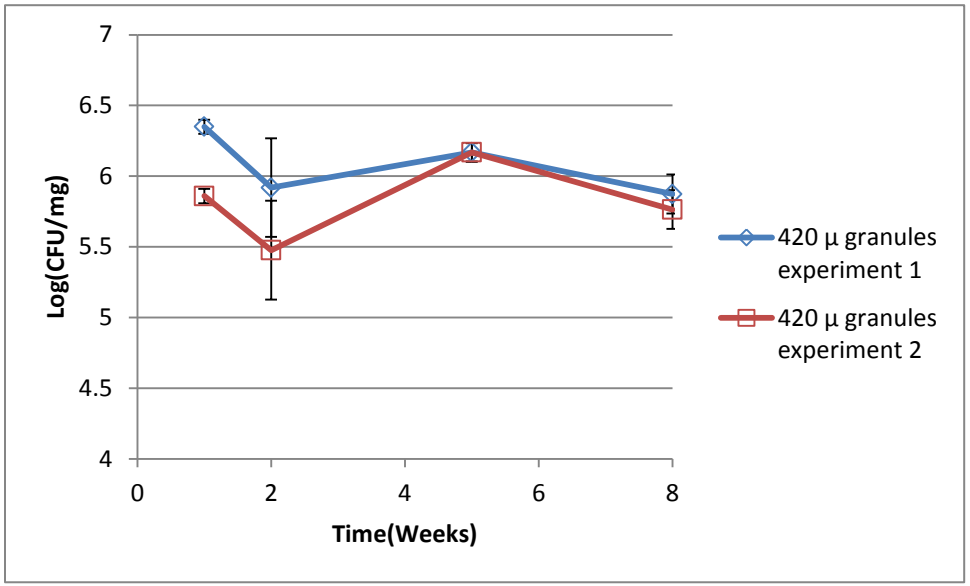

Figure 1. Cell Viability vs. Time of 420 µ granules stored at 4 °C. Each experiment was performed in triplicate.

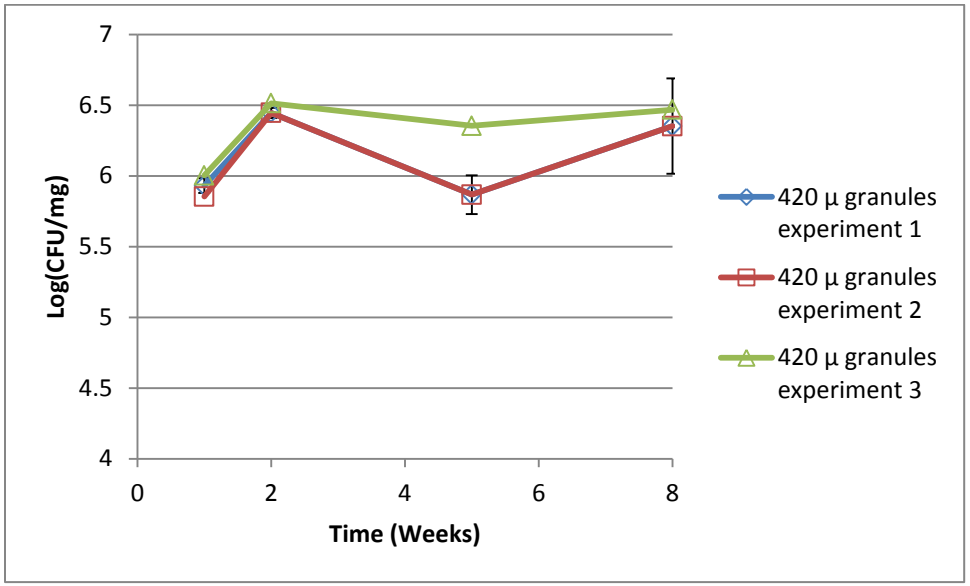

Figure 2. Cell Viability vs. Time of 420 µ granules stored at room temperature (RT). Each experiment was performed in triplicate.

Table 1. Cell viability of *B. pumilus* RI0695 in 420 µ granules (Log(CFU/mg)) over 8 weeks while stored at either room temperature (RT) or 4 °C.

Time (Week)	Exp. 1 (RT)	Exp. 2 (RT)	Exp. 3 (RT)	Exp. 1 (4 °C)	Exp. 2 (4 °C)
1	5.93± 0.05 [b]	5.85±0.03 [b]	6.00± 0.02 [b]	6.34±0.05 [b]	5.86±0.05 [b]
2	6.44±0.04 [a b]	6.44± 0.35 [a b]	6.51± 0.03 [a b]	5.92±0.35	5.48±0.35
5	5.87±0.14 [b]	5.87± 0.05 [b]	6.35± 0.20 [b]	6.17±0.07 [b]	6.17±0.07 [b]
8	6.35± 0.34 [a b]	6.35±0.39 [a b]	6.47± 0.12 [b]	5.87±0.05	5.76±0.05

[a] 1 way ANOVA analysis indicates bioreplicate experiments were significantly different.

[b] 2 way ANOVA analysis indicates that results with 420 µ granule formulation was significantly different from results with the other granule sizes.

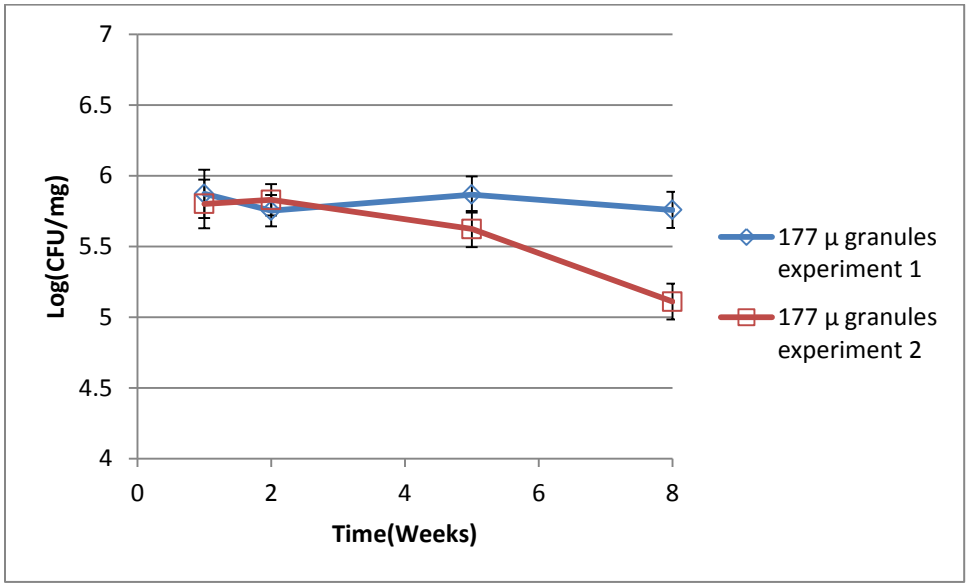

Figure 3. Cell Viability vs. Time of 177 µ granules stored at 4 °C. Each experiment was performed in triplicate.

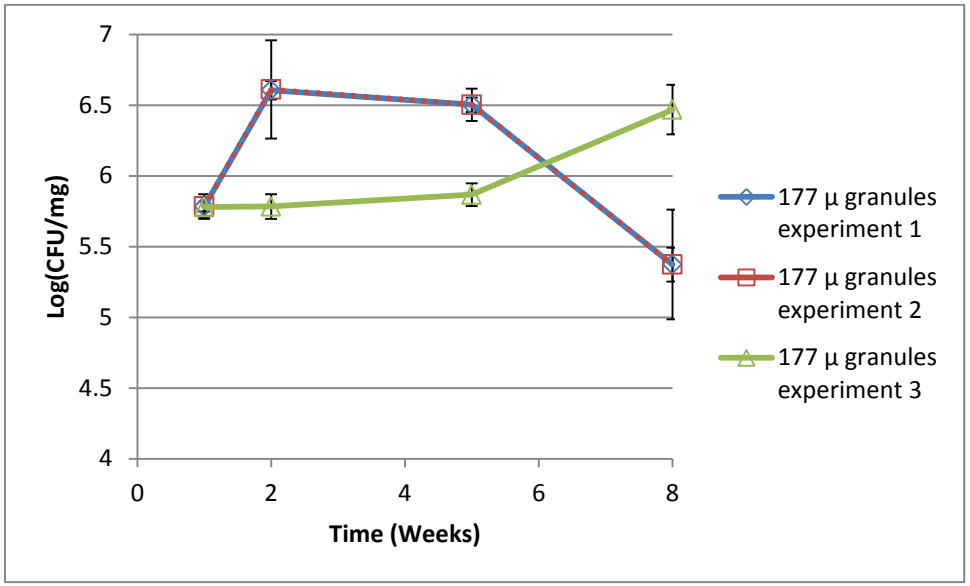

Figure 4. Cell Viability vs. Time of 177 µ granules stored at RT. Each experiment was performed in triplicate.

Table 2. Viability of *B. pumilus* RI0695 177 µ granules (Log(CFU/mg)) over 8 weeks while stored at RT and 4 °C

Time (Week)	Exp. 1 (RT)	Exp. 2 (RT)	Exp. 3 (RT)	Exp. 1 (4 °C)	Exp. 2 (4 °C)
1	5.78± 0.09 [b]	5.78± 0.07 [b]	5.78± 0.05 [b]	5.87± 0.17 [b]	5.80±0.17 [b]
2	6.61 ± 0.07 [a b]	6.61± 0.90 [a b]	5.78± 0.08 [a b]	5.75±0.11	5.83±0.11
5	6.50± 0.11 [b]	6.50± 0.80 [b]	5.87± 0.30 [b]	5.87±0.13	5.62±0.13
8	5.37± 0.1 [b] 2	5.37± 0.17 [b]	6.47± 0.16 [b]	5.76±0.13 [a b]	5.11±0.13 [a b]

[a] 1 way ANOVA analysis indicates bioreplicate experiment was significantly different.

[b] 2 way ANOVA analysis indicates that results with 177 µ granule formulation was significantly different from results with the other granule sizes.

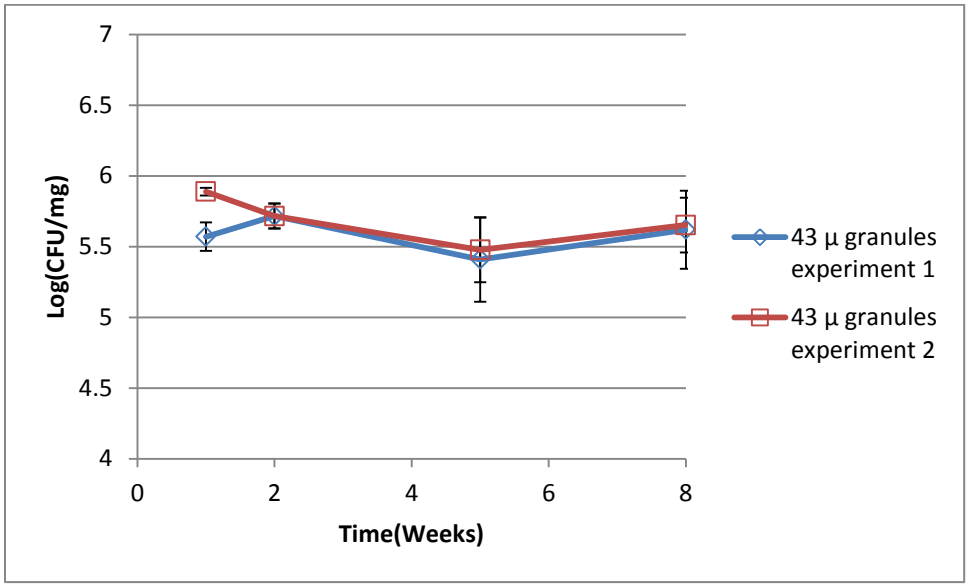

Figure 5 Cell Viability vs. Time of 43 µ granules stored at 4 °C. Each experiment was performed in triplicate.

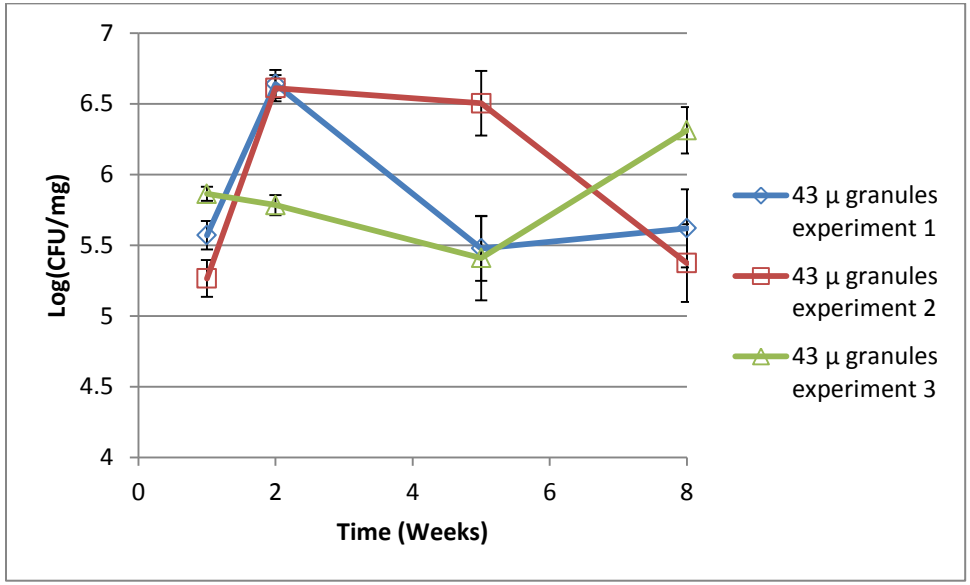

Figure 6. Cell Viability vs. Time of 43 µ granules stored at RT. Each experiment was performed in triplicate.

Table 3. Cell viability of *B. pumilus* RI0695 43 µ granules (Log(CFU/mg)) over 8 weeks while stored at RT and 4 °C

Time (Week)	Exp. 1 (RT)	Exp. 2 (RT)	Exp. 3 (RT)	Exp. 1 (4 °C)	Exp. 2 (4 °C)
1	5.57± 0.10	5.26± 0.13	5.86± 0.5	5.57±0.10	5.89±0.03
2	6.64± 0.10 [a b]	6.61± 0.09 [a b]	5.78± 0.07 [a b]	5.72±0.09 [b]	5.72±0.09 [b]
5	5.48± 0.23 [a b]	6.50± 0.23 [a b]	5.41 ± 0.30 [a b]	5.41±0.30 [a]	5.48±0.23 [a]
8	5.62± 0.28 [a b]	5.37± 0.14 [a b]	6.31± 0.16 [b a]	5.62±0.28 [a b]	5.65±0.19 [a b]

[a] 1 way ANOVA analysis indicates bioreplicate experiment was significantly different.

[b] 2 way ANOVA analysis indicates that results with 43 µ granule formulation was significantly different from results with the other granule sizes.

Effect of granulation on antimicrobial activity and cell morphology

Since antibiotic production by *B. pumilus* RI0695 may be critical to the desired probiotic activity (Socha 2008), the colonies derived from the formulated products were tested for their ability to limit the growth of *V. harveyi* BB120. There were comparable zones of no growth surrounding all the *B. pumilus* colonies, demonstrating that the formulation process had no effect on antibiotic production during subsequent cultivations (Table 4).

Table 4. Spot overlay *in vitro* assay of the all *B. pumilus* RI0695 particle sizes

Granulation	Zone of Inhibition
420 µ	7 mm
177 µ	8 mm
43 µ	8 mm
Control	8 mm

1000 µL of a 5 mg/mL solution of each granule size (420 µ, 177 µ and 43 µ) was inoculated in YP broth for 48 h at 27 °C with shaking at 175 rpm. *B. pumilus* RI0695 from cryostocks served as the control.

Effect of granulation on cell morphology

The resuspended RI0695 formulations all retained the same morphology as the RI0695 grown from cryostock. Each of the resuspended granulated formulations exhibited individual small rods and spores that are consistent with the bacterium *B. pumilus* RI0695 (Figure 7).

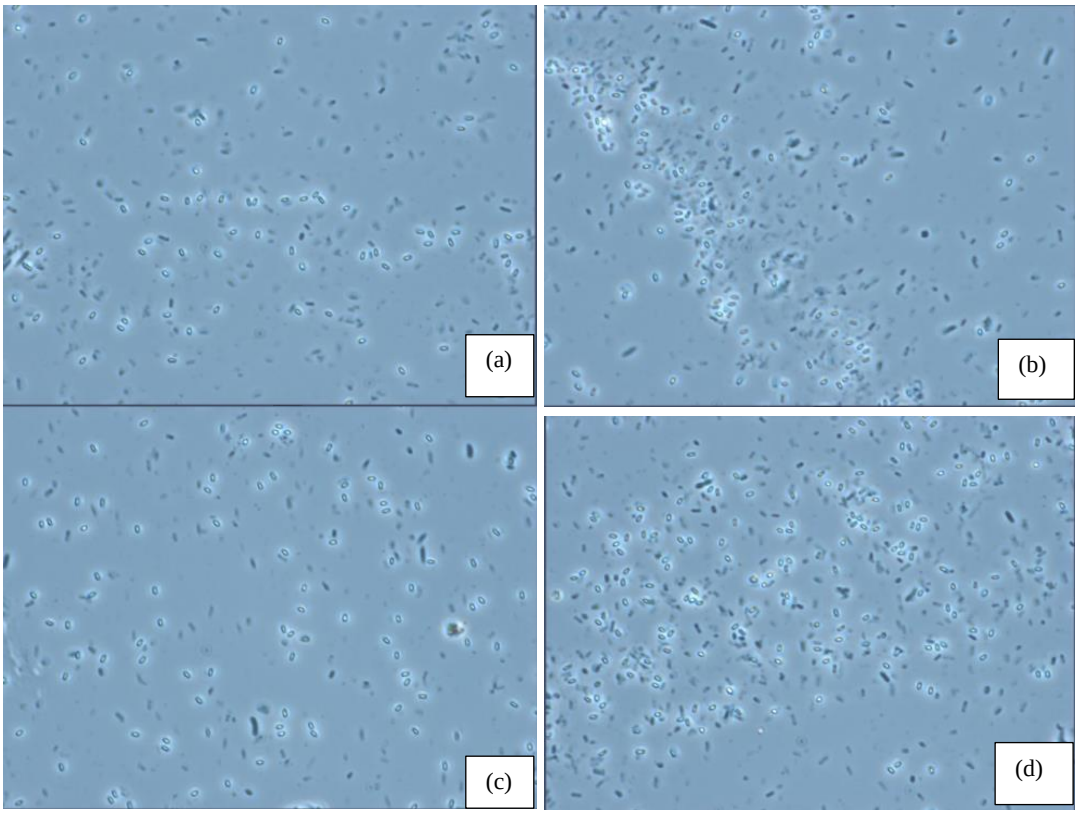

Figure 7. (a) Cell morphology of 420 µ granules resuspended in seawater to 10^5CFU/mL and examined at 100 x magnification (b) Cell morphology of 177 µ granules resuspended in seawater to 10^5 CFU/mL and examined at 100 x magnification (c) Cell morphology of 43 µ formulation resuspended in seawater to 10^5 CFU/mL and examined at 100 x magnification (d) Cell morphology of *B. pumilus* RI0695 (grown from cryostocks)

Oyster larval challenges

The granular formulations were next tested for probiotic activity using *in vivo* oyster larval and juvenile bacterial challenge assays. Two of the particle size formulations (177 µ and 43 µ) were chosen because the oyster larvae and seed in the hatcheries range from approximately 40-200 µ in size. The smaller particles that are easily soluble in water were considered as better candidates for hatchery delivery since

they might not cause sinking of swimming larvae (Karen Tammi, Blount Shellfish hatchery, personal communication). Each formulation was 29 weeks old at the time of the experiment. Cell viabilities of the 177 µ and 43 µ formulations were found to be 5.31 $\pm$ 4.93 log (CFU/mg) and 5.90 $\pm$ 5.21 log (CFU/mg), respectively, and each was added to treated oysters at 10^4 CFU/mL. Neither of the formulations was found to be detrimental to oyster survival (Figure 8). Survival of the oyster larvae after 24 h exposure to pathogen *V. tubiashii* RE22 was reduced to 25$\pm$5%. Oyster larvae pretreated with the 177 µ and 43 µ RI0695 formulations at 10^4 CFU/mL and then exposed to *V. tubiashii* demonstrated greater survival rates than larvae exposed to pathogen alone. The level of protection was similar between formulations (Table 5). The formulations provided larval protection similar to what was previously seen with probiotic treatments of freshly cultured *B. pumilus* RI0695 (10^4 CFU/mL) (Karim, Zhao et al. 2013).

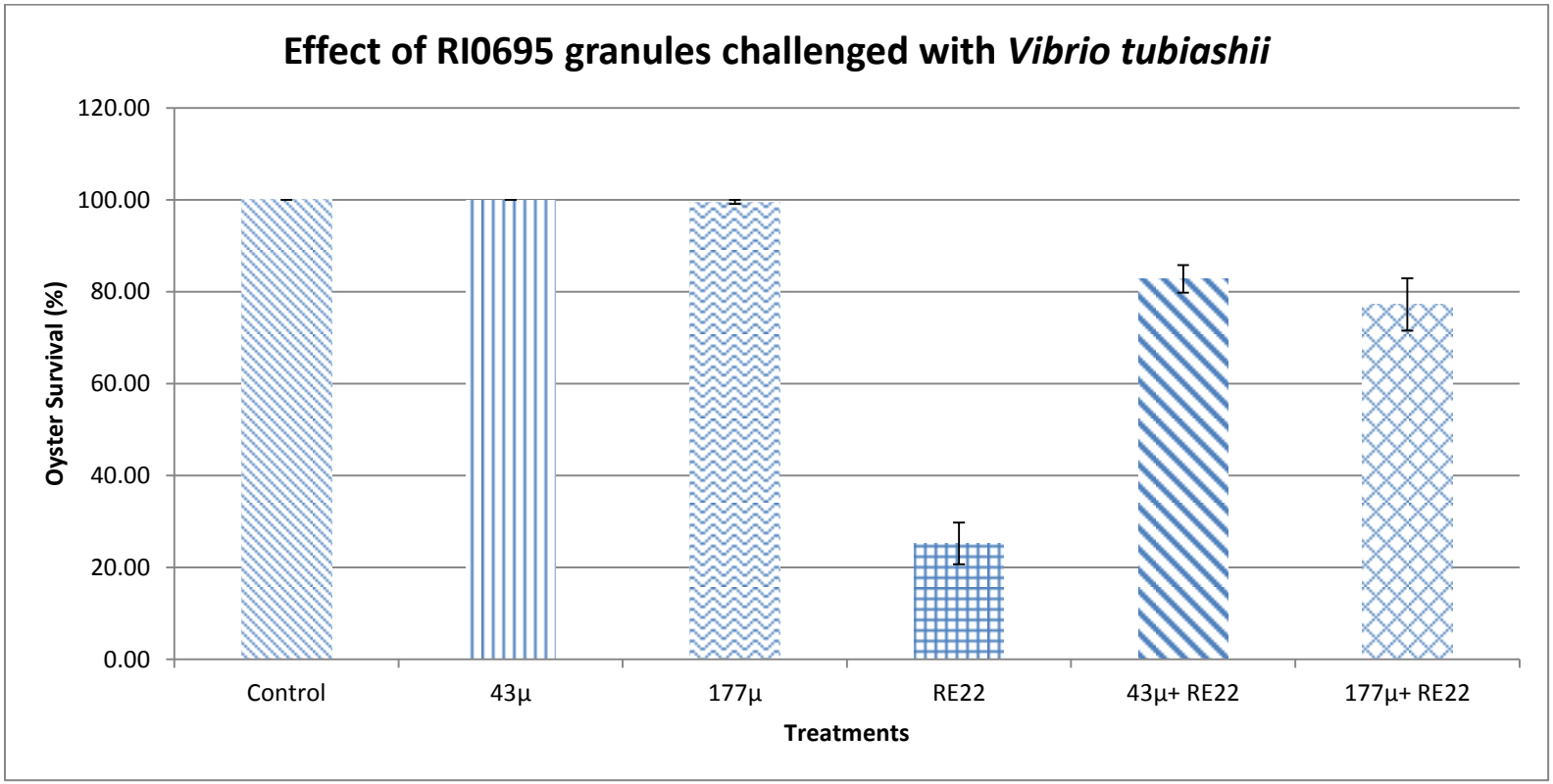

Figure 8. Effect of preincubation of oyster larvae with RI0695 granules (177µ and 43µ) at 10^4 CFU/mg on survival % (±SE) 24 hours after challenge with *V. tubiashii* RE22. Representative of 3 experiments.

Table 5. Effect of pre-incubation with 177 µ and 43 µ RI0695 granules on survival (% survival) of oyster larvae and seed after challenge with bacterial pathogen *Vibrio tubiashii* RE22.

	RE22 only (10^5 CFU/mL)	**Pretreated with probiotic formulation (**10^4 **CFU/mL)**		**Control fresh *Bacillus pumilus* RI0695 (**10^4 **CFU/mL)**
		43 µ	177 µ	
Oyster Larvae	25±5	84±3	83±5	NA
Oyster Seed	53±13	86±14	100±0	85±8

Oyster seed challenges

The 177 µ and 43 µ formulations were next tested for protection of oyster seed against *V. tubiashii* infection. After 7 days, RI0695 granules were harmless to the seed with survival rates (SR) of 100± 0% and 86±14%, respectively, for the 177 µ and 43 µ formulations (Figure 9 and table 5). The protection provided by the 177 µ and 43 µ granules to both oyster larvae and seed was similar to that provided by freshly cultured *B. pumilus* RI0695.

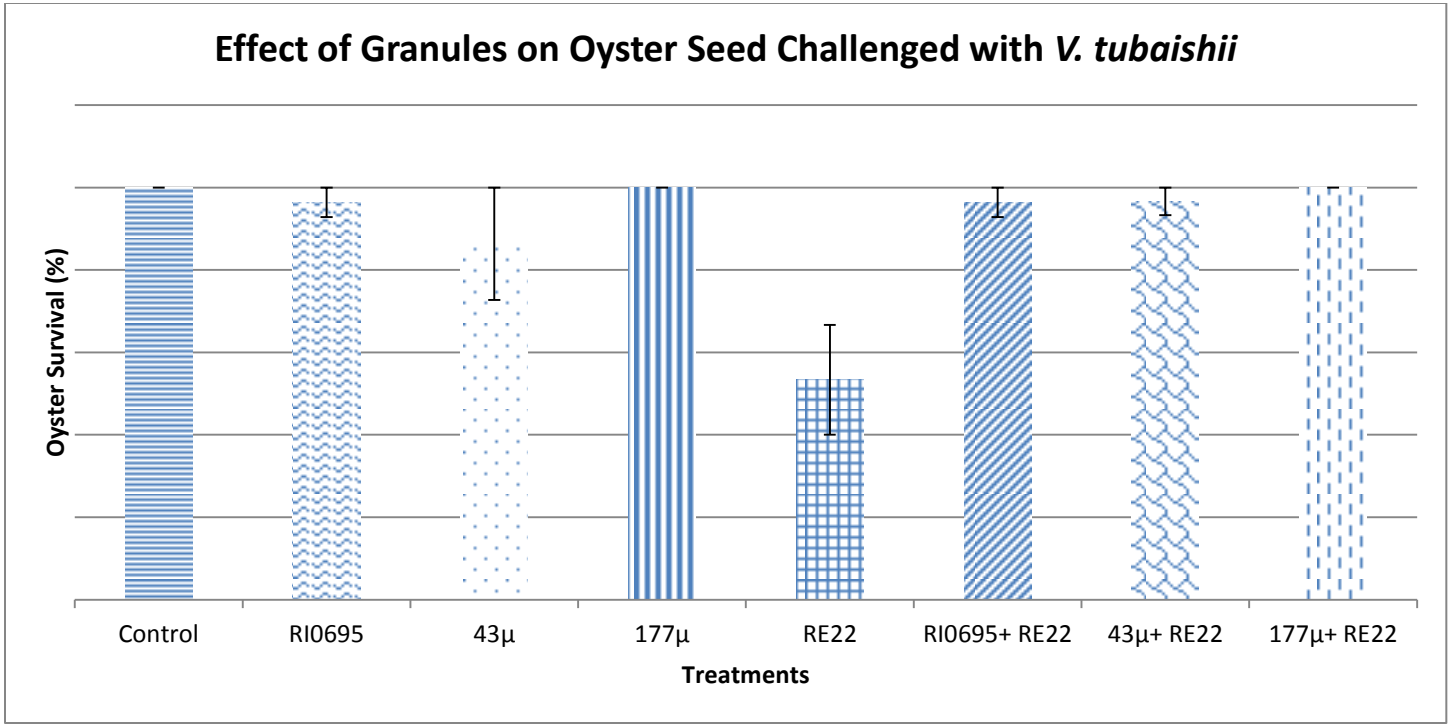

Figure 9. Effect of preincubation on oyster seed with freshly cultured *B. pumilus* (RIO695) and formulated granules (177µ and 43µ) at 10^4 CFU/mL. Controls included seed oysters exposed to no additional bacteria (Control) and oysters exposed to only *V. tubiashii* (RE22). In the challenge experiments, the seed oysters were pretreated with the *B. pumilus* 24 hours prior to addition of the *V. tubiashii* RE22. Representative of 1 experiment.

Discussion

Previous results by Karim and coworkers determined both *Bacillus pumilus* RI0695 and *P. gallaeciensis* S4 exhibited antagonistic effects towards *V. tubiashii in vitro* (Karim, Zhao et al. 2013). More importantly, they were able to protect *C. virginica* larvae when challenged with *V. tubiashii in vivo* (Karim 2013, Karim, Zhao et al. 2013). However, this study measured the probiotic effects of freshly cultured bacteria, an approach that is not feasible at commercial shellfish hatcheries. Granular formulation was chosen for this study because it is a cost effective process. One parameter we sought to measure was cell viability over time. A probiotic formulation might be stored at a hatchery for weeks or months before use, so a successful product must be stable. While the formulations generally appeared to be stable at both temperatures tested, measuring the cell viability of the granular formulations proved to be difficult. There was variability in cell viability in all of the granule sizes formulated based on the log(CFU/mg) data at each time point (1-8 weeks). A large variation in cell viability in the 420 µ granules may be caused by several reasons. First, the granules might not have fully redissolved into seawater, thus causing some heterogeneity in the samples used to determine viable cell counts. Second, the 420 µ granules appear to have had some increases in numbers of viable cells by the second week of storage at room temperature, suggesting that the particles may have absorbed water effecting the weight of the sample. This could cause variations in the cell viability counts. Third, the formulations were tested at different times of the year and thus may have been influenced by changes in humidity. More humid storage

conditions could affect particle clumping and thus size and dissolution. Our results suggest that storage of the granules could be at either 4 °C or RT (~25 °C) since the products were relatively stable at each temperature. However, storage at 4 °C was less variable in cell viability because the relative humidity is more controlled, and therefore might be optimal for hatcheries that experience large ambient temperature fluctuations in the summer.

Based on the cell viability studies of granules up to 8 weeks, the cell viabilities per milligram of granules are in a range sufficient to provide probiotic effects against *V. tubiashii* infection. A probiotic formulation should have shelf-life of at least 12 weeks, since the normal spawning season of wild oysters is June through August (UMCES 2014). The granular formulations satisfy this requirement since both the oyster seed and larval challenges were performed with formulations that were greater than 20 weeks old and yielded similar relative percent survival compared to the freshly cultured *B. pumilus* RI0695 control. Bacteria cultured from the granules retained both antibiotic activity and cell morphology identical to cell cultured from frozen stocks.

Future studies should be directed at measuring the time it takes the dried granules to fully disintegrate. Knowing the conditions to get complete dissolution of the particles would enable more consistent cell viability counts for the formulations. Other potential probiotic formulations could be also explored, such as a lyophilized product or a liquid formulation for ease during scale up for commercial production. However, the granular formulations prepared here provided cell viabilities above 10^5 CFU/mg (log(CFU/mg) = 5.0), suggesting that sufficient bacteria remained viable for

use in treating aquaculture tanks. For example, tanks that are 100 L would require least 10.0 g per treatment. Future pilot studies in commercial hatcheries are now needed to test the suitability of these products for commercial use.

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CHAPTER 4

PREPARATION OF LYOPHILIZED FORMULATION OF *PHAEOBACTER GALLAECIENSIS* S4 FOR USE IN DISEASE MANAGEMENT IN *CRASSOSTREA VIRGINICA* HATCHERIES

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Abstract

Probiotic agents have the potential to mitigate disease outbreaks in aquaculture facilities. However, there are currently no commercially available probiotics for shellfish larviculture. The marine bacterium *Phaeobacter gallaeciensis* S4 was previously reported to provide significant protection of Eastern oyster, *Crassostreae virginica,* larvae against the shellfish pathogen *Vibrio tubiashii.* This investigation aimed to create a stable, lyophilized formulation of *P. gallaeciensis* S4 for potential delivery at shellfish hatcheries. Lyophilized formulations of *P. gallaeciensis* S4 were prepared at both log and stationary growth phases, and two cryoprotectants, sucrose and mannitol, were investigated. For each cyroprotectant, three weight by volume concentrations were tested: 20% (w/v), 30% (w/v) and 40 % (w/v). The formulated bacteria were stored at various temperatures, including 4 °C, room temperature (RT), 30 °C, and 30 °C with 75 % humidity. The best results were achieved with the 30-M (30% mannitol) formulation stored at RT. This formulation provided cell viabilities of 4.39$\pm$3.69 log(CFU/mg) after 1 week of storage, but lost an additional 30% of the viable cells over the following 1 week. Since a goal of the study was to prepare a formulation that exceeded 10^5 CFU/mg and remained relatively stable for at least 12 weeks, none of the formulations were considered a success.

Introduction

To reduce the threat of disease, hatcheries employ various strategies to eliminate pathogens, including seawater filtration systems, ozonolysis and UV treatments of seawater, controlling salinity, selective breeding of the cultured species,

and electrolytic treatments of incoming seawater (Ruiz-Ponte, Samain et al. 1999, Jorquera, Valencia et al. 2002, Meunpol, Lopinyosiri et al. 2003, Park, Kim et al. 2011). Despite these approaches, infectious diseases continue to be problematic for shellfish hatcheries. The use of probiotic bacteria is an attractive alternative to disease management in bivalve hatcheries, but no commercial probiotic products currently exist for this area of aquaculture.

Probiotic bacteria are defined by the World Health Organization as live microorganisms that provide a beneficial effect to the host (FAO 2002). Investigations to identify potential probionts for bivalve aquaculture have shown promise. Ruiz-Point et al determined that *Roseobacter* strain BS 107 was able to improve the survival of *Pecten maximus* when challenged by *Vibrio anguillarum 408* (Ruiz-Ponte, Samain et al. 1999). Gibson et al. demonstrated that probiotic candidate *Aeromonas media* sp. 199 produced an inhibitory substance that contributed to the antagonistic effects *in vitro* as well *in vivo* towards *V. tubiashii* (Gibson, Woodworth et al. 1998). Riquelme et al. demonstrated *Anthrobacter* strain 77 exuded compounds that inhibited *Vibrio esplendidus, Vibrio alginolyticus* and *Aeromonas hidrophyla.* Riquelme additionally determined *Anthrobacter* strain 77, when ingested, was able to colonize the gut was not toxic to Chilean scallop larvae. (Riquelme, Jorquera et al. 2001). *Vibrio* strain OY15, a naturally-occurring bacterium present in the digestive glands of adult *C. virginica,* increased survival of oyster larvae when challenged with pathogen *Vibrio* sp. strain B 183 (Kapareiko, Lim et al. 2011). Karim et al. showed that *P. gallaeciensis* S4 and *Bacillus pumilus* RI0695 exhibited protective effects of

Crassostrea virginica larvae when challenged with *V. tubiashii* both *in vitro* and *in vivo* (Karim, Zhao et al. 2013).

Delivery of freshly cultured bacteria is not a feasible approach for disease management at commercial shellfish hatcheries. Lyophilization as a means to create a stable formulation was chosen for this study because of previously demonstrated long shelf life of stored lyophilized microbes (Leslie, Israeli et al. 1995, Costa, Usall et al. 2000, Miyamoto-Shinohara, Imaizumi et al. 2000, Hubálek 2003, Miyamoto-Shinohara, Sukenobe et al. 2006, Savini, Cecchini et al. 2010). Miyamoto-Shinohara et al. observed that gram negative bacteria containing polar flagella had low to moderate (58.2±25.0 % to 10.7%) cell viability survival rate post lyophilization (Miyamoto-Shinohara, Sukenobe et al. 2008). Sucrose, mannitol and trehalose have been previously chosen when formulating gram negative bacteria for their extracellular cryoprotection (Leslie, Israeli et al. 1995, Costa, Usall et al. 2000, Miyamoto-Shinohara, Imaizumi et al. 2000, Hubálek 2003, Miyamoto-Shinohara, Sukenobe et al. 2006, Miyamoto-Shinohara, Sukenobe et al. 2008, Savini, Cecchini et al. 2010). Mannitol and sucrose were chosen for this study due to their ability to protect the cell membrane of Gram negative bacteria (Costa, Usall et al. 2000, Hubálek 2003, Savini, Cecchini et al. 2010).

An important next step in the development of probiotic bacteria for shellfish aquaculture is the creation of stable and safe formulations. Freeze drying is a commonly used method in microbial culture storage (Leslie, Israeli et al. 1995, Costa, Usall et al. 2000, Miyamoto-Shinohara, Imaizumi et al. 2000, Hubálek 2003, Miyamoto-Shinohara, Sukenobe et al. 2006, Miyamoto-Shinohara, Sukenobe et al.

2008, Savini, Cecchini et al. 2010). In this study, we explored lyophilized preparations of *P. gallaeciensis* S4 for their cell viability over time and protection of oyster larvae against *V. tubiashii* infections.

Methods and Materials

Bacterial cultivation

P. gallaeciensis S4, *R. crassostreae* Cv 919-312^T (Boettcher, Geaghan et al. 2005) and *V. tubiashii* RE22 (Elston, Hasegawa et al. 2008) were provided by Dr. Marta Gómez-Chiarri (University of Rhode Island). *Vibrio harveyi* BB120 was provided by Dr. David Nelson (University of Rhode Island). All bacteria were cultured in a seawater-based yeast extract-peptone culture medium (YP). YP media was prepared using 5 g/L of peptone, 1 g/L of yeast extract, and 30 g/L of Instant Ocean (Blacksburg, VA) in pure, reverse osmosis (RO) water. For YP agar, 15 g of agar per L. *R. crassostreae* were used for *in vitro* antibiotic susceptibility testing, while *V. tubiashii* was used for challenges. *P. gallaeciensis* S4 was cultured at 28 °C and shaking at 175 rpm. Bacterial stocks were stored at −80 °C in YP broth with 25% glycerol until use.

Formulation of Bacterial Cells

Forty-eight 50 mL cultures of S4 were incubated at 28 °C for 48 hours. Bacteria growth was measured by measuring the absorbance (OD_{600}). The cultures were formulated at two time points: (1) OD_{600}= 0.5$\pm$0.1 or ~10^5 CFU/mL, and (2) OD_{600}=0.8 or ~10^8 CFU/mL (personal communication with Dr. Wenjing Zhao). Time point (1) was considered to be exponential growth phase and time point (2) was

considered to be stationary growth phase (personal communication with Dr. Wenjing Zhao). The cells were harvested by centrifugation at 4,500 x *g* for 10 min at 20 °C. The cell pellet was then washed in 5 mL of sterile artificial seawater twice. The resulting bacterial cell pellet was re-suspended in 5 mL of artificial seawater and either (1) 20%, 30% or 40% (w/v) of mannitol (20-M, 30-M, 40-M) or (2) 20%, 30% or 40% (w/v) of sucrose (20-S, 30-S 40-S). Each formulation was given a designation: LGP denotes formulation during exponential growth phase while SGP designates a formulation from S4 in the stationary growth phase (e.g. 30-M SGP). All formulations were thoroughly mixed, frozen at -80 °C overnight, and then lyophilized. A sterilized spatula was used to break up the lyophilized presscake into a free-flowing powder. The formulated products were stored in plastic centrifuge tubes at either room temperature (RT) (approximately 22$\pm$3 °C), 4 °C for 5 weeks, 30 °C for 4 weeks, or 30 °C with 75% relative humidity for 4 weeks.

Colony-Forming Unit (CFU) Viability Assay

The cell viability of probiotic formulations stored at RT and 4 °C was determined at 1, 2, and 5 weeks. Cell viability assay was performed at 2 and 4 weeks for formulations stored at 30 °C and 30 °C with 75% relative humidity. All formulations were reconstituted in 5 mL of sterile reversed osmosis water to a concentration of 200, 300 or 400 mg/mL of 20-M LGP, 30-M LGP and 40-M LGP. 1:10 and 1:100 dilutions ranging from 10^2-10^7 CFU/mL were created from the 5 mL stock of formulations in YP broth in triplicate. 10 µL of each dilution was pipetted onto YP agar plates in triplicate. The YP plates were incubated for 48 h at 27 °C and

then colonies were counted. The assay for each formulation was performed in duplicate.

Antibiotic activity of formulated *P. gallaeciensis* S4 against bacterial pathogens

S4 from each formulation was tested for growth inhibitory activity against *R. crassostreae* (Karim, Zhao et al. 2013). An overnight culture of *R. crassostreae* was diluted to approximately 10^8 CFU/mL in YP media. The diluted bacterium was swabbed onto an YP agar plates. Overnight culture of formulated products (10 µL of 5 mL stock) in YP media and an overnight culture of *P. gallaeciensis* S4 from cryostock (control) were pipetted (10 µL) in triplicate on each YP plate. Each plate was incubated for 48 h at 28 °C. Zones of growth inhibition surrounding the colonies of S4 were measured to the nearest mm. Assays were performed in triplicate.

Characterization of cell morphology of lyophilized products

The cell morphology of the 30-M LGP and 40-M LGP and stationary formulations were examined by phase contrast microscopy magnified at 100x (Zeiss Axio Imager 2 microscope). Three 10^2 CFU/mL dilutions in YP media were prepared for each formulation from their rehydrated 5 mL stock solutions.

Oysters challenge assays

Oyster larvae

10-day old oyster larvae (*C. virginica)* were obtained from Blount Shellfish Hatchery at Roger Williams University, Bristol, RI. The larvae were divided into six wells containing 5 mL of aerated sterile seawater (FSSW) and allowed to settle at 20 °C for 24 h. The larvae were fed algal paste (Reed Mariculture Inc., San Jose, CA. USA daily. The water in each well was changed with FSSW every 48 h.

Probiotic treatments of Oyster Larvae

The experiment consisted of six wells containing larvae with various treatments: a positive control group (no treatment), a negative control group treated with *V. tubiashii*, two 30-M LGP treatment groups and two 40-M LGP treatment groups. The positive control groups contained 147 $\pm$ 40 larvae and were fed commercial algal paste (Reed Mariculture Inc., San Jose, CA. USA) daily only. The negative control group contained 95 $\pm$ 52 larvae and were fed commercial feed and treated with *V. tubiashii* (10^5 CFU/mL) after 24 h. Two wells containing 79 $\pm$ 20 larvae were treated with 30-M LGP (10^4 CFU/mL) and two wells containing 70$\pm$30 larvae were treated with 40-M LGP (10^4 CFU/mL). All treatments were performed in triplicate.

Oyster Larvae Challenge

After 24 h, one well treated with1 week old 30-M LGP and one well treated with the 1 week old 40-M LGP were washed and challenged with *V. tubiashii* (10^5 CFU/mL). The negative control well was treated with *V. tubiashii* (10^5 CFU/mL). After an additional 24 h, all wells were stained with neutral red (200 µL) and live larvae were counted. These experiments were run in triplicate. The relative percent survival was calculated using the following formula:

$$\text{Relative Percent Survival} = \left[1-\frac{\%\ \text{survival challenged control}}{\%\text{survival treatment}}\right] \times 100$$

Statistical analysis

The challenge data were analyzed by one-way analysis of variance (ANOVA

). This data were analyzed using Sigma stat 3.1 software (Systat). Differences were considered to be significant at values of $P < 0.05$.

Results

Effect of cryoprotectant on lyophilization process

After the lyophilization process, all sucrose lyophilized formulations were a solid crystallized mass. In contrast, all mannitol lyophilized products were homogenized into a loose-flowing powder.

Effect of lyophilization on antimicrobial activity

All of colonies arising from the S4 stationary growth phase (SGP) lyophilized powders and the 20-M LGP powder did not produce zones of growth inhibition against *R. crassostreae*. These colonies were white in appearance, which is different compared to the yellow color normally observed with S4 cultures from cryostock (Figure 3). The 30-M LGP and 40-M LGP formulations had comparable zones of no growth surrounding all the S4 colonies, similar to the area of no growth surrounding the freshly cultured S4 (Table 1).

Table 1. Antibiotic activity of formulated *P. gallaeciensis* S4 versus *R. crassostreae.*

Lyophilization	Clear Zone of inhibition
20-M log phase	0 mm
30-M log phase	8 mm
40-M log phase	8 mm
20-M stationary phase	0 mm
30-M stationary phase	0 mm
40-M stationary phase	0 mm
Control (*P. gallaeciensis* S4)	10 mm

10 µL of a 5 mL solution of all lyophilized mannitol (M) formulations was grown in YP broth for 48 h at 27 ° C shaking 175 rpm. Control was *P. gallaeciensis* S4 from cryopreserved stock. 10 µL of the resulting cultures were spotted onto plates inoculated with *R. crassostreae* and incubated at 27 ° C for 24 hours. The diameters of zones of no growth surrounding S4 colonies were measured to the nearest mm.

Impacts of lyophilization on *P. gallaeciensis* S4

The cell viability of the 30-M LGP and 40-M LPG stored at RT and 4 °C was assessed over 5 weeks using by measuring CFU per mg over time. Unfortunately neither formulation produced any viable cells (Table 2 and Table 3). The effect of the lyophilization processes of formulated products retaining antimicrobial activity (30-M LGP and 40-M LPG) were assessed by measuring the cell viability prior to and after formulation. Storage of 30-M LGP and 40-M LGP at 30 °C, both with 75% relative humidity and without humidity, did not produce any viable cells at the first assessment of CFU/mg at 2 weeks. The cell viability of the 30-M LGP and 40-M LPG stored at RT and 4 °C had decreased 2 orders of magnitude post formulation. Additionally the

viability rapidly decreased over 2 weeks. By 5 weeks there were no viable cells when test formulations were assessed by measuring CFU per mg over time. (Table 2 and Table 3).

Table 2. Cell viability of *P. gallaeciensis* S4 30-M log phase lyophilized formulation (CFU/mg) over 2 weeks while stored at RT and 4 °C

Time	Exp. 1, RT (CFU/mg)	Exp. 2, RT	Exp. 1, 4 °C	Exp. 2, 4 °C
1	4.39±3.69	4.22±3.37	3.51±2.31	3.43±2.37
2	3.26±2.25	3.18±2.18	3.69±2.11	2.98±1.91
5	0	0	0	0

Table 3. Cell viability of *P. gallaeciensis* S4 40-M log phase lyophilized formulation (CFU/mg) over 2 weeks while stored at RT and 4 °C

Time	Exp. 1 RT	Exp. 2 RT	Exp. 1 4 °C	Exp. 2 4 °C
1	3.90±3.37	4.12±3.21	3.56±2.43	3.54±2.15
2	3.82±2.97	4.18±3.70	2.72±1.97	3.44±1.87
5	0	0	0	0

Impacts of lyophilization on cell morphology

The 30-M and 40-M LPG, resuspended in 5 mL of DI water and diluted in YP media, appeared as small ovoid cells under phase contrast at 100x (Figure 1), while the 30-M and 40-M SPG had the formation of rosettes. Observed cell morphology from both the LPG and SPG formulations were consistent with *P. gallaeciensis* S4 bacterium previously reported by Karim and coworkers (Karim, Zhao et al. 2013).

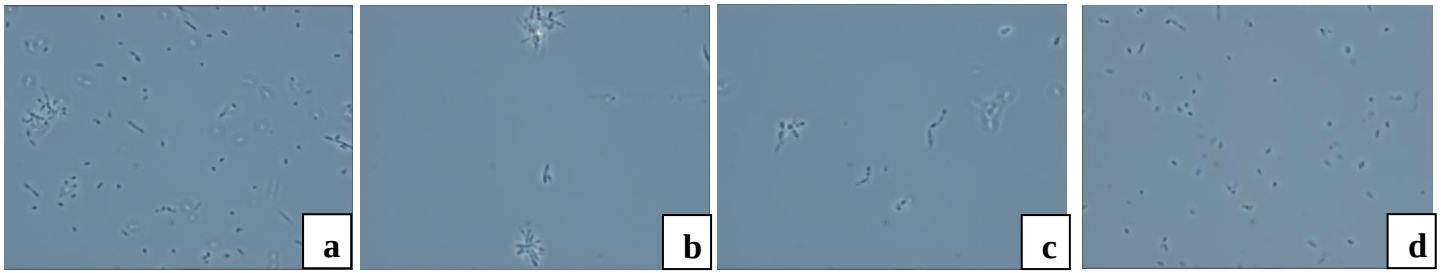

Figure 1. (a) Cell morphology of 30-M SGP formulation using 100 x magnification; (b) Cell morphology of 40-M SGP formulation using 100 x magnification; (c) Cell morphology of 30-M LGP formulation using 100 x magnification; (d) Cell morphology of 40-M LGP formulation using 100 x magnification.

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Oyster larvae challenges

Cell viabilities of the 30-M LPG and 40-LPG were found to be 4.40 $\pm$ 3.70 log (CFU/mg) and 4.11 $\pm$ 3.20 log (CFU/mg), respectively, and each was added to treated oysters at 10^4 CFU/mL. Neither of the formulations were found to be detrimental to oyster survival (Figure 5). Survival of the oyster larvae after 24 h exposure to pathogen *V. tubiashii* RE22 was reduced to 25$\pm$5%. Oyster larvae pretreated with the 30-M LPG and 40-M LPG at 10^4 CFU/mL and then exposed to *V. tubiashii* demonstrated the similar survival rates of 47$\pm$9% and 34$\pm$1%, respectively compared to oysters treated with *V. tubiashii* alone and to each other (Figure 2). Both 30-M LPG and 40-M LPG provided no protection of the oyster larvae. This was significantly different than what was previously seen with probiotic treatments of freshly cultured *P. gallaeciensis* S4 bacterium (10^4 CFU/mL) (Karim, Zhao et al. 2013).

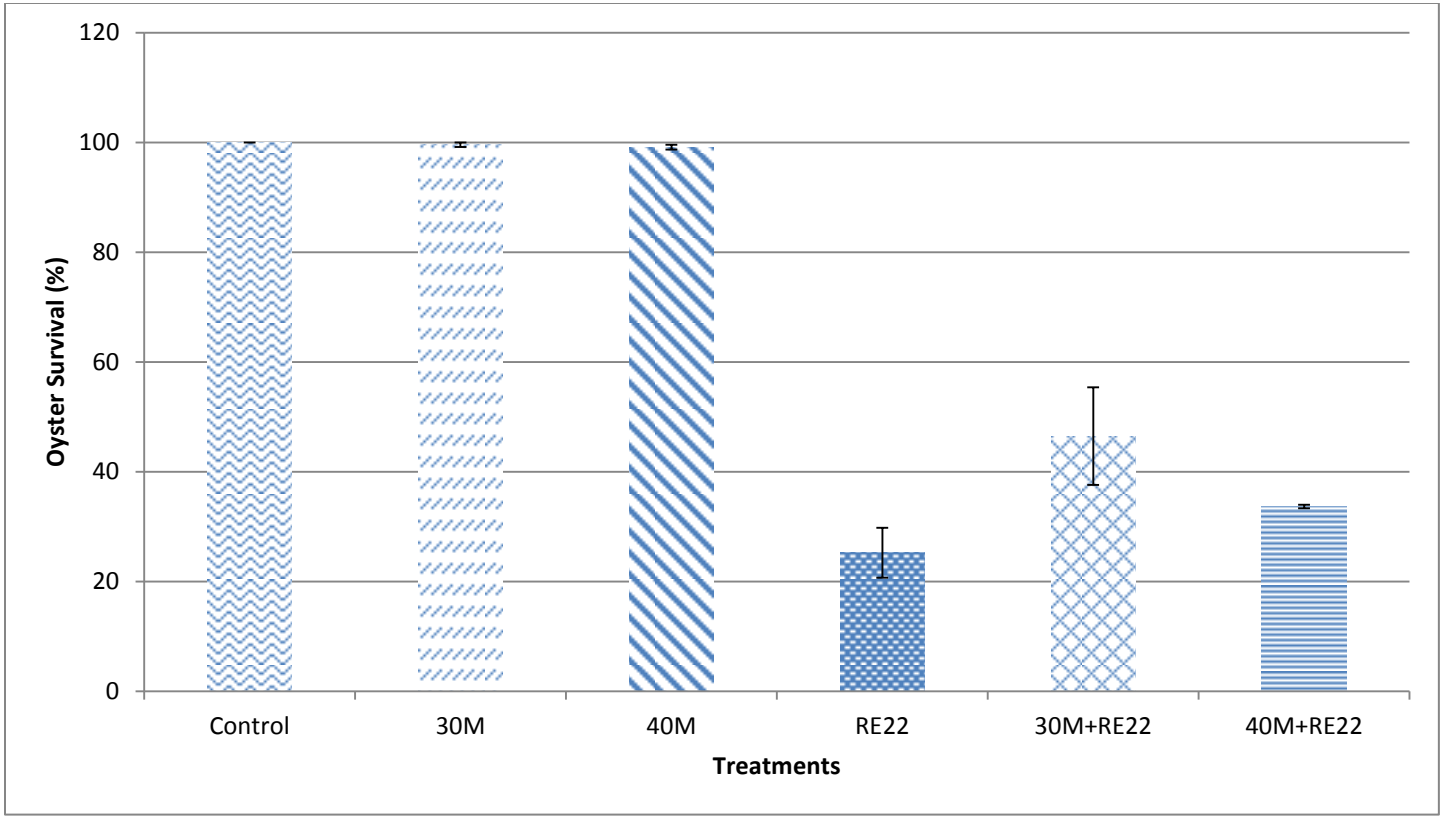

Figure 2. Protection effect of 30-M LGP and 40-LGP lyophilized formulation when challenged with *V. tubiashii* RE22

Oyster larvae was pretreated with rehydrated 30-M LGP and 40-M LGP diluted to 10^4 CFU/mL 24 hours prior to being challenged with *V. tubiashii* RE22. Protection of oyster larvae was measured by percent survival (+standard error). Representative of 3 experiments.

Discussion

Phaeobacter have previously shown promise as probiotic agents for aquaculture. Previous studies have reported several *Roseobacter* isolates that exhibit antagonistic effects against pathogenic *Vibrio* species that are problematic in cod and turbot larviculture(Brinkhoff, Bach et al. 2004, Bruhn, Nielsen et al. 2005, Porsby, Nielsen et al. 2008, Porsby, Webber et al. 2011, D'Alvise, Lillebo et al. 2012). *Roseobacter* isolates have been shown to produce tropodithetic acid, an antibiotic with potent effects against marine and human pathogens (Kintaka, Ono et al. 1984, Kawano, Nakagomi Kazuya. et al. 1998, Liang 2003, Porsby, Webber et al. 2011). Karim and co-workers demonstrated that pre-treatment with fresh culture of *P. gallaeciensis* S4 provided protection to oyster larvae when challenged with *V. tubiashii* RE22.

A successful *P. gallaeciensis* S4 probiotic formulation for potential commercial hatchery use should have cell viability above that required for a probiotic effect. 10^4 CFU/mL was previously determined to be effective using freshly cultured *P. gallaeciensis* S4 bacterium. Furthermore, the formulation should be stable for at least 12 weeks since this the normal spawning season of both wild and aquacultured oysters is June through August (UMCES 2014). Also, the formulation should provide an antagonistic effect towards the pathogen *V. tubiashii* RE22 *in vivo.*

Unfortunately formulating S4 bacterium through lyophilization has proven to be difficult. Lyophilized powders 30-M SGP and 40-M SGP had similar cell morphology to freshly cultured *P. gallaeciensis* S4 bacterium when examined under microscopy 100x. However, both formulations had lost antibiotic activity against *R.*

crassostreae in vitro. While lyophilized powders 30-M LGP and 40-M LGP retained antibiotic activity against *R. crassostreae in vitro,* reduced protection was observed in the oyster larvae in the bacterial challenge with pathogen *V. tubiashii* RE22 (Karim, Zhao et al. 2013). Also, the cell viability of both formulations rapidly decreased (2 weeks). By the 5^{th} week, all formulations had no cell growth when tested. The cell viability may have been adversely impacted by the freezing method or due to short term exposure to the hyperosmotic environment created by the cryoprotectant.

The lyophilization of *P. gallaeciensis* S4 with two different cryoprotectants, sucrose and mannitol, proved to be unsuccessful. It did not meet the criteria necessary for a commercially available probiotic product. (30-M and 40-M) were not of sufficient stability or viability (CFU $>10^6$) for use as a commercial probiotic product. Log phase 30-M and 40-M formulations did not provide protection of *C. virginica* larvae when challenged with *V. tubiashii.* Lyophilization of *P. gallaeciensis* S4 under these conditions are not suitable for prevention of disease in oyster hatcheries. It may be useful to examine if a cryoprotectant is necessary in creating a lyophilized *P. gallaeciensis* S4 formulation. Slower freezing of the sample and less concentrated cryoprotectants might also be beneficial. Other types of formulations should be explored, such as liquid formulations using cells under starvation conditions.

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APPENDICES

Isolation scheme of TDA from *P. gallaeciensis* S4

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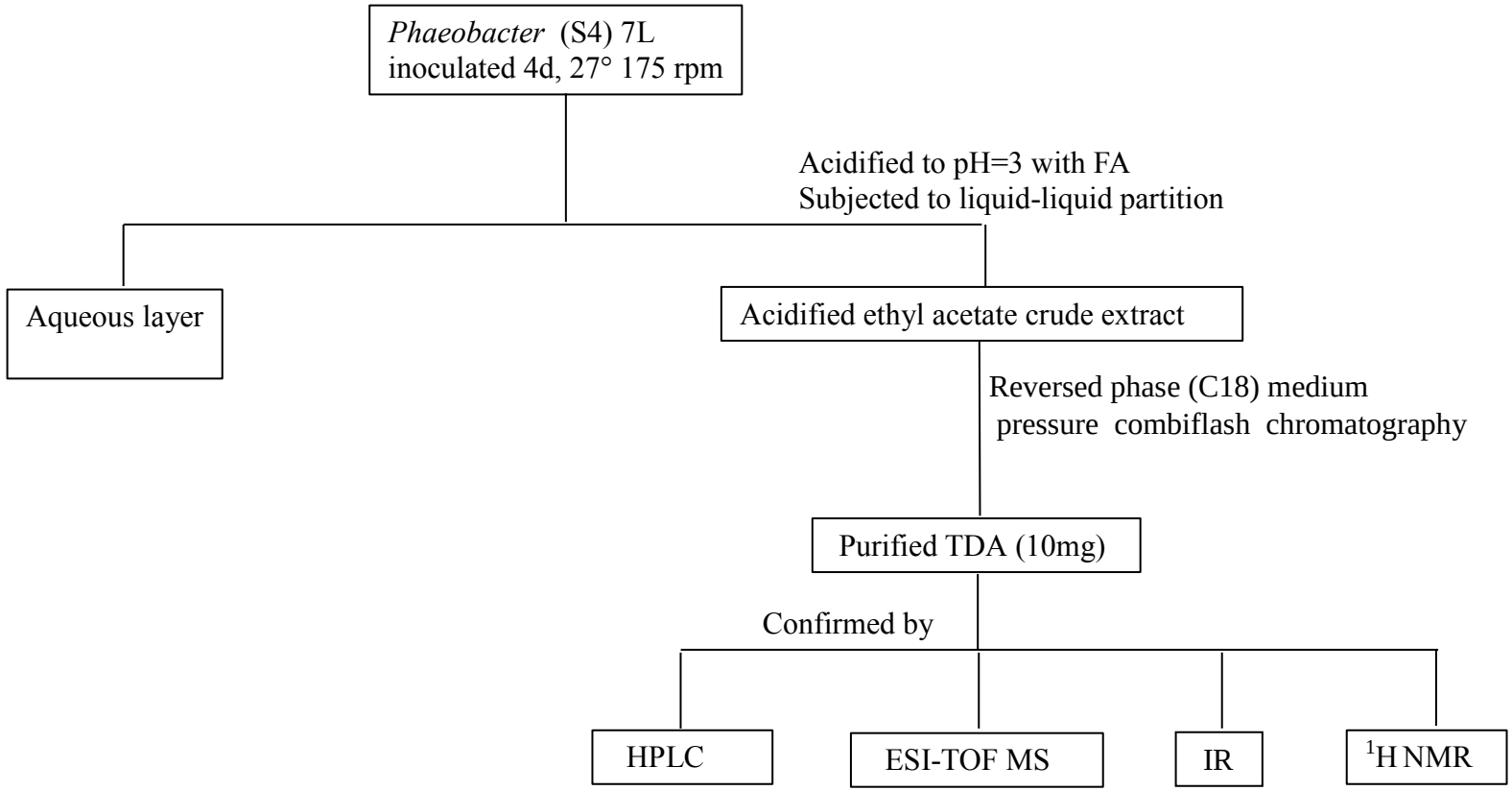

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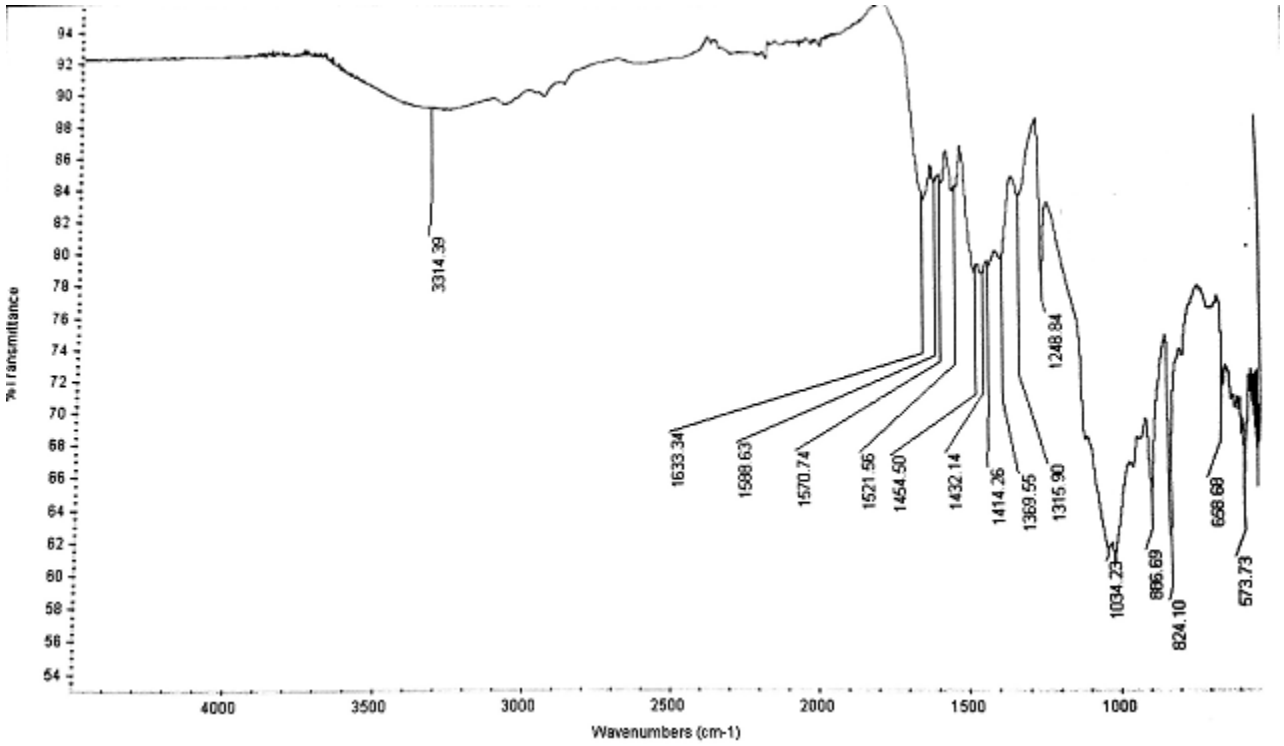

One way ANOVA analysis for cell viability of 420 µ granules 1 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:18:20 PM

Data source: Data 5 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.380)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:18:20 PM

Data source: Data 5 in Notebook 2

Group	N	Missing	Median	25%	75%
A	7	0	5.857	5.813	5.889
B	7	0	6.204	6.020	6.360

H = 9.425 with 1 degrees of freedom. P(est.)= 0.002 P(exact)= <0.001

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
B vs A	48.000	4.337	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 420 µ granules 2 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:23:14 PM

Data source: Data 5 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.734)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:23:14 PM

Data source: Data 5 in Notebook 2

Group	N	Missing	Median	25%	75%
A	4	0	5.476	5.175	5.777
B	5	0	5.924	5.885	5.951

H = 6.000 with 1 degrees of freedom. P(est.)= 0.014 P(exact)= 0.016

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.016)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
B vs A	4.500	2.449	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 420 µ granules 5 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:23:46 PM

Data source: Data 5 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.095)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	6.168	0.0662	0.0221
B	9	0	6.168	0.0662	0.0221

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.000	0.000	0.000	1.000
Residual	16	0.0701	0.00438		
Total	17	0.0701			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 0.048

The power of the performed test (0.048) is below the desired power of 0.800.
Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

1

One way ANOVA analysis for cell viability of 420 µ granules 8 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:22:04 PM

Data source: Data 5 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.541)

Equal Variance Test: Passed (P = 0.106)

Group Name	N	Missing	Mean	Std Dev	SEM
A	8	0	5.792	0.138	0.0489
B	8	0	5.885	0.196	0.0694

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0345	0.0345	1.197	0.292
Residual	14	0.404	0.0288		
Total	15	0.438			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.292).

Power of performed test with alpha = 0.050: 0.067

The power of the performed test (0.067) is below the desired power of 0.800.
Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

1

One way ANOVA analysis for cell viability of 177 µ granules 1 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:26:00 PM

Data source: Data 6 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.093)

Equal Variance Test: Passed (P = 0.169)

Group Name	N	Missing	Mean	Std Dev	SEM
A	8	0	5.800	0.171	0.0606
B	7	0	5.867	0.0493	0.0186

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.0166	0.0166	0.980	0.340
Residual	13	0.220	0.0169		
Total	14	0.237			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.340).

Power of performed test with alpha = 0.050: 0.048

The power of the performed test (0.048) is below the desired power of 0.800.
Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

1

One way ANOVA analysis for cell viability of 177 µ granules 2 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:26:47 PM

Data source: Data 6 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.260)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:26:47 PM

Data source: Data 6 in Notebook 2

Group	N	Missing	Median	25%	75%
A	10	0	5.819	5.778	5.903
B	8	0	5.756	5.716	5.785

H = 3.507 with 1 degrees of freedom. (P = 0.061)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.061)

1

One way ANOVA analysis for cell viability of 177 µ granules 5 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:27:33 PM

Data source: Data 6 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.779)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:27:33 PM

Data source: Data 6 in Notebook 2

Group	N	Missing	Median	25%	75%
A	9	0	5.643	5.518	5.736
B	8	0	5.863	5.792	5.914

H = 12.015 with 1 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
B vs A	8.500	3.464	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 177 µ granules 8 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:28:24 PM

Data source: Data 6 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:28:24 PM

Data source: Data 6 in Notebook 2

Group	N	Missing	Median	25%	75%
A	9	0	5.146	5.035	5.204
B	9	0	5.778	5.602	5.778

H = 13.059 with 1 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
B vs A	81.000	5.058	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 43 µ granules 1 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:30:30 PM

Data source: Data 7 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.208)

Equal Variance Test: Passed (P = 0.069)

Group Name	N	Missing	Mean	Std Dev	SEM
A	7	0	5.893	0.0257	0.00971
B	6	0	5.570	0.101	0.0414

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.337	0.337	66.831	<0.001
Residual	11	0.0554	0.00504		
Total	12	0.392			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
A vs. B	0.323	2	11.561	<0.001	Yes

1

One way ANOVA analysis for cell viability of 43 µ granules 2 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:31:07 PM

Data source: Data 7 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.055)

Equal Variance Test: Passed (P = 1.000)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	5.716	0.0878	0.0293
B	9	0	5.716	0.0878	0.0293

Source of Variation	DF	SS	MS	F	P
Between Groups	1	0.000	0.000	0.000	1.000
Residual	16	0.123	0.00770		
Total	17	0.123			

The differences in the mean values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 1.000).

Power of performed test with alpha = 0.050: 0.048

The power of the performed test (0.048) is below the desired power of 0.800.
Less than desired power indicates you are more likely to not detect a difference when one actually exists. Be cautious in over-interpreting the lack of difference found here.

1

One way ANOVA analysis for cell viability of 43 µ granules 5 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 4:32:04 PM

Data source: Data 7 in Notebook 2

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 4:32:04 PM

Data source: Data 7 in Notebook 2

Group	N	Missing	Median	25%	75%
A	9	0	5.556	5.322	5.648
B	7	0	5.531	5.345	5.584

H = 0.551 with 1 degrees of freedom. (P = 0.458)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.458)

1

One way ANOVA analysis for cell viability of 43 µ granules 8 w stored at 4° C

One Way Analysis of Variance Friday, July 18, 2014, 5:38:26 PM

Data source: Data 1 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:38:26 PM

Data source: Data 1 in Notebook 1

Group	N	Missing	Median	25%	75%
A	7	0	5.857	5.836	5.898
B	4	0	6.480	6.190	6.700
C	4	0	6.531	6.504	6.577

H = 10.563 with 2 degrees of freedom. (P = 0.005)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
C vs A	7.750	2.765	Yes
C vs B	0.500	0.158	No
B vs A	7.250	2.586	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 420 µ granules 1 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:37:41 PM

Data source: Data 1 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.767)

Equal Variance Test: Passed (P = 0.166)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	5.931	0.0522	0.0174
B	6	0	5.854	0.0345	0.0141
C	3	0	6.003	0.0216	0.0125

Source of Variation	DF	SS	MS	F	P
Between Groups	2	0.0473	0.0236	12.358	<0.001
Residual	15	0.0287	0.00191		
Total	17	0.0760			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 0.982

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
C vs. B	0.148	3	6.783	<0.001	Yes
C vs. A	0.0719	3	3.488	0.064	No
A vs. B	0.0764	3	4.688	0.012	Yes

1

One way ANOVA analysis for cell viability of 420 µ granules 2 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:38:26 PM

Data source: Data 1 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:38:26 PM

Data source: Data 1 in Notebook 1

Group	N	Missing	Median	25%	75%
A	7	0	5.857	5.836	5.898
B	4	0	6.480	6.190	6.700
C	4	0	6.531	6.504	6.577

H = 10.563 with 2 degrees of freedom. (P = 0.005)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.005)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
C vs A	7.750	2.765	Yes
C vs B	0.500	0.158	No
B vs A	7.250	2.586	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 420 µ granules 5 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:38:52 PM

Data source: Data 1 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.071)

Equal Variance Test: Passed (P = 0.174)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	5.805	0.138	0.0459
B	5	0	5.867	0.0513	0.0229
C	9	0	6.354	0.198	0.0661

Source of Variation	DF	SS	MS	F	P
Between Groups	2	1.531	0.766	32.105	<0.001
Residual	20	0.477	0.0238		
Total	22	2.008			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
C vs. A	0.549	3	10.656	<0.001	Yes
C vs. B	0.487	3	7.997	<0.001	Yes
B vs. A	0.0615	3	1.009	0.758	No

1

One way ANOVA analysis for cell viability of 420 µ granules 8 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:39:34 PM

Data source: Data 1 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:39:34 PM

Data source: Data 1 in Notebook 1

Group	N	Missing	Median	25%	75%
A	2	0	5.841	5.602	6.079
B	10	0	6.462	6.342	6.556
C	7	0	6.447	6.352	6.518

H = 4.050 with 2 degrees of freedom. (P = 0.132)

The differences in the median values among the treatment groups are not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.132)

1

One way ANOVA analysis for cell viability of 177 µ granules 1 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:40:52 PM

Data source: Data 3 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.397)

Equal Variance Test: Passed (P = 0.278)

Group Name	N	Missing	Mean	Std Dev	SEM
A	8	0	6.624	0.0828	0.0293
B	5	0	5.814	0.0560	0.0250
C	6	0	5.783	0.0498	0.0203

Source of Variation	DF	SS	MS	F	P
Between Groups	2	3.169	1.584	347.805	<0.001
Residual	16	0.0729	0.00456		
Total	18	3.242			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
A vs. C	0.841	3	32.628	<0.001	Yes
A vs. B	0.810	3	29.764	<0.001	Yes
B vs. C	0.0312	3	1.078	0.731	No

1

One way ANOVA analysis for cell viability of 177 µ granules 2 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:44:16 PM

Data source: Data 4 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:44:16 PM

Data source: Data 4 in Notebook 1

Group	N	Missing	Median	25%	75%
A	9	0	6.643	6.556	6.728
B	10	0	5.748	5.663	5.792
C	7	0	5.820	5.720	5.845

H = 15.201 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
A vs B	12.944	3.683	Yes
A vs C	11.016	2.858	Yes
C vs B	1.929	0.512	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One way ANOVA analysis for cell viability of 177 µ granules 5 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:41:57 PM

Data source: Data 3 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.186)

Equal Variance Test: Passed (P = 0.092)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	5.367	0.115	0.0382
B	8	0	6.503	0.0793	0.0280
C	5	0	5.867	0.0513	0.0229

Source of Variation	DF	SS	MS	F	P
Between Groups	2	5.466	2.733	325.440	<0.001
Residual	19	0.160	0.00840		
Total	21	5.626			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
B vs. A	1.136	3	36.065	<0.001	Yes
B vs. C	0.636	3	17.210	<0.001	Yes
C vs. A	0.500	3	13.828	<0.001	Yes

1

One way ANOVA analysis for cell viability of 177 µ granules 8 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:42:28 PM

Data source: Data 3 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.435)

Equal Variance Test: Passed (P = 0.918)

Group Name	N	Missing	Mean	Std Dev	SEM
A	9	0	6.300	0.124	0.0412
B	10	0	5.373	0.174	0.0552
C	9	0	6.468	0.124	0.0414

Source of Variation	DF	SS	MS	F	P
Between Groups	2	6.699	3.349	161.202	<0.001
Residual	25	0.519	0.0208		
Total	27	7.218			

The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001).

Power of performed test with alpha = 0.050: 1.000

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
C vs. B	1.095	3	23.384	<0.001	Yes
C vs. A	0.168	3	3.499	0.052	No
A vs. B	0.927	3	19.794	<0.001	Yes

1

One way ANOVA analysis for cell viability of 43 µ granules 1 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:43:40 PM

Data source: Data 4 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Passed (P = 0.093)

Equal Variance Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:43:40 PM

Data source: Data 4 in Notebook 1

Group	N	Missing	Median	25%	75%
A	9	0	5.602	5.483	5.657
B	9	0	5.301	5.129	5.380
C	9	0	5.881	5.832	5.895

H = 21.500 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparison	Diff of Ranks	q	P<0.05
C vs B	155.500	6.530	Yes
C vs A	87.500	3.675	Yes
A vs B	68.000	2.856	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

One way ANOVA analysis for cell viability of 43 µ granules 2 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:44:16 PM

Data source: Data 4 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:44:16 PM

Data source: Data 4 in Notebook 1

Group	N	Missing	Median	25%	75%
A	9	0	6.643	6.556	6.728
B	10	0	5.748	5.663	5.792
C	7	0	5.820	5.720	5.845

H = 15.201 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
A vs B	12.944	3.683	Yes
A vs C	11.016	2.858	Yes
C vs B	1.929	0.512	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One way ANOVA analysis for cell viability of 43 µ granules 5 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:44:55 PM

Data source: Data 4 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:44:55 PM

Data source: Data 4 in Notebook 1

Group	N	Missing	Median	25%	75%
A	9	0	5.556	5.322	5.648
B	9	0	6.447	6.242	6.562
C	7	0	5.531	5.345	5.584

H = 16.865 with 2 degrees of freedom. (P = <0.001)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = <0.001)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
B vs C	13.500	3.640	Yes
B vs A	11.722	3.379	Yes
A vs C	1.778	0.479	No

Note: The multiple comparisons on ranks do not include an adjustment for ties.

One way ANOVA analysis for cell viability of 43 µ granules 8 w stored at RT

One Way Analysis of Variance Friday, July 18, 2014, 5:45:27 PM

Data source: Data 4 in Notebook 1

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Test execution ended by user request, ANOVA on Ranks begun

Kruskal-Wallis One Way Analysis of Variance on Ranks Friday, July 18, 2014, 5:45:27 PM

Data source: Data 4 in Notebook 1

Group	N	Missing	Median	25%	75%
A	3	0	5.778	5.420	5.778
B	9	0	6.301	6.262	6.411
C	9	0	6.301	6.262	6.411

H = 7.570 with 2 degrees of freedom. (P = 0.023)

The differences in the median values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference (P = 0.023)

To isolate the group or groups that differ from the others use a multiple comparison procedure.

All Pairwise Multiple Comparison Procedures (Dunn's Method) :

Comparison	Diff of Ranks	Q	P<0.05
B vs A	10.500	2.538	Yes
B vs C	0.000	0.000	No
C vs A	10.500	2.538	Yes

Note: The multiple comparisons on ranks do not include an adjustment for ties.

1

Two way ANOVA analysis for cell viability of granules 1 w stored at 4 °C

Two Way Analysis of Variance Friday, July 18, 2014, 4:07:32 PM

Data source: Data 1 in Notebook 2

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Size	2	0.618	0.309	18.014	<0.001
Biological replicate	1	0.0117	0.0117	0.682	0.414
Size x Biological replicate	2	0.779	0.390	22.699	<0.001
Residual	36	0.618	0.0172		
Total	41	1.997	0.0487		

The difference in the mean values among the different levels of Size is greater than would be expected by chance after allowing for effects of differences in Biological replicate. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Biological replicate is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Size. There is not a statistically significant difference (P = 0.414).

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 1.000
Power of performed test with alpha = 0.0500: for Biological replicate : 0.0500
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 1.000

Least square means for Size :

Group	Mean	SEM
420.000	6.028	0.0350
177.000	5.834	0.0339
43.000	5.732	0.0364

Least square means for Biological replicate :

Group	Mean	SEM
A	5.848	0.0280
B	5.881	0.0294

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.850	0.0495
420.000 x B	6.207	0.0495
177.000 x A	5.800	0.0463
177.000 x B	5.867	0.0495

1

Two way ANOVA analysis for cell viability of granules 2 w stored at 4 °C

Two Way Analysis of Variance Friday, July 18, 2014, 4:09:14 PM

Data source: Data 2 in Notebook 2

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Size	2	0.0721	0.0360	2.249	0.119
Biological replicate	1	0.149	0.149	9.327	0.004
Size x Biological replicate	2	0.425	0.212	13.248	<0.001
Residual	39	0.625	0.0160		
Total	44	1.151	0.0262		

The difference in the mean values among the different levels of Size is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Biological replicate. There is not a statistically significant difference (P = 0.119).

The difference in the mean values among the different levels of Biological replicate is greater than would be expected by chance after allowing for effects of differences in Size. There is a statistically significant difference (P = 0.004). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 0.246
Power of performed test with alpha = 0.0500: for Biological replicate : 0.818
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 0.996

Least square means for Size :

Group	Mean	SEM
420.000	5.698	0.0425
177.000	5.791	0.0300
43.000	5.716	0.0298

Least square means for Biological replicate :

Group	Mean	SEM
A	5.674	0.0287
B	5.796	0.0279

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.476	0.0633
420.000 x B	5.919	0.0566
177.000 x A	5.829	0.0400
177.000 x B	5.752	0.0448

1

Two way ANOVA analysis for cell viability of granules 5 w stored at 4 °C

43.000 x A	5.477	0.0545
43.000 x B	5.409	0.0618

All Pairwise Multiple Comparison Procedures (Tukey Test):

Comparisons for factor: **Size**

Comparison	Diff of Means	p	q	P	P<0.050
420.000 vs. 43.000	0.725	3	18.188	<0.001	Yes
420.000 vs. 177.000	0.424	3	10.829	<0.001	Yes
177.000 vs. 43.000	0.302	3	7.456	<0.001	Yes

Comparisons for factor: **Biological replicate**

Comparison	Diff of Means	p	q	P	P<0.050
B vs. A	0.0586	2	1.801	0.209	No

Comparisons for factor: **Biological replicate within 420**

Comparison	Diff of Means	p	q	P	P<0.05
A vs. B	1.776E-015	2	3.261E-014	1.000	No

Comparisons for factor: **Biological replicate within 177**

Comparison	Diff of Means	p	q	P	P<0.05
B vs. A	0.244	2	4.340	0.004	Yes

Comparisons for factor: **Biological replicate within 43**

Comparison	Diff of Means	p	q	P	P<0.05
A vs. B	0.0680	2	1.168	0.413	No

Comparisons for factor: **Size within A**

Comparison	Diff of Means	p	q	P	P<0.05
420.000 vs. 43.000	0.691	3	12.688	<0.001	Yes
420.000 vs. 177.000	0.545	3	10.013	<0.001	Yes
177.000 vs. 43.000	0.146	3	2.675	0.153	No

Comparisons for factor: **Size within B**

Comparison	Diff of Means	p	q	P	P<0.05
420.000 vs. 43.000	0.759	3	13.036	<0.001	Yes
420.000 vs. 177.000	0.302	3	5.374	0.001	Yes
177.000 vs. 43.000	0.457	3	7.648	<0.001	Yes

2

Two way ANOVA analysis for cell viability of granules 8 w stored at 4 °C

Two Way Analysis of Variance Friday, July 18, 2014, 4:11:27 PM

Data source: Data 3 in Notebook 2

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.107)

Source of Variation	DF	SS	MS	F	P
Size	2	4.499	2.249	84.232	<0.001
Biological replicate	1	0.0433	0.0433	1.622	0.209
Size x Biological replicate	2	0.224	0.112	4.187	0.022
Residual	45	1.202	0.0267		
Total	50	5.968	0.119		

The difference in the mean values among the different levels of Size is greater than would be expected by chance after allowing for effects of differences in Biological replicate. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Biological replicate is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Size. There is not a statistically significant difference (P = 0.209).

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = 0.022)

Power of performed test with alpha = 0.0500: for Size : 1.000
Power of performed test with alpha = 0.0500: for Biological replicate : 0.113
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 0.576

Least square means for Size :

Group	Mean	SEM
420.000	6.168	0.0385
177.000	5.745	0.0397
43.000	5.443	0.0412

Least square means for Biological replicate :

Group	Mean	SEM
A	5.756	0.0314
B	5.815	0.0335

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	6.168	0.0545
420.000 x B	6.168	0.0545
177.000 x A	5.623	0.0545
177.000 x B	5.867	0.0578

1

Two way ANOVA analysis for cell viability of 420 µ granules 1 w stored at RT

Two Way Analysis of Variance Friday, July 18, 2014, 5:30:41 PM

Data source: Data 2 in Notebook 1

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Failed (P < 0.050)

Source of Variation	DF	SS	MS	F	P
Size	2	3.166	1.583	205.732	<0.001
Biological replicate	2	1.673	0.837	108.740	<0.001
Size x Biological replicate	4	3.099	0.775	100.674	<0.001
Residual	55	0.423	0.00769		
Total	63	9.239	0.147		

The difference in the mean values among the different levels of Size is greater than would be expected by chance after allowing for effects of differences in Biological replicate. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Biological replicate is greater than would be expected by chance after allowing for effects of differences in Size. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 1.000
Power of performed test with alpha = 0.0500: for Biological replicate : 1.000
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 1.000

Least square means for Size :

Group	Mean	SEM
420.000	5.929	0.0229
177.000	6.074	0.0205
43.000	5.560	0.0169

Least square means for Biological replicate :

Group	Mean	SEM
A	6.036	0.0172
B	5.644	0.0202
C	5.883	0.0229

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.931	0.0292
420.000 x B	5.854	0.0358

1

Two way ANOVA analysis for cell viability of 420 µ granules 2 w stored at RT

Two Way Analysis of Variance Friday, July 18, 2014, 5:32:17 PM

Data source: Data 2 in Notebook 1

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.194)

Source of Variation	DF	SS	MS	F	P
Size	2	0.494	0.247	9.152	<0.001
Biological replicate	2	0.661	0.330	12.247	<0.001
Size x Biological replicate	4	8.637	2.159	80.058	<0.001
Residual	56	1.510	0.0270		
Total	64	11.115	0.174		

The difference in the mean values among the different levels of Size is greater than would be expected by chance after allowing for effects of differences in Biological replicate. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Biological replicate is greater than would be expected by chance after allowing for effects of differences in Size. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 0.961
Power of performed test with alpha = 0.0500: for Biological replicate : 0.994
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 1.000

Least square means for Size :

Group	Mean	SEM
420.000	6.282	0.0439
177.000	6.061	0.0337
43.000	6.079	0.0326

Least square means for Biological replicate :

Group	Mean	SEM
A	6.097	0.0337
B	6.289	0.0372
C	6.036	0.0401

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.861	0.0621
420.000 x B	6.445	0.0821

1

Two way ANOVA analysis for cell viability of 420 µ granules 5 w stored at RT

Two Way Analysis of Variance Friday, July 18, 2014, 5:33:35 PM

Data source: Data 2 in Notebook 1

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.163)

Source of Variation	DF	SS	MS	F	P
Size	2	0.708	0.354	11.593	<0.001
Biological replicate	2	5.857	2.928	95.838	<0.001
Size x Biological replicate	4	4.634	1.158	37.912	<0.001
Residual	61	1.864	0.0306		
Total	69	14.833	0.215		

The difference in the mean values among the different levels of Size is greater than would be expected by chance after allowing for effects of differences in Biological replicate. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The difference in the mean values among the different levels of Biological replicate is greater than would be expected by chance after allowing for effects of differences in Size. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 0.992
Power of performed test with alpha = 0.0500: for Biological replicate : 1.000
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 1.000

Least square means for Size :

Group	Mean	SEM
420.000	6.009	0.0379
177.000	5.912	0.0385
43.000	5.763	0.0352

Least square means for Biological replicate :

Group	Mean	SEM
A	5.550	0.0336
B	6.257	0.0385
C	5.877	0.0393

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.805	0.0583
420.000 x B	5.867	0.0782

1

Two way ANOVA analysis for cell viability of 420 µ granules 8 w stored at RT

Two Way Analysis of Variance Friday, July 18, 2014, 5:35:07 PM

Data source: Data 2 in Notebook 1

General Linear Model

Dependent Variable: CFU/mL

Normality Test: Failed (P < 0.050)

Equal Variance Test: Passed (P = 0.863)

Source of Variation	DF	SS	MS	F	P
Size	2	0.217	0.108	2.423	0.097
Biological replicate	2	2.625	1.312	29.366	<0.001
Size x Biological replicate	4	6.224	1.556	34.821	<0.001
Residual	59	2.637	0.0447		
Total	67	12.221	0.182		

The difference in the mean values among the different levels of Size is not great enough to exclude the possibility that the difference is just due to random sampling variability after allowing for the effects of differences in Biological replicate. There is not a statistically significant difference (P = 0.097).

The difference in the mean values among the different levels of Biological replicate is greater than would be expected by chance after allowing for effects of differences in Size. There is a statistically significant difference (P = <0.001). To isolate which group(s) differ from the others use a multiple comparison procedure.

The effect of different levels of Size depends on what level of Biological replicate is present. There is a statistically significant interaction between Size and Biological replicate. (P = <0.001)

Power of performed test with alpha = 0.0500: for Size : 0.282
Power of performed test with alpha = 0.0500: for Biological replicate : 1.000
Power of performed test with alpha = 0.0500: for Size x Biological replicate : 1.000

Least square means for Size :

Group	Mean	SEM
420.000	6.206	0.0607
177.000	6.047	0.0400
43.000	6.081	0.0525

Least square means for Biological replicate :

Group	Mean	SEM
A	5.920	0.0685
B	6.012	0.0393
C	6.403	0.0426

Least square means for Size x Biological replicate :

Group	Mean	SEM
420.000 x A	5.841	0.149
420.000 x B	6.352	0.0668
420.000 x C	6.427	0.0799

1