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Evaluation of the Efficacy of Candidate Probiotics for Disease Prevention in Shellfish Hatcheries

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EVALUATION OF THE EFFICACY OF CANDIDATE
PROBIOTICS FOR DISEASE PREVENTION IN
SHELLFISH HATCHERIES

BY
SAEBOM SOHN

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
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UNIVERSITY OF RHODE ISLAND

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DOCTOR OF PHILOSOPHY DISSERTATION
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ABSTRACT

In the United States of America, oyster production is an important component of the seafood economy in many communities in coastal states. The severe impact of disease outbreaks and mass mortality of oyster larvae in hatcheries impacts production, since the oyster industry is largely dependent on hatchery and nursery production. The use of probiotics has been proposed as a potential preventative measure to limit the impact of bacterial diseases in shellfish hatcheries. In previous laboratory studies, the probiotic bacteria *Phaeobacter inhibens* S4 and *Bacillus pumillus* RI06-95 improved the survival of eastern oyster (*Crassostrea virginica*) larvae against the pathogens *Vibrio tubiashii* RE22 (now *V. coralliilyticus*) and *Roseovarius crassostreae* CV919-312^T (now *Alliroseovarius crassostreae*). The aim of this study is to evaluate the efficacy of candidate probiotics *P. inhibens* S4 and *B. pumillus* RI06-95 for disease prevention in shellfish hatcheries.

Chapter 1 provides an overview of bacterial disease in marine bivalves and the use of probiotics for disease prevention in bivalve hatcheries. Chapter 2 describes that the daily application of *P. inhibens* S4 and *B. pumillus* RI06-95 mixed with algal feed to culture tanks in the hatchery increased survival of oyster larvae to experimental challenge with *V. coralliilyticus* RE22. The levels of total *Vibrios* in water and surfaces of tanks treated with probiotics were significantly decreased ($p < 0.05$) compared to non-treated tanks, whereas there were no significant differences between treatments in levels of *Vibrios* in oysters. These probiotic strains had no significant impact on oyster larvae growth and survival rate at the hatchery.

Chapter 3 evaluates the safety and efficacy of candidate probiotic bacteria strains, *P. inhibens* S4 and *B. pumilus* RI06-95, in four bivalve species, including hard clams *Mercenaria mercenaria*, bay scallops *Argopecten irradians*, blue mussels *Mytilus edulis*, and razor clams *Ensis directus*. Pre-exposure of larvae to 10^4 CFU/ml of probiotics for 24 h in the laboratory did not protect these bivalve species to challenge with *V. coralliilyticus* RE22, but pre-exposure to 10^6 CFU/ml probiotics S4 and a mixture of S4 and RI did confer some protection to bay scallop larvae (RPS; 69 ± 4 %). Daily application of 10^4 CFU/ml probiotics to tanks with bay scallop larvae at the hatchery offered partial protection against bacterial infection without impacting levels of *Vibrios* in tank surfaces, water, and larvae. However, although daily probiotic treatment of tanks containing hard clam larvae led to a decrease in the levels of *Vibrio* sp. in rearing water and larvae, it provided no consistent protection to bacterial challenge.

Chapter 4 evaluates the effects of formulation methods on the viability and efficacy of two formulations of *B. pumilus* RI06-95, including a granulated (43 μ m in size) and a lyophilized (containing 100 mM sucrose as a cryoprotectant) formulation. Granulation led to a decrease in cell viability from 10^8 CFU/mg to 10^5 CFU/mg. This level of viability was maintained for up to 8 weeks of storage. Lyophilization in the presence of 100 mM sucrose did not significantly impact the cell viability of RI06-95, but exposure of oyster larvae to this lyophilized formulation resulted in decreased survival compared to non-treated controls in a small-scale experiment. Furthermore, pretreatment of oyster larvae with the lyophilized formulation did not increase larval

survival to challenge with the pathogen *V. coralliilyticus* RE22. More work needs to be done to develop effective probiotic formulations for shellfish hatcheries.

Chapter 5 characterized the microbial community of rearing water, tank surface, and oyster larvae during a pilot-hatchery trial using 16S rDNA-based MiSeq sequencing. The impact of treatment with probiotic *B. pumilus* RI06-95 on the microbial community at the oyster hatchery also described. *Proteobacteria* was the most abundant phylum in all collected samples at the hatchery trials. The proportion of bacterial groups at the phylum level was different for sources of collected sample (water, tank surfaces, and larvae). No shift was detected in the composition of the microbiome within/between treatments (probiotic and control) and time points. Therefore, application of probiotic *B. pumilus* RI06-95 at the oyster hatchery may not significantly impact on bacterial community as detected by 16S rDNA sequencing.

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PREFACE

This dissertation was written 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 four manuscripts:

1. Probiotic strains for disease management in hatchery larviculture of the eastern oyster *Crassostrea virginica*
2. Efficacy of probiotics in preventing vibriosis in the larviculture of different species of bivalve shellfish
3. Development of formulations of *Bacillus pumilus* RI06-95 for use in larviculture of the eastern oyster *Crassostrea virginica*
4. Impact of treatment of probiotic *Bacillus pumilus* RI06-95 on bacterial communities in an oyster hatchery

TABLE OF CONTENTS

ABSTRACT	ii
ACKNOWLEDGMENTS	v
PREFACE	vii
TABLE OF CONTENTS	viii
LIST OF TABLES	ix
LIST OF FIGURES	x
CHAPTER 1	1
CHAPTER 2	16
CHAPTER 3	46
CHAPTER 4	77
CHAPTER 5	117
APPENDICES	147

LIST OF TABLES

TABLE	PAGE
Table 1-1 Bacterial pathogens causing Vibriosis to bivalve larvae.	15
Table 2-2. Effect of probiotic treatment in the hatchery on the ability of larvae to survive a laboratory bacterial challenge with the bacterial pathogen <i>V. coralliilyticus</i> RE22. Data is expressed as Relative Percent Survival (RPS, average % $\pm$ SEM) of challenged oysters from tanks exposed to probiotics in the hatchery relative challenged oysters from tanks not exposed to probiotics in the hatchery (n = 3 - 4 tanks per treatment and time point). * indicates statistical significance compare to control challenged group within each trial ($p < 0.05$).	39
Table 3-1. Effect of pre-exposure to candidate probiotic bacteria, <i>Phaeobacter inhibens</i> S4 and <i>Bacillus pumilus</i> RI06-95 in the laboratory on larval survival 24 h after challenge with the bacterial pathogen <i>Vibrio coralliilyticus</i> RE22.	71
Table 3-2. Effect of treatment of probiotic bacteria, <i>Phaeobacter inhibens</i> S4 and <i>Bacillus pumilus</i> RI06-95 in the hatchery on larval survival 24 h after challenge with the bacterial pathogen <i>Vibrio coralliilyticus</i> RE22.	76
Table 4-1 Design of hatchery trials for testing the formulated probiotics.	105
Table 4-2. Effect of pre-incubation of oyster larvae for 24 h with RI06-95 formulated products on survival (RPS, % $\pm$ SEM) after challenge with <i>V. coralliilyticus</i> RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. Data is expressed as Relative Percent Survival (RPS, % $\pm$ SEM) of challenged oysters exposed to probiotics compared to <i>V. coralliilyticus</i> RE22 challenged control. Abbreviations: RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = <i>V. coralliilyticus</i> RE22.	108

LIST OF FIGURES

FIGURE	PAGE
Figure 2-1. Effect of probiotics on amount (number of larvae per tank) of larvae at selected time points after fertilization. A) Trial I; B) Trial II; C) Trial III. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95; S4 = <i>Phaeobacter inhibens</i> S4; S4+RI = <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).	40
Figure 2-2. Effect of probiotics on mean size ($\mu\text{m} \pm \text{SEM}$) of larval oysters at selected time points after fertilization. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95; S4 = <i>Phaeobacter inhibens</i> S4; S4+RI = <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).	41
Figure 2-3. Effect of probiotics on total vibrio levels ($\log_{10}\text{CFU/mL}$) in water (A, C, E) and tank surface (B, D, F) samples at selected time points after fertilization. (A, B) Trial I; (C, D) Trial II; (E, F) Trial III. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95; S4 = <i>Phaeobacter inhibens</i> S4; S4+RI = <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).	42
Figure 2-4. Effect of probiotics on total vibrio levels ($\log_{10}\text{CFU}/1000$ oysters) in oyster larval samples at selected time points after fertilization. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95; S4 = <i>Phaeobacter inhibens</i> S4; S4+RI = <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95. *	

indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$)..... 44

Figure 2-5. Effect of probiotic treatment in the hatchery on the ability of larvae to survive a laboratory bacterial challenge with the bacterial pathogen *V. coralliilyticus* RE22. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$)..... 45

Figure 3-1. Survival of larvae from four different bivalve species after a 24 h challenge with various concentrations of *Vibrio coralliilyticus* RE22. An asterisk (*) indicates significant differences between bivalve species (mean $\pm$ SEM, $p < 0.05$). . 67

Figure 3-2. Effect of a 24 h exposure to *Vibrio coralliilyticus* RE22 on larval hard clams *Mercenaria mercenaria* (A, B) and bay scallops *Argopecten irradians* (D, E) compared to non-exposed larvae of hard clam (C) and bay scallop (F). Signs of bacterial infection included: a) presence of cell debris around the larvae, b) clumping of cilia, c) empty shells due to larval mortality, and d) tissue necrosis or detachment of cells. Magnification: 20X. Scale bar: 100 μ m. 68

Figure 3-3. Effect of a 24 h probiotic treatment on survival of larvae from 4 bivalve species to bacterial challenge. Larvae of hard clams (A), bay scallops (B), razor clams (C), and blue mussels (D) were exposed to probiotics for 24 h exposure and then challenged with *Vibrio coralliilyticus* RE22 for an additional 24 h. Different letters indicate significant differences between treatments (mean $\pm$ SEM, $p < 0.05$). Abbreviations: C = no probiotic provided; RI = *B. pumilus* RI06-95 added; S4 = *P.*

<i>inhibens</i> S4 added; S4+RI = a combination of <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95 added; RE22 = <i>V. coralliilyticus</i> RE22 added.	70
Figure 3-4. Effect of daily probiotic treatment in the hatchery on mean size ($\mu\text{m} \pm \text{SEM}$) of bay scallop (A) and hard clam (B) at selected sampling days after fertilization. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95 added; S4 = <i>Phaeobacter inhibens</i> S4 added; S4+RI = a combination of <i>B. pumilus</i> RI06-95 and <i>P. inhibens</i> S4 added.	72
Figure 3-5. Effect of daily probiotic treatment in the hatchery on survival ($\% \pm \text{SEM}$) of live larvae of bay scallop (A) and hard clam (B) at selected time points after fertilization. Abbreviations: C = no probiotic provided; RI = <i>Bacillus pumilus</i> RI06-95 added; S4 = <i>Phaeobacter inhibens</i> S4 added; S4+RI = a combination of <i>B. pumilus</i> RI06-95 and <i>P. inhibens</i> S4 added.	72
Figure 3-6. Effect of daily probiotic treatment in the hatchery on total vibrio levels (Log CFU/ml) in water (A, B), tank surface (C, D) and larvae (E, F) samples for bay scallops (A, C, E) and hard clams (B, D, F). An asterisk (*) indicates significant differences between treatments (mean $\pm \text{SEM}$, $p < 0.05$). Abbreviations: C = no probiotic provided; RI = <i>B. pumilus</i> RI06-95 added; S4 = <i>P. inhibens</i> S4 added; S4+RI = a combination of <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95 added.	73
Figure 3-7. Effect of daily exposure to probiotics in the hatchery on survival ($\% \pm \text{SEM}$) of larval bay scallop (A, B) and hard clam (C, D) 24 h after laboratory challenge with <i>Vibrio coralliilyticus</i> RE22. A, C) on day 8 of trials; B, D) on day 11 of trials. A different letter indicates a significant difference ($p < 0.05$). Abbreviations: C = no probiotic provided; RI = <i>B. pumilus</i> RI06-95 added; S4 = <i>P. inhibens</i> S4 added;	

S4+RI = a combination of <i>P. inhibens</i> S4 and <i>B. pumilus</i> RI06-95 added; RE22 = <i>V. coralliilyticus</i> RE22 added.....	75
Figure 4-1. Impact of formulation processing (granulation or lyophilization) and temperature storage on the stability of <i>Bacillus pumilus</i> RI06-95. Cell count in the reconstituted formulation after storage for up to 8 weeks was determined using a plating method. Data expressed as $\log_{10}(\text{CFU/mg}) \pm \text{SEM}$ for the granulated formulation and $\log_{10}(\text{CFU/mL}) \pm \text{SEM}$ for the lyophilized formulation.	106
Figure 4-2. Effect of pre-incubation of oyster larvae with <i>Bacillus pumilus</i> RI06-95 formulated products for 24 h on survival ($\% \pm \text{SEM}$) after challenge with <i>V. coralliilyticus</i> RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. (A) Exposure to a granulated product of <i>Bacillus pumilus</i> RI06-95; (B), (C), and (D) Exposure to lyophilized formulations (representative experiments). Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = <i>V. coralliilyticus</i> RE22. Different letters indicates statistical significant differences between the treatments.	107
Figure 4-3. Effect of daily treatment with different formulations of <i>Bacillus pumilus</i> RI06-95 of larval eastern oysters (<i>Crassostrea virginica</i>) in the hatchery on mean larval size ($\mu\text{m} \pm \text{SEM}$) at selected time points. (A) Trial I; (B) Trial II; (C) Trial III; and (D) Trial IV. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = <i>V. coralliilyticus</i> RE22. * indicates statistical significances compared to controls.	109

Figure 4-4. Effect of daily treatment with probiotics in the hatchery on interval survival ($\% \pm \text{SEM}$) of oyster larvae between selected time points. (A) Trial I; (B) Trial II; (C) Trial III; and (D) Trial IV. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates statistical significances between treatments..... 110

Figure 4-5. Effect of daily treatment with probiotics on total vibrio levels ($\text{Log}_{10}(\text{CFU/ml}) \pm \text{SEM}$) in water (A, B, C) and tank surfaces (D, E, F) in a hatchery. (A and D) Trial I (no bacteria were detected in tank surfaces in Trail I); (B and E) Trial II; and (C and F) Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean $\pm \text{SEM}$, $p < 0.05$; Two-way ANOVA)..... 112

Figure 4-6. Effect of daily treatment with probiotics on total vibrio levels ($\text{Log}_{10}(\text{CFU/ml}) \pm \text{SEM}$) on oyster larvae in the hatchery. (A) Trial I; (B) Trial II; and (C) Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean $\pm \text{SEM}$, $p < 0.05$; Two-way ANOVA)..... 113

Figure 4-7. Effect of daily probiotic treatment in the hatchery on larval survival to a laboratory challenge with the pathogen *Vibrio coralliilyticus* RE22. Larvae were brought to the laboratory and survival was measured 24 h after challenge with RE22.

(A) Larvae collected on Day 3 after fertilization on Trial I; (B) Day 10 on Trial I; (C) Day 5 on Trial II; D) Day 12 on Trial II; E) Day 5 on Trial III; F) Day 12 on Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. A different letter indicates a significant difference between treatments (One-way ANOVA; $p < 0.05$). 114

Figure 5-1. Heat map analysis of the taxonomic composition of samples from an oyster hatchery. Samples were collected from water, swab, and oyster before and after treatment with RI06-95 at oyster hatchery during 2 different trials (H1 and H2). The relative abundance of clustered OTUs at 97 % similarity was illustrated using heat map. 140

Figure 5-2. Relative abundance of dominant bacterial phyla in microbiota of samples collected from an oyster hatchery. Data for the most abundant phyla is expressed as percentage of the total community. W = water, S = swab from tank surface, O = oyster larvae, E = fertilized eggs, C = control (no probiotic), R = *Bacillus pumilus* RI06-95 treated, B = before probiotic treatment, A = after probiotic treatment (5, 8, and 12 days after fertilization for Trial II and 12 days after fertilization for Trial III). 141

Figure 5-3. Proportion of the seven major bacterial phyla in microbiota of each sample at an oyster hatchery. Samples were collected from (A) water, (B) tank surface, and (C) larval oyster during 2 different hatchery trials. 142

Figure 5-4. Relative abundance of a single microbial phyla feature detected by LEfSe as biomarker. Abbreviation: W = water, S = swab from tank surface, O = oyster larvae, NO PROBIOTIC = samples from non-treated tanks (control, red bars), and

PROBIOTIC = samples from *Bacillus pumilus* RI06-95 treated tanks (green). Each bar indicates the averages of two trials (Trial II and III) at each time points (1, 5, 7, 8, 10 and 12 days after fertilization). 143

Figure 5-5. Chao1 rarefaction diversity samples including water, swab, larval oyster, and egg tank (negative control) before or/and after treatment with RI06-95 at oyster hatchery (H1 and H2). Chao diversity was calculated from sequence distribution A) based on source of sample; B) based on treatments..... 145

Figure 5-6. Beta diversity in microbial communities in shellfish hatcheries (binary Jaccard measures). A) Before probiotic treatment. B) After probiotic treatment. Bigger nodes correspond to samples treated with *B. pumillus* RI06-95 at the hatchery in 2 different trials. Each node color indicates following; Yellow /Green = water; Blue/Purple = Swab from tank surface; Red/Orange = Larvae oyster..... 146

CHAPTER 1

LITERATURE REVIEW:

PROBIOTICS FOR BACTERIAL DISEASE MANAGEMENT IN BIVALVE LARVICULTURE

Bivalves

Bivalves belong to the phylum Mollusca, one of the largest and the most diverse groups of animals. Two-part hinged shell valves are the main characteristic of bivalves. Examples of bivalve species are oysters, scallops, clams, and mussels. Bivalves have a free-swimming larval stage, but they have different life styles during the adult stages. For example, sedentary species such as oysters and mussels attach themselves to a substrate, whereas others such as clams or scallops burrow and move around on the bottom, or live on the water bottom and swim for short distance, respectively (Gosling 2015). Most bivalves are filter feeders. They gain nutrients from microorganisms such as plankton, detritus, and bacteria by pumping and filtering large volumes of water through the gills (Jørgensen 1996).

Bivalve shellfish aquaculture

The bivalve shellfish aquaculture industry is an important and expanding area of world aquaculture production. Global mollusk production in 2012 was 15.17 million tons, which is equivalent to 22.8 % of total aquaculture production, and represents the second most important aquaculture product (FAO 2014a). The bivalve aquaculture industry in the United States comprises the production of mollusks for human consumption and the production of seed for the farming of those bivalves. A variety of bivalve species including oysters, clams, scallops, and mussels have been produced commercially. Oysters and clams are the primary mollusk species with production worth \$136 million and \$99 million, respectively (NOAA Fisheries 2014), followed by scallops and mussels in 2012 (FAO 2014b).

Hatchery production is the main source of seed (juveniles) for bivalve shellfish grow-out farms and restoration projects. Hatcheries produce fertilized eggs, larvae and small juveniles and culture them until they are large enough for deployment in estuaries. It is important to ensure an inexpensive, constant and sufficient supply of seed for sustainability of bivalve shellfish cultivation (Gosling 2015). In addition, availability of a diverse set of species of bivalve shellfish would contribute to the sustainable development of the aquaculture industry. To date, the eastern oyster, *Crassostrea virginica*, is the most common bivalve species produced in hatcheries along the east coast of United States. The hard clam, *Mercenaria mercenaria*, is also widely cultured. To a lesser degree of hatchery production, the blue mussel, *Mytilus edulis*, and bay scallop, *Argopecten irradians*, have been successfully cultured (Helm et al. 2004). The razor clam, *Ensis directus*, has been getting attention in recent years as a potential candidate shellfish species for aquaculture (da Costa & Martínez-Patiño 2009, da Costa et al. 2011, Flanagan 2013).

Bacterial disease in bivalve larvae

Disease outbreaks in bivalve larvae caused by bacterial pathogens are a main constraint to the growth and sustainability of bivalve aquaculture because they cause high losses in hatcheries. Vibriosis, caused by a variety of *Vibrio* species, is reported as the most common disease in association with mass mortality in bivalve hatcheries (Paillard et al. 2004, Beaz-Hidalgo et al. 2010, da Costa et al. 2011, Gosling 2015, Travers et al. 2015). Bacteria belonging to the genus *Vibrio* are gram-negative rods which have a single, rigid curve or are straight (Goldman & Green 2015), and include

some opportunistic pathogenic strains to invertebrates (Thompson et al. 2004, Paillard et al. 2004). For example, *Vibrio alginolyticus*, *V. tubiashii*, and *V. anguillarum* are the causative agents of bacillary necrosis in larvae of hard clams, oysters, bay scallops (Guillard 1959, Tubiash et al. 1965, 1970). Although very few diseases have been described for razor clams, a recent study suggested that *V. splendidus*-like isolates were associated with mortalities in the hatchery culture of the razor clam, *Solen marginatus* (Pulteney) (Prado et al. 2014). A list of the main causative agents of vibriosis in bivalve shellfish is summarized in Table 1-1.

Bacillary necrosis is characterized by loss of velar epithelial cells, clumped cilia, abnormal swimming behavior, and a high mortality in a short time. Bacteria initially attach and colonize on the external shell surface. Infection of the mantle epithelium and soft tissues of the larvae result in tissue necrosis and death (Tubiash et al. 1965, Elston 1999, Elston et al. 2008). The strain *V. tubiashii* is one of the most important causative agents of bacillary necrosis as described on the east/west coast of America, England, France, Spain, and other countries (Tubiash et al. 1965, Hada et al. 1984, Lodeiros et al. 1987, Elston et al. 2008, Travers et al. 2015). Unfortunately, many bacteria belonging to the genus *Vibrio* are often misidentified due to their similarity and close taxonomic relationship with other *Vibrio* species. The strain *V. tubiashii* RE22, RE98, LMG 1095 and ATCC19105 has recently been reclassified as *V. coralliilyticus* (Wilson et al. 2013, Richards et al. 2015), making *V. coralliilyticus* one of the potential pathogens affecting a wide range of hosts including bivalves.

Various species of bacteria in the genera *Pseudomonas*, *Aeromonas*, and *Altermonas* also have been described as pathogenic for bivalve larvae including

oysters (*O. edulis*, *C. virginica*), clams (*M. mercenaria*, *Tridacna giga*), and scallops (*A. purpuratus*, *Euvola ziczac*). In most cases, however, they act as pathogens together with bacteria from the genus *Vibrio* to cause mass mortality in larval cultures (Brown 1973, Garland et al. 1983, Elston 1984, Hada et al. 1984, Lodeiros et al. 1987, 1991, Olafsen et al. 1993, Sutton & Garrick 1993, Romalde & Barja 2010).

Methods of controlling bacterial disease in bivalve aquaculture

Controlling bacterial disease in bivalve culture is a complex but important issue for the sustainability of shellfish aquaculture. Maintaining a healthy rearing environment is crucial for preventing disease outbreak in shellfish hatcheries. Some sources of pathogenic bacteria are introduced into the bivalve hatchery system through contaminated food, incoming water, rearing tank, brood stock, and equipment (Elston et al. 2008).

Appropriate husbandry methods are essential to prevent mortality outbreaks in hatcheries. This includes improved hygiene practices such as sanitary disposal of dead animals, as well as sterilization of equipment such as container surfaces, water lines, and air hoses at the hatchery. Maintaining appropriate animal densities and providing uncontaminated microalgae for diet are also important husbandry practices. Movement regulations of seed and brood stock are also effective in minimizing the transfer of disease agents (Shumway 2011). The maintenance of optimum water quality is very important for the prevention and management of disease outbreak in hatcheries. There are physical, chemical, and biological water treatment systems to reduce or eliminate potential pathogenic bacteria. For instance, ultraviolet light treatment, ozone

disinfection, filtration, and the addition of silver (AgNO_3) to water are used in shellfish hatcheries (Verschuere et al. 2000). Although treatment with antibiotic such as neomycin is suggested for disease control in shellfish hatchery (Karunasagar et al. 1994, Nicolas et al. 1996), it is highly regulated by some governments because it may lead to the development of antibiotic resistance in pathogens and the elimination of beneficial microorganisms (Weston 1996, Kemper 2008, Blogoslawski et al. 2009).

Probiotics in bivalve shellfish

The use of probiotics has been proposed as a promising alternative method to manage a good larval rearing environment and to prevent bacterial disease in shellfish aquaculture. The term “probiotic” refers to bacteria first described for their ability to as produce substances secreted by one organism which inhibit the growth of another (Lilly & Stillwell 1965). It is now defined as any live microorganism which, when administered in adequate amounts, confers a health benefit on the host (FAO & WHO 2006).

Although most studies on the use of probiotics in aquaculture are focused on fish, their use in bivalve aquaculture has also been studied. The potential probiotics commonly used in aquaculture include gram-positive lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, gram-positive bacteria such as *Enterococcus* and *Bacillus*, and gram-negative bacteria such as *Vibrio* and *Pseudomonas*. They are used for growth promotion, pathogen inhibition, and improvement of nutrient digestion, water quality, tolerance for stress, and reproduction in aquaculture (Prado et al. 2010, Martínez Cruz et al. 2012a).

For example, *Alteromonas* sp. CA2 (Douillet & Langdon 1993, 1994) and *Aeromonas media* A199 showed a beneficial effect on the growth and survival of oyster (*C. gigas*) larvae (Gibson et al. 1998). The probiotic strains *Bacillus pumilus* RI06-95 and *Phaeobacter inhibens* S4 offered some protection against *V. tubiashii* RE22 (recently reclassified as *V. coralliilyticus*) in eastern oysters (*C. virginica*) (Karim et al. 2013). Application of *Vibrio* sp. OY15 also improved survival of larval eastern oysters (Kapareiko et al. 2011, Lim et al. 2011). *Phaeobacter gallaeciensis* PP-154, isolated from hatcheries of flat oysters (*Ostrea edulis*) and clams (*Ruditapes decussatus* and *Venerupis pullastra*), showed inhibitory activity against pathogens, mainly *Vibrio* sp., on the European flat oyster (Prado et al. 2009). In addition, some studies reported the effect of exposure to probiotics in various species of scallops. *Pseudoalteromonas* sp. X153 (Longeon et al. 2004) and *Roseobacter* (*Phaeobacter*) *gallaeciensis* BS107 (Ruiz-Ponte et al. 1999), for example, protected larval great scallop (*P. maximus*) against mortality. The strain *Flavobacterium* sp. P14 also showed antibacterial activity in the tropical scallop (*Pecten ziczac*) (Lodeiros et al. 1991). The inoculation of mixtures of strains *Pseudomonas* sp. 11, *Vibrio* sp. C33, *Bacillus* sp. B2 protected Chilean scallops from bacterial mortality and also provided their antibacterial activity against *V. anguillarum*-like bacteria (Riquelme et al. 1997, 2001).

Development of probiotic products for bivalve shellfish

At present, there are no commercial probiotics products specifically designed for bivalve aquaculture, although some formulated probiotic products are

commercially available for fish and/or shrimp culture as food additives (Queiroz & Boyd 1998, Moriarty 1998, Verschuere et al. 2000, Wang et al. 2005, Martínez Cruz et al. 2012a). For example, a commercial product containing *Bacillus* sp., *Saccharomyces cerevisiae*, *Nitrosomonas* sp., and *Nitrobacter* sp. showed a beneficial influence on water quality in shrimp (*Penaeus vannamei*) ponds (Wang et al. 2005). Most commercial probiotic products include *Bacillus* spp. (e.g. Verschuere et al. 2000, Martínez Cruz et al. 2012a).

In order to develop safe and effective probiotics for bivalve aquaculture, the potential probiotic should not be pathogenic or toxic to the host and other live organisms in the system, and have a beneficial effect on the host in the environmental conditions in which the host is most commonly cultured (Verschuere et al. 2000). Commercial probiotic products are available in liquid or powder form for a simple and easy way of storage and transport (Austin et al. 1995, Schisler et al. 2004, Salinas et al. 2006, Savini et al. 2010, Dagá et al. 2013). Optimizing the formulation process is important to maintain and/or increase the viability of probiotics and to induce safe and protective effects to the host.

Goals of this study

The overall goal of the research is to evaluate the effects of candidate probiotics, *Phaeobacter inhibens* S4 and *Bacillus pumillus* RI06-95, as an alternative management tool to combat bacterial disease outbreaks in bivalve shellfish aquaculture.

The first objective of the research was to determine the safety and the

effectiveness of the delivery of probiotics to the eastern oyster, *Crassostrea virginica* larvae under pilot-scale hatchery culture condition and their protective effects on the survival of oyster larvae when exposed to the pathogen *Vibrio coralliilyticus* RE22. The second objective of the research was to investigate ability of these probiotics to protect other species of cultured larval shellfish, including the hard clam *Mercenaria mercenaria*, the bay scallop *Argopecten irradians*, the blue mussel *Mytilus edulis*, and the razor clam *Ensis directus*. The third objective of the research was to develop effective and simple forms of candidate probiotic *Bacillus pumillus* RI06-95, and test the safety and efficacy of developed formulations of RI06-95 on oyster larvae for delivery in hatcheries as disease management tools. Lastly, the forth objective of the research was to evaluate the effect of probiotic treatment on the composition of the microbial community in tank surfaces, water and oyster larvae at the hatchery.

LITERATURE CITED

- Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* 18:93–96
- Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ (2010) Diversity and pathogenecity of *Vibrio* species in cultured bivalve molluscs: *Vibrio* spp., bivalve molluscs, pathogens. *Environ Microbiol Rep* 2:34–43
- Blogoslawski WJ, Stewart ME, Rhodes EW (2009) BACTERIAL DISINFECTION IN SHELLFISH HATCHERY DISEASE CONTROL. *Proc Annu Meet - World Maric Soc* 9:587–602
- Brown C (1973) The effects of some selected bacteria on embryos and larvae of the American oyster, *Crassostrea virginica*. *J Invertebr Pathol* 21:215–223
- Costa F da, Darriba S, Martínez-Patiño D, Guerra A (2011) Culture possibilities of the razor clam *Ensis arcuatus* (Pharidae: Bivalvia): Culture possibilities of the razor clam *E. arcuatus*. *Aquac Res* 42:1549–1557
- Costa F da, Martínez-Patiño D (2009) Culture potential of the razor clam *Solen marginatus* (Pennant, 1777). *Aquaculture* 288:57–64
- Dagá P, Feijoo G, Moreira MT, Costas D, Villanueva AG, Lema JM (2013) Bioencapsulated probiotics increased survival, growth and improved gut flora of turbot (*Psetta maxima*) larvae. *Aquac Int* 21:337–345
- Douillet P, Langdon CJ (1993) Effects of marine bacteria on the culture of axenic oyster *Crassostrea gigas* (Thunberg) larvae. *Biol Bull* 184:36–51
- Douillet PA, Langdon CJ (1994) Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). *Aquaculture* 119:25–40
- Elston RA (1984) Prevention and management of infectious diseases in intensive mollusc husbandry. *J World Maric Soc* 15:284–300
- Elston RA (1999) Health management, development and histology of seed oysters. World Aquaculture Society, Louisiana, USA
- Elston R, Hasegawa H, Humphrey K, Polyak I, Häse C (2008) Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. *Dis Aquat Organ* 82:119–134
- FAO (2014a) The State of world fisheries and aquaculture: opportunities and challenges. Food and Agriculture Organization of the United Nations, Rome

- FAO (2014b) Fao Yearbook. Fishery and Aquaculture Statistics 2012. Food and Agriculture Organization of the United Nations, Rome, Italy
- FAO, WHO (Eds) (2006) Probiotics in food: health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization of the United Nations : World Health Organization, Rome, Italy
- Flanagan MP (2013) Investigation of Early Development and Importance of Sediment Choice in the Hatchery Production of Razor Clams, *Ensis Directus*. Honors College, Paper 111
- Garland C, Nash G, Summer C, McMeekin T (1983) Bacterial pathogens of oyster larvae (*Crassostrea gigas*) in a Tasmanian hatchery. *Mar Freshw Res* 34:483–487
- Gibson L., Woodworth J, George A. (1998) Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. *Aquaculture* 169:111–120
- Goldman E, Green LH (2015) Practical handbook of microbiology: edited by Emanuel Goldman, Lorrence H Green. CRC Press, Taylor & Francis Group, Boca Raton; London; New York
- Gosling E (2015) Marine bivalve molluscs, Second edition. Wiley Blackwell, Chichester, West Sussex, UK ; Hoboken, NJ, USA
- Guillard RR (1959) Further evidence of the destruction of bivalve larvae by bacteria. *Biol Bull* 117:258–266
- Hada HS, West PA, Lee JV, Stemmler J, Colwell RR (1984) *Vibrio tubiashii* sp. nov., a Pathogen of Bivalve Mollusks. *Int J Syst Bacteriol* 34:1–4
- Jørgensen CB (1996) Bivalve filter feeding revisited. *Mar Ecol Prog Ser*:142–287
- Kapareiko D, Lim HJ, Schott EJ, Hanif A, Wikfors GH (2011) Isolation and Evaluation of New Probiotic Bacteria for use in Shellfish Hatcheries: II. Effects of a *Vibrio* sp. Probiotic Candidate Upon Survival of Oyster Larvae (*Crassostrea virginica*) in Pilot-Scale Trials. *J Shellfish Res* 30:617–625
- Karim M, Zhao W, Rowley D, Nelson D, Gomez-Chiarri M (2013) Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. *J Shellfish Res* 32:401–408
- Karunasagar I, Pai R, Malathi GR, Karunasagar I (1994) Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* 128:203–209

- Kemper N (2008) Veterinary antibiotics in the aquatic and terrestrial environment. *Ecol Indic* 8:1–13
- Lilly DM, Stillwell RH (1965) Probiotics: growth-promoting factors produced by microorganisms. *Science* 147:747–748
- Lim HJ, Kapareiko D, Schott EJ, Hanif A, Wikfors GH (2011) Isolation and Evaluation of New Probiotic Bacteria for use in Shellfish Hatcheries: I. Isolation and Screening for Bioactivity. *J Shellfish Res* 30:609–615
- Lodeiros C, Bolinches J, Dopazo CP, Toranzo AE (1987) Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. *Aquaculture* 65:15–29
- Lodeiros C, Freitas L, Velez A (1991) [Bacillary necrosis in larvae of the bivalve *Euvola ziczac* (Linnaeus, 1758) caused by a *Pseudomonas* sp.]. *Acta Cient Venez* 43:154–158
- Longeon A, Peduzzi J, Barthelemy M, Corre S, Nicolas J-L, Guyot M (2004) Purification and Partial Identification of Novel Antimicrobial Protein from Marine Bacterium *Pseudoalteromonas* Species Strain X153. *Mar Biotechnol* 6:633–641
- Martínez Cruz P, Ibáñez AL, Monroy Hermosillo OA, Ramírez Saad HC (2012) Use of Probiotics in Aquaculture. *ISRN Microbiol* 2012:1–13
- Moriarty DJ. (1998) Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture* 164:351–358
- Nicolas J-L, Corre S, Gauthier G, Robert R, Ansquer D (1996) Bacterial problems associated with scallop *Pecten maximus* larval culture. *Dis Aquat Organ* 27:67–76
- NOAA Fisheries (2014) Fisheries of the United States 2013. NOAA Fisheries, Silver Spring, MD
- Olafsen JA, Mikkelsen HV, Giæver HM, Hansen GH (1993) Indigenous bacteria in hemolymph and tissues of marine bivalves at low temperatures. *Appl Environ Microbiol* 59:1848–1854
- Paillard C, Le Roux F, Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquat Living Resour* 17:477–498
- Prado S, Dubert J, Costa F da, Martínez-Patiño D, Barja JL (2014) Vibrios in hatchery cultures of the razor clam, *Solen marginatus* (Pulteney). *J Fish Dis* 37:209–217
- Prado S, Montes J, Al E (2009) Inhibitory activity of *Phaeobacter* strains against aquaculture pathogenic bacteria. *Int Microbiol*:107–114

- Prado S, Romalde JL, Barja JL (2010) Review of probiotics for use in bivalve hatcheries. *Vet Microbiol* 145:187–197
- Queiroz JF, Boyd CE (1998) Effects of a Bacterial Inoculum in Channel Catfish Ponds. *J World Aquac Soc* 29:67–73
- Richards GP, Watson MA, Needleman DS, Church KM, Hase CC (2015) Mortalities of Eastern and Pacific Oyster Larvae Caused by the Pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Appl Environ Microbiol* 81:292–297
- Riquelme C, Araya R, Vergara N, Rojas A, Guaita M, Candia M (1997) Potential probiotic strains in the culture of the Chilean scallop *Argopecten purpuratus* (Lamarck, 1819). *Aquaculture* 154:17–26
- Riquelme CE, Jorquera MA, Rojas AI, Avendaño RE, Reyes N (2001) Addition of inhibitor-producing bacteria to mass cultures of *Argopecten purpuratus* larvae (Lamarck, 1819). *Aquaculture* 192:111–119
- Romalde J, Barja J (2010) Bacteria in molluscs: good and bad guys. *Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol Mendez-Vilas Ed Formatex Res Cent*:136–147
- Ruiz-Ponte C, Samain JF, Sánchez JL, Nicolas JL (1999) The Benefit of a *Roseobacter* Species on the Survival of Scallop Larvae. *Mar Biotechnol* 1:52–59
- Salinas I, Díaz-Rosales P, Cuesta A, Meseguer J, Chabrillón M, Moriñigo MÁ, Esteban MÁ (2006) Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). *Vet Immunol Immunopathol* 111:279–286
- Savini M, Cecchini C, Verdenelli MC, Silvi S, Orpianesi C, Cresci A (2010) Pilot-scale Production and Viability Analysis of Freeze-Dried Probiotic Bacteria Using Different Protective Agents. *Nutrients* 2:330–339
- Schisler D, Slininger P, Behle R, Jackson M (2004) Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 94:1267–1271
- Shumway S (Ed) (2011) *Shellfish aquaculture and the environment*. Wiley-Blackwell, Ames, Iowa
- Sutton D, Garrick R (1993) Bacterial disease of cultured giant clam *Tridacna gigas* larvae. *Dis Aquat Organ* 16:47–53
- Thompson FL, Iida T, Swings J (2004) Biodiversity of *Vibrios*. *Microbiol Mol Biol Rev* 68:403–431

- Travers M-A, Boettcher Miller K, Roque A, Friedman CS (2015) Bacterial diseases in marine bivalves. *J Invertebr Pathol* 131:11-31
- Tubiash HS, Chanley PE, Leifson E (1965) Bacillary necrosis, a disease of larval and juvenile bivalve mollusks I. Etiology and epizootiology. *J Bacteriol* 90:1036–1044
- Tubiash HS, Colwell RR, Sakazaki R (1970) Marine vibrios associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. *J Bacteriol* 103:271
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic Bacteria as Biological Control Agents in Aquaculture. *Microbiol Mol Biol Rev* 64:655–671
- Wang Y, XU Z, XIA M (2005) The effectiveness of commercial probiotics in northern white shrimp *Penaeus vannamei* ponds. *Fish Sci* 71:1036–1041
- Weston D (1996) Environmental considerations in the use of antibacterial drugs in aquaculture. In *Aquaculture and Water Resource Management*. (Baird, D. J., Beveridge, M. C. M., Kelly, L. A., et al. Eds). Blackwell Science, Oxford
- Wilson B, Muirhead A, Bazanella M, Huete-Stauffer C, Vezzulli L, Bourne DG (2013) An Improved Detection and Quantification Method for the Coral Pathogen *Vibrio coralliilyticus* (F Fabiano, Ed.). *PLoS ONE* 8:e81800

Table 1-1 Bacterial pathogens causing Vibriosis to bivalve larvae.

*Abbreviations: C = *Crassostrea*, M = *Mercenaria*, O = *Ostrea*, P= *Pecten*, R = *Ruditapes*, A = *Argopecten*

Pathogenic species	Bivalve larvae		Symptom	Mortality	Reference
<i>V. alginolyticus</i>	Mediterranean mussel	<i>Mytilus galloprovincialis</i>	A reduced velum	32% in 24 h	(Anguiano-Beltrán et al. 2004)
	Carpet shell clam	<i>R. decussatus</i>	Mantle, disorganization of muscles fibers	48–60% in 30 days	(Gomez-Leon et al. 2005)
	European flat oyster;	<i>O. edulis</i> ;	Bacillary necrosis		(Tubiash et al. 1970)
	Hard clam;	<i>M. mercenaria</i> ;			
	Eastern oyster	<i>C. virginica</i>			
<i>Vibrio</i> sp.	Pacific oyster;	<i>C. gigas</i> ; <i>O. edulis</i>	A deformed velum (clumped cilia), and exhibited circular swimming movements on their sides.	100% after 72h	(Estes et al. 2004)
	European flat oyster				
	Hard clam	<i>M. mercenaria</i>	Disruption of the velum and internal tissues	70% of the population	(Guillard 1959)
<i>V. neptunius</i> ; <i>Vibrio</i> sp.	European flat oyster	<i>O. edulis</i>	Growth depression, reduction of motility, abnormal swimming, velum deformation in the larval stages or the clearance of the spat mass	98.5 to 100% in 72 to 96 h	(Prado et al. 2005)
<i>V. pectenica</i>	Great scallop	<i>P. maximus</i>	Interrupt the digestive transit and degrade the tissues	37% in 24-48 h	(Lambert et al. 1998, Sandlund et al. 2006)
<i>V. splendidus</i> biovar II	Carpet shell clam	<i>R. decussatus</i>	Velum and necrosis of tissues	62% recorded during outbreak	(Gomez-Leon et al. 2005)
	Japanese oyster	<i>C. gigas</i>	Bacillary necrosis	100% in 24 h	(Sugumar et al. 1998)
<i>V. splendidus</i> -like(or related)	Great scallop	<i>P. maximus</i>	Velar damage with necrosis and detachment of velar cells	100% in 3–5 days	(Nicolas et al. 1996, Sandlund et al. 2006)
<i>V. coralliilyticus</i> ; <i>V. neptunius</i>	Green-lipped Mussel	<i>Perna canaliculus</i>	Irregular movements, detachment of cilia, aggregation of bacteria around the velum and deterioration of soft tissues	75% in 7 days	(Kesarcodi-Watson et al. 2009a)
<i>V. tubiashii</i>	Pacific Oyster; Eastern Oyster; Great scallop; Bay scallop	<i>C. gigas</i> ; <i>C. virginica</i> ; <i>O. edulis</i> ; <i>. irradians</i>	Soft-tissue necrosis; bacillary necrosis	100% in 12 h	(Tubiash et al. 1965)

CHAPTER 2

PROBIOTIC STRAINS FOR DISEASE MANAGEMENT IN HATCHERY LARVICULTURE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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ABSTRACT

Bacterial pathogens are a major cause of mortality in bivalve hatcheries, and outbreaks can result in shortages of seed supply to the grow-out industry. The use of probiotic bacteria is a potential preventative measure to limit the impact of bacterial diseases. We previously showed that the marine bacteria *Phaeobacter inhibens* S4 (S4) and *Bacillus pumilus* RI06-95 (RI) protect larval eastern oysters (*Crassostrea virginica*) when challenged with the pathogens *Vibrio tubiashii* RE22 (now *V. coralliilyticus* RE22) and *Roseovarius crassostreae* CV919-312^T. In the present study, we tested these probiotic bacteria under hatchery conditions. Daily addition of S4 and RI (10⁴ CFU/mL) to 100 L culture tanks resulted in a significant decrease in the levels of total *Vibrios* in water and tank surfaces ($p < 0.05$), but not in oysters. Larval growth and survival was unaffected by the probiotic treatments. Larvae treated with probiotics in the hatchery showed significantly less mortality than larvae from control tanks when exposed to 10⁵ CFU/mL of *V. coralliilyticus* RE22 for 24 hours in a laboratory challenge. These results suggest that *P. inhibens* S4 and *B. pumilus* RI06-95 are safe and potentially effective tools to limit disease outbreaks in oyster hatcheries.

INTRODUCTION

The shellfish industry is an important and rapidly expanding area of world aquaculture production. In the United States of America, marine aquaculture production increased about 10 percent annually from 2008 to 2012. The primary marine aquaculture species produced in the U.S. include oysters and clams, which rely mainly on seed supplied by hatcheries (NOAA Fisheries 2014). For example, a report on the shellfish aquaculture industry in Virginia demonstrated that seed oyster sales from hatcheries increased approximately four fold from 2008 to 2010 (Hudson and Murray, 2015). Rearing of larvae is a crucial step to ensure constant and sufficient supply of seed to support of the aquaculture industry (Helm et al., 2006).

Bacterial diseases, particularly vibriosis, continue to be a major cause of mortality in hatcheries and nurseries, resulting in major losses and great expenditure for producers (e.g. Estes et al., 2004). Bacteria belonging to the genus *Vibrio* are both numerous and ubiquitous in marine environments, and are harbored within many diverse marine organisms, such as mollusks, shrimp, fish, cephalopods, and corals (Thompson et al., 2004). Bivalve shellfish larvae infected with pathogenic *Vibrio* spp., including *V. alginolyticus*, *V. splendidus*, and *V. tubiashii*, show clumping of the cilia, soft tissue necrosis, a rapid reduction in larval motility and ultimately mortality (Tubiash et al., 1965). *Vibrio tubiashii* re-emerged in 2006 and has since been considered responsible for mass larval mortalities of Pacific oysters, *Crassostrea gigas* in the Pacific coast of the United States (Elston et al., 2008). One of the *Vibrio tubiashii* strains (RE22) isolated from disease outbreaks in Pacific oysters (Estes et al., 2004) has recently been reclassified as *Vibrio coralliilyticus* (Richards et al., 2015;

Wilson et al., 2013). *V. coralliilyticus* and *V. tubiashii* strains are pathogenic to a variety of marine invertebrates, including oysters, clams, and corals (Elston et al., 2008).

Probiotics are desirable tools for mitigating disease outbreaks and for maintaining a healthy larval rearing environment in shellfish hatcheries. Probiotics are live, non-pathogenic microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO/WHO, 2006). They can be used to eradicate harmful bacteria (Balcazar et al., 2006; Kesarcodi-Watson et al., 2008) and to improve the digestive and immune systems of the host (Castex et al., 2009). In aquaculture, probiotics can be administered either as a food supplement or as an additive to the water (Moriarty, 1998). Studies have demonstrated significantly improved survival of probiotic-treated animals when subsequently challenged by pathogenic bacteria (Castex et al., 2009; Gibson et al., 1998; Kumar et al., 2006; Rengpipat et al., 1998; Ruiz-Ponte et al., 1999; Taoka et al., 2006). Probiotic microbes are a desirable alternative to the use of antibiotics in aquaculture systems, since use of the latter can lead to the development of drug resistant strains (Karunasagar et al., 1994; Kemper, 2008; Weston, 1996).

Probiotic bacteria have shown promise in bivalve aquaculture, although few studies have tested candidate strains in hatchery scale experiments. For example, gram-positive lactic acid bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, gram-positive bacteria such as *Enterococcus* and *Bacillus*, and gram-negative bacteria such as *Vibrio* and *Pseudomonas* are commonly used as potential probiotics in aquaculture (Kesarcodi-Watson et al., 2008; Verschuere et al., 2000).

The benefits of probiotics have been studied in the Pacific oyster *C. gigas* (Douillet and Langdon, 1994), the great scallop *Pecten maximus* (Ruiz-Ponte et al., 1999), the peruvian scallop *Argopecten purpuratus* (Riquelme et al., 2000), the pearl oyster *Pinctada mazatlanica* (Aguilar-Macías et al., 2010), the green-lipped mussel *Perna canaliculus* (Kesarcodi-Watson et al., 2009), and the Manila clam *Ruditapes philippinarum* (Castro et al., 2002). In larviculture of the eastern oyster *Crassostrea virginica*, probiotic candidate *Vibrio* sp. OY15 provides a beneficial effect to larvae, both in the presence and absence of the shellfish pathogen *Vibrio* sp. B183 (Kapareiko et al., 2011).

We previously showed that the marine bacteria *Phaeobacter inhibens* S4 (S4) and *Bacillus pumilus* RI06-95 (RI) protect larval eastern oysters when challenged with the pathogens *Vibrio coralliilyticus* RE22 and *Roseovarius crassostreae* CV919-312^T (Karim et al., 2013). The goal of the present study was to test the safety and efficacy of these two candidate probiotics in larviculture of *C. virginica* at pilot-scale hatchery culture conditions. Measurements included the impact of probiotic treatments on larval survival, larval growth, total *Vibrios* in the tank surfaces, water and larvae, and the survival of larvae following exposure to *V. coralliilyticus* RE22 in laboratory challenges.

METHODS

Mollusk larvae

Adult eastern oysters *C. virginica* were spawned at the Luther H. Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA) following standard

procedures (Helm et al., 2004). Larvae (1 day old) were distributed and maintained in 120 L conical tanks and fed with live microalgae. The microalgae strains used throughout the trial were *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea* (CCMP1324; formerly *Isochrysis* sp., Tahitian strain), *Pavlova pinguis* (CCMP609), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weissflogii* (CCMP1336).

Bacterial strains

Bacterial strain *V. coralliilyticus* RE22 (Estes et al., 2004) was supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University (USA). *P. inhibens* S4 and *B. pumilus* RI06-95 were isolated by our group as previously described (Karim et al., 2013). All bacteria were maintained and stored in 50 % glycerol stocks at -80 °C until use. 16S rDNA sequencing was used to confirm strain identity prior to use in experiments (GenBank accession nos. KC625490, KC625491, and CP009264.2).

Preparation of bacterial isolates for pilot-scale trials and challenge test

The probiotic candidates and pathogen were cultured in marine medium YP30 (5 g L⁻¹ of peptone, 1 g L⁻¹ of yeast extract, 30 g L⁻¹ of ocean salt, Red Sea Salt, Israel) at 28 °C with shaking for 48 h and 24 h, respectively. Bacteria were pelleted at 2,300 × g for 10 min and then twice re-suspended in filtered sterile seawater (FSSW, 28 psu) and centrifuged to harvest the cells. The cell pellet was re-suspended in FSSW and the bacterial density was determined by measuring optical density at 550 nm (Synergy™

HT, BioTek, USA). Bacterial suspensions were diluted to the target concentration in FSSW for hatchery delivery. Additionally, serial dilution and spot plating on YP30 agar plates was used to determine cell viability and cell concentration.

Design of pilot-scale hatchery trials

Larvae were maintained in triplicate 120 L conical tanks per treatment in static conditions until reaching the pediveliger stage. Tanks were randomly assigned to following treatments: no probiotics (control), candidate probiotic S4, candidate probiotic RI, or a combination treatment comprised of both candidate probiotics S4 and RI. Each treatment was mixed with algal feed to achieve the effective dose of 10^4 CFU/mL in the tank (Karim et al., 2013), and then poured directly into individual tanks. Treatments in trials I and III were performed in triplicate, whereas those in trial II were performed in quadruplicate. Tanks were drained-down (emptying of tanks to perform 100% water changes) every other day and the day the larvae were produced (day of fertilization) was defined as day 0. Frequency and timing of treatment for each trial are described in **Table 2-1**. Sampling time was adjusted for each trial to accommodate hatchery schedule.

Effect of probiotic treatment on larval growth and survival in shellfish

During the drain-down process, the water containing larvae from each tank was screened using 2 different sized mesh screens: a large screen (75 μ m or 105, 125, 150 μ m, depending on the age of the larvae) and a small screen (40 μ m). Larvae retained on each screen were carefully rinsed out of the screen with a fixed volume of

seawater and placed in containers with a final volume of 1 - 5 L (depending on larval density). Three 1 mL aliquots from each screen were placed in Sedgewick Rafter counting chambers (Graticules ® S50) and fixed with two to five drops of Lugol's Iodine. Live and dead larvae were then counted using a compound microscope and 50 larvae from each tank (25 from top screen, 25 from bottom screen) were randomly selected from the slides, photographed with the Olympus BX51 microscope (Olympus) and measured using an Olympus DP25 camera and CellSens Standard 1.6 image software (Olympus).

Effect of probiotic treatment on levels of *Vibrio* spp.

The number of total *Vibrio* sp. in larvae, water, and tank surface samples were evaluated using a serial dilution and plating method (Miles et al., 1938). Larvae from each tank were collected from the drain-down sieves, rinsed with FSSW, and 10 mL of larvae from each tank were placed into a sterile tube. The larvae were filtered through a 48 µm nylon membrane, re-suspended in 1 mL of FSSW, homogenized using a sterile pestle and serial 1:10 dilutions of the larval homogenate were created. Next, triplicate 10 µL samples of each of the dilutions were spotted onto thiosulfate citrate bile salts sucrose agar (TCBS, Difco) plates. The inoculated plates were incubated for 16 - 20 h at 28 °C and CFU were counted. *Vibrio* abundance was quantified based on the presence of bacteria in the lowest dilution. Meanwhile, 10 mL of water samples from the drain down were collected into sterile falcon tubes. Then, water samples were diluted, plated and incubated using the same method as above. Lastly, three different sides of each tank were swabbed with three sterile cotton swabs,

each covering a non-overlapping line of approximately 48 cm in length. Each cotton swab was placed into 1 mL of FSSW and vigorously mixed, and CFU were determined as described above. Results are expressed as CFU/mL, where 1 mL corresponds to 1 mL of water in the tank, 1 mL of swab suspension (or 24 cm² of tank wall), or 1 mL of water containing about 10,000 larvae.

Effect of probiotic treatment on larval oyster survival after bacterial challenge

Laboratory experimental challenges were performed as previously described (Gómez-León et al., 2008) with minor modifications. Larvae from each of the experimental tanks in the hatchery were collected in individual sterile 50 mL Falcon tubes after selected drain down events and immediately transported to the laboratory. Larvae from each tank were placed in separate triplicate wells, each containing 5 mL FSSW, of a 6-well plate and kept at 22 - 23 °C with gentle rocking throughout the experiment. *V. coralliilyticus* RE22 was added to each well to achieve 10⁵ CFU/mL, a concentration previously determined to cause 50 - 80 % mortality (Karim et al., 2013). In order to promote ingestion of the pathogen, commercial algal paste (20,000 cells mL⁻¹; Reed Mariculture Inc., San Jose, CA, USA) was also added to each well at the time of challenge. Survival of oyster larvae was determined using the neutral red technique (Gómez-León et al., 2008). Percent larval survival for each well was calculated by using the formula:

$$\text{Survival (\%)} = 100 \times (\text{number of live larvae} / \text{total number of larvae}).$$

Results are expressed as average % $\pm$ SEM larval survival in each treatment (n = 3 tanks per treatment and time point for Trials I and III, n = 4 for Trial II)

The relative percent survival (RPS) (Amend 1981) conferred by the probiotic (treatment) with respect to the challenged larvae (control) was calculated using the formula: $RPS = [1 - (\% \text{ Mortality treatment} / \% \text{ Mortality control})] \times 100$.

Results are expressed as Relative Percent Survival (RPS, average % $\pm$ SEM) of challenged oysters from tanks exposed to probiotics in the hatchery relative challenged oysters from tanks not exposed to probiotics in the hatchery (n = 3 tanks per treatment and time point for Trials I and III, n = 4 for Trial II).

Statistical Analysis

Statistical analysis was carried out with Graphpad Prism, version 6.0 (Graphpad Software, Inc.). Two-way (with time and treatment as factors) and one-way (treatment within each time point) analysis of variance (ANOVA) was used to determine significance between groups. The Tukey's multiple comparison test was used for post-hoc pairwise comparisons. Larval oyster survival data was subjected to arc sine square root transformation before ANOVA. A p-value <0.05 was considered to be statistically significant.

RESULTS

Effect of probiotic treatment on larval growth and survival in the hatchery

No significant differences were observed between treatments in the quantity of live oyster larvae within each time point for Trials I and III (One-way ANOVA within each day, $p > 0.05$; Figure 2-1). In addition, probiotic treatment did not negatively impact the size of live larvae on Trials I and II (One-way ANOVA, $p > 0.05$; Figure

2). On the other hand, treatment with S4 led to a significant decrease in larval survival compared to the other treatments in Trial II due to one outlier tank experiencing high mortality (60 %) on day 13 (Figure 2-1). The mean size of live oyster larvae treated in the hatchery with a mixture of S4 and RI was significantly smaller ($142 \pm 20 \mu\text{m}$) than other groups ($174 \pm 19 \mu\text{m}$ and $183 \pm 19 \mu\text{m}$) at day 9 on Trial III (one-way ANOVA; $p < 0.05$; Figure 2-2). This result was driven by one outlier tank showing relatively low proportion of larvae in large screen ($150 \mu\text{m}$) (34 %) compared to other tanks ($> 98 \%$) on day 9 at the hatchery. No significant differences on larval survival or size were detected between treatments if the outlier tanks mentioned above are removed from the analyses (not shown).

Effect of probiotic treatment on the amount of total *Vibrio* spp. in the hatchery

In general, treatment of hatchery larval tanks with probiotics significantly influenced the numbers of *Vibrios* present in water and tank surfaces compared to untreated controls, although variability in the duration and level of the impact was seen between trials and treatments (Figure 2-3). Two-way ANOVA analysis of levels of *Vibrios* in water in each of the trials indicated there were significant time ($p < 0.05$) and treatment ($p < 0.05$) effects, but no treatment $\times$ time interaction ($p > 0.05$) for Trials I and II (Appendix A). No significant impact of probiotic treatment on *Vibrio* levels in water was seen in trial III ($p > 0.05$; Figure 2-3 E), probably due to the relatively lower levels of *Vibrios* present in water during winter (less than 10^2 CFU/mL). The level of *Vibrios* in water, in particular, was significantly lower in tanks treated with probiotic S4 than in other treatments during Trials I and II (Figure 2-3 A,

C). While the effect of S4 on levels of *Vibrios* in the water persisted for the length of Trial I (Figure 2-3 A), a significant reduction was only seen on day 6 in trial II (Figure 2-3 C). The probiotic RI or a mix of RI and S4 had no significant effect on levels of *Vibrios* in water in any of the trials (Figure 2-3 A, C, E).

Two-way ANOVA analysis of levels of *Vibrios* in tank surface indicate there were significant time ($p < 0.05$), treatment ($p < 0.05$) effects, and treatment $\times$ time interaction ($p < 0.05$) for Trials II and III (Appendix A). Treatment of tanks with probiotics S4 and RI led to a significant reduction in the levels of *Vibrios* on tank surfaces in Trials II and III ($p > 0.05$; Figure 2-3 D, F), but not on Trial I ($p > 0.05$; Figure 2-3 B). In these trials, the impact of S4 was significantly higher than the effect of RI (Figure 2-3 D, F). In Trial III (winter trial), a significant effect of probiotic treatment was seen on day 6, the only day in which *Vibrios* were detected in tank surfaces (Figure 2-3 F). Interestingly, treatment with the combination of the two probiotics did not have a significant impact on the level of *Vibrios* in the water or the tank surfaces in the two trials in which this combination treatment was tested (Trials I and II), even when individual probiotic treatments had a significant effect in Trial III (Figure 2-3 D, F). While the impact of probiotics on *Vibrios* in water was only significant on day 6, a significant effect on *Vibrios* in tank surfaces was also seen on day 10 in Trial II (Figure 2-3 C, D). Treatment with probiotics did not have a significant effect on the level of *Vibrios* in oyster larvae in Trials II and III ($p < 0.05$; Figure 2-4). The only treatment that showed a significant impact on levels of *Vibrios* in larvae compared to controls was S4 on day 12 of Trial I but the effect was transient (Figure 2-4 A).

Effect of probiotic treatment on larval survival to experimental bacterial challenge

Exposure to probiotics in the hatchery significantly improved survival of larval oysters to bacterial challenge in the laboratory, although high levels of variability between tanks within treatments were observed on the levels of protection, as reflected in high standard errors (Table 2-2). Survival rates of non-challenged larvae from all hatchery treatments (control and probiotics) and all experiments ranged between 92 - 99 % in the laboratory (not shown). Survival at 24 h after challenge with RE22 in the laboratory of larvae from the control tanks ranged from 16 to 60 %, while survival of probiotic-treated larvae ranged between 20 to 83 % (not shown). Two-way ANOVA analysis of survival of oyster larvae in each of the trials (Appendix A) indicated that a significant increase in survival after challenge compared to non-treated oysters was seen for all treatments and sampling points with the exception of all treatments on day 6 on Trial I, RI treated group on day 6 and both S4 and RI treated group on day 12 on Trial II, and S4 treated group and the combination group on day 6 on Trial III (Figure 2-5).

Levels of protection conferred by the mixture of S4 and RI probiotic treatments relative to control challenged larvae ranged between an RPS of 65 ± 0 % during summer (Trial I) and -40 ± 60 % in the winter (Trial III) (Table 2-2). The most protection against bacterial infection were observed for the RI treated group on Trial I and II, which showed RPS of 63 ± 4 % on day 12 and RPS of 52 ± 11 % on day 6 respectively. Effects of probiotic treatment in the hatchery on protection against challenge were variable between time points. Overall, the most protection to oyster

larvae against bacterial infection were shown on day 6 on Trial II and III. In Trial I, the highest levels of protection were seen in oyster larvae collected on day 12, which is one day after treated with probiotic at the hatchery. The negative value of RPS seen Trial III reflects lower survival after challenge of probiotic-treated larvae from some of the tanks than the non-treated larvae. For example, RPS for each of the tanks treated with S4 on day 6 was -61%, 33%, and 8%, and for each of the tanks treated with S4 and RI was -24%, -58%, and -91%.

DISCUSSION

Pilot trials were conducted to assess the safety and efficacy of two candidate probiotic strains, *P. inhibens* S4 and *B. pumilus* RI06-95 in oyster larvae produced in a hatchery. These experiments confirmed that the beneficial probiotic effects observed in laboratory studies in a previous study (Karim et al., 2013) could be translated to hatchery production conditions. In general, probiotic treatment in the hatchery: 1) had no significant impact on larval growth and survival; 2) significantly decreased the total levels of *Vibrios* in water and tank surfaces; 3) significantly increased survival of larvae treated in the hatchery to an experimental bacterial challenge in the laboratory as compared to non-treated larvae. However, some levels of variability in efficacy were observed between tanks within treatments in a trial and between trials, suggesting that conditions of delivery need to be optimized to achieve consistent results in hatchery conditions.

Probiotic additives may be helpful in controlling microbial populations in aquaculture systems, but the mechanisms by which they accomplish this outcome have

yet to be intensively investigated. A possible probiotic mechanism is that these microbes serve as nutrients for the larvae (Verschuere et al., 2000). In this study, oyster larvae were counted and measured during the hatchery's drain-down procedure. With a couple of exceptions, the numbers and size of live larvae were not statistically different to those in control tanks. Thus, it indicates that the delivery of probiotics may not be served as an additional nutrient source that impacted larval growth. The methods used to measure larval growth during these trials, however, are not very sensitive, and further experiments need to be done to determine the potential impact of these probiotics on larval growth and nutritional composition.

Another possible probiotic mechanism includes improved water quality and competition with harmful microorganisms (Verschuere et al., 2000). Total numbers of *Vibrios* were measured in the rearing seawater, tank surfaces and on the bivalve larvae. The average concentration of *Vibrio* counts during these hatchery trials was relatively low ($2.2 \pm 1.1 \log_{10}\text{CFU/mL}$, as compared to $3.03 \log_{10}\text{CFU/mL}$ reported in a survey of bivalve hatcheries, Elston et al., 2008). This was due to the fact that this hatchery uses filtration and UV treatment on incoming water to limit the introduction of pathogens. These background *Vibrio* levels in water and tank surfaces, however, varied widely between tanks and trials, probably due to the impact of handling and season on levels of *Vibrios* (Elston et al., 2008). Interestingly, although some of the probiotic treatments significantly reduced the levels of *Vibrios* in water and tank surfaces, vibrio counts on oyster larvae were not significantly decreased. Although these experiments measured total *Vibrios*, operationally defined as bacteria that grow on selective TCBS agar, and not specifically *Vibrio* pathogens, these results indicate

that probiotic treatments may lead to a decrease in the chances of an outbreak in hatchery conditions through decreasing levels of *Vibrios* in the hatchery system but without significantly impacting the vibrio bacterial community inside oysters.

Some differences in efficacy were seen between trials, which may be attributed to differences in environmental conditions between trials. Trials I and II were performed during summer, while Trial III was performed during winter. Bacterial communities in temperate coastal waters are known to significantly change with season, with a decrease in overall bacterial abundance during the winter (e.g. Staroscik and Smith, 2004). High levels of variability in the impact of probiotics were seen, which might be due to the potential impact of handling. Nevertheless, these results suggest the potential of probiotics for reducing the threat of *Vibrio* infections in bivalve larviculture. Further research should explore the effect of probiotic treatment on microbial communities in the hatchery environment using non-culture methods such as high-throughput sequencing on 16S rDNA libraries.

Using *in vivo* bacterial challenge assays, Karim et al. (2013) demonstrated that both probiotic strains provided a strong protective effect for larval oysters when oysters were challenged right after the probiotic was removed from the water. However, the protective effect was substantially diminished when larvae were challenged 48 or 96 h after removing the probiotic from the water (Karim et al., 2013). The hatchery studies here were consistent this result. Probiotics were added every other day during Trial I. Protective effects were higher when larvae were sampled less than 24 h post-treatment (on days 12 and 15), and not when sampled about 48 h following exposure (day 6). Differences in levels of protection at different sampling

time points during a trial could also have been due to a cumulative effect from repeated treatments or to the increased age of the larvae. However, we did not see evidence of increased protection at the later sampling points compared to earlier sampling points during trials II and III. These results suggest that daily treatments are required in hatcheries for protection against infections. Short residence times have been observed for other probiotic bacteria tested for aquaculture use. For example, a short residence time was observed for *Phaeobacter* sp. 24-7 when tested with turbot larvae (Planas et al., 2006) or rotifers (Pintado et al., 2010), as well as in the seawater of rearing tanks. Bivalve larvae are may be sensitive to bacterial growth and probiotics are eliminated after a short transit time (Gatesoupe, 1999).

In conclusion, *P. inhibens* S4 and *B. pumilus* RI06-95 are promising candidates to manage the impact of vibriosis in oyster. Our results show that daily treatments are safe to larvae and provide partial protection when larvae are subjected to challenge with the oyster pathogen *V. coralliilyticus* RE22. Probiotic efficacy of these bacteria in other cultured bivalve species, such as scallops and clams, remains to be determined. Further, these bacteria will need to be formulated for effective shipping and hatchery delivery. Research in our labs is underway to address these issues.

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LITERATURE CITED

- Aguilar-Macías, O.L., Ojeda-Ramírez, J.J., Campa-Córdova, A.I., Saucedo, P.E., 2010. Evaluation of natural and commercial probiotics for improving growth and survival of the pearl oyster, *Pinctada mazatlanica*, during late hatchery and early field culturing. J. World Aquac. Soc. 41, 447–454. doi:10.1111/j.1749-7345.2010.00386.x
- Balcazar, J., Blas, I., Ruizzarzuela, I., Cunningham, D., Vendrell, D., Muzquiz, J., 2006. The role of probiotics in aquaculture. Vet. Microbiol. 114, 173–186. doi:10.1016/j.vetmic.2006.01.009
- Castex, M., Lemaire, P., Wabete, N., Chim, L., 2009. Effect of dietary probiotic *Pediococcus acidilactici* on antioxidant defences and oxidative stress status of shrimp *Litopenaeus stylirostris*. Aquaculture 294, 306–313. doi:10.1016/j.aquaculture.2009.06.016
- Castro, D., Pujalte, M.J., Lopez-Cortes, L., Garay, E., Borrego, J.J., 2002. *Vibrios* isolated from the cultured manila clam (*Ruditapes philippinarum*): numerical taxonomy and antibacterial activities. J. Appl. Microbiol. 93, 438–447. doi:10.1046/j.1365-2672.2002.01709.x
- Douillet, P.A., Langdon, C.J., 1994. Use of a probiotic for the culture of larvae of the Pacific oyster (*Crassostrea gigas* Thunberg). Aquaculture 119, 25–40.
- Elston, R., Hasegawa, H., Humphrey, K., Polyak, I., Häse, C., 2008. Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. Dis. Aquat. Organ. 82, 119–134. doi:10.3354/dao01982
- Estes, R., Friedman, C., Elston, R., Herwig, R., 2004. Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. Dis. Aquat. Organ. 58, 223–230. doi:10.3354/dao058223
- FAO/WHO (Eds.), 2006. Probiotics in food: health and nutritional properties and guidelines for evaluation. FAO Food and Nutrition Paper no. 85. Food and Agriculture Organization of the United Nations : World Health Organization, Rome.
- Gatesoupe, F., 1999. The use of probiotics in aquaculture. Aquaculture 180, 147–165. doi:10.1016/S0044-8486(99)00187-8
- Gibson, L., Woodworth, J., George, A., 1998. Probiotic activity of *Aeromonas media* on the Pacific oyster, *Crassostrea gigas*, when challenged with *Vibrio tubiashii*. Aquaculture 169, 111–120. doi:10.1016/S0044-8486(98)00369-X
- Gómez-León, J., Villamil, L., Salger, S., Sallum, R., Remacha-Triviño, A., Leavitt, D., Gómez-Chiarri, M., 2008. Survival of eastern oysters *Crassostrea virginica*

- from three lines following experimental challenge with bacterial pathogens. Dis. Aquat. Organ. 79, 95–105. doi:10.3354/dao01902
- Helm, M., Bourne, N., Lovatelli, A., 2006. Bivalve culture in hatcheries: a practical manual. FAO Tech. Doc. 471, 182.
- Helm, M.M., Bourne, N., Lovatelli, A., 2004. Hatchery culture of bivalves: a practical manual. FAO Fisheries Technical Paper no. 471. Food and Agriculture Organization of the United Nations, Rome.
- Hudson, K., Murray, T., 2015. Virginia shellfish aquaculture situation and outlook report, in: VSG-15-01, VIMS Marine Resources Report No. 2015–3.
- Kapareiko, D., Lim, H.J., Schott, E.J., Hanif, A., Wikfors, G.H., 2011. Isolation and evaluation of new probiotic bacteria for use in shellfish hatcheries: II. Effects of a *Vibrio* sp. probiotic candidate upon survival of oyster larvae (*Crassostrea virginica*) in pilot-scale trials. J. Shellfish Res. 30, 617–625. doi:10.2983/035.030.0304
- Karim, M., Zhao, W., Rowley, D., Nelson, D., Gomez-Chiarri, M., 2013. Probiotic strains for shellfish aquaculture: Protection of eastern oyster, *Crassostrea virginica*, larvae and juveniles against bacterial challenge. J. Shellfish Res. 32, 401–408. doi:10.2983/035.032.0220
- Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128, 203–209. doi:10.1016/0044-8486(94)90309-3
- Kemper, N., 2008. Veterinary antibiotics in the aquatic and terrestrial environment. Ecol. Indic. 8, 1–13. doi:10.1016/j.ecolind.2007.06.002
- Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2009. Screening for probiotics of Greenshell™ mussel larvae, *Perna canaliculus*, using a larval challenge bioassay. Aquaculture 296, 159–164.
- Kesarcodi-Watson, A., Kaspar, H., Lategan, M.J., Gibson, L., 2008. Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. Aquaculture 274, 1–14. doi:10.1016/j.aquaculture.2007.11.019
- Kumar, R., Mukherjee, S.C., Prasad, K.P., Pal, A.K., 2006. Evaluation of *Bacillus subtilis* as a probiotic to Indian major carp *Labeo rohita* (Ham.). Aquac. Res. 37, 1215–1221.
- Miles, A.A., Misra, S.S., Irwin, J.O., 1938. The estimation of the bactericidal power of the blood. J. Hyg. (Lond.) 38, 732. doi:10.1017/S002217240001158X
- Moriarty, D.J., 1998. Control of luminous *Vibrio* species in penaeid aquaculture ponds. Aquaculture 164, 351–358. doi:10.1016/S0044-8486(98)00199-9

NOAA Fisheries, 2014. Fisheries of the United States 2013. NOAA Fisheries.

- Pintado, J., Pérez-Lorenzo, M., Luna-González, A., Sotelo, C.G., Prol, M.J., Planas, M., 2010. Monitoring of the bioencapsulation of a probiotic *Phaeobacter* strain in the rotifer *Brachionus plicatilis* using denaturing gradient gel electrophoresis. *Aquaculture* 302, 182–194.
- Planas, M., Pérez-Lorenzo, M., Hjelm, M., Gram, L., Uglenes Fiksdal, I., Bergh, Ø., Pintado, J., 2006. Probiotic effect in vivo of *Roseobacter* strain 27-4 against *Vibrio* (Listonella) *anguillarum* infections in turbot (*Scophthalmus maximus* L.) larvae. *Aquaculture* 255, 323–333. doi:10.1016/j.aquaculture.2005.11.039
- Rengpipat, S., Phianphak, W., Piyatiratitivorakul, S., Menasveta, P., 1998. Effects of a probiotic bacterium on black tiger shrimp *Penaeus monodon* survival and growth. *Aquaculture* 167, 301–313. doi:10.1016/S0044-8486(98)00305-6
- Richards, G.P., Watson, M.A., Needleman, D.S., Church, K.M., Hase, C.C., 2015. Mortalities of eastern and pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Appl. Environ. Microbiol.* 81, 292–297. doi:10.1128/AEM.02930-14
- Riquelme, C., Araya, R., Escribano, R., 2000. Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture* 181, 25–36. doi:10.1016/S0044-8486(99)00225-2
- Ruiz-Ponte, C., Samain, J.F., Sánchez, J.L., Nicolas, J.L., 1999. The benefit of a *Roseobacter* species on the survival of scallop larvae. *Mar. Biotechnol.* 1, 52–59. doi:10.1007/PL00011751
- Staroscik, A.M., Smith, D.S., 2004. Seasonal patterns in bacterioplankton abundance and production in Narragansett Bay, Rhode Island, USA. *Aquatic Microbial Ecology* 35, 275–282. doi:10.3354/ame035275
- Taoka, Y., Maeda, H., JO, J., KIM, S., PARK, S., Yoshikawa, T., Sakata, T., 2006. Use of live and dead probiotic cells in tilapia *Oreochromis niloticus*. *Fish. Sci.* 72, 755–766.
- Thompson, F.L., Iida, T., Swings, J., 2004. Biodiversity of *Vibrios*. *Microbiol. Mol. Biol. Rev.* 68, 403–431. doi:10.1128/MMBR.68.3.403-431.2004
- Tubiash, H.S., Chanley, P.E., Leifson, E., 1965. Bacillary necrosis, a disease of larval and juvenile bivalve mollusks I. Etiology and epizootiology. *J. Bacteriol.* 90, 1036–1044.

- Verschuere, L., Rombaut, G., Sorgeloos, P., Verstraete, W., 2000. Probiotic bacteria as biological control agents in aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671. doi:10.1128/MMBR.64.4.655-671.2000
- Weston, D., 1996. Environmental considerations in the use of antibacterial drugs in aquaculture. In *Aquaculture and Water Resource Management*. (Baird, D. J., Beveridge, M. C. M., Kelly, L. A., et al. Eds). Blackwell Science, Oxford.
- Wilson, B., Muirhead, A., Bazanella, M., Huete-Stauffer, C., Vezzulli, L., Bourne, D.G., 2013. An improved detection and quantification method for the coral pathogen *Vibrio coralliilyticus*. *PLoS ONE* 8, e81800. doi:10.1371/journal.pone.0081800

Table 2-1. Design of the hatchery trials.

Trial Number	Treatment Type	Period	Probiotic provided (days after fertilization)
Trial I	C, S4, RI, S4+RI	07/11/12 - 07/30/12	2, 4, 6, 8, 11, 14, 16, 18
Trial II	C, S4, RI	07/31/12 - 08/14/12	Daily from 2 - 13
Trial III	C, S4, RI, S4+RI	01/09/13 - 01/18/13	Daily from 2 - 9

Abbreviations: C = controls (no probiotic provided); S4 = *Phaeobacter inhibens*; RI = *Bacillus pumilus* RI06-95; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95.

Table 2-2. Effect of probiotic treatment in the hatchery on the ability of larvae to survive a laboratory bacterial challenge with the bacterial pathogen *V. coralliilyticus* RE22. Data is expressed as Relative Percent Survival (RPS, average % $\pm$ SEM) of challenged oysters from tanks exposed to probiotics in the hatchery relative challenged oysters from tanks not exposed to probiotics in the hatchery (n = 3 - 4 tanks per treatment and time point). * indicates statistical significance compare to control challenged group within each trial ($p < 0.05$).

		Relative Percent Survival (RPS, % $\pm$ SEM)				
Days after fertilization		6	9	12	14	15
Trial I (n = 3)	RI06-95	6 $\pm$ 29	-	63 $\pm$ 4*	-	51 $\pm$ 6*
	S4	21 $\pm$ 22	-	45 $\pm$ 9*	-	37 $\pm$ 3*
	S4+RI06-95	14 $\pm$ 2	-	65 $\pm$ 0*	-	52 $\pm$ 2*
Trial II (n = 4)	RI06-95	52 $\pm$ 11	-	-	33 $\pm$ 13	-
	S4	59 $\pm$ 6*	-	-	24 $\pm$ 16	-
Trial III (n = 3)	RI06-95	39 $\pm$ 6*	24 $\pm$ 12	-	-	-
	S4	-7 $\pm$ 49	12 $\pm$ 12	-	-	-
	S4+RI06-95	34 $\pm$ 10	-40 $\pm$ 60	-	-	-

(-) Not tested. RPS (%) = $[1 - (\% \text{ survival control} / \% \text{ survival treatment})] \times 100$.

Figure 2-1. Effect of probiotics on amount (number of larvae per tank) of larvae at selected time points after fertilization. A) Trial I; B) Trial II; C) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).

Figure 2-2. Effect of probiotics on mean size ($\mu\text{m} \pm \text{SEM}$) of larval oysters at selected time points after fertilization. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).

Figure 2-3. Effect of probiotics on total vibrio levels ($\log_{10}$ CFU/mL) in water (A, C, E) and tank surface (B, D, F) samples at selected time points after fertilization. (A, B) Trial I; (C, D) Trial II; (E, F) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).

Figure 2-4. Effect of probiotics on total vibrio levels ($\log_{10}$ CFU/1000 oysters) in oyster larval samples at selected time points after fertilization. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).

Figure 2-5. Effect of probiotic treatment in the hatchery on the ability of larvae to survive a laboratory bacterial challenge with the bacterial pathogen *V. coralliilyticus* RE22. (A) Trial I; (B) Trial II; (C) Trial III. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95; S4 = *Phaeobacter inhibens* S4; S4+RI = *P. inhibens* S4 and *B. pumilus* RI06-95. * indicates statistical significance between the treatments connected by the labeled bracket ($p < 0.05$).

CHAPTER 3

EFFICACY OF PROBIOTICS IN PREVENTING VIBRIOSIS IN THE LARVICULTURE OF DIFFERENT SPECIES OF BIVALVE SHELLFISH

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ABSTRACT

Shellfish aquaculture is an important industry in many countries, especially in coastal and estuarine environments. Hatcheries are the main source of seed for bivalve mollusk culture. However, these facilities can suffer from disease outbreaks that result in high loss of production stocks. In this study, we investigated the effectiveness of candidate probiotic bacteria strains, *Phaeobacter inhibens* S4 (S4) and *Bacillus pumilus* RI06-95 (RI), previously shown to protect the larvae of eastern oysters, *Crassostrea virginica*, against bacterial challenge, in protecting larvae of four other bivalve species, including hard clams *Mercenaria mercenaria*, bay scallops *Argopecten irradians*, blue mussels *Mytilus edulis*, and razor clams *Ensis directus*. Experiments evaluating the optimal dose for experimental challenges showed that hard clam larvae were less susceptible to *V. coralliilyticus* RE22 than the other bivalves. Pretreatment of larvae with 10^4 CFU/ml probiotics for 24 h did not protect any of the bivalve species tested against bacterial challenge, whereas pretreatment of 10^6 CFU/ml probiotics S4 and a mixture of S4 and RI protected bay scallop larvae (RPS; 69 ± 4 %). In pilot-scale hatchery trials, daily addition of candidate probiotics to the water of tanks containing hard clam or bay scallop larvae had no significant impact on larval growth and survival. Daily treatment of tanks with the probiotic RI led to significantly lower levels of *Vibrio* spp. levels in water and larvae compared to control group on day 8 of the hard clam hatchery trial ($p < 0.05$) but not on the bay scallop trial. Lastly, exposure of bay scallop larvae to probiotics S4 and a mixture of S4 and RI in the hatchery provided partial protection to experimental challenge (RPS: 55 ± 14 % and 54 ± 4 % respectively). Treatment of hard clam larvae with probiotics in the hatchery

did not lead to increased protection to bacterial challenge. Therefore, these candidate probiotic strains appear to have species-specific protective effects for shellfish larvae.

INTRODUCTION

Shellfish aquaculture is an important economic activity and growing area of aquaculture production in many countries. The world production of bivalves has been steadily increasing since the 1990s and reached 15.17 million tones in 2012 (FAO 2014a). The main bivalve species used in marine aquaculture include oysters, clams, scallops and mussels. In particular, the top two marine aquaculture species in the United States are oysters and clams, with an estimated aquaculture production worth \$ 136 million and \$ 99 million, respectively, in 2012 (NOAA Fisheries 2014).

Shellfish production is influenced by environmental conditions and disease outbreaks. In particular, larval mollusks in hatcheries are vulnerable to infection with bacterial pathogens. For example, Vibriosis is one of the most serious diseases affecting bivalve shellfish larvae, causing bacillary necrosis (Paillard et al. 2004). This disease is characterized by loss of cilia and velar epithelial cells, soft-tissue necrosis, and high mortality rates in bivalve mollusk larvae (Tubiash et al. 1965, 1970, Lodeiros et al. 1987). *Vibrio tubiashii* is one of the main causative agents of larval bivalve mollusk mortalities in hatcheries. Vibriosis caused by *V. tubiashii* has been reported from larval hard clam (*Mercenaria mercenaria*), eastern oyster (*Crassostrea virginica*), Pacific oysters (*Crassostrea gigas*), Kumamoto oysters (*Crassostrea sikamea*), and geoduck clams (*Panope abrupta*) (Tubiash et al. 1965, Hada et al. 1984, Elston et al. 2008). Recently, some of the strains *V. tubiashii*, such as RE22, RE98,

LMG 1095 and ATCC19105, has been reclassified as *V. coralliilyticus* (Wilson et al. 2013, Richards et al. 2015)., which is a marine pathogen causing disease in several marine organisms, including corals.

The impact of disease outbreaks in hatcheries may result in a sudden shortage of sufficient seed supply for aquaculture industry. In order to achieve a sustainable production of mollusk larvae and provide a consistent product on long-term basis, management of bacterial disease outbreaks in the hatchery is crucial for the bivalve shellfish industry. The application of probiotics has been suggested as a natural means for combating disease outbreaks in aquaculture. Probiotics are live, non-pathogenic microorganisms which, when administered in adequate amounts, confer a health benefit on the host (FAO & WHO 2006). The candidate probiotic strains *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95 have been successfully used to prevent larval oyster mortality and render protection against bacterial infection in laboratory experiments (Karim et al. 2013). Moreover, we have demonstrated that these probiotics are safe and potentially effective methods to control bacterial disease outbreak in oyster hatcheries (Sohn et al. submitted; Chapter 2 of this dissertation).

The objectives of this study are to: 1) investigate the pathogenicity of *V. coralliilyticus* RE22 (formerly named *V. tubiashii* RE22) in larvae of hard clams *Mercenaria mercenaria*, bay scallops *Argopecten irradians*, blue mussels *Mytilus edulis*, and razor clams *Ensis directus*; 2) determine the effect of pre-incubation with candidate probiotic bacteria S4 and RI06-95 on the survival of four species of cultured larva shellfish to *in vivo* challenge with bacterial pathogen *V. coralliilyticus* RE22; and 3) investigate the safety and the effectiveness of the delivery of probiotic candidates

S4 and RI06-95 to hard clams *Mercenaria mercenaria* and bay scallops *Argopecten irradians* under pilot-scale hatchery culture conditions. These experiments will determine if these candidate probiotics have the potential for preventing bacterial disease in a broad range of bivalve species.

METHODS

Bivalve larvae

Larvae from the 4 bivalve target species were obtained from selected hatcheries in New England, including the Aquaculture Research Corporation (ARC) in Dennis, MA (hard clams, 4 d old), Martha's Vineyard Shellfish Group in Oak Bluffs, MA (bay scallops, 6 - 8 d old), and the University of Maine's Darling Marine Center hatchery in Walpole, ME (razor clams and blue mussels, 4 - 6 d old). Larvae were shipped overnight to the University of Rhode Island. Upon arrival to the laboratory, larvae were washed with filtered sterile seawater (FSSW) on a 48 um nylon screen to reduce residual environmental bacteria and small parasites. Larvae were maintained at room temperature (approximately 23 °C) and fed instant algae Shellfish Diet 1800™ (Reed Mariculture Inc., San Jose, CA. USA) for the duration of the experiments.

For hatchery trials, hard clams and bay scallops adults were spawned at the Blount Hatchery at Roger Williams University (Bristol, RI, USA) and fed with a diet of *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea* (CCMP1324; formerly *Isochrysis* sp., Tahitian strain), *Pavlova pinguis* (CCMP609), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weissflogii* (CCMP1336) at the hatchery.

Bacterial strains

The *Vibrio tubiashii* RE22 strain (Estes et al. 2004), recently reclassified as *Vibrio coralliilyticus* (Wilson et al. 2013, Richards et al. 2015), was used for bacterial challenges. The *Phaeobacter inhibens* S4 strain and *Bacillus pumilus* RI06-95 strains (Karim et al. 2013) were used as candidate probiotics. Bacterial strains were cultured in yeast peptone with 3% NaCl (YP30) media (5 g L⁻¹ of peptone, 1 g L⁻¹ of yeast extract, 30 g L⁻¹ of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 170 rpm and stored in 50% glycerol stocks at -80 °C until use. The cultures were centrifuged at 2,300 × g for 10 min and washed twice with FSSW to harvest the cells. The OD₅₅₀ was measured to estimate bacterial cell concentration using a spectrophotometer (SynergyTM HT, BioTek, USA) and used to determine the volumes needed for the probiotic treatments. Additionally, serial dilution and spot plating on YP30 agar plate were used to determine the colony forming units (CFU) of actual bacterial suspension. Bacterial suspensions were diluted to the target concentration in FSSW for challenge assay.

Bacterial Challenge assay

Health status of bivalve larvae was checked before use to determine that they showed active swimming and cilia movement. Approximately 50 to 100 larvae were placed into each well of 6 well plates containing 5 ml of FSSW (28 – 30 psu). For the pathogenicity assay, *V. coralliilyticus* RE22 was added to the challenged larval groups of pathogen (10⁴, 10⁵, and 10⁶ CFU/mL). Larval control groups were not inoculated

with pathogen. All plates were incubated for 24 h at 22-23 °C with gentle rocking. Each concentration of the pathogen was tested in triplicate. Larval survival was determined at 24 hours after adding pathogen using the neutral red technique (Gómez-León et al. 2008). Larvae were photographed using the EVOS microscope (Advanced Microscopy Group, Bothell, WA). Larval percent survival was calculated using the formula:

$$\text{Survival (\%)} = 100 \times (\text{number of live larvae} / \text{total number of larvae}).$$

Three challenge experiments were performed for each bivalve species with each treatment tested in triplicate.

Effect of pretreatment with probiotics on the survival of bivalve larvae from different species after experimental challenge with *V. coralliilyticus* RE22

Larvae (50 – 100) were placed in 5 mL of FSSW in each well of 6 well plates and treated for 24 h before challenge. Treatments included: control with no probiotics, S4, RI06-95, and a mixture of S4 and RI06-95. Probiotics were tested at two different concentrations (10^4 and 10^6 CFU/mL). After 24 hours of incubation with the probiotic treatments, larvae were pipetted on top of a 48 um nylon mesh screen, gently rinsed with 5 mL of FSSW, and placed back in the corresponding well in 5 mL of fresh FSSW. Challenge assays were performed as described above. Hard clam larvae were challenged using a final concentration of 10^6 CFU/mL *V. coralliilyticus* RE22 while the larvae from the other three bivalve species were challenged using a final concentration of 10^5 CFU/mL RE22. Each assay was conducted in triplicate for each treatment.

The relative percent survival (RPS) (Amend 1981) conferred by the probiotic (treatment) with respect to the challenged larvae (control) was calculated using the formula : $RPS = [1 - (\% \text{ Mortality treatment} / \% \text{ Mortality control})] \times 100$.

Pilot-scale hatchery trials for hard clams and bay scallops

Hatchery trials using hard clams and bay scallops were carried out at the Blount Shellfish Hatchery at Roger Williams University (Bristol, RI, USA) following the methods of Sohn et al. submitted (Chapter 2 of this dissertation). Larvae were distributed into 12 x 120 L tanks one day after fertilization and maintained in static conditions until termination of the trial (day 11 after fertilization). Treatments were randomly assigned to 3 tanks each and included: a control with no probiotic, S4, RI06-95, a mixture of S4 and RI06-95. The pilot-scale hatchery trials were performed as previously described (Sohn et al. submitted, Chapter 2 of this dissertation). Probiotics were mixed with algal feed and added daily for 2 weeks to the water in each of the treatment tanks at a final concentration of 10^4 CFU/mL (Karim et al. 2013). Tanks were drained every other day and the day of fertilization was defined as day 0. The pilot-scale hatchery trial for each species carried out once.

Effect of probiotic treatment on larval growth and survival in shellfish

Size and survival of larvae in each of the tanks were measured at selected time points during routine drain-down events using Sedgewick Rafter counting chambers (Graticules ® S50) after being fixed with Lugol's iodine. Larvae from each tank were separated into various sized mesh screens including 40, 105, or 120 µm depend on

their age and size. Bivalve larvae retained in each of the drain-down sieves was gently rinsed with seawater on the sieve, and then placed into a container with 1 – 5 L of seawater (depending on larval numbers). Live and dead larvae were counted under a compound microscope as described before (Sohn et al. submitted; Chapter 2 of this dissertation). A total 50 larvae from each tank were photographed with an Olympus BX51 microscope (Olympus) and measured the size using CellSens Standard 1.6 image software and an Olympus DP25 camera (Olympus).

Effect of probiotic treatment on levels of *Vibrio* spp.

Levels of *Vibrios* in the rearing water, tank surface, and reared larvae were determined as previously described (Sohn et al. submitted, Chapter 2 of this dissertation). Briefly, 10 mL of rearing water from each tank was collected during the drain-down. Three different sides of each tank (approximately 48 cm in length) were swabbed with sterile cotton swabs. Each swab was placed into 1 mL of FSSW and rinsed vigorously. Larvae from each tank were collected from the drain-down sieves, rinsed with FSSW, and 10 mL of larvae from each tank were placed into a sterile tube. The larvae were filtered through a 48 µm nylon membrane, re-suspended in 1 mL of FSSW, homogenized using a sterile pestle and serial 1:10 dilutions of the larval homogenate were created.

Next, triplicate 10 µL samples of each of the dilutions were spotted onto thiosulfate citrate bile salts sucrose agar (TCBS, Difco) plates. The inoculated plates were incubated for 16 - 20 h at 28 °C and CFU were counted. *Vibrio* abundance was quantified based on the presence of bacteria in the lowest dilution.

Effect of probiotic treatment on larval oyster survival after bacterial challenge

Bacterial challenges were performed as described above. Briefly, larvae treated with probiotics in the hatchery were transported to the laboratory at URI and placed in wells of 6 well plates. Larvae from each tank and treatment were challenged using RE22 in triplicate and survival was determined 24 h after challenge.

Statistical Analysis

Percent survival data were subjected to the arcsine of the square root-transformed before a standard one- and two-way analysis of variance (ANOVA). ANOVA was used to determine significance between groups with time and treatment as factors (two-way) or treatment within each time point (one-way). The Tukey's multiple comparison was used for pairwise comparisons. A p-value < 0.05 was considered to be statistically significant. Statistical analysis was performed with Graphpad Prism, version 6.0 (Graphpad Software, Inc.).

RESULTS

Bacterial Challenge assay

Larvae from the four bivalve species used in these experiments, including hard clams *M. mercenaria*, bay scallops *A. irradians*, blue mussels *M. edulis*, and razor clams *E. directus*, showed susceptibility to experimental challenge with *V. coralliilyticus* RE22. Differences in susceptibility to bacterial challenge were observed between species, with hard clam larvae showing significantly higher survival than

larvae from any of the other bivalve species after exposure for 24 h to 10^5 and 10^6 of *V. coralliilyticus* RE22 ($p < 0.05$; Figure 3-1). Larval survival at 24 h of exposure at the concentration 5.0×10^5 CFU/ml of *V. coralliilyticus* RE22 ranged from 91 ± 3 % for hard clams to 26 ± 18 % for bay scallops. Survival of control groups was maintained above 93 % on all bivalve larvae (Figure 3-1). Larvae from all four bivalve species showed the classic clinical signs of bacillary necrosis 24 h after exposure to *V. coralliilyticus* RE22 strain, including clumping of cilia, cell debris on the margins of the larvae, necrosis of tissue, and empty shells (**Figure 3-2**).

Efficacy of probiotics on bivalve larvae

Treatments with two different concentrations (10^4 and 10^6 CFU/mL) of probiotics had no adverse effects on larval survival for each of the 4 target species (Figure 3-3). Pretreatment of larvae with 10^4 CFU/mL of probiotics for 24 h offered no significant protection to experimental bacterial challenge with *V. coralliilyticus* RE22 (One-way ANOVA; $p > 0.05$; Figure 3-3). In the case of razor clams, probiotic treatment led to increased mortality to bacterial challenge compared to non-treated larvae (negative RPS; Table 3-1), even if treatment with probiotic alone for 24 h did not affect larval survival (Figure 3-3C). A high level of variability was observed in these experiments. In particular, survival at 24 h after challenge with RE22 in the laboratory of razor clam larvae from the control tanks ranged from 44 to 56 %, while survival of S4 and RI mixed-treated larvae ranged between 7 to 37 % (not shown). Pretreatment with 10^6 CFU/mL probiotics, however, led to partial protection for bay scallops (One-way ANOVA; $p < 0.05$; Figure 3-3 B). In particular, pretreatment of

bay scallop larvae with 10^6 CFU/mL of S4 or a mixture of S4 and RI led to significantly increased survival to bacterial challenge as compared to non-treated challenged controls (RPS; 60 ± 6 % and 69 ± 4 %, respectively; Table 3-1).

Hatchery trials

Effect of daily treatment with probiotics in the hatchery on larval growth and survival

Daily treatment of larvae in the hatchery with all probiotics did not significantly impact the mean size or survival of larval hard clams and bay scallops compared to control non-treated larvae throughout each of the hatchery trials (One-way ANOVA; $p > 0.05$; Figure 3-4 and Figure 3-5).

*Effects of daily treatment with probiotics in the hatchery on levels of total *Vibrio* spp.*

In general, daily treatment of bay scallop hatchery larval tanks with probiotics did not significantly impact the levels of *Vibrio* spp. in water, tank surfaces, and larvae compared to the control tanks at any of the time points sampled (One-way ANOVA; $p > 0.05$) although variability in duration and level of impact was seen between tanks and treatments (Figure 3-6 A, C, E). Two-way ANOVA analysis of levels of *Vibrio* spp. in water and bay scallop larvae during the hatchery trial indicated there were significant time effects ($p < 0.05$), but no treatment and/or treatment $\times$ time interaction effects ($p > 0.05$). It also indicated that there were no significant time, treatment, and/or treatment $\times$ time interaction effects in swab samples collected from the tank surfaces ($p > 0.05$) (Appendix B).

On the other hand, treatment of hard clam larval rearing tanks in the hatchery

with probiotic RI and a mixture of S4 and RI led to a significant decrease in the levels of *Vibrio* spp. in water collected on day 8 after fertilization as compared to control (One-way ANOVA; $p < 0.05$; Figure 3-6 B). The levels of *Vibrio* spp. in hard clam larvae treated with probiotics were lower than levels in untreated tanks (One-way ANOVA; $p < 0.05$; Figure 3-6 F). Two-way ANOVA analysis of levels of *Vibrio* spp. in water, tank surfaces, and hard clam larvae during the hatchery trial indicated there were significant time effects ($p < 0.05$), but no treatment and/or treatment $\times$ time interaction effects ($p > 0.05$) (Appendix B). The levels of *Vibrio* spp. in swab for tank surfaces were barely detectable and no significant differences compare to control during both pilot-scale hatchery trials ($p > 0.05$; Figure 3-6 C, D).

Effect of daily treatment with probiotics in the hatchery on larval survival to bacterial challenge

Larval bay scallop and hard clam treated with probiotics at the hatchery for either 8 or 11 days showed similar survival after 48 h of incubation in the laboratory than untreated larvae (Figure 3-7). Bay scallop larvae exposed to S4 and a mixture of S4 and RI06-95 daily in the hatchery for either 8 or 11 days showed significantly higher survival to bacterial challenge (RE22) than non-treated challenged larvae ($p < 0.05$, Figure 3-7 A, B). Levels of protection (relative percent survival) provided by probiotic S4 and the mix of S4 and RI were similar and ranged between 54 and 63 % (Table 3-2). On the other hand, no significant differences in survival of hard clam larvae after bacterial challenge were observed between treatments ($p > 0.05$; Figure 3-7 C, D; Table 5).

DISCUSSION

The aim of this study was to determine if there were differences between bivalve species on the ability of two probiotics to protect larvae against bacterial challenge with a single broad host range pathogen, *V. coralliilyticus* RE22. Challenge of different host species with a single pathogen would allow determining the potential role of host-probiont (versus pathogen-probiont) interactions on the mechanisms of action of these probiotic species. This study demonstrated that: 1) the pathogen *V. coralliilyticus* RE22 causes mortality of all four bivalve species but hard clam larvae were relatively less susceptible than other bivalves; 2) pretreatment with probiotic S4 and a mixture of S4 and RI06-95 (10^6 CFU/mL) for 24 h provides protection against challenge with the pathogen *V. coralliilyticus* RE22 in bay scallops, but not in the 3 other species tested; 3) there were no adverse effects on larval survival and growth by application of probiotics at the bay scallop and hard clam hatchery; 4) application of probiotics at the hatchery led to a significant decrease in on the levels of *Vibrio* spp. in water and hard clam larvae during a hard clam hatchery trial, but not during the bay scallop trial; 5) daily treatment with probiotic S4 and a combination of S4 and RI06-95 at the hatchery conferred partial protection to bay scallop larvae to experimental bacterial challenge, but not to hard clams. Our results indicate that these probiotics appear to have species-specific protective effects for shellfish larvae.

As expected based on the previously reported broad host range of *V. coralliilyticus* and *V. tubiashii* strains (Tubiash et al. 1965, Elston et al. 2008, Gosling 2015), the 4 bivalve species tested in this study showed susceptibility to this pathogen. Three of these species, bay scallops, razor clams, and blue mussels, showed similar

levels of susceptibility as eastern oysters (*C. virginica*) (Karim et al. 2013). Hard clam larvae, however, were relatively less susceptible than the other species in the conditions used in these experiments. Some species of bivalve shellfish such as manila clams (*Venerupis philippinarum*) appear to be relatively more resistant to vibriosis than other species (Elston et al. 2008). The pathogenesis of vibriosis caused by RE22 in these species was similar to that reported during vibrio hatchery outbreaks and experimental challenges (Tubiash et al. 1965, Gómez-León et al. 2008). There are several potential reasons for differences in disease susceptibility between bivalves. First, these differences could be due to differences in defense mechanisms among these bivalve species to bacterial infection (Auffret 1985, Tripp 1992, Harris-Young et al. 1993, Canesi et al. 2002, Song et al. 2010, Shumway & Parsons 2011, Gosling 2015). However, little is known about immune response to bacterial infection in early stages of bivalves. Second, these differences could also be due to differences in susceptibility to the effects of virulence factors from the pathogen. *V. tubiashii* produces several potential virulence factors associated with its pathogenic capacity including extracellular metalloproteases and toxins such as haemolysins, cytotoxins, and siderophores (Hasegawa et al. 2008, Mersni-Achour et al. 2015). Disease susceptibility might be related to combinations of multiple factors and how they may interact with host factors (Prieur 1981, Pruzzo et al. 2005).

Probiotics are known to confer benefits through different mechanisms such as water quality improvement, production of antimicrobial compounds, enhancement of nutrition to host species, competition with pathogenic bacteria, and stimulation of immune response on host species. The bacterium *P. inhibens* S4 inhibits a wide range

of potential pathogens (Ruiz-Ponte et al. 1999) and provides protection against pathogen challenge in larvae of various molluscan species, including scallops, flat oysters, and Pacific oysters (Kesarcodi-Watson et al. 2012). The potential mechanisms of probiotic *P. inhibens* S4 involve contributions from both biofilm formation and the production of the antibiotic tropodithietic acid (TDA) (Zhao et al. 2016). There is also evidence that three N-acyl homoserin lactones (AHLs) present in S4Sm culture supernatant may disrupt the quorum-sensing pathway that activates metalloprotease (a virulence factor) transcription in *V. coralliilyticus* RE22 (Zhao 2014). However, these studies also indicate that mechanisms of action of S4 are complex and that other yet uncharacterized factors may contribute to probiotic activity (Zhao et al. 2016).

The lack of protection by *P. inhibens* S4 seen in other bivalves may be a result of differences on host-microbe interactions, including: 1) the ability of the different hosts to ingest the microbes, 2) differences between hosts in immune responses to the probiotic and rates of elimination, and/or 3) differences in the patterns of colonization of host surfaces by the probiotic. Differences in the mechanisms of feeding (or selective ingestion of microalgae and microbes) have been observed between bivalve species, including adult hard clams *M. mercenaria*, larval or adult eastern oysters *C. virginica* (Shumway et al. 1985, Baldwin 1995, Beals 2004), larval blue mussels *M. edulis*, and larval scallop *A. purpuratus* (Prieur 1981, Riquelme et al. 2000). If feeding mechanisms are shown to impact probiotic uptake and efficacy in further research, these differences could be exploited to improve probiotic development and methods of delivery.

Differences between hosts and experimental variability within hosts in the

ability to confer protection by these 2 candidate probiotics may also be due to the health quality of different batches/sources of larvae and other environmental conditions. Each bivalve species were obtained from different hatchery facilities and varied in rearing conditions and microbial communities. Once they were transported to the laboratory, environmental factors such as temperature and/or salinity (which were kept constant between experiments but may have different effects depending on larval species) may influence the species-related variation in larval shellfish. It is well known that environmental factors such as temperature and salinity can influence the physiological state of bivalves and their susceptibility to bacterial infection (Maugeri et al. 2000, Paillard et al. 2004, Garnier et al. 2007, Beaz-Hidalgo et al. 2010).

The successful development of probiotics for bivalve larvae should be verified by testing the biological effects of probiotics on bivalve larvae under controlled experimental conditions as well as on larger scale environment such as in bivalve hatcheries. In this study, pilot-scale hatchery trials were performed on hard clams and bay scallops only, based on results from the laboratory trials. Similarly to prior hatchery trials (Sohn et al. submitted, Chapter 2 of this dissertation), a high level of variability was observed on the impact of probiotic treatment on levels of *Vibrio* spp. in water, tank surfaced and bivalve larvae between the 2 hatchery trials. The diversity and composition of microbial populations in aquaculture facilities is very complex and influenced by environmental factors. Variation in environmental parameters, as well as in interactions between probiotics and resident microorganisms in the hatchery system, may have led to variability in the impact of the probiotic treatment (Thompson et al. 2004).

In conclusion, our results indicate that probiotic strains *P. inhibens* S4 and *B. pumillus* RI06-95 may be useful in the management of vibriosis in oyster and bay scallop hatcheries, but not hard clam, razor clam, or blue mussel hatcheries. Our research also points to the importance of host-probiotic-pathogen interactions on probiotic efficacy. In order to develop effective and simple means of probiotic delivery for commercial use in shellfish hatcheries, further research is needed to determine mechanism of action in different species.

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LITERATURE CITED

- Amend DF (1981) Potency testing of fish vaccines, p. 447-454. In D. P. Anderson and W. Hennessen (ed.), Fish biologics: serodiagnostics and vaccines. S. Karger, Basel, Switzerland
- Auffret M (1985) Morphologie comparative des types hémocytaires chez quelques mollusques bivalves d'intérêt commercial.”. PhD Thesis, University of Bretagne Occidentale, Brest, France
- Baldwin BS (1995) Selective particle ingestion by oyster larvae (*Crassostrea virginica*) feeding on natural seston and cultured algae. *Mar Biol* 123:95–107
- Beals CD (2004) Clearance rates and particle selectivity in the hard clam, *Mercenaria mercenaria*, from warm water habitats. M.S. Thesis, University of Florida, Gainesville
- Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ (2010) Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs: *Vibrio* spp., bivalve molluscs, pathogens. *Environ Microbiol Rep* 2:34–43
- Canesi L, Gallo G, Gavioli M, Pruzzo C (2002) Bacteria-hemocyte interactions and phagocytosis in marine bivalves. *Microsc Res Tech* 57:469–476
- Elston R, Hasegawa H, Humphrey K, Polyak I, Häse C (2008) Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and management. *Dis Aquat Organ* 82:119–134
- FAO (2014) The State of world fisheries and aquaculture: opportunities and challenges. Food and Agriculture Organization of the United Nations, Rome
- FAO, WHO (Eds) (2006) Probiotics in food: health and nutritional properties and guidelines for evaluation. Food and Agriculture Organization of the United Nations : World Health Organization, Rome
- Garnier M, Labreuche Y, Garcia C, Robert M, Nicolas J-L (2007) Evidence for the Involvement of Pathogenic Bacteria in Summer Mortalities of the Pacific Oyster *Crassostrea gigas*. *Microb Ecol* 53:187–196
- Gómez-León J, Villamil L, Salger S, Sallum R, Remacha-Triviño A, Leavitt D, Gómez-Chiarri M (2008) Survival of eastern oysters *Crassostrea virginica* from three lines following experimental challenge with bacterial pathogens. *Dis Aquat Organ* 79:95–105
- Gosling E (2015) Marine bivalve molluscs, Second edition. Wiley Blackwell, Chichester, West Sussex, UK ; Hoboken, NJ, USA

- Hada HS, West PA, Lee JV, Stemmler J, Colwell RR (1984) *Vibrio tubiashii* sp. nov., a Pathogen of Bivalve Mollusks. *Int J Syst Bacteriol* 34:1–4
- Harris-Young L, Tamplin ML, Fisher WS, Mason JW (1993) Effects of physicochemical factors and bacterial colony morphotype on association of *Vibrio vulnificus* with hemocytes of *Crassostrea virginica*. *Appl Environ Microbiol* 59:1012–1017
- Hasegawa H, Lind EJ, Boin MA, Hase CC (2008) The Extracellular Metalloprotease of *Vibrio tubiashii* Is a Major Virulence Factor for Pacific Oyster (*Crassostrea gigas*) Larvae. *Appl Environ Microbiol* 74:4101–4110
- Karim M, Zhao W, Rowley D, Nelson D, Gomez-Chiarri M (2013) Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. *J Shellfish Res* 32:401–408
- Kesarcodi-Watson A, Miner P, Nicolas J-L, Robert R (2012) Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). *Aquaculture* 344-349:29–34
- Lodeiros C, Bolinches J, Dopazo CP, Toranzo AE (1987) Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. *Aquaculture* 65:15–29
- Maugeri TL, Caccamo D, Gugliandolo C (2000) Potentially pathogenic vibrios in brackish waters and mussels. *J Appl Microbiol* 89:261–266
- Mersni-Achour R, Cheikh YB, Pichereau V, Doghri I, Etien C, Degremont L, Saulnier D, Fruitier-Arnaudin I, Travers M-A (2015) Factors other than metalloprotease are required for full virulence of French *Vibrio tubiashii* isolates in oyster larvae. *Microbiology* 161:997–1007
- NOAA Fisheries (2014) Fisheries of the United States 2013. NOAA Fisheries, Silver Spring, MD
- Paillard C, Le Roux F, Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquat Living Resour* 17:477–498
- Prieur D (1981) Experimental studies of trophic relationships between marine bacteria and bivalve molluscs. *Kiel Meeresforsch Sonderh* 5:376–383
- Pruzzo C, Gallo G, Canesi L (2005) Persistence of vibrios in marine bivalves: the role of interactions with haemolymph components: *Vibrio* interactions with marine bivalve haemolymph. *Environ Microbiol* 7:761–772

- Richards GP, Watson MA, Needleman DS, Church KM, Hase CC (2015) Mortalities of Eastern and Pacific Oyster Larvae Caused by the Pathogens *Vibrio coralliilyticus* and *Vibrio tubiashii*. *Appl Environ Microbiol* 81:292–297
- Riquelme C, Araya R, Escribano R (2000) Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture* 181:25–36
- Ruiz-Ponte C, Samain JF, Sánchez JL, Nicolas JL (1999) The Benefit of a *Roseobacter* Species on the Survival of Scallop Larvae. *Mar Biotechnol* 1:52–59
- Shumway SE, Cucci TL, Newell RC, Yentsch CM (1985) Particle selection, ingestion, and absorption in filter-feeding bivalves. *J Exp Mar Biol Ecol* 91:77–92
- Shumway SE, Parsons GJ (2011) *Scallops: Biology, Ecology and Aquaculture: Biology, Ecology and Aquaculture*. Elsevier, Amsterdam
- Song L, Wang L, Qiu L, Zhang H (2010) Bivalve immunity. In: *Invertebrate immunity*. Springer, p 44–65
- Thompson FL, Iida T, Swings J (2004) Biodiversity of *Vibrios*. *Microbiol Mol Biol Rev* 68:403–431
- Tripp MR (1992) Phagocytosis by hemocytes of the hard clam, *Mercenaria mercenaria*. *J Invertebr Pathol* 59:222–227
- Tubiash HS, Chanley PE, Leifson E (1965) Bacillary necrosis, a disease of larval and juvenile bivalve mollusks I. Etiology and epizootiology. *J Bacteriol* 90:1036–1044
- Tubiash HS, Colwell RR, Sakazaki R (1970) Marine vibrios associated with bacillary necrosis, a disease of larval and juvenile bivalve mollusks. *J Bacteriol* 103:271
- Wilson B, Muirhead A, Bazanella M, Huete-Stauffer C, Vezzulli L, Bourne DG (2013) An Improved Detection and Quantification Method for the Coral Pathogen *Vibrio coralliilyticus* (F Fabiano, Ed.). *PLoS ONE* 8:e81800
- Zhao W (2014) Characterization of the probiotic mechanism of *Phaeobacter gallaeciensis* S4 against bacterial pathogens. Doctoral dissertation, University of Rhode Island
- Zhao W, Dao C, Karim M, Gomez-Chiarri M, Rowley D, Nelson DR (2016) Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter inhibens*. *BMC Microbiol* 16(1):1

Figure 3-1. Survival of larvae from four different bivalve species after a 24 h challenge with various concentrations of *Vibrio coralliilyticus* RE22. An asterisk (*) indicates significant differences between bivalve species (mean $\pm$ SEM, $p < 0.05$).

Figure 3-2. Effect of a 24 h exposure to *Vibrio coralliilyticus* RE22 on larval hard clams *Mercenaria mercenaria* (A, B) and bay scallops *Argopecten irradians* (D, E) compared to non-exposed larvae of hard clam (C) and bay scallop (F). Signs of bacterial infection included: a) presence of cell debris around the larvae, b) clumping of cilia, c) empty shells due to larval mortality, and d) tissue necrosis or detachment of

cells. Magnification: 20X. Scale bar: 100 μm .

Figure 3-3. Effect of a 24 h probiotic treatment on survival of larvae from 4 bivalve species to bacterial challenge. Larvae of hard clams (A), bay scallops (B), razor clams (C), and blue mussels (D) were exposed to probiotics for 24 h exposure and then challenged with *Vibrio coralliilyticus* RE22 for an additional 24 h. Different letters indicate significant differences between treatments (mean $\pm$ SEM, $p < 0.05$). Abbreviations: C = no probiotic provided; RI = *B. pumilus* RI06-95 added; S4 = *P. inhibens* S4 added; S4+RI = a combination of *P. inhibens* S4 and *B. pumilus* RI06-95 added; RE22 = *V. coralliilyticus* RE22 added.

Table 3-1. Effect of pre-exposure to candidate probiotic bacteria, *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95 in the laboratory on larval survival 24 h after challenge with the bacterial pathogen *Vibrio coralliilyticus* RE22.

Relative Percent Survival (RPS, % $\pm$ SEM) compared to RE22 challenged control						
Bivalve \ Probiotic	S4		RI06-95		S4 + RI06-95	
	10 ⁴	10 ⁶	10 ⁴	10 ⁶	10 ⁴	10 ⁶
Hard Clam	24 $\pm$ 7	31 $\pm$ 21	17 $\pm$ 38	-20 $\pm$ 6	46 $\pm$ 15	22 $\pm$ 10
Bay Scallop	43 $\pm$ 12	60 $\pm$ 6	24 $\pm$ 6	-4 $\pm$ 6	56 $\pm$ 10	69 $\pm$ 4
Razor Clam	-41 $\pm$ 20	-2 $\pm$ 14	-61 $\pm$ 9	-48 $\pm$ 27	-38 $\pm$ 24	-261 $\pm$ 204
Blue Mussel	-6 $\pm$ 24	2 $\pm$ 17	23 $\pm$ 9	7 $\pm$ 26	28 $\pm$ 12	31 $\pm$ 11

Figure 3-4. Effect of daily probiotic treatment in the hatchery on mean size ($\mu\text{m} \pm \text{SEM}$) of bay scallop (A) and hard clam (B) at selected sampling days after fertilization. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95 added; S4 = *Phaeobacter inhibens* S4 added; S4+RI = a combination of *B. pumilus* RI06-95 and *P. inhibens* S4 added.

Figure 3-5. Effect of daily probiotic treatment in the hatchery on survival ($\% \pm \text{SEM}$) of live larvae of bay scallop (A) and hard clam (B) at selected time points after fertilization. Abbreviations: C = no probiotic provided; RI = *Bacillus pumilus* RI06-95 added; S4 = *Phaeobacter inhibens* S4 added; S4+RI = a combination of *B. pumilus* RI06-95 and *P. inhibens* S4 added.

Figure 3-6. Effect of daily probiotic treatment in the hatchery on total vibrio levels (Log CFU/ml) in water (A, B), tank surface (C, D) and larvae (E, F) samples for bay scallops (A, C, E) and hard clams (B, D, F). An asterisk (*) indicates significant differences between treatments (mean $\pm$ SEM, $p < 0.05$). Abbreviations: C = no

probiotic provided; RI = *B. pumilus* RI06-95 added; S4 = *P. inhibens* S4 added; S4+RI
= a combination of *P. inhibens* S4 and *B. pumilus* RI06-95 added.

Figure 3-7. Effect of daily exposure to probiotics in the hatchery on survival (% $\pm$ SEM) of larval bay scallop (A, B) and hard clam (C, D) 24 h after laboratory challenge with *Vibrio coralliilyticus* RE22. A, C) on day 8 of trials; B, D) on day 11 of trials. A different letter indicates a significant difference ($p < 0.05$). Abbreviations: C = no probiotic provided; RI = *B. pumilus* RI06-95 added; S4 = *P. inhibens* S4 added; S4+RI = a combination of *P. inhibens* S4 and *B. pumilus* RI06-95 added; RE22 = *V. coralliilyticus* RE22 added.

Table 3-2. Effect of treatment of probiotic bacteria, *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95 in the hatchery on larval survival 24 h after challenge with the bacterial pathogen *Vibrio coralliilyticus* RE22.

		Relative Percent Survival (RPS, % $\pm$ SEM) compared to RE22 challenged control					
Probiotic		S4		RI06-95		S4 + RI06-95	
Bivalve	Day	8	11	8	11	8	11
Hard Clam		6 $\pm$ 25	10 $\pm$ 18	35 $\pm$ 12	1 $\pm$ 15	21 $\pm$ 26	-18 $\pm$ 8
Bay Scallop		63 $\pm$ 10	55 $\pm$ 14	26 $\pm$ 22	23 $\pm$ 5	62 $\pm$ 4	54 $\pm$ 4

CHAPTER 4

FORMULATIONS OF *BACILLUS PUMILUS* RI06-95 FOR USE IN LARVICULTURE OF THE EASTERN OYSTER *CRASSOSTREA VIRGINICA*

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ABSTRACT

Vibriosis is a major disease affecting larval eastern oysters, *Crassostrea virginica*, which could lead to shortages in the supply of oyster seed for the industry when hatcheries are impacted. Probiotics are a suitable tool for controlling the impact of bacterial pathogens in shellfish hatcheries. The efficacy of a candidate probiotic strain, *Bacillus pumilus* RI06-95, was previously investigated and found to prevent mortality due to bacterial disease in oyster larvae and juveniles. In this study, we have developed two types of formulations (granulated and lyophilized) of *B. pumilus* RI06-95 designed for use in shellfish hatcheries. The cell viability of both RI06-95 formulations remained above 10^5 CFU/ml for up to 8 weeks of storage in spite of a significant decrease in cell viability after granulation. In a small-scale laboratory challenge experiment, a granulated formulation had no adverse impacts on larval oyster survival. However, treatment of larval oysters with a lyophilized formulation in which sucrose was used as a cryoprotectant led to a significant decrease in survival compared to non-treated controls. The granulated formulation provided protection to challenge with *Vibrio coralliilyticus* RE22 (RPS; 69 ± 1 %), while the lyophilized formulation afforded no protection. In 3 of 4 pilot-scale hatchery trials, daily addition of either the granulated or the lyophilized formulations of RI06-95 did not significantly impact oyster survival and growth. In contrast with the effect previously observed with fresh probiotics, the levels of *Vibrio* spp. on water, tank surface, and oyster larvae were not significantly affected by the formulated probiotic treatments. In addition, treatment of oyster larvae in the hatchery with the granulated or the lyophilized probiotic failed to protect larvae to laboratory challenge with the pathogen

RE22. Further research is needed to develop formulations of *B. pumilus* for commercial use in shellfish hatcheries.

INTRODUCTION

The bivalve shellfish (oysters, clams, scallops, and mussels) industry is an important and rapidly expanding area of worldwide aquaculture production. A primary requisite for the aquaculture of most bivalve shellfish species is an abundant, reliable and inexpensive supply of seed/small juveniles (Helm et al. 2004b). Shellfish larvae, however, are prone to infectious diseases, which can result in a rapid and high rate of larval mortality in commercial hatcheries (Elston 1998), leading to substantial economic losses. For instance, pathogenic strains from several *Vibrio* spp. including *V. alginolyticus*, *V. anguillarum*, *V. coralliilyticus*, *V. ordalii*, *V. splendidus*, *V. tubiashii*, and others cause bacillary necrosis on larval bivalve shellfish. Clinical signs of vibriosis in bivalves include necrosis of mantle epithelium, clumping of the cilia, and rapid mortality (Tubiash et al. 1965, Berthe 2004, Gomez-Leon et al. 2005, Kesarcodi-Watson et al. 2009a).

The use of probiotics is one of the most promising management strategies for shellfish disease prevention and control (Elston 1998, Verschueren et al. 2000, Prado et al. 2010). Probiotics are defined as live, non-pathogenic microorganisms which, when administered in adequate amounts, confer a health benefit to the host (World Health Organization 2006). The most widely used probiotics in human and animal health are belonging to *Bacillus* spp., *Bifidobacterium* spp., and lactic-acid bacteria such as *Lactobacillus* spp. (Hong et al. 2005). In particular, *Bacillus* spp. are attractive for

commercial products because they are aerobic, spore-forming bacteria. Spores are capable of surviving extreme conditions such as the high temperatures and pressure conditions sometimes used for processing a commercial product. Products of *Bacillus* spp. are stable for long periods without significant loss in viability because of the ability of spores to survive in harsh environmental conditions until germination and proliferation when placed in a more favorable environment (Lalloo et al. 2010, Cutting 2011, de Azevedo & Tavares Brag 2012, Sorokulova 2013, Edna et al. 2014).

Several *Bacillus* spp. have been commonly studied as probiotic bacteria that improve host survival, growth, and development in aquaculture (Queiroz & Boyd 1998, Luis-Villaseñor et al. 2011, Martínez Cruz et al. 2012b, Li et al. 2014). Additionally, bacilli have shown an antagonistic effect against several *Vibrio* spp. pathogenic to invertebrates (Vaseeharan & Ramasamy 2003, Decamp & Moriarty 2006). We have previously shown that a Rhode Island marine isolate, *Bacillus pumilus* RI06-95, producer of the antibiotic amicoumacin (Socha 2008), exhibited antagonistic effects towards the shellfish pathogen *V. coralliilyticus* RE22 *in vitro* and protected eastern oyster *Crassostrea virginica* and bay scallop *Argopecten irradians* larvae against experimental challenge with RE22 (Karim et al. 2013, Sohn et al. in preparation, Chapter 3 of this dissertation). It was also shown that daily treatment of larval rearing tanks in a hatchery with RI06-95 led to a decrease the levels of *Vibrio* spp. on the tank surfaces and an increase in the survival of larval oysters to bacterial challenge (Sohn et al. submitted, Chapter 2 of this dissertation).

Although many studies have shown promising results for the use of probiotics in shellfish aquaculture, no commercial probiotic products are available that have been

specifically shown to be effective in bivalve larviculture. Probiotics typically comprise live bacterial cells, and are available in several types of commercial probiotic formulations, including dry products (such as wettable powders, dusts, and granules) and liquid products (such as cell suspensions in water, oils, and emulsions) (Austin et al. 1995, Schisler et al. 2004, Salinas et al. 2006, Savini et al. 2010, Dagá et al. 2013). The appropriate formulation of probiotics may offer several advantages, including: the stabilization of microbial agents during distribution and storage; facilitating the handling and application of the product; the protection of the agent from adverse environmental factors; and enhanced activity of microbial agents when used in the field. Therefore, the successful development of a formulation of a candidate probiotic strain showing efficacy in bivalve hatcheries is a necessary step for the development of a commercial product that can be mass produced for use in larviculture facilities.

The objective of this study was to develop a formulation of *B. pumilus* RI06-95 that is safe, stable, efficacious, and easy-to-use in bivalve shellfish hatcheries as a disease management tool. In this study, two types of formulations, one granulated and one freeze-dried (lyophilized), of *B. pumilus* RI06-95 were developed and their viability and stability after processing were evaluated. The safety and efficacy of the probiotic formulations were also determined in small scale and in pilot-scale hatchery trials, using oyster larvae challenged with the pathogen *V. coralliilyticus* RE22.

METHODS

Oyster larvae

For the bacterial challenge experiments, eastern oysters, *C. virginica*, (4 - 6 day old) were obtained from the Blount Shellfish Hatchery at Roger Williams University (Bristol, RI, USA). Oyster larvae were transported to the laboratory at the University of Rhode Island (Kinston, RI, USA) and acclimated at room temperature for at least 24 h before treatment. The larvae were fed instant algae Shellfish Diet 1800™ (Reed Mariculture Inc., San Jose, CA, USA) during the experiments.

Adult eastern oysters were spawned at the Blount Shellfish Hatchery for Trials I, II, and III and at the VIMS Shellfish Hatchery at the Aquaculture Genetics & Breeding Technology Center (ABC), Virginia Institute of Marine Science (VIMS) (Wachapreague, VA, USA) for Trial IV. Larval oysters were distributed into 120 L conical tanks at the Blount Shellfish Hatchery 2 days after fertilization and fed live microalgae, a mix of *Tisochrysis lutea* (CCMP1324; formerly *Isochrysis* sp., Tahitian strain) and *Pavlova lutheri* (CCMP1325), daily. Larvae were distributed into 60 L tanks at the VIMS Shellfish hatchery, and fed *Pavlova* sp. days 1 - 4 and a mix of *Pavlova* sp. and *Chaetocerus gracilis* from day 5 on.

Bacterial isolates

The candidate probiotic strain *B. pumilus* RI06-95 was isolated from a marine sponge from Narrow River in Rhode Island by the Natural Product Laboratory at the University of Rhode Island (Teasdale et al. 2009). *Vibrio coralliilyticus* RE22 (Estes et al. 2004) was supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University (USA). All bacteria were cultured on yeast peptone with 3% NaCl (YP30) media (5 g L⁻¹ of peptone, 1 g L⁻¹ of yeast extract, 30 g L⁻¹ of ocean salt

(Red Sea Salt, Ohio, USA)) at 28 °C with shaking and stored in 50% glycerol stocks at -80 °C until use.

Formulation of *B. pumilus* RI06-95

1. Lyophilized Product Formulation

1.1. Formulation for *in vivo* small-scale challenge test and viability test

B. pumilus RI06-95 was incubated in 2.25% NaCl (YP22.5) broth (yeast extract 1 g/L, peptone 5 g/L, 22.5 g/L ocean salt, Instant Ocean) at 25 °C and 175 rpm. An initial 10 mL culture was incubated for 2 d, then transferred to 1 L of YP22.5 and incubated for 4 d. The culture was partitioned into 50 mL sterile centrifuge tubes and the tubes were then centrifuged for 10 min at $2,350 \times g$. After discarding the liquid supernatant, 25 mL of Sugar Salt Solution (SSS) (2.5 g/L Instant Ocean, 200 mM sucrose, filtered deionized (DI) water (prefiltered through a 0.2 μ m filter)) was added to each of the tubes, and the cell pellet was re-suspended using a vortex. The re-suspended cells were frozen at -20 °C for 12 h, and then lyophilized for 48 h with a Labconco FreeZone 4.5 lyophilizer (Kansas City, MO, USA). The tubes were then stored at 4 °C until use. Lyophilized formulation products containing 50, 100, and 200 mM of either sucrose (Sigma-Aldrich) or trehalose (Sigma-Aldrich) were prepared to determine the most suitable type and concentration of cryoprotectants.

1.2. Formulation for Pilot-scale hatchery trial and stability test

The formulation procedure in 1.1 was followed, except for the following changes: the culture was partitioned into 15 mL sterile centrifuge tubes, 10 mL each

and each tube received 5 mL of SSS (100 mM sucrose) after centrifuging and media removal. Individual tubes were intended for single-use for a 100 L larval tank to reach the appropriate concentration of CFU/mL in the tank.

2. Granulated Product Formulation

2.1. Formulation for *in vivo* small-scale and viability test

B. pumilus RI06-95 was grown and centrifuged according to section 1.1. After centrifuging, the cell pellets were transferred into a sterile petri dish (100 × 15 mm) using a sterile spatula. 25 mL of the previously decanted media was then placed in the centrifuge tubes and centrifuged for 20 min at $2,350 \times g$ to ensure that all cells were recovered from the remaining media and tube surface. All media except for a remaining ca. 0.5 mL was then decanted, and any remaining cells and media were transferred to the petri dish surface. A total of four tubes were emptied onto one petri dish. The dishes were swirled to ensure that the surfaces were completely covered in cells. The dishes were then covered with single ply, light duty paper (Kimwipes) and then placed in a convection oven at 30 °C with constant airflow. After 24 h, the dry cell mass was extruded through three particle size (40s, 80s, and 325s) USA standard sieve stainless steel screen (Cole Palmer, Illinois, USA), yielding a product with an average size particle size of 43, 177, and 420 µm. The resulting granulated product was transferred into sterile glass vials and stored at 4 °C.

2.2. Formulation for Pilot-scale hatchery trial

The granulated formulations were scaled up for pilot-scale testing following

the same formulation procedure as stated in section 2.1, except for the following changes: bacterial cultures were centrifuged at $9,300 \times g$ for 10 min, the cell pellet was twice re-suspended in filtered sterile seawater (FSSW, 2.8 %) to wash away small molecule impurities, waste and unspent media, and then re-pelleted, and the final cell pellet was dried at room temperature (22 ± 3 °C) for approximately 2 days.

Viability and stability testing of formulated products

Bacterial viability and stability were measured by counting colony forming units (CFU) using standard agar plate methods. An aliquot of the culture, directly before centrifugation, was taken to determine the pre-formulation cell concentration in CFU/mL. The lyophilized product was re-suspended in 50 mL FSSW, the original volume of the cell pellet, and the granulated product was prepared at a 5 mg/mL concentration in FSSW, allowed to dissolve for 10 min and then vortexed for 1 min. Serial dilutions of the stock in FSSW were prepared and were spread onto YP22.5 agar plates in triplicate. The YP plates were incubated for 24 - 48 h at 27 - 28 °C and then colonies were counted. The percent cell viability in the formulations was calculated as follows:

$$\% \text{ Viability} = [(sample \text{ CFU/mL}) / (pre\text{-}formulation \text{ CFU/mL})] \times 100\%$$

The lyophilized formulation was stored at 4 °C, while samples of the granulated formulation were stored at either room temperature (RT) or 4 °C. The stability of the formulated probiotics was measured immediately after formulation (t = 0) and 1, 2, 5 and 8 weeks after formulation with serial sampling. Each assay was performed in triplicate.

In vivo small-scale challenge test

Experimental challenges were performed according to a previously established protocol (Karim et al. 2013). Treatments included: control with no probiotics pretreatment and no pathogen challenge; control with probiotics pretreatment; pathogen-challenged without probiotics pretreatment; and pathogen-challenged with probiotics pretreatment. In order to investigate the influence of sucrose (lyophilization cryoprotectant, present in the lyophilized formulation) on larval survival, a control containing only 100 mM sucrose was also assessed. The assay using the granulated formulation product was run once with each treatment tested in triplicate.

Larval oysters were placed in six well plates with 5 ml of FSSW. Formulated or fresh probiotics were added to the larvae at a concentration of 10^4 CFU/ml and incubated at room temperature with gentle shaking. After 24 h, the larvae were placed onto a 42 μ m nylon mesh and washed gently using FSSW. Larvae were placed back into the original wells using 5 ml of FSSW and *V. coralliilyticus* RE22 was added to each well with the exception of the non-challenged controls at a final concentration of 10^5 CFU/ml. Larval survival was quantified 24 h after adding pathogen, using the neutral red technique (Gómez-León et al. 2008). Survival was calculated by using the formula:

$$\text{Survival (\%)} = 100 \times (\text{number of live larvae} / \text{total number of larvae}).$$

The relative percent survival of probiotics pretreatment compared to the challenged control was calculated using the formula:

$$\text{Relative Percent Survival (\%)} = [1 - (\% \text{ survival challenged control treatment} / \% \text{ survival challenged treatment})] \times 100.$$

Pilot-scale hatchery trial

Larval culture tanks (120 L or 60 L) were stocked with larvae on day 2 after fertilization and were randomly assigned to a treatment. The granulated (Trial I) or the lyophilized (Trial II, III, and IV) probiotics were mixed with seawater vigorously by shaking, and then added to the water in the assigned tank at the time of feeding. Trial I (granulated formulation) included the following treatments: control with no probiotic (4 tanks), and a granulated formulation (3 tanks). Trial II and III (lyophilized formulation) included: a control with no probiotics, a control with sucrose and no probiotic, unformulated RI06-95 cells, and a lyophilized formulation (3 tanks each). Trial IV (lyophilized formulation) included: a control with no probiotics, and a lyophilized formulation (6 tanks each). Treatments were applied daily from day 2 after fertilization until day 10 (Trial I), 12 (Trial II and III), or 14 (Trial IV) after fertilization (Table 4-1). Culture tanks were drained every other day. For each tank, the effect of treatment on larval growth and survival was determined at selected time points for all trials. In addition, levels of total *Vibrios* on larvae, water, and tank surfaces, and survival to bacterial challenge were determined in Trial I, II, and III, as previously described on Chapter 2 of this dissertation (Sohn et al., submitted).

1. Effect of probiotic treatment in the hatchery on larval growth and survival

Larval growth and survival in each tank were monitored at selected time points during the pilot-scale hatchery trial following the methods of Sohn et al. (submitted, Chapter of this dissertation). When water was changed at the hatchery, oyster larvae were passed through different sized mesh screens (35, 55, 75, and/or 105 μ m for Trial

I, II, III; 35, 48, or 63 μm for Trial IV) depending on the age and size of the larvae. Oyster larvae retained in each of the screens were collected in a container, seawater was adjusted to a fixed volume (1 – 5 L depending on the amount of larvae), and aliquot samples (1 mL each) were placed in Sedgewick Rafter counting chambers (Graticules ® S50). Larvae were fixed with Lugol's iodine (Trial I, II, III) or temporarily immobilized with a 2:1 mixture of freshwater: 70% isopropyl alcohol (Trial IV). Larvae were counted under a microscope and the presence of live and dead larvae were recorded. After counting, 50 larvae from each tank (25 from top screen, 25 from bottom screen) on Trial I and 25 larvae from each tank on Trial II and III were randomly selected from the slides and photographed with an Olympus BX51 microscope (Olympus) and measured using an Olympus DP25 camera and CellSens Standard 1.6 image software (Olympus). On Trial IV, 5 larvae from each tank were randomly selected and measured on a Nikon E200 microscope. A random sample from each culture was photographed using a Nikon DS-Fi2 camera and DS-L3 camera control unit. Interval survival rate was determined by dividing the number of live larvae at each time point by the number of live larvae returned to the tank on the previous time point.

*2. Effect of probiotic treatment in the hatchery on levels of total *Vibrio* spp.*

Total number of *Vibrio* spp. was evaluated using a plating count method on Thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco)(Sohn et al., submitted, Chapter 2 of this dissertation). The samples were collected from water in the rearing tank, tank surfaces, and oysters when the tanks were drained. Briefly, 10 ml of water

samples were taken from each tank. Ten-fold serial dilutions of each water samples were prepared in triplicate, and then triplicate 10 μ L of each dilution were plated on TCBS agar plate. Swab samples of tank surfaces were collected from three different sections of each tank and each section is approximately 48 cm in length. Each cotton swab was placed into sterile falcon tubes containing 1 ml of FSSW and then mixed vigorously. Serial dilutions of each swab samples were also prepared and plated on TCBS agar plate. Approximately 1,000 oyster larvae were collected from the drain-down by passing through the sieves. Oyster larvae were rinsed with FSSW, and then homogenized using a sterile pestle and suspended in FSSW. After a ten-fold serial dilution, 10 μ L samples of each of the dilutions were spotted evenly onto TCBS agar plates in triplicate. The inoculated plates were incubated for 16 - 20 h at 28 °C and the colony forming units (CFU) were calculated. Results are expressed as CFU/ml, where 1 mL corresponds to 1 mL corresponds to 1 mL of water in the tank, 1 mL of swab suspension, or 1 mL of water contacting about 1,000 larvae.

3. Effect of probiotic treatment in the hatchery on larval survival after bacterial challenge

An aliquot of larvae from each tank collected at selected drain-down events were transported to the laboratory at URI. Oysters (about 40 – 50 larvae) were placed in six well plates and then challenged with *V. coralliilyticus* RE22 at a final concentration of 10^5 CFU/ml. Survival of oyster larvae was monitored at 24 h after challenge by staining with neutral red. Percent survival and the relative percent survival were calculated as described above. Oyster larvae from Trial IV could not

examined since very low number of oyster larvae was left in the probiotic treated groups at the hatchery.

Statistical Analysis

Statistical analysis of survival data was carried out with Graphpad Prism, version 6.0 (Graphpad Software, Inc.). Larval oyster survival data were subjected to arcsine square root transformation prior to statistical analysis. One-way analysis of variance (ANOVA) was used to determine significance between treatments within each time point. Two-way ANOVA was also used to determine significance between groups with time and treatment as factors. The Tukey's or Sidak's multiple comparison tests were used for post-hoc pairwise comparisons. A p-value < 0.05 was considered to be statistically significant.

Formulation cell viability data were analyzed by two-way ANOVA followed by Tukey's Test for each temperature and each time point. All statistical analyses were performed using Graphpad Prism, version 6.0 (Graphpad Software, Inc.). Differences were considered to be significant at values of $p < 0.05$.

RESULTS

Viability and stability of formulated products

Probiotic cultures before formulation showed an average cell count of 3.99×10^8 CFU/mL. Lyophilization of RI06-95 with trehalose (50, 100, and 200 mM) and sucrose (50, 100, and 200 mM) led to a significant reduction in cell numbers compared to counts prior to formulation (data not shown). The smallest reduction in

cell number due to the process of lyophilization was observed when cells were lyophilized in the presence of 100 mM sucrose (from 3.99×10^8 CFU/mL to 2.89×10^8 CFU/mL, a $25 \pm 16\%$ reduction), so this product was selected for further testing. The average viability of the product lyophilized in 100 mM sucrose remained above 10^8 CFU/mL for up to 8 weeks after formulation and storage at 4 °C (Figure 4-1)

RI06-95 cultures (1 L) grown for four days provided a total of 155.1 mg of 43 µm granules. The process of formulation by drying and granulation led to a loss in cell counts (from 3.99×10^8 CFU/mg to 1.27×10^8 CFU/mg, a $60 \pm 16\%$ reduction) (Figure 4-1). The average viability of the granulated products significantly decreased to around 10^5 - 10^6 CFU/mg within 1 week of storage at either 4 or 27 °C ($p < 0.05$), remaining stable during the rest of the 8 week storage period (Figure 4-1).

***In vivo* small-scale challenge test**

Both the lyophilized and the granulated formulation were tested for probiotic activity using *in vivo* oyster larval bacterial challenge assays. Cell viabilities of the lyophilized and granulated formulations were found to be an average of 2.61×10^8 CFU/mL and 8.9×10^7 CFU/mg at the time of the challenge experiments, respectively, and each was added to treated oysters at 10^4 CFU/mL.

The granulated probiotic had no significant effect on larval oyster survival compared to non-treated controls or controls pre-treated with the unformulated probiotic (One-way ANOVA; $p > 0.05$; Figure 4-2 A). Oyster larvae pretreated with the granulated RI06-95 and then exposed to *V. coralliilyticus* RE22 showed significantly greater survival rates ($83 \pm 3 \%$) than larvae exposed to the pathogen

alone (25 ± 5 %). Relative percent survival (RPS) provided by the granulated formulation was 69 ± 1 % (Table 4-2).

Pretreatment of oyster larvae with sucrose (100 mM) or the lyophilized formulation led to a significant decrease in larval survival in one (One-way ANOVA; $p < 0.05$; Figure 4-2 B, lyophilized group I in Table 4-2) out of 5 individual experiments, while in the other 3 experiments it did not affect larval survival (One-way ANOVA; $p > 0.05$; a representative experiment of these 3 is shown in Figure 4-2 C, lyophilized group II in table 4-2) and in another one it provided some protection (One-way ANOVA; $p < 0.05$; Figure 4-2 D, lyophilized group III in table 4-2). In the single experiment in which the sucrose control led to a significant decrease in larval survival, survival of larvae pretreated with the lyophilized probiotic was similar to the survival of larvae treated with sucrose ($p > 0.05$) and significantly lower than that of control larvae ($p < 0.05$, Figure 4-2 B). As a result of this variability in outcomes, larvae pretreated with the lyophilized probiotic provided a wide range of RPS between -93 ± 86 % to 74 ± 1 % compared to the non-probiotic challenged larvae (Table 4-2). Probiotic treatments with unformulated RI06-95 also had a variable effect on larval survival to bacterial challenge, but it always provided some level of protection (RPS between 22 and 56 %, Table 4-2).

Pilot-scale hatchery trials

Effect of daily treatment with probiotics in the hatchery on larval growth and survival

At the beginning of each trial, the size and number of larval oysters was similar in all tanks. Larval oysters on the control groups showed steady growth during the

hatchery trials, and no unusual decreases in larval survival were seen. At the time the experiments were terminated on day 10 on Trial I, day 12 on Trial II and III, and day 14 on Trial IV, larval size for the control groups averaged 116 ± 17 , 143 ± 21 , 197 ± 36 , and 227 ± 8 μm , respectively (Figure 4-3). In Trials I and III, larval oysters displayed similar and stable rates of growth on all treatment over the pilot-scale trial period and there were no significant differences between control groups and the groups treated with each probiotic (One-way ANOVA; $p > 0.05$; Figure 4-3 A, C). In comparison to the control group, the average size of oyster larvae in the group treated with fresh RI06-95 culture on Trial II was significantly bigger at day 12 (179 ± 34 μm) (Figure 4-3 B). The average size of oyster larvae in the group treated with lyophilized RI06-95 product on Trial IV was significantly smaller compared to control group from day 7 (Figure 4-3 D).

In Trials II and III, there were no significant differences on survival of oyster larvae among treatment at each time point (One-way ANOVA; $p > 0.05$; Figure 4-4 B, C). However, application of granulated *B. pumilus* RI06-95 on Trial I resulted in a significant decrease on survival of oyster on day 7 (Figure 4-4 A). The application of lyophilized *B. pumilus* RI06-95 on Trial IV also resulted in a significant decrease on survival on day 14 (Figure 4-4 D). The remainder of the larvae not used in the experiments reached the pediveliger stage and settled within 15 – 17 days after fertilization.

Effect of daily treatment with probiotics in the hatchery on levels of total Vibrio spp.

In general, daily treatment of tanks with either the granulated or the lyophilized *B. pumilus* RI06-95 did not lead to a significant decrease in the levels of total *Vibrio* spp. in water, tank surfaces, or oyster larvae as compared to control groups at each of the time points (Figure 4-5 and Figure 4-6). High levels of variability were observed between tanks and trials within treatments. For instance, the levels of the *Vibrios* in tank surface ranged from 0 - 2.18×10^4 CFU/ml between the lyophilized *B. pumilus* RI06-95 treated tanks in Trial II, while it ranged from 5.33×10^2 – 1.60×10^4 CFU/ml between the lyophilized *B. pumilus* RI06-95 treated tanks in Trial III.

In trials II and III, total *Vibrio* spp. in water and larval oysters ranged between 10^3 and 10^4 CFU/ml, but total *Vibrio* spp. in swab reached up to 10^6 CFU/ml, (Figure 4-5 and Figure 4-6, respectively). Interestingly, levels of *Vibrios* in the water were lower than 10^3 CFU/ml in Trial I (Figure 4-5 A) and no viable yellow and/or green colonies were detected on TCBS when inoculated with samples from the tank swab samples (not shown). Trial I was performed in January, a month in which lower levels of *Vibrios* are present in coastal waters in the region (and therefore in water being pumped into the hatchery) (Duan & Su 2005, Parveen et al. 2008). Levels of *Vibrios* in tanks, surfaces, and larvae were not measured during Trial IV.

Two-way ANOVA analysis of levels of *Vibrios* in water indicated there were significant time ($p < 0.05$) effects but no treatment and/or time $\times$ treatment ($p > 0.05$) effects for Trial I and Trial III, whereas there were significant time ($p < 0.05$) and treatment ($p < 0.05$) effects, but no time $\times$ treatment ($p > 0.05$) effects for Trial II (Appendix C). In Trials I and III, there was a significant decrease of *Vibrio* spp. levels with time (Figure 4-5 A, C). In Trial II, tanks treated with the fresh probiotic showed

significantly lower levels of *Vibrio* spp. than tanks treated with the lyophilized RI (day 8 and 11) and the sucrose vehicle (day 8) (Figure 4-5 B).

Two-way ANOVA analysis of levels of *Vibrio* spp. in tank surfaces indicate that there were significant time ($p < 0.05$) and treatment ($p < 0.05$) effects, but no time $\times$ treatment ($p > 0.05$) effects for Trial II, whereas there were significant time ($p < 0.05$) effects but no treatment and/or time $\times$ treatment ($p > 0.05$) effects for Trial III (Appendix C). In trial II, tanks treated with the non-formulated RI showed significantly lower levels of vibrios compared to tanks treated with the lyophilized RI and the sucrose vehicle on day 5 (Figure 4-5 E).

Two-way ANOVA analysis of levels of *Vibrio* spp. in oyster larvae indicate that there were no significant time ($p > 0.05$), treatment ($p > 0.05$), and time $\times$ treatment ($p > 0.05$) effects for Trial I. While there were significant time significant time ($p < 0.05$) and treatment ($p < 0.05$) effects but no time $\times$ treatment ($p > 0.05$) effects for Trial II, there were significant time significant time ($p < 0.05$) effects but no treatment and/or time $\times$ treatment ($p > 0.05$) effects for Trial III (Appendix C). In Trial II, levels of vibrios in oyster larvae decreased through time. Moreover, tanks treated with the non-formulated RI showed significantly lower levels of vibrios than other tanks on day 5 (Figure 4-6 B). However, RI treatment did not lead to a significant decrease in vibrio levels in larvae on Trials I or III (Figure 4-6 A C).

Effect of daily treatment with probiotics in the hatchery on larval survival to bacterial challenge

Larval oysters treated with the granulated RI06-95 at the hatchery for 7 d showed significantly lower survival than larvae from control tanks after 48 h of incubation in the laboratory (Figure 4-7 B). No significant differences in larval survival after the 48 h incubation in the laboratory were detected between larvae from the different hatchery treatments for any of the other trials or time points. Larvae exposed to the granulated or lyophilized probiotics in the hatchery did not show significantly higher survival to a 24 h bacterial (RE22) challenge as compared to non-treated challenged larvae (One-way ANOVA; $p > 0.05$; Figure 4-7). On the other hand, a fresh culture of RI06-95 offered some protection on day 12 in Trial II (One-way ANOVA; $p < 0.05$; Figure 4-7 D). Relative percent survival (RPS) provided by the fresh culture of RI06-95 in this trial was $36 \pm 6 \%$ on day 12 (Table 4-3).

DISCUSSION

In order to develop a feasible commercial approach for using probiotics in shellfish hatcheries, this study identified two formulation methods, which may be used to prepare the probiotic *B. pumilus* strain RI06-95 for transportation, storage, and use in a shellfish hatchery. This study demonstrated that: 1) the granulation process caused a loss in viability right after formulation and one week after storage at both 4 and 27 °C; 2) formulated probiotics increased oyster larvae survival against bacterial infection in some of our laboratory challenge experiments, but the results were not consistent; 3) daily treatment of larvae in hatcheries with the 2 formulated probiotics had detrimental effects on larval survival and growth in some of our experiments and did not lead to beneficial impacts on total levels of *Vibrio* spp. in water, tank surfaces, and

oyster larvae nor provided the protective effects on oyster larvae against bacterial infection previously seen with the unformulated (freshly cultured) probiotic. Therefore, our efforts to provide a formulation method for RI06-95 were unsuccessful. A traditional approach for producing formulating microorganisms is air-convective drying, which is a cost-effective process for the dehydration of microorganisms (Fu & Chen 2011, Guergoletto et al. 2012). Granulation after an air-convective drying is necessary to prevent segregation of the constituents of the powder and to provide regular amount of probiotic product in particulate form. The loss of viability of probiotic bacteria during granulation is associated with granulation operating conditions such as temperature, mechanical and moisture stress, and the characteristics of the selected microorganisms (Hiolle et al. 2010). Drying processes are a major cause of loss of viability of probiotics, making the cell membrane more susceptible to damage. While the mechanisms of cell inactivation have not been fully elucidated, the dehydration of bacterial cells may pose serious physiological challenges to the survival of cells, such as conformational and chemical changes in structural proteins and membrane lipids (Ananta 2005, Santivarangkna et al. 2008, Ohtake & Wang 2011). Storage conditions such as temperature and humidity can also affect the stability of the granulated probiotic product after granulation (Ananta 2005). Mortality of probiotic bacteria during storage is associated with various stress factors such as temperature, oxygen/air, light, moisture/humidity, and package material, a combination of which tends to damage or destroy cells (Wang et al. 2004, Ananta 2005, Chávez & Ledebor 2007). Our results, however, suggest that, beyond an initial decrease in viability, the granulated product of RI06-95 could be stored at either 4 °C

or room temperature by showing relatively stable viability up to for 8 weeks. The stability of the granulated RI06-95 during storage may be due to the adaptation of *Bacillus* spp. to extreme environmental stress during storage due to their spore-forming characteristics (Desmond et al. 2002, Driks 2002, Hong et al. 2005, Cutting 2011)

In contrast to granulation, our results showed that lyophilization of RI06-95 did not significantly impact cell viability after the formulation process. Lyophilization has been investigated as a way of preserving and formulating *Bacillus* spp. as probiotic products (Henn et al. 2015). In order to ensure sufficient viability after freeze-drying, a disaccharide cryoprotectant such as sucrose or trehalose is typically added to provide structural support to cell membranes and proteins (Leslie et al. 1995). Our results indicate that 100 mM sucrose provided the highest level of stability over time, and was considered the best cryoprotectant candidate (as compared to trehalose) after the stability essays.

Experiments using our short term exposure (24 h) laboratory challenge model showed inconsistent results among individual experiments regarding probiotic activity of the granulated and lyophilized formulation of RI06-95, while treatments using the unformulated RI06-95 provided similar levels of protection in these experiments to what was previously seen (Karim et al., 2013, Sohn et al. submitted, Chapter 2 of this dissertation). Results from the hatchery trials, in which larvae were exposed to probiotics daily for at least 6 days, showed that the probiotic activity (as determined by survival of larvae to bacterial challenge and effect of treatment on levels of *Vibrios* in water and tank surfaces) of RI06-95 was eliminated by the formulation. Moreover,

exposure of larvae to the lyophilized formulation or the vehicle (small concentrations of sucrose) had a negative impact on larval growth and survival. These results suggest that lyophilized formulations of probiotics may not be adequate for use in bivalve aquaculture. Based on the variable results obtained with the granulated formulation, more research needs to be done to determine if the efficacy of these granulated formulations can be improved.

As seen in previous hatchery experiments (Sohn et al., submitted, Chapter 2), high levels of variability were seen between tanks and trials within a treatment. As discussed in the previous chapter, the variation in results within and/or between experiments in this study could be due to several factors: (1) a different quality and health status of larvae from each spawning; (2) the impact of various environmental and biological factors such as salinity, pH, temperature/season at the hatchery; (3) variability in the characteristics of different rearing systems, such as tank, source or treatment of water, and location of hatchery (Balcazar et al., 2006; Gatesoupe, 1999; Martínez Cruz et al., 2012; Utting and Millican, 1997); and 4) the effect of variability in the composition of microbial communities and how these communities may interact with the probiotic.

In conclusion, further research is needed to develop formulations for oyster hatcheries. The identification of protective agents that enhance probiotic cellular survival during storage, preserve probiotic activity, and have no negative impacts on larval survival is a key challenge. The adequate administration method of the formulated probiotics also remains to be determined.

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LITERATURE CITED

- Ananta E (2005) Impact of environmental factors on viability and stability and high pressure pretreatment on stress tolerance of *Lactobacillus rhamnosus* GG (ATCC 53103) during spray drying. Dr. Ing. Thesis, Prozesswissenschaften der Technischen Universität, Berlin
- Austin B, Stuckey LF, Robertson PAW, Effendi I, Griffith DRW (1995) A probiotic strain of *Vibrio alginolyticus* effective in reducing diseases caused by *Aeromonas salmonicida*, *Vibrio anguillarum* and *Vibrio ordalii*. *J Fish Dis* 18:93–96
- Azevedo RV de, Tavares Brag LG (2012) Use of Probiotics in Aquaculture. In: Rigobelo E (ed) *Probiotic in Animals*. InTech, DOI: 10.5772/50056
- Balcazar J, Blas I, Ruizzarzuela I, Cunningham D, Vendrell D, Muzquiz J (2006) The role of probiotics in aquaculture. *Vet Microbiol* 114:173–186
- Berthe F (2004) Report about mollusc diseases. *Mediterr Aquac Diagn Lab* 49:33–48
- Chávez BE, Ledebor AM (2007) Drying of Probiotics: Optimization of Formulation and Process to Enhance Storage Survival. *Dry Technol* 25:1193–1201
- Cutting SM (2011) *Bacillus* probiotics. *Food Microbiol* 28:214–220
- Dagá P, Feijoo G, Moreira MT, Costas D, Villanueva AG, Lema JM (2013) Bioencapsulated probiotics increased survival, growth and improved gut flora of turbot (*Psetta maxima*) larvae. *Aquac Int* 21:337–345
- Decamp O, Moriarty D (2006) Probiotics as alternative to antimicrobials: limitations and potential. *World Aquac* 37(4):60-62
- Desmond C, Stanton C, Fitzgerald GF, Collins K, Paul Ross R (2002) Environmental adaptation of probiotic lactobacilli towards improvement of performance during spray drying. *Int Dairy J* 12:183–190
- Driks A (2002) Overview: development in bacteria: spore formation in *Bacillus subtilis*. *Cell Mol Life Sci CMLS* 59:389–391
- Duan J, Su Y-C (2005) Occurrence of *Vibrio parahaemolyticus* in Two Oregon Oyster-growing Bays. *J Food Sci* 70:M58–M63
- Edna M, Roets Y, Gardiner N, Lalloo R (2014) The Use and Benefits of *Bacillus* Based Biological Agents in Aquaculture. In: Hernandez-Vergara M (ed) *Sustainable Aquaculture Techniques*. InTech, Murcia, Spain

- Elston R (1998) Shellfish Diseases and Their Management in Commercial Recirculating Systems. In: Proceedings of The Second International Conference on Recirculating Aquaculture, Roanoke, Virginia.
- Estes R, Friedman C, Elston R, Herwig R (2004) Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. *Dis Aquat Organ* 58:223–230
- Fu N, Chen XD (2011) Towards a maximal cell survival in convective thermal drying processes. *Food Res Int* 44:1127–1149
- Gatesoupe F. (1999) The use of probiotics in aquaculture. *Aquaculture* 180:147–165
- Gomez-Leon J, Villamil L, Lemos ML, Novoa B, Figueras A (2005) Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from Aquacultured Carpet Shell Clam (*Ruditapes decussatus*) Larvae Associated with Mass Mortalities. *Appl Environ Microbiol* 71:98–104
- Gómez-León J, Villamil L, Salger S, Sallum R, Remacha-Triviño A, Leavitt D, Gómez-Chiarri M (2008) Survival of eastern oysters *Crassostrea virginica* from three lines following experimental challenge with bacterial pathogens. *Dis Aquat Organ* 79:95–105
- Guergoletto KB, Tsuruda AY, Hirooka EY, Martins EP, Souza JCB de, Sivieri K, Roig SM, Garcia S (2012) Dried Probiotics for Use in Functional Food Applications, Food Industrial Processes - Methods and Equipment, Dr. Benjamin Valdez (Ed.), ISBN: 978-953-307-905-9, InTech, Available from: <http://www.intechopen.com/books/food-industrial-processes-methods-and-equipment/dried-probiotics-for-use-in-functional-food-applications>
- Helm MM, Bourne N, Lovatelli A (2004) Hatchery culture of bivalves: a practical manual. Food and agriculture organization of the United Nations, Rome
- Henn MR, Von Maltzahn G, D'onofrio AM, Litcofsky KD, Berry DA, Cook DN, Afeyan NB, Aunins JG (2015) Synergistic bacterial compositions and methods of production and use thereof. U.S. Patent Application No. 14/592,481
- Hiolle A, Sampsonis C, Auclair E, Lenoir C (2010) Stable probiotic granulate, and method for preparing same. U.S. Patent Application No. 13/255,110
- Hong HA, Duc LH, Cutting SM (2005) The use of bacterial spore formers as probiotics: Table 1. *FEMS Microbiol Rev* 29:813–835
- Karim M, Zhao W, Rowley D, Nelson D, Gomez-Chiarri M (2013) Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. *J Shellfish Res* 32:401–408

- Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2009) Two pathogens of GreenshellTM mussel larvae, *Perna canaliculus*: *Vibrio splendidus* and a *V. coralliilyticus/neptunius* -like isolate. J Fish Dis 32:499–507
- Lalloo R, Maharajh D, Görgens J, Gardiner N (2010) A downstream process for production of a viable and stable *Bacillus cereus* aquaculture biological agent. Appl Microbiol Biotechnol 86:499–508
- Leslie SB, Israeli E, Lighthart B, Crowe JH, Crowe LM (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. Appl Environ Microbiol 61:3592–3597
- Li J, Xu Y, Jin L, Li X (2014) Effects of a probiotic mixture (*Bacillus subtilis* YB-1 and *Bacillus cereus* YB-2) on disease resistance and non-specific immunity of sea cucumber, *Apostichopus japonicus* (Selenka). Aquaculture Research 46(12), 3008-3019
- Luis-Villaseñor IE, Macías-Rodríguez ME, Gómez-Gil B, Ascencio-Valle F, Campa-Córdova ÁI (2011) Beneficial effects of four *Bacillus* strains on the larval cultivation of *Litopenaeus vannamei*. Aquaculture 321:136–144
- Martínez Cruz P, Ibáñez AL, Monroy Hermosillo OA, Ramírez Saad HC (2012) Use of Probiotics in Aquaculture. ISRN Microbiol 2012:1–13
- Ohtake S, Wang YJ (2011) Trehalose: Current use and future applications. J Pharm Sci 100:2020–2053
- Parveen S, Hettiarachchi KA, Bowers JC, Jones JL, Tamplin ML, McKay R, Beatty W, Brohawn K, DaSilva LV, DePaola A (2008) Seasonal distribution of total and pathogenic *Vibrio parahaemolyticus* in Chesapeake Bay oysters and waters. Int J Food Microbiol 128:354–361
- Prado S, Romalde JL, Barja JL (2010) Review of probiotics for use in bivalve hatcheries. Vet Microbiol 145:187–197
- Queiroz JF, Boyd CE (1998) Effects of a Bacterial Inoculum in Channel Catfish Ponds. J World Aquac Soc 29:67–73
- Salinas I, Díaz-Rosales P, Cuesta A, Meseguer J, Chabrillón M, Morínigo MÁ, Esteban MÁ (2006) Effect of heat-inactivated fish and non-fish derived probiotics on the innate immune parameters of a teleost fish (*Sparus aurata* L.). Vet Immunol Immunopathol 111:279–286
- Santivarangkna C, Higl B, Foerst P (2008) Protection mechanisms of sugars during different stages of preparation process of dried lactic acid starter cultures. Food Microbiol 25:429–441

- Savini M, Cecchini C, Verdenelli MC, Silvi S, Orpianesi C, Cresci A (2010) Pilot-scale Production and Viability Analysis of Freeze-Dried Probiotic Bacteria Using Different Protective Agents. *Nutrients* 2:330–339
- Schisler D, Slininger P, Behle R, Jackson M (2004) Formulation of *Bacillus* spp. for biological control of plant diseases. *Phytopathology* 94:1267–1271
- Socha AM (2008) Chemistry of Antibiotics from Atlantic Actinomycete and *Bacillus* bacteria. Doctoral dissertation, University of Rhode Island
- Sorokulova I (2013) Modern Status and Perspectives of *Bacillus* Bacteria as Probiotics. *J Probiotics Health* 1:1-5
- Teasdale ME, Liu J, Wallace J, Akhlaghi F, Rowley DC (2009) Secondary Metabolites Produced by the Marine Bacterium *Halobacillus salinus* That Inhibit Quorum Sensing-Controlled Phenotypes in Gram-Negative Bacteria. *Appl Environ Microbiol* 75:567–572
- Tubiash HS, Chanley PE, Leifson E (1965) Bacillary necrosis, a disease of larval and juvenile bivalve mollusks I. Etiology and epizootiology. *J Bacteriol* 90:1036–1044
- Utting SD, Millican PF (1997) Techniques for the hatchery conditioning of bivalve broodstocks and the subsequent effect on egg quality and larval viability. *Aquaculture* 155:45–54
- Vaseeharan B, Ramasamy P (2003) Control of pathogenic *Vibrio* spp. by *Bacillus subtilis* BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett Appl Microbiol* 36:83–87
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic Bacteria as Biological Control Agents in Aquaculture. *Microbiol Mol Biol Rev* 64:655–671
- Wang Y-C, Yu R-C, Chou C-C (2004) Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. *Int J Food Microbiol* 93:209–217
- World Health Organization (2006) Probiotics in food: health and nutritional properties and guidelines for evaluation. *FAO Food Nutr. Pap.* 85, Food and Agriculture Organization of the United Nations and World Health Organization, Rome.

Table 4-1 Design of hatchery trials for testing the formulated probiotics.

Trial	Treatment Type	Period	Probiotic provided (days after fertilization)	Hatchery Location
Trial I	C, RI-G	01/03/14 – 01/24/14	Daily from 2 - 10	RWU
Trial II	C, ConwS, RI, RI-L	01/29/15 – 02/10/15	Daily from 2 - 12	RWU
Trial III	C, ConwS, RI, RI-L	02/22/15 – 03/06/15	Daily from 2 - 12	RWU
Trial IV	C, RI-L	06/24/15 – 07/08/15	Daily from 2 - 14	VIMS

Abbreviations: C = controls (no probiotic provided); ConwS = 100 mM sucrose (no probiotic); RI-G = granulated *Bacillus pumilus* RI06-95; RI-L = lyophilized *Bacillus pumilus* RI06-95 (in 100 mM sucrose); RI = fresh *Bacillus pumilus* RI06-95; RWU = Roger Williams University; VIMS = Virginia Institute of Marine Science.

Figure 4-1. Impact of formulation processing (granulation or lyophilization) and temperature storage on the stability of *Bacillus pumilus* RI06-95. Cell count in the reconstituted formulation after storage for up to 8 weeks was determined using a plating method. Data expressed as $\log_{10}(\text{CFU/mg}) \pm \text{SEM}$ for the granulated formulation and $\log_{10}(\text{CFU/mL}) \pm \text{SEM}$ for the lyophilized formulation.

Figure 4-2. Effect of pre-incubation of oyster larvae with *Bacillus pumilus* RI06-95 formulated products for 24 h on survival (% $\pm$ SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. (A) Exposure to a granulated product of *Bacillus pumilus* RI06-95; (B), (C), and (D) Exposure to lyophilized formulations (representative experiments). Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. Different letters indicates statistical significant differences between the treatments.

Table 4-2. Effect of pre-incubation of oyster larvae for 24 h with RI06-95 formulated products on survival (RPS, % $\pm$ SEM) after challenge with *V. coralliilyticus* RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. Data is expressed as Relative Percent Survival (RPS, % $\pm$ SEM) of challenged oysters exposed to probiotics compared to *V. coralliilyticus* RE22 challenged control. Abbreviations: RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22.

	Treatments	Relative Percent Survival (RPS, % $\pm$ SEM)	
Granulated RI06-95	RI-G + RE22	69 $\pm$ 1	(Figure 4-2 A)
Lyophilized RI06-95 I	RI + RE22	26 $\pm$ 5	(Figure 4-2 B)
	RI-L + RE22	-93 $\pm$ 86	
Lyophilized RI06-95 II	RI + RE22	22 $\pm$ 11	(Figure 4-2 C)
	RI-L + RE22	25 $\pm$ 6	
Lyophilized RI06-95 III	RI + RE22	56 $\pm$ 4	(Figure 4-2 D)
	RI-L + RE22	74 $\pm$ 1

Figure 4-3. Effect of daily treatment with different formulations of *Bacillus pumilus* RI06-95 of larval eastern oysters (*Crassostrea virginica*) in the hatchery on mean larval size ($\mu\text{m} \pm \text{SEM}$) at selected time points. (A) Trial I; (B) Trial II; (C) Trial III; and (D) Trial IV. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. * indicates statistical significances compared to controls.

Figure 4-4. Effect of daily treatment with probiotics in the hatchery on interval survival ($\% \pm \text{SEM}$) of oyster larvae between selected time points. (A) Trial I; (B) Trial II; (C) Trial III; and (D) Trial IV. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates statistical significances between treatments.

Figure 4-5. Effect of daily treatment with probiotics on total vibrio levels ($\text{Log}_{10}(\text{CFU/ml}) \pm \text{SEM}$) in water (A, B, C) and tank surfaces (D, E, F) in a hatchery. (A and D) Trial I (no bacteria were detected in tank surfaces in Trail I); (B and E) Trial II; and (C and F) Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean $\pm$ SEM, $p < 0.05$; Two-way ANOVA).

Figure 4-6. Effect of daily treatment with probiotics on total vibrio levels ($\text{Log}_{10}(\text{CFU/ml}) \pm \text{SEM}$) on oyster larvae in the hatchery. (A) Trial I; (B) Trial II; and (C) Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. An asterisk (*) indicates significant differences between treatments (mean $\pm$ SEM, $p < 0.05$; Two-way ANOVA).

Figure 4-7. Effect of daily probiotic treatment in the hatchery on larval survival to a laboratory challenge with the pathogen *Vibrio coralliilyticus* RE22. Larvae were brought to the laboratory and survival was measured 24 h after challenge with RE22.

(A) Larvae collected on Day 3 after fertilization on Trial I; (B) Day 10 on Trial I; (C) Day 5 on Trial II; D) Day 12 on Trial II; E) Day 5 on Trial III; F) Day 12 on Trial III. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh RI06-95; RE22 = *V. coralliilyticus* RE22. A different letter indicates a significant difference between treatments (One-way ANOVA; $p < 0.05$).

Table 4-3. Effect of daily exposure to formulations of *B. pumilus* RI06-95 in the hatchery on larval oyster survival (%) 24 h after challenge with *Vibrio coralliilyticus* RE22. Data is expressed as Relative Percent Survival (RPS, % $\pm$ SEM) of challenged oysters exposed to probiotics compared to *V. coralliilyticus* RE22 challenged control. Abbreviations: C = no probiotic; ConwS = 100 mM sucrose; RI-G = granulated RI06-95; RI-L = lyophilized (in 100 mM sucrose) RI06-95; RI = fresh/unformulated RI06-95; RE22 = *V. coralliilyticus* RE22

Trial	Treatments	Relative Percent Survival (RPS, % $\pm$ SEM)	
	Time	Day 3	Day 7
Trial I	RI-G+RE22	-10 $\pm$ 2	-78 $\pm$ 88
Trial II	Time	Day 5	Day 12
	RI+RE22	36 $\pm$ 9	36 $\pm$ 6
	RI-L+RE22	46 $\pm$ 3	2 $\pm$ 5
Trial III	RI+RE22	2 $\pm$ 13	16 $\pm$ 3
	RI-L+RE22	- 36 $\pm$ 28	26 $\pm$ 1

CHAPTER 5

IMPACT OF TREATMENT OF PROBIOTIC *BACILLUS PUMILUS* RI06-95 ON THE BACTERIAL COMMUNITIES IN AN OYSTER HATCHERY

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ABSTRACT

The efficacy of probiotic treatment in a bivalve hatchery may be mediated by impacts on the microbial community. The composition and diversity of the microbial community in rearing water, tank surface, and oyster larvae at an oyster hatchery was analyzed using 16S rRNA-based MiSeq sequencing in order to understand the impact of probiotic treatment. In pilot-scale hatchery trials, daily addition probiotic *Bacillus pumilus* RI06-95 did not affect the levels of total *Vibrios* in water, tank surface, and oyster larvae ($p > 0.05$), but offered some protection as late as 12 day ($p < 0.05$; RPS 36 ± 6 %). In total, 56 phyla were identified in hatchery samples, and *Proteobacteria* was the most abundant phylum in all samples comprising 53 – 85 % of OTU's. The *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria*, *Planctomycetes*, and/or *Firmicutes* were major phylum represented in hatchery samples, demonstrating that a few taxa constituted the majority of the bacterial community at this oyster hatchery. There were significant differences on the composition of the bacterial community between sample sources. While *Proteobacteria* and *Bacteroidetes* constituted in total 84 % of OTU's in water, *Proteobacteria* and *Cyanobacteria* constituted 83 % of OTU's in swabs of tank surfaces. Lastly, *Proteobacteria* mainly constituted 85 % of OTU's in oyster larvae. *Firmicutes* were detected in oyster larval samples in relatively higher proportion than in other samples, constituting 4 % of OTU's detected in larvae. No significant changes or shifts in microbial community were observed either between treatments and/or time points when probiotic *Bacillus pumilus* RI06-95 was applied daily for 12 days at the oyster hatchery. This study is a first step in understanding the role of the microbial community on the effect of a probiotic in a bivalve hatchery.

INTRODUCTION

Bacterial pathogens, mainly members of genus *Vibrio*, are responsible for significant economic losses in bivalve hatcheries (Paillard et al. 2004, Romalde & Barja 2010, Beaz-Hidalgo et al. 2010). In order to prevent disease outbreaks, a healthy environment has to be provided and maintained during larval culture at the hatcheries through conventional methods such as the maintenance of optimum water quality and culture density. The use of probiotics, which are non-pathogenic microorganisms that are beneficial to finfish and shellfish, has been proposed as an alternative tool for the management of disease in aquaculture. The use and selection of probiotic bacteria for use in the culture of larval aquatic organisms is very important due to their potential to enhance production and promote animal health (Gatesoupe 1999, Kesarcodi-Watson et al. 2008, 2012, Prado et al. 2010). Previous studies demonstrated that candidate probiotic bacteria *Phaeobacter inhibens* S4 and *Bacillus pumilus* RI06-95, could significantly increase the survival of larval oysters in an *in vivo* bacterial challenge assay (Karim et al. 2013). Based on these promising results, the effect of probiotic treatment in the hatchery was tested using larvae of oyster *Crassostrea virginica*, hard clam *Mercenaria mercenaria*, and bay scallops *Argopecten irradians*. Tanks treated with either *P. inhibens* S4 and/or *B. pumilus* RI06-95 showed dramatic reductions in total vibrio counts, especially in the seawater (Sohn et al. submitted; Chapter 2 of this dissertation). Moreover, daily treatment of oyster and bay scallop larvae in the hatchery with these probiotics led to increased survival to a bacterial challenge (Sohn et al. in preparation; Chapter 3 of this dissertation)

However, there is still a lack of knowledge about the exact modes of action involved in probiotic effects. Laboratory challenge experiments have shown that the mechanisms of probiotic action of *P. inhibens* S4 appear to be complex, involving the production of an antibiotic compound, the ability to form thick biofilms, and the ability to produce signaling molecules that inhibit the production of virulence factors by vibrios, as well as other unknown mechanisms (Karim 2012, Zhao 2014, Zhao et al. 2016). These mechanisms of action indicate that probiotic treatment may have significant impacts on the microbial communities in bivalve hatcheries.

Bivalves are filter feeders, which means they can ingest many different kinds of microorganisms by filtering to gain nourishment from the water. Changes in the microbial composition of water could have an impact on the host health (Sakowski 2015, Lokmer & Mathias Wegner 2015). For example, the microbial communities in the digestive tracts of fish are reflective of microbes that present in the surrounding environment, and can influence health and survival (Cahill 1990). Investigation of the effect of probiotics on the diversity and changes of bacterial and algal communities in shellfish hatchery is needed due to the interactive relationship of larval health with the animals' external environment. Furthermore, evaluation of the effect of probiotics on microbial communities in shellfish hatcheries could provide clues on the mechanisms of action of probiotics (Verschuere et al. 2000).

Microbial communities in hatcheries have been studied for decades using culture-based and culture-independent approaches (Sandaa et al. 2003, Schulze et al. 2006, Powell et al. 2013). With the advent of high-throughput sequencing technologies, metagenomic studies became feasible to studying microbial

communities (Simon & Daniel 2011), leading to exhaustive characterization of the Human (Turnbaugh et al. 2007) and Earth (Gilbert et al. 2010) microbiomes. These technologies have allowed for a deeper understanding of the host-microbiome interaction in health and disease and the microbial ecology. Furthermore, manipulation of microorganisms by probiotics and prebiotics has been suggested as an alternative approach to improve and maintain health (Gatesoupe 1999, Preidis & Versalovic 2009, Ringø et al. 2010, Gareau et al. 2010).

Relatively little is known, however, about the composition of microbial communities in oysters. Bacterial communities in oyster stomach and gut varied between tissues and sampling sites (King et al. 2012). Bacterial communities in the coastal environments in which oysters are cultured change in response to seasonal environmental conditions, such as temperature (Kirchman et al. 2010) or other factors including salinity, dissolved oxygen and nutrients (Hill et al. 2012). Recently, changes in bacterial communities in the water used in an oyster hatchery have been studied using tag-encoded pyrosequencing. Changes in some groups of bacterial communities were observed during the study, although there was no clear relationship between production outcomes (such as production losses due to disease outbreaks) and the overall bacterial community structure (Powell et al. 2013).

In this study, the microbial community in rearing water, tank surface, and oyster larvae at the oyster hatchery was characterized using 16S rRNA-based MiSeq sequencing (Caporaso et al. 2012) in order to understand the healthy composition or diversity of microbial community at the oyster hatchery. Changes in microbial community following introduction of candidate probiotic *Bacillus pumilus* RI06-95 in

oyster hatchery was also investigated to find out the potential influence of probiotics on hatchery microbial communities.

METHODS

Oyster larvae

Eastern oysters (*C. virginica*) were spawned at the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA) on January, 2015 (Trial II on Chapter 4 of this dissertation) and February, 2015 (Trial III on Chapter 4 of this dissertation). Larvae were distributed 2 days after fertilization and maintained in triplicate 120 L conical tanks per treatment. The microalgae strains used throughout the trials were *Tisochrysis lutea* (CCMP1324; formerly *Isochrysis* sp., Tahitian strain), *Pavlova lutheri* (CCMP1325), *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Pavlova pinguis* (CCMP609), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weissflogii* (CCMP1336).

Bacterial isolates

The *Bacillus pumilus* RI06-95 strain was previously isolated from a marine sponge from Narrow River in Rhode Island (Teasdale et al. 2009) and selected as a candidate probiotic strain by showing protection of eastern oyster larvae against bacterial challenge *Vibrio coralliilyticus* RE22 (Karim et al. 2013). *Vibrio coralliilyticus* RE22 (Estes et al. 2004) was supplied by H. Hasegawa, Department of Biomedical Sciences, Oregon State University (USA). All bacteria were cultured in yeast peptone with 3% NaCl (YP30) media (5 g L⁻¹ of peptone, 1 g L⁻¹ of yeast

extract, 30 g L⁻¹ of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 170 rpm. The OD₅₅₀ was measured to estimate bacterial cell concentration using a spectrophotometer (SynergyTM HT, BioTek, USA) and used to determine the amount needed for the probiotic treatments at the hatchery. Additionally, serial dilution and spot plating on YP30 agar plate were used to determine the colony forming units (CFU) of the bacterial suspension used in the challenge.

Collecting samples from hatchery trial

Samples for microbiome analysis were collected from two experiments described in Chapter 4 of this dissertation (Trials II and III), in which larval tanks had been treated daily with either control (no treatment), unformulated, or formulated probiotic *Bacillus pumilus* RI06-95. Briefly, larval oyster culture tanks (120 L) were stocked with larvae on day 2 after fertilization and were randomly assigned to treatments including no probiotics (control, 3 tanks) or candidate probiotic RI06-95 (formulated and unformulated, 3 tanks each). Samples from tanks treated with the formulated probiotic did not collected to investigate the microbial community at the hatchery. Probiotic RI06-95 was mixed with algal feed and applied daily in each of the treatment tanks at a final concentration of 10⁴ CFU/mL (Sohn et al. in preparation, Chapter 4 of this dissertation). Tanks were drained every other day and the day of fertilization was defined as day 0.

Samples from selected tanks (control and unformulated probiotics) and time points (5, 8, and 12 days after fertilization for Trial II and 12 days after fertilization for Trial III) were collected for microbiome analyses. Rearing water (1 – 4 L) was

collected in triplicate from each treatment during the drain-down and then filtered through a 0.22 µm Sterivex filter (Millipore, Millford, MA, USA). The Sterivex filters were immediately stored frozen at - 20 °C until required for DNA extraction. Swab samples were collected from each tank by swabbing inside of tank surface (approximately 24 cm² of tank wall) with a sterile cotton swab. Each swab was placed into tubes containing cell lysis solution (solution C1, MO BIO PowerSoil ® DNA Isolation Kit (Carlsbad, CA, USA)). Oyster larvae were collected on a 55 µm sieve at each drain-down water changes at the hatchery and 10 ml of oyster larvae from each tank (about 150 - 1500 larvae) were placed into a sterile tube and transported to the laboratory at University of Rhode Island. In the laboratory, oyster larvae were placed on a 40 µm nylon membrane and rinsed with filtered sterile seawater (FSSW) to reduce residual non-attached environmental bacteria. Samples of swab and oyster larvae were then immediately placed in liquid nitrogen for flash freezing and were stored in a - 80 °C freezer until used.

DNA extraction

Microbial DNA from water samples was extracted from the Sterivex filters using the PowerWater® Sterivex™ DNA Isolation Kit (MoBio laboratories, Carlsbad, CA, USA) according to manufacturer recommendations. In addition, microbial DNA from the swabs and oyster larvae was extracted using a MO BIO PowerSoil ® DNA Isolation Kit (MoBio laboratories, Carlsbad, CA, USA) with the following modifications. In brief, oyster larvae were ground in a mortar with sterile pestle and then placed into bead tubes for extraction. The cotton tops of swabs were

cut off directly into bead tubes. Bead tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 10 min using the MO BIO vortex adaptor. The remaining steps were performed as directed by the manufacturer. Extracted DNA samples were stored at -20 °C until further use.

PCR amplification of 16S ribosomal DNA (rDNA) and Library preparation

A two-step PCR protocol is used to prepare PCR products for sequencing according to a modification of Illumina's 16S Metagenomic Sequencing Library Preparation protocol. A first round of PCR was performed using primers contained adapter overhang sequences; forward 515F (5'-TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGGTGYCAGCMGCCGCGGTAA-3') and reverse 806R (5'-GTCTCG TGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT-3'). These primers target the V4 variable region of 16S rDNA (Caporaso et al. 2012). The first round of PCR conditions consisted of 94 °C for 3 min for initial denaturation, followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s, and elongation at 72 °C for 90 s, and a final elongation step at 72 °C for 10 min. Negative controls (no DNA) were included. PCR products were checked by gel electrophoresis for a product of 300 - 350 bp and then submitted to the Rhode Island Genomics and Sequencing Center (RIGSC) of the University of Rhode Island (RI, USA) for the index PCR and running on the Illumina MiSeq high throughput sequencer.

In brief, PCR products from the first PCR were cleaned with Ampure XP (Beckman Coulter, Pasadena, CA) and then visualized by agarose gel electrophoresis.

A second round of PCR (50 ng of template DNA, 5 cycles) was performed to attach Nextera indices and adapters using Illumina Nextera® Index Kit (Illumina, San Diego, CA). PCR products from the second PCR were cleaned with Ampure XP (Beckman Coulter, Pasadena, CA) and analyzed by agarose gel electrophoresis and using the Agilent BioAnalyzer DNA1000 chip. Quantification was performed on all samples prior to pooling using Qubit fluorometer (Invitrogen, Carlsbad, CA, USA), and the final pooled library were quantified using qPCR in a Roche LightCycler480 with the KAPA Biosystems Illumina Kit (KAPA Biosystems, Woburn, MA) prior to loading on the MiSeq flow cell. Samples were analyzed for a 250 bp paired-end sequencing on an Illumina MiSeq (Illumina, San Diego, CA)

Data analysis using QIIME

For analysis of amplicons generated from environmental gDNA, sequences were initially demultiplexed using FastQC version 0.11.4 (Andrews 2010). The Illumina reads were merged, and subsequently quality trimmed to remove low-quality reads and those less than 200 bp using Trimmomatic v 0.32 (Bolger et al. 2014). The remaining sequences were exported as FASTA files and processed with the Quantitative Insights Into Microbial Ecology (QIIME v.1.9.0) software package (Caporaso et al. 2010). Sequences were screened for chimeras using the usearch61 algorithm (Edgar et al. 2011) and putative chimeric sequences were completely removed from the dataset. Subsequently, every sample sequence was sub-sampled to the smallest sample size to avoid analytical issues associated with variable library size. Sub-sampled data were then pooled and the associated metadata was added to the

files.

The sequences were clustered into operational taxonomic units (OTU) at 97% similarity. Representative sequences from each OTU were extracted, and these sequences were classified using the “assign_taxonomy” algorithm implementing the RDP classifier, with the Greengenes reference OTU build (DeSantis et al. 2006). A biological observation matrix (BIOM) table was generated at taxonomic levels from phylum to genus using the “make_OTU_table” algorithm. The BIOMs were used to generate heatmap at the phylum level to compare the observed number of OTU’s in the dataset. Alpha and beta diversity was calculated over the rarefied samples and various diversity indices were used including chao1 and binary_jaccard, respectively. In addition, a metagenomic biomarker discovery approach was employed with LEfSe (linear discriminant analysis (LDA) coupled with effect size measurements), which performs a nonparametric Wilcoxon sum-rank test followed by LDA analysis to assess the effect size of each differentially abundant taxon (Segata et al. 2011).

Statistical Analysis

Student’s t-test was used to test a difference in the prevalence of OTU’s. The Analysis of variance (ANOVA) test was used to identify OTU’s whose abundance is different within/between treatments and time points using QIIME. A level of $P < 0.05$ was determined as statistically significant.

RESULTS

Composition of bacterial community at the oyster hatchery

In total, 56 phyla were identified in this study by the V4 region of 16S rDNA sequences. Significant differences in microbial composition were observed between sources of samples including rearing water, swab from tank surface, and eastern oyster larvae ($p < 0.05$) but not between treatments (probiotics and control) and time points (5, 8, and 12 days after fertilization for Trial II and 12 days after fertilization for Trial III; $p > 0.05$). A heat map analysis showed the relative abundance of each bacterial phylum (defined at 97 % similarity) across all samples (Figure 5-1) and the relative abundance of most abundant OTU's in each sample type as percentages illustrated on an area chart (Figure 5-2). Visual examination of these figures indicated that samples from the same sources (water, swab, or oyster) were more similar to each other than samples from different treatments or hatchery trials. It also indicated that there were no significant time effects in each sample sources ($p > 0.05$). Although only few samples from oyster egg tanks were collected, overall microbial community composition was similar in eggs and larvae from same sources (water, swab, or egg). It suggested that oyster egg and larvae share the core bacterial communities.

The proportion of bacterial groups at the phylum level was also investigated. *Proteobacteria* was the most abundant phylum in most samples including rearing water, swab from tank surface and oyster larvae (Figure 5-3). Besides *Proteobacteria*, five phyla including *Bacteroidetes*, *Cyanobacteria*, *Actinobacteria*, *Planctomycetes*, and *Firmicutes* were also present in most samples. In all rearing water samples, 53 % of OTU's were assigned to *Proteobacteria* and *Bacteroidetes* was the second major phylum with 31 % of OTU's abundance. *Cyanobacteria* and

Actinobacteria represented 6 % and 5 % of OTU's, respectively, while *Planctomycetes* represented 1 % of OTU's (Figure 5-3 A).

In swab samples from tank surface, *Cyanobacteria* was the second major phylum with 18 % of OTU's while *Proteobacteria* constituted 66 % of OTU's. Other phyla detected included *Bacteroidetes* (8 % of OTU's), as well as *Planctomycetes*, *Actinobacteria*, and *Firmicutes* (less than 2 % of OTU's each; Figure 5-3 B). Interestingly, 85 % of the OTU's in oyster samples were assigned to *Proteobacteria*, with *Bacteroidetes* and *Firmicutes* being the next most abundant, each of them constituting 4 % of OTU's (Figure 5-3 C).

Effect of probiotic treatment in the hatchery on bacterial community

Daily application of probiotics to tanks in the oyster hatchery did not result in significant changes of bacterial community compared to control, non-treated tanks ($p > 0.05$). There was a significant difference in the relative abundance of the three dominant bacterial phyla, *Proteobacteria*, *Bacteroidetes*, and *Cyanobacteria* between samples sources ($p < 0.05$) but not between treatments ($p > 0.05$; Figure 5-4).

Alpha and beta diversity

Chao1-based rarefaction curves were created to estimate the species richness in population with the total numbers of OTU's present in order to compare the species richness in the bacterial communities at the oyster hatchery. Rarefaction curves indicated that among all sample sources collected from hatchery trials, oyster larvae have lower diversity of bacterial communities than water and swab samples (Figure

5-5 A). There was no significant difference between control groups and probiotic treated group (Figure 5-5 B). Beta-diversity using binary Jaccard illustrated that there is no shifts in bacterial communities between treatments and time points, as shown by samples clustered mainly by source of collected samples from the hatchery (Figure 5-6).

DISCUSSION

This study of the effect of probiotic treatment on microbial communities in an oyster hatchery demonstrated that: 1) only a few taxa constituted the majority of bacterial community at the oyster hatchery; 2) there was a significant difference in composition of core microbial communities between samples of tank water, tank surfaces, and larval oysters reared in the tank; and 3) daily application of probiotic *B. pumilus* RI06-95 at oyster hatchery system had no significant impact on composition and diversity of microbial community in rearing water, tank surface, and larval oyster at the hatchery.

The bacterial communities in this study were dominated by the phyla *Proteobacteria*, *Bacteroidetes*, and *Cyanobacteria*. The phyla *Proteobacteria* was most common in all samples including rearing water, swabs from tank surface, and oyster larvae, constituting 53 – 85 % of OTU's. *Proteobacteria* has been reported as the largest and most phenotypically diverse phylum in oyster microbiota (Hernandez-Zarate & Olmos-Soto 2006, Trabal Fernández et al. 2014). Several members of the *Proteobacteria*, including *Vibrio* and *Roseobacter* are often associated with shellfish diseases such as Vibriosis, Roseovarius Oyster Disease (ROD), previously known as

Juvenile Oyster Disease (JOD), respectively (Paillard et al. 2004, Boettcher 2005). In our hatchery experiments levels of the cultivable *Vibrio* spp. ranged from 10^0 to 10^4 CFU/ml during the hatchery trial period (Sohn et al., in preparation, Chapter 4).

In rearing water at the hatchery, *Bacteroidetes* was the second most abundant phylum. These phylum, which includes *Bacteroides* and *Flavobacterium*, usually dominate marine environments (Thomas et al. 2011, Gilbert et al. 2012, Trabal Fernández et al. 2014), often associated with biofilms on surfaces (Edwards et al. 2010) and macroalgae (Staufenberger et al. 2008) or on the aggregated particles such as marine snow (DeLong et al. 1993). On tank surfaces, *Cyanobacteria* were detected as the second most abundant phylum, which is also known as blue-green bacteria. The phylum *Cyanobacteria* is often related to harmful algal blooms (HABs) by producing toxins that can cause adverse effects in animals (Landsberg 2002, Hudnell 2008).

Differences in microbial composition between sample types at the oyster hatchery indicate that tank surfaces and oysters are selectively colonized by certain species in the surrounding seawater, mainly from the phylum *Proteobacteria*. *Bacteroidetes* and *Cyanobacteria* were relatively less abundant on tank surfaces and oyster samples than in water samples, at the expense of *Proteobacteria*, which became relatively more abundant in these samples.

Interestingly, in oyster larvae, the phylum *Firmicutes* were observed as the second abundant phylum (but only as 4 % in overall bacterial community) but there were no significant differences between treatments ($p > 0.05$). The genus *Bacillus* is a member of the phylum *Firmicutes*, which is another common component of oyster microbiota. The phylum *Firmicutes* have been suggested to play an important role in

biodegradation of oyster shells (Math et al. 2010). Interestingly, this phylum was not detected in rearing water and tank surface in spite of daily application of *B. pumilus* RI06-95 in the hatchery. This could be because the amount of collected samples from each tank was not enough to detect single species using this method.

Although there were no significant differences statistically on the diversity of bacteria community within/between oyster larvae, water and swab from tank surface had relatively diverse bacterial communities, but not on oyster larvae (Figure 5-6). Interestingly, oysters at the egg stage were found to have a more diverse bacterial community than larval oysters. Such changes in composition of bacterial community in early stage may be due to the elimination of transient microbial communities by larvae as they develop, the development of the shell, and/or changes in the physiology as feeding strategies evolve from relying on maternal reserves on active selective filter feeding (Shumway et al. 1985, Baldwin 1995, Prieur 1981, Riquelme et al. 2000). Higher diversity and richness were shown in the post-larvae oyster compare to adult larvae (Trabal Fernández et al. 2014). It will be interesting to determine if the bacterial community of eggs is more similar to the originating oyster brood stock at each particular spawning event than to oyster larvae.

These studies may provide some preliminary insights into mechanisms of action of RI06-95 on oyster larvae. Mechanisms of action of probiotics and complex and variable between species, including the production of antimicrobial compounds that inhibit the growth of pathogens, competition with the pathogens for colonization sites and nutrients in the host, improvement of water quality, enhancement of growth and survival by providing a favorable surrounding environment, and enhancement of

nutrition and host immunity (Verschuere et al. 2000). The potential mechanisms of action *B. pumilus* RI06-95 in preventing disease in bivalve oysters are unknown, but previous research has shown that daily treatment of oyster larval tanks with *B. pumilus* RI06-95 may lead to a decrease in the chances of an bacterial disease outbreak by decreasing levels of *Vibrio* spp. in water and tank surfaces (Sohn et al. submitted, Chapter 2 of this dissertation). This effect may be mediated by the production of the antibiotic amicoumacin, which has inhibitory activity against *Roseovarius crassostreae in vitro* (Karim et al. 2013). Interestingly, in these trials, exposure of oyster larvae to probiotic *B. pumilus* RI06-95 in the hatchery provided beneficial probiotic effects on oyster larvae showing increased protection of larvae to challenge with a bacterial pathogen (Sohn et al. in preparation; Chapter 4 of this dissertation). However, no effect of probiotic treatment was seen on levels of total *Vibrio* spp. in water, oyster, or swab samples (Sohn et al. in preparation; Chapter 4 of this dissertation) or on microbial community composition or diversity.

These results indicate that the effect of probiotic *B. pumilus* RI06-95 on the larvae is due to mechanisms other than major effects on the composition of the microbial community at the hatchery. However, this analysis has been done only at the phylum level and only for probiotic RI06-95. Therefore, further analysis is needed to determine the impact of probiotic treatment on particular species of interest, such as *Vibrio* spp., as well as determining the role of other potential mechanisms of action. Based on previous research in our laboratories, other potential mechanisms of action include immunomodulation of the host (as indicated by species-specific effects, Sohn et al. in preparation; Chapter 3 of this dissertation), as well as other potential impacts

on larval physiology, as seen for other probiotic bacilli (Aly et al. 2008, C. De et al. 2014).

In conclusion, this study provides a first step in the characterization of microbial communities in bivalve hatcheries and the potential impact of probiotic treatment on these communities. Further research should be done to see if other probiotics, such as *Phaeobacter inhibens* S4 (Karim et al, 2013, Zhao et al. 2016, Sohn et al. in preparation, this dissertation) show effects on microbial communities in bivalve hatcheries.

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LITERATURE CITED

- Aly SM, Abdel-Galil Ahmed Y, Abdel-Aziz Ghareeb A, Mohamed MF (2008) Studies on *Bacillus subtilis* and *Lactobacillus acidophilus*, as potential probiotics, on the immune response and resistance of *Tilapia nilotica* (*Oreochromis niloticus*) to challenge infections. *Fish Shellfish Immunol* 25:128–136
- Andrews S (2010) FastQC: A quality control tool for high throughput sequence data. Ref Source
- Beaz-Hidalgo R, Balboa S, Romalde JL, Figueras MJ (2010) Diversity and pathogenicity of *Vibrio* species in cultured bivalve molluscs: *Vibrio* spp., bivalve molluscs, pathogens. *Environ Microbiol Rep* 2:34–43
- Boettcher KJ (2005) *Roseovarius crassostreae* sp. nov., a member of the *Roseobacter* clade and the apparent cause of juvenile oyster disease (JOD) in cultured Eastern oysters. *Int J Syst Evol Microbiol* 55:1531–1537
- Bolger AM, Lohse M, Usadel B (2014) Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30:2114–2120
- Cahill MM (1990) Bacterial flora of fishes: a review. *Microb Ecol* 19:21–41
- Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley GA, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone CA, McDonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters WA, Widmann J, Yatsunenko T, Zaneveld J, Knight R (2010) QIIME allows analysis of high-throughput community sequencing data. *Nat Methods* 7:335–336
- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J* 6:1621–1624
- C. De B, Meena DK, Behera BK, Das P, Das Mohapatra PK, Sharma AP (2014) Probiotics in fish and shellfish culture: immunomodulatory and ecophysiological responses. *Fish Physiol Biochem* 40(3):921–971
- DeLong EF, Franks DG, Alldredge AL (1993) Phylogenetic diversity of aggregate-attached vs. free-living marine bacterial assemblages. *Limnol Oceanogr* 38:924–934
- DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL (2006) Greengenes, a Chimera-Checked 16S rRNA Gene Database and Workbench Compatible with ARB. *Appl Environ Microbiol* 72:5069–5072

- Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* 27:2194–2200
- Edwards JL, Smith DL, Connolly J, McDonald JE, Cox MJ, Joint I, Edwards C, McCarthy AJ (2010) Identification of Carbohydrate Metabolism Genes in the Metagenome of a Marine Biofilm Community Shown to Be Dominated by Gammaproteobacteria and Bacteroidetes. *Genes* 1:371–384
- Estes R, Friedman C, Elston R, Herwig R (2004) Pathogenicity testing of shellfish hatchery bacterial isolates on Pacific oyster *Crassostrea gigas* larvae. *Dis Aquat Organ* 58:223–230
- Gareau MG, Sherman PM, Walker WA (2010) Probiotics and the gut microbiota in intestinal health and disease. *Nat Rev Gastroenterol Hepatol* 7:503–514
- Gatesoupe F. (1999) The use of probiotics in aquaculture. *Aquaculture* 180:147–165
- Gilbert JA, Meyer F, Jansson J, Gordon J, Pace N, Tiedje J, Ley R, Fierer N, Field D, Kyrpides N (2010) The Earth Microbiome Project: Meeting report of the “1st EMP meeting on sample selection and acquisition” at Argonne National Laboratory October 6th 2010. *Stand Genomic Sci* 3:249
- Gilbert JA, Steele JA, Caporaso JG, Steinbrück L, Reeder J, Temperton B, Huse S, McHardy AC, Knight R, Joint I, Somerfield P, Fuhrman JA, Field D (2012) Defining seasonal marine microbial community dynamics. *ISME J* 6:298–308
- Hernandez-Zarate G, Olmos-Soto J (2006) Identification of bacterial diversity in the oyster *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. *J Appl Microbiol* 100:664–672
- Hill PG, Heywood JL, Holland RJ, Purdie DA, Fuchs BM, Zubkov MV (2012) Internal and External Influences on Near-Surface Microbial Community Structure in the Vicinity of the Cape Verde Islands. *Microb Ecol* 63:139–148
- Hudnell HK (2008) in: K.H. Hudnell (Ed.), *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs*, *Advances in Experimental Medicine and Biology*, vol. 619, vol. XXIV (2008), p. 950
- Karim M, Zhao W, Rowley D, Nelson D, Gomez-Chiarri M (2013) Probiotic Strains for Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica*, Larvae and Juveniles Against Bacterial Challenge. *J Shellfish Res* 32:401–408
- Kesarcodi-Watson A, Kaspar H, Lategan MJ, Gibson L (2008) Probiotics in aquaculture: The need, principles and mechanisms of action and screening processes. *Aquaculture* 274:1–14
- Kesarcodi-Watson A, Miner P, Nicolas J-L, Robert R (2012) Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves:

- Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). *Aquaculture* 344-349:29–34
- King GM, Judd C, Kuske CR, Smith C (2012) Analysis of Stomach and Gut Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA (J Neufeld, Ed.). *PLoS ONE* 7:e51475
- Kirchman DL, Cottrell MT, Lovejoy C (2010) The structure of bacterial communities in the western Arctic Ocean as revealed by pyrosequencing of 16S rRNA genes: Arctic Bacteria in Winter and Summer. *Environ Microbiol* 12:1132–1143
- Landsberg JH (2002) The Effects of Harmful Algal Blooms on Aquatic Organisms. *Rev Fish Sci* 10:113–390
- Lokmer A, Mathias Wegner K (2015) Hemolymph microbiome of Pacific oysters in response to temperature, temperature stress and infection. *ISME J* 9:670–682
- Math RK, Islam SMA, Hong SJ, Cho KM, Kim JM, Yun MG, Cho JJ, Kim EJ, Lee YH, Yun HD (2010) Metagenomic characterization of oyster shell dump reveals predominance of Firmicutes bacteria. *Microbiology* 79:509–519
- Paillard C, Le Roux F, Borrego JJ (2004) Bacterial disease in marine bivalves, a review of recent studies: Trends and evolution. *Aquat Living Resour* 17:477–498
- Powell SM, Chapman CC, Bermudes M, Tamplin ML (2013) Dynamics of Seawater Bacterial Communities in a Shellfish Hatchery. *Microb Ecol* 66:245–256
- Prado S, Romalde JL, Barja JL (2010) Review of probiotics for use in bivalve hatcheries. *Vet Microbiol* 145:187–197
- Preidis GA, Versalovic J (2009) Targeting the Human Microbiome With Antibiotics, Probiotics, and Prebiotics: Gastroenterology Enters the Metagenomics Era. *Gastroenterology* 136:2015–2031
- Prieur D (1981) Experimental studies of trophic relationships between marine bacteria and bivalve molluscs. *Kiel Meeresforsch Sonderh* 5:376–383
- Ringø E, Olsen RE, Gifstad T ø., Dalmo RA, Amlund H, Hemre G-I, Bakke AM (2010) Prebiotics in aquaculture: a review. *Aquac Nutr* 16:117–136
- Riquelme C, Araya R, Escribano R (2000) Selective incorporation of bacteria by *Argopecten purpuratus* larvae: implications for the use of probiotics in culturing systems of the Chilean scallop. *Aquaculture* 181:25–36

- Romalde J, Barja J (2010) Bacteria in molluscs: good and bad guys. *Curr Res Technol Educ Top Appl Microbiol Microb Biotechnol Mendez-Vilas Ed Formatex Res Cent*:136–147
- Sakowski E (2015) The microbiome of the eastern oyster, *Crassostrea virginica*, in health and disease. Doctoral dissertation, University of Delaware
- Sandaa R-A, Magnesen T, Torkildsen L, Bergh Ø (2003) Characterisation of the Bacterial Community Associated with Early Stages of Great Scallop (*Pecten maximus*), Using Denaturing Gradient Gel Electrophoresis (DGGE). *Syst Appl Microbiol* 26:302–311
- Schulze AD, Alabi AO, Tattersall-Sheldrake AR, Miller KM (2006) Bacterial diversity in a marine hatchery: Balance between pathogenic and potentially probiotic bacterial strains. *Aquaculture* 256:50–73
- Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, Huttenhower C (2011) Metagenomic biomarker discovery and explanation. *Genome Biol* 12:R60
- Simon C, Daniel R (2011) Metagenomic Analyses: Past and Future Trends. *Appl Environ Microbiol* 77:1153–1161
- Staufenberger T, Thiel V, Wiese J, Imhoff JF (2008) Phylogenetic analysis of bacteria associated with *Laminaria saccharina*: Bacteria associated with *Laminaria saccharina*. *FEMS Microbiol Ecol* 64:65–77
- Teasdale ME, Liu J, Wallace J, Akhlaghi F, Rowley DC (2009) Secondary Metabolites Produced by the Marine Bacterium *Halobacillus salinus* That Inhibit Quorum Sensing-Controlled Phenotypes in Gram-Negative Bacteria. *Appl Environ Microbiol* 75:567–572
- Thomas F, Hehemann J-H, Rebuffet E, Czejek M, Michel G (2011) Environmental and Gut Bacteroidetes: The Food Connection. *Front Microbiol* 2:93
- Trabal Fernández N, Mazón-Suástegui JM, Vázquez-Juárez R, Ascencio-Valle F, Romero J (2014) Changes in the composition and diversity of the bacterial microbiota associated with oysters (*Crassostrea corteziensis* , *Crassostrea gigas* and *Crassostrea sikamea*) during commercial production. *FEMS Microbiol Ecol* 88:69–83
- Turnbaugh PJ, Ley RE, Hamady M, Fraser-Liggett CM, Knight R, Gordon JI (2007) The Human Microbiome Project. *Nature* 449:804–810
- Verschuere L, Rombaut G, Sorgeloos P, Verstraete W (2000) Probiotic Bacteria as Biological Control Agents in Aquaculture. *Microbiol Mol Biol Rev* 64:655–671

- Zhao W (2014) Characterization of the probiotic mechanism of *Phaeobacter gallaeciensis* S4 against bacterial pathogens. Doctoral dissertation, University of Rhode Island
- Zhao W, Dao C, Karim M, Gomez-Chiarri M, Rowley D, Nelson DR (2016) Contributions of tropodithietic acid and biofilm formation to the probiotic activity of *Phaeobacter inhibens*. *BMC Microbiol* 16(1):1

Figure 5-1. Heat map analysis of the taxonomic composition of samples from an oyster hatchery. Samples were collected from water, swab, and oyster before and after treatment with RI06-95 at oyster hatchery during 2 different trials (H1 and H2). The relative abundance of clustered OTUs at 97 % similarity was illustrated using heat map.

Figure 5-2. Relative abundance of dominant bacterial phyla in microbiota of samples collected from an oyster hatchery. Data for the most abundant phyla is expressed as percentage of the total community. W = water, S = swab from tank surface, O = oyster larvae, E = fertilized eggs, C = control (no probiotic), R = *Bacillus pumilus* RI06-95 treated, B = before probiotic treatment, A = after probiotic treatment (5, 8, and 12 days after fertilization for Trial II and 12 days after fertilization for Trial III).

Figure 5-3. Proportion of the seven major bacterial phyla in microbiota of each sample at an oyster hatchery. Samples were collected from (A) water, (B) tank surface, and (C) larval oyster during 2 different hatchery trials.

Figure 5-4. Relative abundance of a single microbial phyla feature detected by LEfSe as biomarker. Abbreviation: W = water, S = swab from tank surface, O = oyster

larvae, NO PROBIOTIC = samples from non-treated tanks (control, red bars), and PROBIOTIC = samples from *Bacillus pumilus* RI06-95 treated tanks (green). Each bar indicates the averages of two trials (Trial II and III) at each time points (1, 5, 7, 8, 10 and 12 days after fertilization).

A **B**

Figure 5-5. Chao1 rarefaction diversity samples including water, swab, larval oyster, and egg tank (negative control) before or/and after treatment with RI06-95 at oyster hatchery (H1 and H2). Chao diversity was calculated from sequence distribution A) based on source of sample; B) based on treatments.

Figure 5-6. Beta diversity in microbial communities in shellfish hatcheries (binary Jaccard measures). A) Before probiotic treatment. B) After probiotic treatment. Bigger nodes correspond to samples treated with *B. pumillus* RI06-95 at the hatchery in 2 different trials. Each node color indicates following; Yellow /Green = water; Blue/Purple = Swab from tank surface; Red/Orange = Larvae oyster.

APPENDICES

Appendix A - Chapter 2

Two-way ANOVA for the levels of *Vibrios* in water, tank surface, and oyster on each trials.

< Trial I – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	8.738	6	1.456	F (6, 16) = 1.134	P = 0.3873
Time	9.824	2	4.912	F (2, 16) = 3.824	P = 0.0439
Treatment	11.71	3	3.903	F (3, 8) = 3.505	P = 0.0693
Subjects (matching)	8.909	8	1.114	F (8, 16) = 0.8670	P = 0.5625
Residual	20.55	16	1.285		

< Trial I – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.819	6	0.4698	F (6, 16) = 0.7882	P = 0.5920
Time	8.728	2	4.364	F (2, 16) = 7.321	P = 0.0055
Treatment	8.497	3	2.832	F (3, 8) = 1.710	P = 0.2418
Subjects (matching)	13.25	8	1.657	F (8, 16) = 2.779	P = 0.0390
Residual	9.537	16	0.5961		

< Trial I – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6.826	6	1.138	F (6, 16) = 1.197	P = 0.3571
Time	15.15	2	7.576	F (2, 16) = 7.971	P = 0.0040
Treatment	33.84	3	11.28	F (3, 8) = 15.20	P = 0.0011
Subjects (matching)	5.939	8	0.7423	F (8, 16) = 0.7811	P = 0.6256
Residual	15.21	16	0.9504		

< Trial II – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.2907	2	0.1454	F (2, 9) = 0.2012	P = 0.8214
Time	34.37	1	34.37	F (1, 9) = 47.56	P < 0.0001
Treatment	2.731	2	1.365	F (2, 9) = 4.880	P = 0.0367
Subjects (matching)	2.518	9	0.2798	F (9, 9) = 0.3872	P = 0.9131
Residual	6.503	9	0.7226		

< Trial II – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	18.35	4	4.587	F (4, 18) = 7.880	P = 0.0007
Time	7.346	2	3.673	F (2, 18) = 6.309	P = 0.0084

Treatment	32.95	2	16.47	F (2, 9) = 24.83	P = 0.0002
Subjects (matching)	5.971	9	0.6634	F (9, 18) = 1.140	P = 0.3866
Residual	10.48	18	0.5822		

< Trial II – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.899	4	0.7248	F (4, 18) = 1.146	P = 0.3670
Time	10.80	2	5.402	F (2, 18) = 8.539	P = 0.0025
Treatment	16.97	2	8.487	F (2, 9) = 5.694	P = 0.0252
Subjects (matching)	13.42	9	1.491	F (9, 18) = 2.356	P = 0.0581
Residual	11.39	18	0.6326		

< Trial III – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.799	3	0.9332	F (3, 8) = 0.8868	P = 0.4881
Time	1.191	1	1.191	F (1, 8) = 1.132	P = 0.3184
Treatment	11.64	3	3.882	F (3, 8) = 2.589	P = 0.1254
Subjects (matching)	11.99	8	1.499	F (8, 8) = 1.425	P = 0.3141
Residual	8.418	8	1.052		

< Trial III – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	13.02	3	4.341	F (3, 8) = 14.04	P = 0.0015
Time	21.07	1	21.07	F (1, 8) = 68.16	P < 0.0001
Treatment	13.02	3	4.341	F (3, 8) = 14.04	P = 0.0015
Subjects (matching)	2.473	8	0.3092	F (8, 8) = 1.000	P = 0.5000
Residual	2.473	8	0.3092		

< Trial III – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	3.301	3	1.100	F (3, 8) = 1.908	P = 0.2068
Time	0.01723	1	0.01723	F (1, 8) = 0.02987	P = 0.8671
Treatment	3.593	3	1.198	F (3, 8) = 1.251	P = 0.3541
Subjects (matching)	7.660	8	0.9575	F (8, 8) = 1.660	P = 0.2446
Residual	4.614	8	0.5767		

Two-way ANOVA for larval survival to experimental bacterial challenge on each trial

< Trial I >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.7952	14	0.05680	F (14, 32) = 11.88	P < 0.0001
Time	0.4654	2	0.2327	F (2, 32) = 48.69	P < 0.0001
Treatment	9.281	7	1.326	F (7, 16) = 200.0	P < 0.0001
Subjects (matching)	0.1061	16	0.006628	F (16, 32) = 1.387	P = 0.2097
Residual	0.1529	32	0.004779		

< Trial II >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.5105	5	0.1021	F (5, 18) = 7.020	P = 0.0008
Time	0.005325	1	0.005325	F (1, 18) = 0.3662	P = 0.5527
Treatment	7.755	5	1.551	F (5, 18) = 113.0	P < 0.0001
Subjects (matching)	0.2470	18	0.01372	F (18, 18) = 0.943	P = 0.5485
Residual	0.2618	18	0.01454		

< Trial III >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.2667	7	0.03810	F (7, 16) = 3.264	P = 0.0237
Time	0.05137	1	0.05137	F (1, 16) = 4.401	P = 0.0521
Treatment	5.633	7	0.8048	F (7, 16) = 42.76	P < 0.0001
Subjects (matching)	0.3011	16	0.01882	F (16, 16) = 1.613	P = 0.1745
Residual	0.1867	16	0.01167		

Appendix B - Chapter 3

Two-way ANOVA for the levels of *Vibrios* in water, tank surface, and bay scallop on the hatchery trial.

< Bay scallop Hatchery Trial – Bay scallop larvae >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.4077	6	0.06794	F (6, 16) = 0.5143	P = 0.7890
Time	2.250	2	1.125	F (2, 16) = 8.516	P = 0.0030
Treatment	0.2074	3	0.06912	F (3, 8) = 0.1804	P = 0.9068
Subjects (matching)	3.066	8	0.3833	F (8, 16) = 2.901	P = 0.0332
Residual	2.114	16	0.1321		

< Bay scallop Hatchery Trial – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.7932	6	0.1322	F (6, 16) = 0.3741	P = 0.8848
Time	1.670	2	0.8349	F (2, 16) = 2.362	P = 0.1262
Treatment	0.2443	3	0.08143	F (3, 8) = 0.2645	P = 0.8492
Subjects (matching)	2.463	8	0.3079	F (8, 16) = 0.8712	P = 0.5595
Residual	5.654	16	0.3534		

< Bay scallop Hatchery Trial – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.779	6	0.4631	F (6, 16) = 0.9244	P = 0.5034
Time	29.12	2	14.56	F (2, 16) = 29.06	P < 0.0001
Treatment	0.7095	3	0.2365	F (3, 8) = 0.3043	P = 0.8217
Subjects (matching)	6.217	8	0.7771	F (8, 16) = 1.551	P = 0.2162
Residual	8.016	16	0.5010		

Two-way ANOVA for the levels of *Vibrios* in water, tank surface, and hard clam larvae on the hatchery trial.

< Hard clam Hatchery Trial – Hard clam larvae >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5.677	6	0.9462	F (6, 16) = 2.387	P = 0.0770
Time	17.89	2	8.946	F (2, 16) = 22.56	P < 0.0001
Treatment	14.20	3	4.732	F (3, 8) = 2.218	P = 0.1638
Subjects (matching)	17.07	8	2.133	F (8, 16) = 5.381	P = 0.0021
Residual	6.343	16	0.3965		

< Hard clam Hatchery Trial – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.053	6	0.1755	F (6, 16) = 1.441	P = 0.2598
Time	43.09	2	21.55	F (2, 16) = 176.9	P < 0.0001
Treatment	0.7668	3	0.2556	F (3, 8) = 3.677	P = 0.0625
Subjects (matching)	0.5560	8	0.06950	F (8, 16) = 0.5708	P = 0.7869
Residual	1.948	16	0.1218		

< Hard clam Hatchery Trial – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	6.078	6	1.013	F (6, 16) = 1.494	P = 0.2424
Time	5.759	2	2.880	F (2, 16) = 4.247	P = 0.0332
Treatment	10.67	3	3.557	F (3, 8) = 3.351	P = 0.0762
Subjects (matching)	8.494	8	1.062	F (8, 16) = 1.566	P = 0.2117
Residual	10.85	16	0.6781		

Two-way ANOVA for larval survival to experimental bacterial challenge on each trial

< Bay scallop Hatchery Trial >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.571	14	0.1122	F (14, 128) = 3.269	P = 0.0002
Time	1.644	2	0.8220	F (2, 128) = 23.95	P < 0.0001
Treatment	5.108	7	0.7298	F (7, 64) = 24.40	P < 0.0001
Subjects (matching)	1.914	64	0.02991	F (64, 128) = 0.8716	P = 0.7270
Residual	4.393	128	0.03432		

< Hard clam Hatchery Trial >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.615	7	0.2308	F (7, 64) = 10.56	P < 0.0001
Time	1.289	1	1.289	F (1, 64) = 59.01	P < 0.0001
Treatment	27.05	7	3.864	F (7, 64) = 247.2	P < 0.0001
Subjects (matching)	1.000	64	0.01563	F (64, 64) = 0.7155	P = 0.9084
Residual	1.398	64	0.02185		

Appendix C - Chapter 4

Two-way ANOVA for the levels of *Vibrios* in water, tank surface, and oyster on each trials with RI formulations.

< Trial I – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	9.009	2	4.505	F (2, 10) = 2.278	P = 0.1530
Time	1.689	2	0.8446	F (2, 10) = 0.4271	P = 0.6638
Treatment	0.03975	1	0.03975	F (1, 5) = 0.07956	P = 0.7892
Subjects (matching)	2.498	5	0.4996	F (5, 10) = 0.2526	P = 0.9289
Residual	19.78	10	1.978		

< Trial I – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	2.834	2	1.417	F (2, 10) = 2.879	P = 0.1029
Time	4.138	2	2.069	F (2, 10) = 4.204	P = 0.0473
Treatment	0.05357	1	0.05357	F (1, 5) = 0.03503	P = 0.8589
Subjects (matching)	7.647	5	1.529	F (5, 10) = 3.107	P = 0.0599
Residual	4.922	10	0.4922		

< Trial II – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4.051	6	0.6752	F (6, 16) = 1.467	P = 0.2512
Time	46.39	2	23.19	F (2, 16) = 50.39	P < 0.0001
Treatment	8.178	3	2.726	F (3, 8) = 4.766	P = 0.0344
Subjects (matching)	4.576	8	0.572	F (8, 16) = 1.243	P = 0.3372
Residual	7.364	16	0.4603		

< Trial II – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	5.513	6	0.9188	F (6, 16) = 0.4252	P = 0.8515
Time	58.79	2	29.39	F (2, 16) = 13.60	P = 0.0004
Treatment	20.56	3	6.854	F (3, 8) = 4.529	P = 0.0389
Subjects (matching)	12.11	8	1.513	F (8, 16) = 0.7004	P = 0.6872
Residual	34.57	16	2.161		

< Trial II – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	0.9064	6	0.1511	F (6, 16) = 0.2986	P = 0.9286
Time	7.833	2	3.916	F (2, 16) = 7.740	P = 0.0045
Treatment	18.74	3	6.247	F (3, 8) = 12.66	P = 0.0021
Subjects (matching)	3.948	8	0.4936	F (8, 16) = 0.9755	P = 0.4883
Residual	8.095	16	0.506		

< Trial III – Oyster >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4.149	6	0.6914	F (6, 16) = 1.599	P = 0.2112
Time	3.533	2	1.766	F (2, 16) = 4.086	P = 0.0369
Treatment	0.6853	3	0.2284	F (3, 8) = 0.2769	P = 0.8406
Subjects (matching)	6.599	8	0.8248	F (8, 16) = 1.908	P = 0.1292
Residual	6.918	16	0.4324		

< Trial III – Tank Surface >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	4.142	6	0.6904	F (6, 16) = 0.6954	P = 0.6571
Time	67.07	2	33.54	F (2, 16) = 33.78	P < 0.0001
Treatment	7.267	3	2.422	F (3, 8) = 0.6960	P = 0.5801
Subjects (matching)	27.84	8	3.48	F (8, 16) = 3.506	P = 0.0157
Residual	15.88	16	0.9927		

< Trial III – Water >

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Interaction	1.432	6	0.2387	F (6, 16) = 0.2925	P = 0.9318
Time	6.147	2	3.074	F (2, 16) = 3.766	P = 0.0457
Treatment	7.227	3	2.409	F (3, 8) = 1.383	P = 0.3163
Subjects (matching)	13.93	8	1.741	F (8, 16) = 2.134	P = 0.0937
Residual	13.06	16	0.8161		