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PROBIOTICS FOR EASTERN OYSTER HATCHERIES: COMMERCIAL FORMULATIONS AND EFFECT ON MICROBIAL COMMUNITIES

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PROBIOTICS FOR EASTERN OYSTER HATCHERIES: COMMERCIAL FORMULATIONS AND EFFECT ON MICROBIAL COMMUNITIES

BY

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ABSTRACT

In the United States of America, oyster production is an important component of the seafood industry in many coastal communities. Oysters provide ecological, economic, and cultural services. Several hatcheries providing eastern oyster, *C. virginica,* seed to oyster farms face significant losses owing to *Vibrio* spp. infections causing massive larval mortalities. Probiotics have been proposed as a potential preventive measure to limit the impact of bacterial diseases in shellfish hatcheries. The probiotic bacterium *Phaeobacter inhibens* S4 has been shown to protect *C. virginica* larvae from *Vibrio coralliilyticus* RE22 (RE22) infection. A liquid formulation of this probiotic has been developed for ease of use in commercial hatcheries. The overall goal of this dissertation is to evaluate the efficacy and safety of the formulated probiotic S4 as an alternative management tool in disease prevention in oyster hatcheries.

Chapter 1 provides an overview of the importance of oysters in aquaculture, the use of probiotics for disease prevention in bivalve hatcheries, and the potential role that the relationships between the environment, microbial communities, and probiotics could play on the larval oyster host.

Chapter 2 describes the impact of treatment with a formulation of probiont *Phaeobacter inhibens* S4 on the growth and survival of oysters in several hatchery trials. Daily application of an S4 formulation mixed with algal feed to culture tanks in the hatchery consistently increased the survival of oyster larvae to experimental challenge with the bacterial pathogen *V. coralliilyticus* RE22, but had no detectable impact on larval growth and survival in the hatchery in the absence of a bacterial challenge. Treatment with S4 had no significant effect on the levels of total culturable vibrios in the larvae. This result

suggests that the novel S4 formulation is safe, easy to use, and an effective tool in preventing larval losses to vibriosis.

Chapter 3 characterized the effect of treatment with the S4 formulation on the bacterial community of oyster larvae in the hatchery through several trials spanning different hatcheries, years and seasons using 16S rDNA sequencing. *Proteobacteria* was consistently the most abundant phylum in oyster larval samples. Larval bacterial communities significantly differed mainly by hatchery and trial, and, to a lesser extent, by probiotic treatment. The addition of the S4 formulation caused subtle but significant changes in the structure of oyster larval bacterial communities but did not affect bacterial diversity. Probiotic treatment had a targeted impact on the relative abundance of a few selected taxa in oyster larvae, amplifying *Alteromonas* and decreasing *Pseudomonas*. This shows that the effect of probiont S4 is subtle and targeted to a few selected taxa (*i.e.* does not cause dysbiosis).

Chapter 4 analyzed data collected from larval performance (survival and growth), microbial community, and environmental parameters during the trials reported in previous chapters to determine the variables that may impact larval performance in the hatchery. As previously described, variations in environmental parameters were associated with changes in larval survival, with the best larval performance being observed at temperatures of 25 - 26^{o}C, salinities of 14 – 17 psu, and pH of 8.2-8.3. A principal component analysis (PCA) showed correlations between bacterial community composition, environmental variables, and larval growth and survival. Several taxa whose relative abundance correlated with changes in larval performance at the hatchery were identified. Bacterial taxa showing correlations with temperature and salinity also overlapped with larval performance,

suggesting a complex interplay between temperature, salinity, microbial community composition, and larval performance in the hatchery. Results from this dissertation provide essential foundational knowledge for better understanding of the use of the probiotic S4 formulation in the hatchery and its interaction with the larval host.

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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 the following four manuscripts and a summary chapter:

1. "Effect of a formulation of Probiont *Phaeobacter inhibens* S4 on oyster larval performance in the hatchery" prepared for submission to the *Journal of Aquaculture Research*

2. "Effect of probiotic treatment on bacterial microbiomes of larval eastern oysters, *Crassostrea virginica,* raised in different hatcheries" prepared for submission to *Frontiers in Microbiology*.

3. "The relationship between microbial composition, environmental parameters, and larval performance in eastern oyster hatcheries" prepared for submission to *Frontiers in Microbiology*.

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CHAPTER I: LITERATURE REVIEW

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The importance of oysters in aquaculture

Various bivalve species, including oysters, clams, scallops, and mussels, are commercially produced in aquaculture. The farming of shellfish is vital to supplying protein for food and feed for the growing world population(FAO/WHO, 2006). Aquaculture of the eastern oyster *Crassostrea virginica* is a rapidly expanding and economically important industry on the Atlantic coasts of North America (Arfken et al., 2021; Yeh et al., 2020). Oyster production through aquaculture in the United States totaled 219 million dollars (USD) in 2018 (NOAA Fisheries, 2019). Hatcheries are the facilities that produce fertilized eggs, larvae, and small juveniles and culture them until they are large enough for deployment in grow-out farms. One primary constraint to the growth and sustainability of the bivalve larvae in the hatchery is the loss of larvae to disease outbreaks caused by bacterial pathogens, especially in the genus *Vibrio*.

Vibriosis disease is caused by various *Vibrio* species and is reported as the most common disease in association with mass mortality in bivalve hatcheries (Beaz-Hidalgo et al., 2010; Paillard et al., 2004; Thompson et al., 2004; Travers et al., 2015). It has been reported that these pathogenic bacteria are introduced into the bivalve hatchery system through contaminated food, incoming water, rearing tank, broodstock, and equipment (Elston, 2008; Dubert et al., 2017). Appropriate methods such as water treatment systems(filtration, ultraviolet light, water pasteurization), best management practices (equipment disinfection, microbiological testing), and other labor-intensive biosecurity measures are often used to avoid the introduction of pathogens and mortality outbreaks in the hatcheries (Dubert et al., 2017). However, Gray et al. (2022) found that *Vibrio* species were not detected on plate culture during a mortality event in the hatchery, suggesting that

other pathogens could elicit larval mortalities. One disadvantage of using plate culture for pathogen identification is the inability to identify bacteria that are not culturable. However, relying on 16S sequencing enables ease in bacteria identification. Antibiotic usage as a disease prevention tool is discouraged because of bacteria's potential development of resistance and negative impacts on healthy microbiota (Lokmer et al., 2016; Prado et al., 2010). Other alternative approaches, such as probiotics to manage disease outbreaks in the bivalve hatcheries, have been proposed.

The use of probiotics in bivalve shellfish aquaculture

As defined by Food and Agricultural Organization and World Health Organization, probiotics are live microorganisms that confer a health benefit to the host (FAO/WHO, 2006). In aquaculture, probiotics are administered as either food supplements or as an additive to the rearing water (Cha et al., 2013; Gioacchini et al., 2010; Hai, 2015; Zhou et al., 2009). Candidate probiotics that have been identified for use in invertebrate aquaculture include a variety of gram-negative, gram-positive bacteria, yeast, and unicellular algae(Hasan & Banerjee, 2020). Examples of common bacteria species used in probiotics are *Bacillus subtilis, Lactobacillus helveticus*, *Enterococcus faecium*, *Phaeobacter inhibens*, *Vibrio*, *Pseudomonas*, *Plesiomonas*, and *Aeromonas*. Many studies on probiotics usage in aquaculture described a variety of mechanisms of action such as host growth promotion, competition for nutrients with pathogens, improvement of water quality, pathogen inhibition, secretion of antimicrobials, and immunomodulation (Kesarcodi-Watson et al., 2012; Martínez Cruz et al., 2012; Modak & Gomez-Chiarri, 2020; Prado et al., 2010; Sánchez et al., 2017; Yeh et al., 2020). For example, probiotic strains *Bacillus pumilus RI06-95* and *Phaeobacter inhibens* S4 protected eastern oyster larvae against *V.*

coralliilyticus RE22 pathogen (Karim et al., 2013; Sohn, 2016). *Phaeobacter gallaeciensis PP-154* showed inhibitory activity against the European flat oyster (Prado et al., 2009) and *Vibrio* sp. OY15 improved the survival of eastern larval oysters (Kapareiko et al., 2011, Lim et al., 2011). Other studies have shown the protective efficacy of probiotics against bacterial pathogens in other bivalves such as scallops and shrimps (Longeon et al., 2004; Riquelme et al., 2001; Tan et al.,2016).

Various experimentally approved commercial probiotics are available in the market for use in aquaculture. Formulated probiotic products (a mixture prepared according to a particular formula) are commercially available for fish and shellfish culture. Formulations offer advantages such as ease and convenience in storage, handling, and delivery at the hatchery. They mainly include dry products such as wettable powders, dust, granules, and liquid products such as cell suspensions in water, oils, and emulsions (Martínez Cruz et al., 2012; Verschuere et al., 2000; Wang et al., 2016). Examples of commercially formulated probiotics include Prosol (*Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus rhamnosus*, *Lactobacillus salivarius*, *Lactobacillus Plantarum*), Engest Probiotics (*Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus megaterium*) for shrimp (Engest ®: Aquamimicry, 2002; Gupta & Dhawan, 2011) and Bio plus (*Bacillus subtilis*, *Bacillus licheniformis*) for rainbow trout (Bagheri et al., 2008). Most commercial probiotics are formulated from a mixture of gram-positive bacteria that show high survival after freeze-drying. However, it has been suggested that both gram-positive and gram-negative marine bacteria are possible candidate probiotics for commercial hatchery settings. 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).

The effect of probiotics on marine microbiomes

Marine organisms interact with their associated microbiomes, which influence many aspects of host and microbial fitness (Bahrndorff et al., 2016). This interaction may be mutualistic, parasitic, or a commensal relationship with the host, impacting the host and microbial fitness (Hammer et al., 2019). Marine host-associated microbiomes perform many beneficial functions for their hosts, including nutrient sharing and cycling (Fiore et al., 2010; Kneip et al., 2007; Yellowlees et al., 2008; Zhang et al., 2015), protection against disease (Egan and Gardiner, 2016; Janssens et al., 2018; Longford et al., 2019; Vonaesch et al., 2018), acclimation to the environment (Carrier and Reitzel, 2018), and contributions to host fitness, performance and survival (Parfrey et al., 2018b; Thompson et al., 2017). On the other hand, marine microbes can also serve as disease, stress, and decay agents, especially in susceptible hosts (Groner et al., 2016; Lafferty et al., 2015). It is known that the microbiome associated with an organism significantly contributes to its health. Oysters are filter feeders and ingest many different kinds of microorganisms, making them vulnerable to changes in their microbial composition and potentially impacting oyster health. Therefore, it is important to study the effects of probiotics directly on the health and protection of the oysters and also the bacterial communities, as probiotics can result in bacterial dysbiosis (a shift in the microbial community), which could ultimately impact their health positively or negatively. Studies have shown that oyster microbiota performs a variety of beneficial functions such as providing nutrition, influencing immune responses, reducing or preventing detrimental microorganisms from proliferating and causing disease

by creating competition for nutrients, reducing space for settlement, or producing antimicrobials (Castro et al., 2002; Gomez-Gil et al., 2000; Kesarcodi-Watson et al., 2012; Prado et al., 2010b; Sanches-Fernandes et al., 2021; Schulze et al., 2006). Studies show that probiotics alter the microbial community composition by causing changes in the relative abundances of bacterial taxa and enhance host health in host species such as microalgae, oysters and shrimps (Dittmann et al., 2019; Sánchez et al., 2017; García Bernal et al., 2017; Restrepo et al., 2021; Stevick et al., 2019; Majzoub et al., 2019). Others are also known to cause a decrease in microbial diversity in the digestive gland in Nile Tilapia (Merrifield & Carnevali, 2014). Altogether, these studies indicate that probiotic effects on microbial community structure are highly dependent on the host species and the probiotics.

Impact of environmental conditions on host and microbiome

Environmental conditions such as temperature, salinity, and pH contribute to species performance, such as an organism's metabolism, mortality, and growth (Brown et al., 2004; Lowe et al., 2017; Pusack et al., 2018). The optimal temperatures of 25°C–27°C and the salinity of 10psu–32psu are known to support oyster larval growth and survival (Helm et al., 2004). Studies have confirmed that the early, unshelled larval life stage is the most vulnerable to environmental perturbations (Barton et al., 2012; Ragg et al., 2019), and exposure to conditions outside an oyster's optimal range can have negative consequences for their growth or survival (Heilmayer et al., 2008; Munroe et al., 2017; Rybovich et al., 2016). High temperatures have been identified as one of the main factors that affect the severity and prevalence of diseases such as Vibriosis, causes summer mortality, and lower hemocyte counts in *M. edulis* (Burge et al., 2014; Mackenzie et al., 2014). Lower salinities (<10) have also been shown to restrict the intrusion of predators

and diseases but can lead to decreased growth in oysters (La Peyre et al., 2003). Larval bay scallops (*Argopecten irradians*) displayed increased mortality under acidic conditions exacerbated by hypoxia (DePasquale et al., 2014).

Environmental conditions are also known to affect microbial community structure and function. For example, bacterial communities in the coastal environments where oysters are cultured change in response to temperature, salinity, dissolved oxygen, and nutrients (Kirchman et al., 2004; Hill et al., 2012; Neulinger et al., 2009; Amoo & Babalola, 2019; Arroyo et al., 2015; Tripathi et al., 2013; Rath et al.,2019). Given that environmental parameters could directly influence the host and microbial communities of the host, it is important to tease apart the relationship between environmental variables, host-associated microbial communities, and the host performance to determine the factors that influence larval performance and the interactions between these factors.

Goals of this study

Host organisms interact with their associated microbiomes, which influence many aspects of the host fitness (Bahrndorff et al., 2016). Also, environmental conditions or perturbations such as probiotics are known to affect host fitness or compromise mutualistic microbes that may be instrumental in maintaining host health and survival. Despite the wealth of studies and reviews on oyster development, growth, host-microbiome, and the effect of biological and environmental factors on oyster production, the interactions between oyster larval performance, environmental conditions, and host-associated microbiota are unexplored. Therefore, the knowledge gap remains in understanding the crosstalk between these variables and how they impact larval performance in the hatchery.

The overall goal of this dissertation is to evaluate the efficacy of probiotics as an alternative management tool in disease prevention in the oyster larvae in the hatchery. The bacteria *Phaeobacter inhibens* S4 (S4) has been demonstrated as effective probiotic conferring health benefits to larval oysters against bacterial pathogen *V. coralliilyticus* RE22 (Karim et al., 2013; Sohn, 2016). A liquid formulation of these probiotics has been developed for use in the hatcheries at a commercial scale based on the promising effect of the freshly cultured S4 bacteria.

The first objective of this study is to determine the safety and efficacy of the formulated probiotic S4 to the eastern oyster in different hatcheries and its protective effects on the survival of oyster larvae when exposed to the pathogen RE22. This will aid in optimizing the commercially-produced S4 formulation in protecting larval oysters against Vibriosis and enhance our knowledge and understanding of the use of this probiotic in aquaculture and its ability to improve commercial hatchery production of the eastern oysters.

The second objective is to determine the effect of probiotic S4 treatment on larval microbiomes of eastern oyster raised in different hatcheries by characterizing 1) the bacterial community of eastern oyster larvae grown in different hatcheries; and 2) how community changes following exposure to the S4 formulation using 16S rRNA gene sequencing analysis. The research will illuminate how the probiont may alter the larval oyster bacterial community and determine if there is a potential for dysbiosis.

The third objective was to use data collected from the hatcheries to determine the relationship between microbial community composition, environmental parameters, and

larval performance in the hatchery using various multivariate analyses. This will help explain the interactions between these variables, and help uncover the dynamics in larval performance in the hatchery.

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Figures

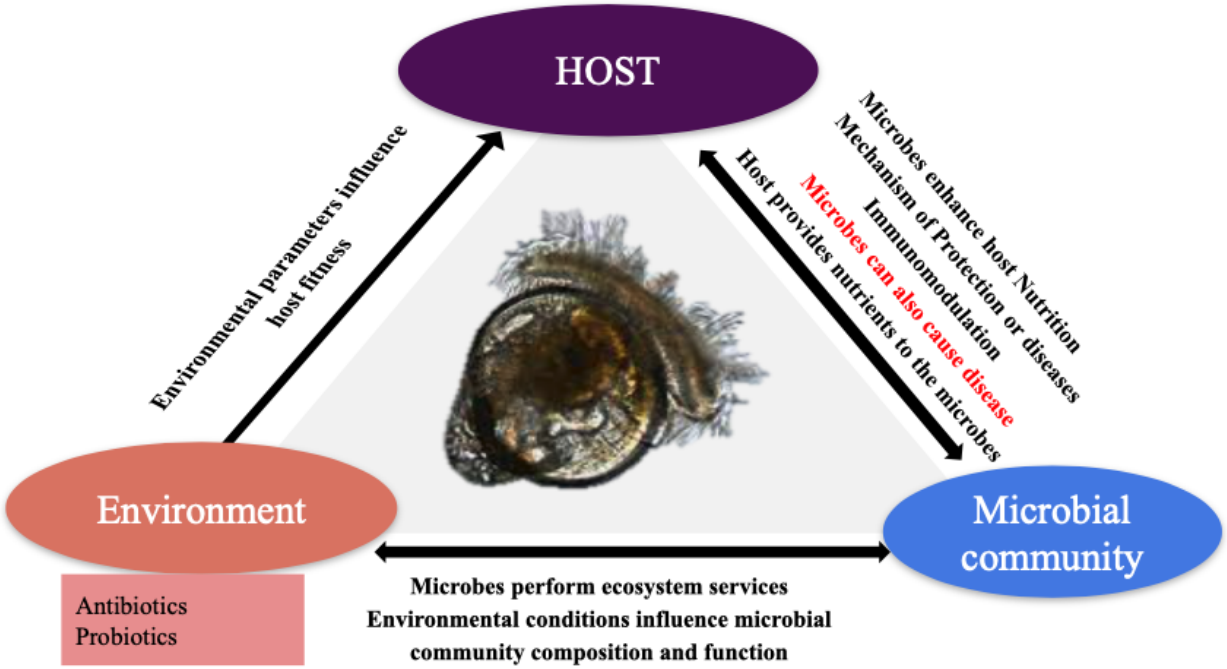

Figure I-1. The interactions between the host, probiotic and microbial community. Microbial communities can have complex associations and effects on the oyster larval host. Both the host and the microbial communities are affected by environmental conditions. These complex interplay between hosts, microbial communities, and the environment can be influenced by biotic (e.g. probiotics) and abiotic external factors.

CHAPTER II: EFFECT OF A FORMULATION OF PROBIONT *PHAEOBACTER INHIBENS* S4 ON OYSTER LARVAL PERFORMANCE IN THE HATCHERY

BY

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Abstract

Larval eastern oysters (*Crassostrea virginica*) grown in shellfish hatcheries are susceptible to bacterial diseases, particularly Vibriosis. Probiotics are beneficial microbes that confer health benefits to the host and have been identified as promising tools to manage diseases in aquaculture. The marine bacterium *Phaeobacter inhibens* S4 protects larval eastern oysters against challenge with the bacterial pathogen *V. coralliilyticus* RE22. A liquid formulation of probiont S4 has been developed for commercial use in shellfish hatcheries. The safety and efficacy of the formulation was tested in six different trials in two hatcheries with different production methods. The S4 formulation (10^4 colony forming units, CFU/mL) was added to *C. virginica* larvae culture tanks daily from day 1 post spawning to day 6, 12 or 14. Treatment of larvae in the hatchery with the S4 formulation did not significantly affect the survival and growth of the larvae. Treatment led to a significant increase in Relative Percent Survival (RPS) when larvae were subsequently exposed to the pathogen RE22 (10^5 CFU/mL) for 24 hours in a laboratory challenge as compared to untreated larvae (Range of RPS = 46 - 74%, $p < 0.05$). These results suggest that this novel S4 formulation is a safe, easy to use, and an effective tool in preventing larval losses to Vibriosis.

Keywords

probiotic, formulation, bivalve, larvae, hatchery, vibriosis

Introduction

The eastern oyster, *Crassostrea virginica*, is a bivalve species of the Gulf of Mexico and Atlantic coasts of North America that has significant economic and ecological value (Arfken et al., 2021; Yeh et al., 2020). Oyster production through aquaculture in the United States totaled 219 million dollars(USD) in 2018 (NOAA Fisheries, 2019). Hatchery production of oyster seed is crucial for ensuring a constant and sufficient supply of juveniles to support the oyster industry. However, changes in environmental conditions and disease outbreaks are limiting factors for the growth of aquaculture production(Sohn et al., 2016; Stentiford et al., 2012). Vibriosis, disease caused by the pathogenic bacteria in the genus *Vibrio*, has been an issue of particular concern in bivalve hatcheries. Various strains of *Vibrio* spp. that are pathogenic to oyster larvae lead to a rapid and high rate of larval mortality in hatcheries, resulting in substantial economic loss to the oyster industry. Techniques for managing disease outbreaks in hatcheries currently include the use of water treatment systems such as filtration, ultraviolet light, water pasteurization, and other labor-intensive biosecurity measures such as cleaning of equipment to avoid the introduction of pathogens(Dubert et al., 2017). Antibiotic usage is discouraged because of the potential development of resistance by bacteria and negative impacts on healthy microbiota (Lokmer et al., 2016; Prado et al., 2010). Despite significant efforts to treat the water supply, pathogenic vibrios are still detected in shellfish hatcheries(Dubert et al., 2017).

The use of probiotics has emerged as a potential tool to reduce mortalities in the rearing of aquatic organisms and manage disease outbreaks in aquaculture (Newaj-Fyzul et al., 2014; Verschuere et al., 2000, Cruz et al., 2012). Probiotics are defined as live, non-pathogenic microorganisms which, when administered in adequate amounts, confer a health benefit to the host (FAO/WHO, 2006). In aquaculture, probiotics are administered

as either food supplement or as an additive to the water(Cha et al., 2013; Gioacchini et al., 2010; Hai, 2015; Zhou et al., 2009). Candidate probiotics for use in invertebrate aquaculture include a variety of gram-negative and gram-positive bacteria, yeast, and unicellular algae. Depending on the probiotic species, these health benefits are derived from a variety of complementary mechanisms including improvement of water quality, enhancement of nutrition of host species through the production of supplemental digestive enzymes, competition for space with pathogenic bacteria, production of antimicrobial compounds, host immunomodulation, and modulation of microbial community structure to promote health (Kesarcodi-Watson et al., 2008; Macey & Coyne, 2005; Modak & Gomez-Chiarri, 2020; Nandi et al., 2018; Nayak, 2010, Cruz et al., 2012).

The marine bacterium *Phaeobacter inhibens* S4(S4) is a gram-negative alpha-Proteobacterium in the *Rhodobacter* clade. Several *Phaeobacter* species exhibit inhibitory activity against a wide variety of marine pathogens such as *V. coralliilyticus* RE22, *V. anguillarum, V. tubiashii* and *R. crassostreae* (Belas et al., 2009; D'Alvise et al., 2012; Grotkjær et al., 2016; Karim et al., 2013; Sonnenschein et al., 2021) and have been shown to effectively colonize surfaces forming dense biofilms (Zhao et al., 2016) Previous studies have shown the probiotic ability of S4 to prevent larval eastern oyster mortality against bacterial infection in laboratory and hatchery experiments (Karim et al., 2013; Sohn et al., 2016). Previously studied mechanisms of action of the probiont S4 include biofilm formation, secretion of the antibiotic tropodithietic acid (TDA), quorum quenching by which S4 represses gene expression of virulence factors in the shellfish pathogen *V. coralliilyticus* RE22, and host immune modulation (Modak & Gomez-Chiarri, 2020; Zhao et al., 2016, 2019). Although the probiont S4 demonstrated promising results for limiting

Vibrio infections in bivalve aquaculture hatcheries, the daily preparation of fresh cultures in the hatchery is impractical. Standardized formulation of S4 would offer advantages such as ease and convenience in storage, handling, and delivery at the hatchery. Commercially formulated probiotics mostly include dry products such as wettable powders, dusts, granules, and liquid products such as cell suspensions in water, oils, and emulsions (Cruz et al., 2012). Most commercial probiotics available in the market for aquaculture are formulated from a mixture of gram-positive bacteria that show high survival after freeze drying. Examples include Prosol (*Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus plantarum*), Engest Probiotics (*Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium*) for shrimp (*Engest ®: Aquamimicry*, 2002; Gupta & Dhawan, 2011) and Bioplus (*B. subtilis, B. licheniformis*) for rainbow trout (Bagheri et al., 2008). To the best of our knowledge, the only gram-negative bacteria commercially formulated is Eco-Pro (*Rhodopseudomonas palustris*), used for improving the quality of ponds (Hasan & Banerjee, 2020).

Based on previous research showing its efficacy and safety as a probiont in bivalve larval culture (Sohn et al., 2016; Sohn et al., 2016b), the present study focused on developing a novel liquid formulation for the gram-negative bacterium S4, allowing for ease of application in the hatchery at a commercial scale. This novel liquid formulation was tested for its safety, efficacy, host protection, and ease in handling and delivery in bivalve hatchery facilities. The results indicate that the formulation showed similar performance in the hatchery as previously reported for the freshly cultured S4 and pre-treatment in the hatchery consistently protected eastern oyster larvae from experimental challenge with the pathogen RE22.

Methods

Bacterial strains

Bacterial strains *Phaeobacter inhibens* S4Sm (probiont) and *Vibrio coralliilyticus* RE22Sm (pathogen) (both are streptomycin resistant strains by spontaneous mutation) were maintained as stocks in 50% glycerol at -80°C until use. Bacteria were cultured on yeast peptone with 3% sea salt (mYP30) media (5 g /L of peptone, 1 g/L of yeast extract, 30 g/L of ocean salt (Red Sea Salt, Ohio, USA)) at 27°C with shaking at 175 rpm as described in Karim et al. (2013) unless otherwise indicated.

Development of a Liquid Probiotic Formulation

Bacteria from glycerol stock stored at -80°C were streaked for isolation on a mYP30 agar plate and incubated at 27°C for 24 - 48 hrs. A single S4 colony was inoculated into Luria Broth with 3% sea salt (mLB30, pH 7) growth medium and incubated at 27°C with shaking for 48 hr, until reaching high density (10^8 – 10^9 CFU/mL). Four different formulation methods were tested for viability after storage for 6 weeks: (1) S4 mLB30 broth cultures stored at 4°C (S4_4^{o}C); (2) S4 mLB30 broth cultures diluted 1:1 with 3% artificial seawater (ASW) and stored at 4°C (S4_ASW_4^{o}C); (3) S4 mLB30 broth culture diluted 1:1 with ASW and stored at 22°C (S4_ASW_22^{o}C); and (4) S4 mLB30 broth culture diluted 1:1 with mYP30 broth media and stored at 4°C (S4_mYP30_4^{o}C). The viability (CFU/mL) of the formulations were determined at 0, 2, 4 and 6 weeks in each of the storage conditions by spot plating serial dilutions in triplicate on mYP30 agar plates and counting colony forming units (CFU/ml) after 24 – 48 hrs of growth.

Laboratory challenge experiment of fresh cultured and formulated S4 probiotic

Laboratory challenge assays were conducted following protocols as described by Karim et al. (2013). Briefly, eastern oyster larvae (7 days old, 100 – 150 μm in size) were obtained from the Aquaculture Breeding Center, Virginia Institute of Marine Sciences (VIMS) hatchery. Oyster larvae (50-100 per well) were placed into 6-well plates with 5 mL of filtered sterile artificial sea water (FSSW, 2.8% salinity). Larval oysters were fed with commercial algal paste (20,000cells/mL; Reed Mariculture Inc., San Jose, CA) prior to addition of probiotics to enhance the ingestion of probiotics. Freshly cultured or formulated S4 were added to larvae in wells designated for each probiotic treatment at a concentration of 10^4 CFU/mL and incubated at room temperature with gentle shaking. After 24 hr, the pathogen RE22 was added to each well at a final concentration of 10^5 CFU/mL. Control wells included unchallenged larvae (no S4 no RE22) and larvae incubated with S4 but without the pathogen. Each treatment was run in triplicate. Larval survival was determined ~ 24 hr after the pathogen was added using the neutral red technique (Gómez-León et al., 2008). Survival was calculated by using the formula: Survival (%) = 100 x (number of live larvae/total number of larvae). The relative percent survival (RPS) of probiotic pretreatment compared to the challenged control was calculated using the formula: RPS (%) = [1 - (% Mortality treatment / % Mortality control)] x 100 (Karim et al., 2013).

Hatchery trial set up

Hatchery experiments were conducted at the Aquaculture Breeding Center, Virginia Institute of Marine Sciences (VIMS) hatchery (Gloucester, Virginia, USA) and Mook Sea Farms hatchery (MOOK; Walpole, Maine, USA). At VIMS, four independent trials (Trials 1-4) were set up, and, for each trial, 60 L conical larval rearing tanks were used (Table 1). Tanks (minimum of 3 per treatment) were randomly assigned to the following treatments: no probiotics (control) or S4 formulation (probiotic treatment). Broodstock were spawned at the hatchery for each trial and were initiated by adding 3.6×10^5 to 6×10^5 larvae (6.2-10 larvae/ml) to each conical static tank 1-2 days after fertilization. Larvae in each tank were fed with a hatchery-reared microalgal diet consisting of *Pavlova pingus*, *Chaetoceros negrocile,* and *Tetraselmis chui*. In Trial 5 conducted at MOOK, 5.2×10^7 larvae (17.3 larvae/ml) were raised in each of two single 3000 L static tanks (one control, one treated with S4) from day 1 to day 8 after spawning, and then larvae from each tank were distributed into 3 × 200 L flowthrough tanks from day 9 to day 12. In Trial 6 at MOOK, larvae were raised in 15 L buckets from days 1 to 12. Larvae in each tank were fed with a hatchery-reared microalgal diet consisting of *Pavlova lutheri* (CCMP1325), *Tetraselmis sp*. (CCMP892), *Tisochrysis lutea* (CCMP1324), and *Chaetoceros muelleri* (CCMP1316). Probiotic formulations were added daily at a dose of 10^4 CFU/mL at the time of algal feeding from day 1 (24 hr after spawning) until the termination of the trial, either at day 12 or 14 (just prior to larval setting, Trials 1, 3, 5 and 6) or earlier if larval performance was low (Trial 2) or the hatchery needed the tanks for routine production (Trial 4). Larval tanks were drained down every other day for size grading of larvae and maintenance of water quality (Helm et al., 2004).

Evaluation of the effect of S4 formulation on larval growth and survival during hatchery trials

Data for each trial were collected every 2 days during the trial period at the time of drain down. Larvae from each tank were collected on nylon mesh screens during drain down, rinsed, and transferred to 150 L tanks filled with 100 L in trial5 and 400 mL beakers filled with filtered seawater to 200 mL in trial 1, 2, 3, 4 and 6. At MOOK, with gentle stirring of the water to evenly distribute larvae, a micropipette was used to collect six samples of 1000 µL larval samples into six well plates and then immobilized with 70% isopropyl alcohol and counted using a dissecting microscope. Larval sizes were estimated based on the percentage of larvae retained on standard mesh sizes (325, 270, 230, 200, 170, 140, 120, 100, 80, 70, 75 micrometer) which measures based on the shortest axis of the larval shell. At VIMS, a micropipette was used to collect four 50 µL larval samples. Each sample was placed on a gridded Sedgewick Rafter counting cell installed on the microscope stage. Larvae were initially observed under a 4x objective for motility, overall shape, and gut coloration to make a health assessment and were assigned a health rating from 1 (Poor health) to 3 (good health). Larvae were then temporarily immobilized with a 2:1 mixture of freshwater and 70% isopropyl alcohol. Larvae were counted under a microscope and percentage survival was calculated and recorded. Larval sizes were observed under 10x objective magnification and an ocular micrometer was used to measure the longest axis of each larval shell. Larval specific growth rate at the end of each trial was calculated from the larval sizes using the formula: SGR (Specific Growth Rate (%)) = ((LnWt - LnWo)/t) x 100 where LnWt = ln final body size (µm), LnWo = ln initial body size (µm), and t = feeding time (day) (Nimrat et al., 2011).

Determination of levels of Vibrio spp. in hatchery larval samples

Total number of culturable *Vibrio* spp. was determined for trials 3 and 4 using a plate count method on thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco) (Sohn et al., 2016). Samples were collected from water in the rearing tank (10 mL) and oysters (~1,000) during drain down periods in the hatchery. Oyster larvae were rinsed with ASW, homogenized using a sterile pestle, and suspended in ASW. Samples were serially diluted and 10µL of each dilution were spot plated on TCBS agar plates in triplicate. The inoculated plates were incubated for 16 - 20 hrs at 28 °C and colonies were counted. Results were expressed as CFU/mL. Controls included 3 wells of non-challenged larvae per tank and treatment.

Laboratory pathogen challenge of probiotic-treated larvae from hatchery

Since pathogens could not be introduced into the hatcheries, a subsample of about 1000 larvae from each tank were collected during drain down periods and shipped overnight on ice to the laboratory at University of Rhode Island. Oyster larvae (~50-100 per well) were placed in 6-well plates and challenged with the pathogen RE22 at a final concentration of 10^5 CFU/mL following the methods described in the laboratory challenge section above.

Statistical Analysis

All statistical analyses were performed in the R statistical computing environment, version 4.0.2 (Martin, 2021). Larval oyster percent survival was 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 trial and also between trials. The Tukey's multiple comparison tests were used for post-hoc pairwise comparisons. A p-value < 0.05 was considered to be statistically significant.

Results

Viability of formulated S4 under various storage conditions

The viability of the different S4 formulation methods described were assessed to determine the concentration and stability of the bacteria for a period of time. The viability was assessed biweekly during storage for 6 weeks (Figure 1). The S4 formulations at the end of the 6 weeks varied and were statistically significant (One way ANOVA; $p < 0.05$; Figure 1), with the S4_ASW_4^{o}C formulation and S4_mYP30_4^{o}C formulation showing significantly higher viability at the end of the 6 weeks (declines of 0.52 log and 0.6 log) as compared to S4_ASW_22^{o}C or S4_4^{o}C formulations (declines of 1.32 and 1.34 log, respectively). Of the four formulations, the S4_mYP30_4^{o}C formulation was used in the hatchery trials.

Laboratory comparison of fresh and formulated S4 at promoting larval survival after pathogen challenge

Pretreatment of larvae with freshly cultured S4 or formulated S4 (S4_mYP30_4^{o}C) had no detrimental effect on larval survival (i.e., in the absence of pathogen challenge) over a 48hr period. Challenge of larvae with the pathogen RE22 led to a significant decrease in larval survival (90% decrease, One Way ANOVA, $p < 0.05$). Larvae treated with either the fresh or formulated S4 probiont showed a range of 40% – 60% increase in survival after RE22 pathogen challenge (One Way ANOVA, $p < 0.05$). Pretreatment with both the fresh

and formulated S4 significantly increased larval survival following RE22 challenge as compared to non-treated controls and the levels of survival did not differ between the two (One Way ANOVA; $p > 0.05$; Figure 2).

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

Based on the protection conferred by the formulation to the bacterial challenge in the laboratory trials, the formulation (S4_mYP30_4^{o}C) was tested in hatchery conditions. Variability in larval growth and survival between trials within a hatchery and between hatcheries was observed, with one trial (Trial 2; VIMS, July 2019) showing extremely survival (*i.e.* larval crash) for both control and probiotic-treated tanks. Daily treatment of larvae with the formulation in the hatcheries did not have a significant impact on larval survival or growth (specific growth rate) in any of the trials in the hatcheries (One-way ANOVA; $p > 0.05$; Figure 3).

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

Daily treatment of larval tanks with the probiotic formulation did not significantly decrease the total number of culturable vibrios in the oyster larvae compared to control treated tanks in any of the hatchery trials, ($p > 0.05$; Figure 5). Variability in *Vibrio* counts between tanks within treatments and trials was observed. Culturable vibrios in water samples were very low to the level of undetectable in all the trials and hatcheries (less than 10^2 CFU/mL, data not shown).

Effect of probiotic treatment on larval survival from the hatchery to experimental RE22 challenge

Exposure of larval oysters to probiotics formulations in the hatchery significantly increased larval survival during subsequent bacterial challenges in the laboratory (Trials 3, 4, and 6; One-way ANOVA; $p < 0.05$, Figure 6). Bacterial challenge assays for Trials 1, 2, and 5 were not performed because they were designed to confirm the safety of the probiotic formulation in each of the hatcheries before conducting further studies. In the laboratory assays, survival rates ranged between 72% - 92 % for larvae from both control and probiotic treated tanks. For larvae challenged with the pathogen, survival from the control ranged from 37% - 41% while survival of probiotic-treated larvae ranged between 60% - 76%, leading to a relative percent survival increase of 46% to 74% (Table S1). Larvae treated with the S4 formulation in the hatchery demonstrated significant increase in survival following RE22 challenge, compared to untreated oyster larvae, for all trials (One Way ANOVA, $p < 0.05$, Figure 6).

Discussion

A novel liquid formulation was developed for the commercial delivery of the gram-negative probiont S4 in oyster hatcheries. The product was found to be stable and maintained viability at 1×10^8 CFU/mL or more for a period of six weeks when stored at 4°C in an airtight container in the dark. The performance of the S4 formulation compared favorably to the freshly cultured S4 in all laboratory experiments and hatchery trials. Larvae treated with the probiotic formulation showed improved survival when experimentally challenged with the pathogen RE22. The study demonstrated that the formulation is safe and effective for use in eastern oyster hatcheries. The applicability

of several *P. inhibens* strains as probiotics for marine aquaculture has been assessed in several studies (Belas et al., 2009; D'Alvise et al., 2012; Grotkjær et al., 2016; Karim et al., 2013; Sonnenschein et al., 2021). However, until now, no suitable formulations have been described for use in hatcheries. As a gram-negative, non-spore forming bacterium, S4 does not survive spray drying procedures commonly used to formulate gram-positive bacteria such as *Bacillus* spp. Our novel approach to formulation of this bacterium takes advantage of prior knowledge in the mechanisms allowing planktonic marine bacteria to survive in the oligotrophic conditions sometimes observed in coastal and oceanic waters. The novelty of the formulated S4 for applications in commercial aquaculture is that it is easily delivered as a live, actively metabolizing bacteria in a liquid medium. Bacteria in the formulation remain highly viable over a period of at least 6 weeks when stored at 4^{o}C. This formulation method differs from other commonly used techniques, such as freeze or spray drying, that put bacteria in a state of dormancy.

The probiotic formulation concept requires that the bacterial strains are not harmful to the growth and yield of cultured larvae. The present study showed that there was no significant difference between fresh S4 and the formulated S4. Both were safe and effective in preventing vibriosis in the oyster larvae. The probiotic formulation did not cause any detrimental effects to the larvae in six trials performed at two different hatcheries, confirming its safety to the larvae at the provided dose. The study also determined that daily treatment of larvae in the hatchery with the S4 formulation did not significantly decrease the level of culturable vibrios in the larvae in any of the hatcheries. These results are consistent with previous studies with other *P. inhibens* strains, showing that probiotic treatment is safe to larvae and does not impact abundance of culturable

vibrios in larvae (Grotkjær et al., 2016; Porsby et al., 2008; Sohn et al., 2016). Interestingly, probiotic treatment did not lead to a significant decrease in the levels of culturable vibrios in larvae. Previously, microbiome analysis performed in a different study showed that *Bacillus pumilus* RI0695 treatment in the hatchery leads to an increase in *Vibrio* diversity and a shift in the composition of the *Vibrio* community to non-pathogenic species, indicating a subtle beneficial effect on larval microbial communities (Stevick et al., 2019). The effect of S4 treatment in the hatchery on microbial composition in these trials is currently being examined to determine if S4 has a similar effect on the *Vibrio* community in treated larvae.

This study also observed that exposure to the probiotic formulation in the hatcheries significantly improved survival of larval oysters when challenged with the pathogen RE22. This confirms results from the previous laboratory and hatchery in vivo challenge assays utilizing freshly cultured S4 administered prophylactically to the oyster larvae (Karim et al., 2013; Sohn et al., 2016). Other *Phaeobacter* spp. have been shown to display a wide range of inhibitory activity against aquaculture pathogens, especially against members of the genus *Vibrio*, which is responsible for most larval mortalities in aquaculture (Kesarcodi-Watson et al., 2012; Planas et al., 2006; Porsby et al., 2008; Prado et al., 2010b; Prol et al., 2009).

Our study also showed that the probiotic treatment did not significantly increase the survival of larvae in any of the hatchery trials, which spanned different environmental conditions and hatchery protocols. Since larval survival was high in most of the trials, and levels of culturable *Vibrio* cells in larvae were low, it was not expected that probiotic treatment would provide a major improvement in survival. However, S4 treatment was not

able to prevent larval losses in the trial performed in July 2019 at VIMS, despite the consistent effect of S4 treatment protecting larvae against challenge with RE22. Most eastern oyster hatcheries try to avoid spawning in July and August, since larval performance is known to be low at this time of the year in most hatcheries due to decreased water quality (personal communications from hatchery managers) and other potential causes such as environmental conditions, adverse physiological conditions, and specific pathogens (Ashton et al., 2020). Also, we observed that S4 treatment did not increase the growth of the larvae in any of the trials, suggesting that probiont S4 may not provide growth enhancement benefits. Other probionts have been shown to provide direct nutritional benefits to the host through increased digestion through the release of digestive enzymes, or as a direct nutritional source, competition for space with pathogenic bacteria, production of antimicrobial compounds, host immunomodulation, and modulation of host microbial community (Bagheri et al., 2008; Campa-Córdova et al., 2009; Hamdan et al., 2016; Modak & Gomez-Chiarri, 2020; Tan et al., 2016). It is not known if the effect of S4 on bacterial communities in the hatchery is due to indirect effects on the larval microbiome exerted through the previously antibiotic, quorum quenching, and immunomodulatory actions of S4 (Modak & Gomez-Chiarri, 2020; Zhao et al., 2016, 2019). Additional research is warranted to determine the effect of S4 on the larval microbiome.

As seen in previous hatchery experiments with the fresh S4 culture (Sohn et al., 2016) levels of variability in growth, survival and culturable vibrios were seen between tanks within treatment, between trials within a hatchery and between hatcheries. Results from this study show that environmental conditions at the hatchery, mainly temperature and salinity, have a major effect on larval survival and growth, but not necessarily on

probiotic efficacy, as measured by the ability to protect larvae to RE22 experimental challenge. Variability in larval performance between tanks within treatments in a trial could be due to handling and husbandry activities in the hatcheries. The frequent handling of each tank during the frequent drain down effects needed to sort the larvae and maintain water quality likely led to the introduction of slightly different bacterial communities in each tank (Arfken et al., 2021; Asmani et al., 2016; Stevick et al., 2019). Variability in the growth and survival of the larvae between hatcheries could be due to differences in location, culture systems, water filtration methods, feeding methods, spawning events, genetic variations in broodstock and environmental conditions, to mention a few. Despite the variability in environmental conditions and performance between trials and hatcheries, there was consistency in the safety and ability to protect the larvae against bacteria challenge when the probiotic S4 formulation was applied in the hatcheries, suggesting that S4 provides a benefit towards protecting against the effect of *Vibrio coralliilyticus* RE22 infection.

Conclusion

This research provides evidence on the effectiveness of a newly developed approach to formulation of marine gram-negative bacteria for use as probiotics in aquaculture. This formulation approach may be useful for developing formulations of other probionts, especially gram-negative bacteria for use in marine aquaculture. The S4 formulation was shown to be safe, easy to handle, and stable to use in the hatchery environment, and it may help manage the impact of vibriosis when used prophylactically in oyster hatcheries, although it may not offer protection against other largely

uncharacterized causes of larval mortality seen in summer months. Future research should focus on identifying the effect of S4 formulation on the microbial community of larvae, water and rearing tanks in the hatchery and combining the use of S4 with other candidate probionts or management methods to provide additional benefits to the larvae and/or prevent other causes of larval mortality.

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Figures and Tables

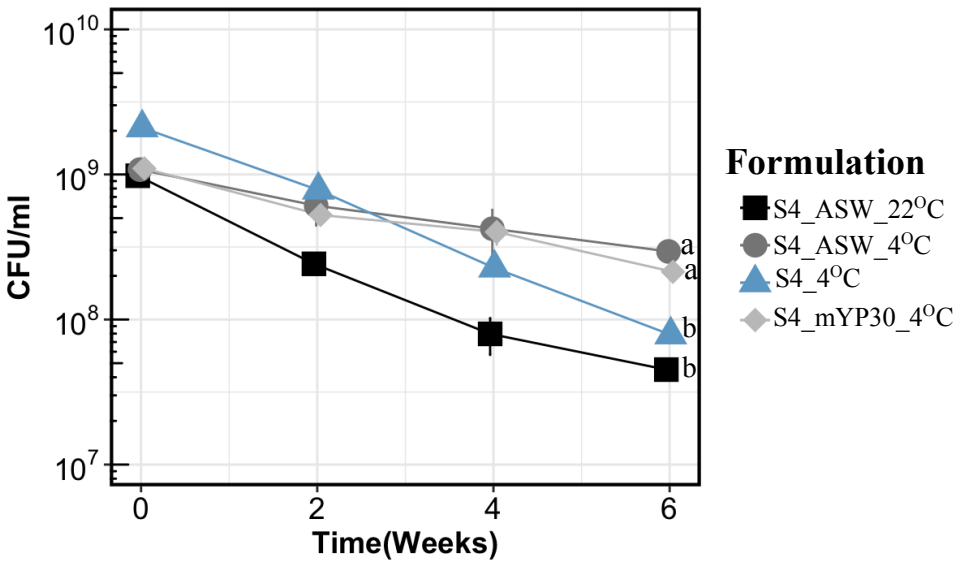

Figure II-1. Impact of formulation method on the viability of S4. Bacterial cultures were stored for 6 weeks in four different conditions and sampled biweekly. Data expressed as mean ± SD of CFU/mL of S4, ASW: Artificial Seawater; mYP30: yeast peptone agar + 3% sea salt. Different letters indicate statistically significant differences based on Tukey's pairwise comparisons (One Way ANOVA, $p < 0.05$).

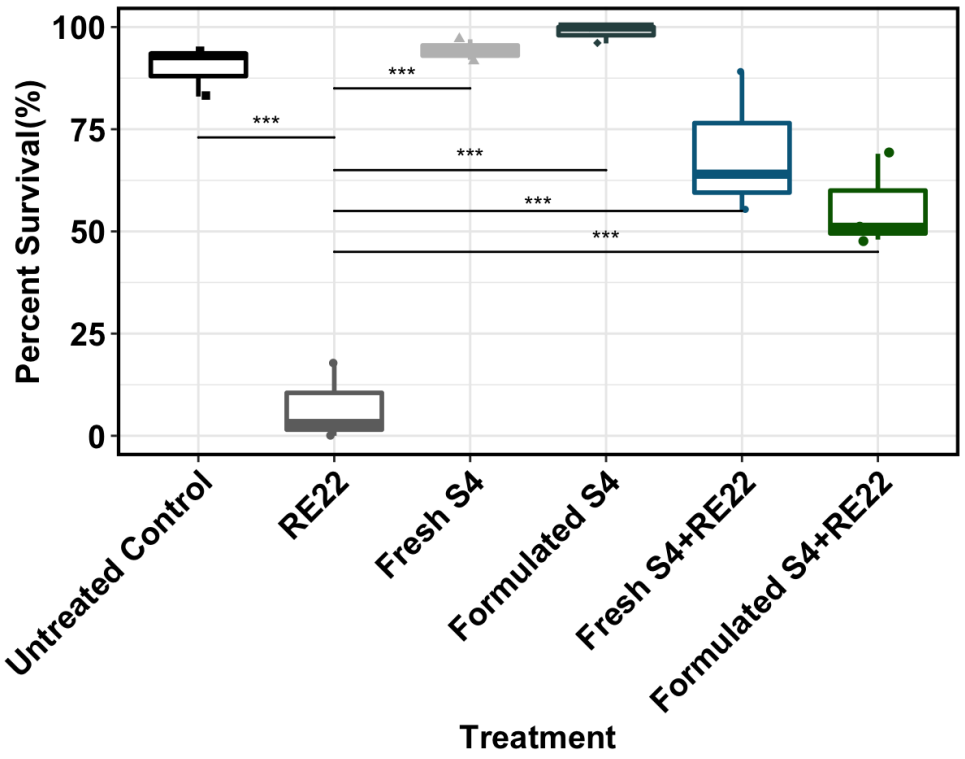

Figure II- 2. Treatment of larvae with S4 formulation in the laboratory led to increased larval survival to a challenge with the pathogen RE22. Effect of pre-incubation of oyster larvae with S4 fresh culture and formulation on survival (% ± SD) after challenge with RE22. Survival was measured 24 h after challenge and 48 h after addition of the probiotic. Fresh S4 = freshly cultured S4; Formulated S4 = S4_mYP30_4ºC; RE22 = *V. coralliilyticus* RE22. *** indicate statistically significant differences between the treatments (One Way ANOVA, $p < 0.05$).

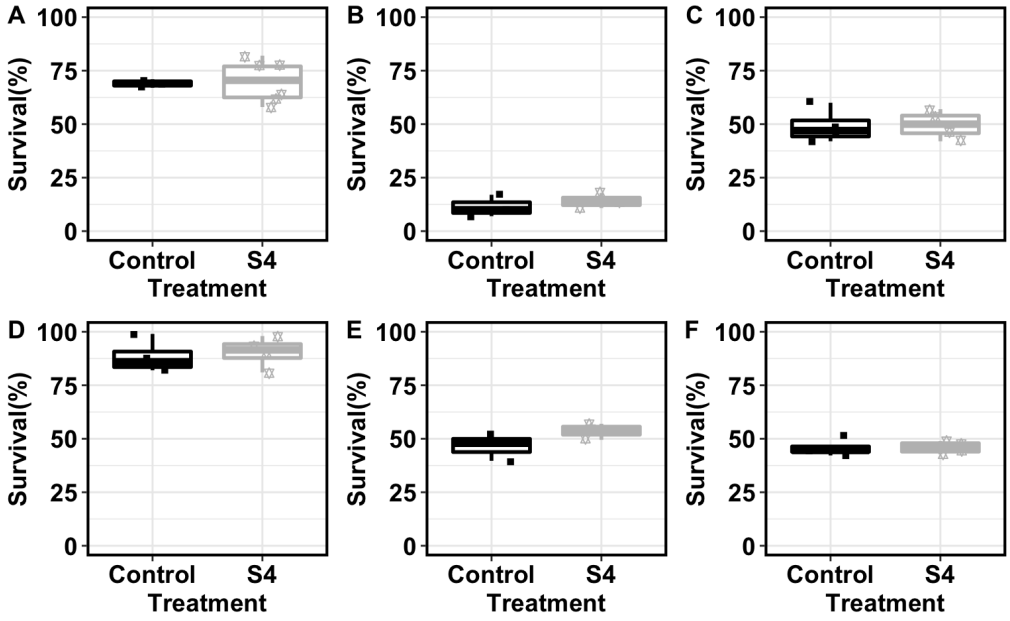

Figure II- 3. Effect of S4 formulation treatment on larval survival at the hatchery. Cumulative Percentage Survival (% ± standard deviation SD) of larval oysters at the end of each trial period is shown. (A)Trial 1; (B)Trial 2; (C)Trial 3; (D)Trial 4; (E)Trial 5; (F)Trial 6. Abbreviations: Control = no probiotic provided; S4 = S4 formulation.

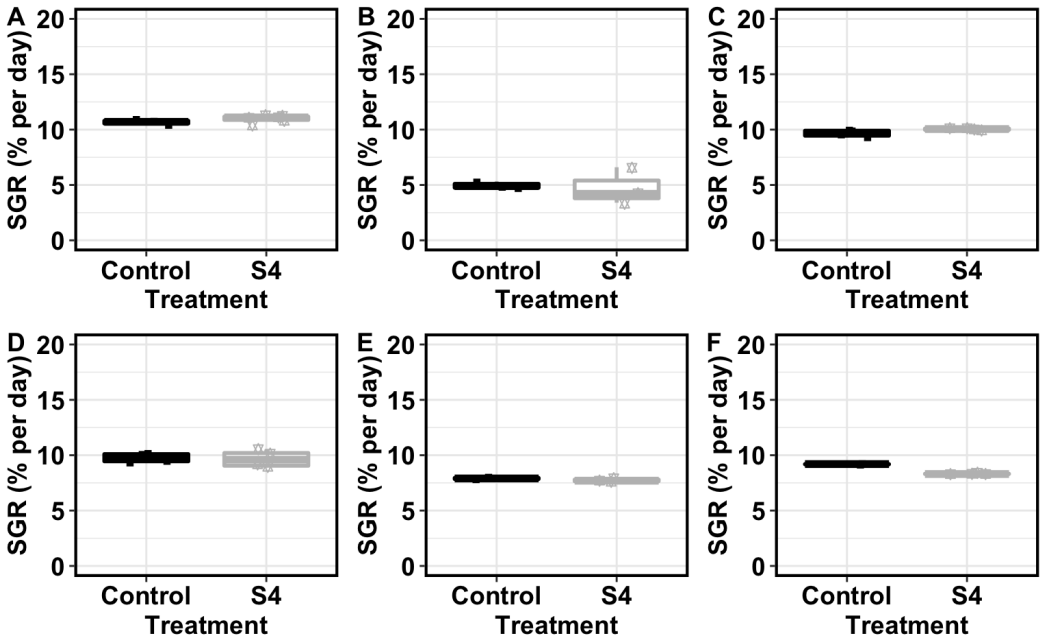

Figure II- 4. Effect of S4 formulation treatment in the hatchery on larval growth. Specific growth rate (%± SD) of larval oysters at the end of each trial is shown. (A) Trial 1; (B) Trial 2; (C) Trial 3; (D) Trial 4; (E) Trial 5; (F) Trial 6. Abbreviations: Control = no probiotic provided; S4 = S4 formulation

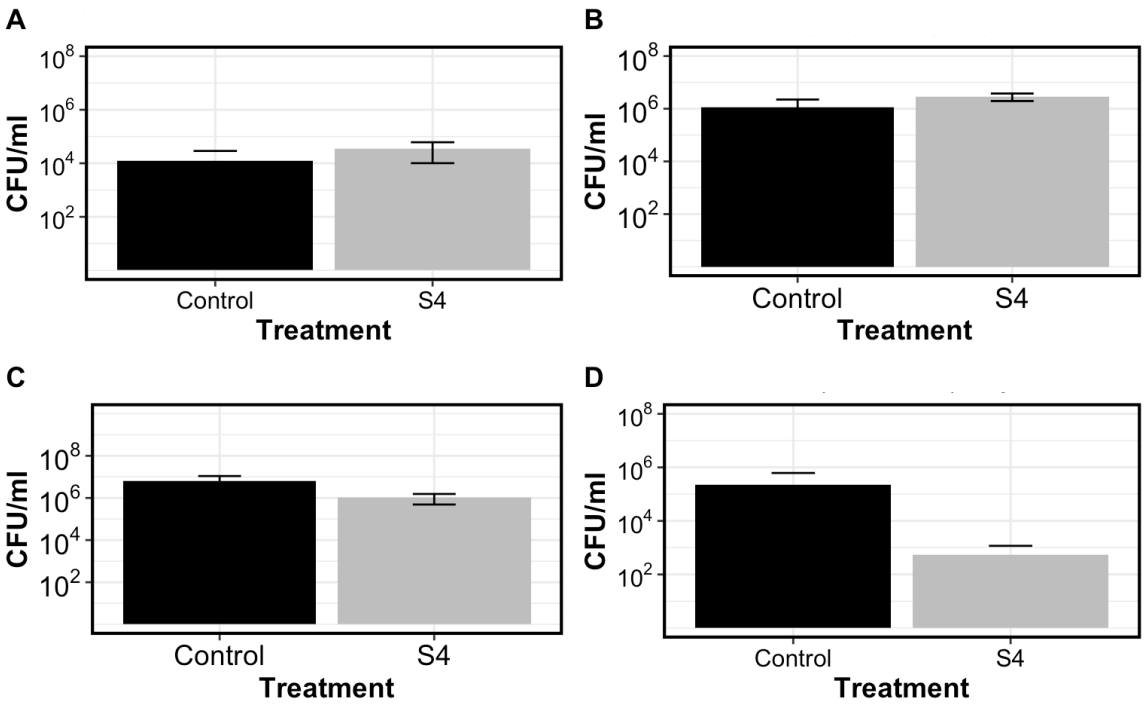

Figure II- 5. Effect of S4 formulation on total culturable *Vibrio* levels in larvae. Vibrio levels (CFU/mL larvae ± SD) in oyster larval samples were measured at the end of: (A) Trial 2; (B) Trial 3;(C) Trial 4 and (E) Trial 6. Abbreviations: Control = no probiotic provided; S4 = S4 formulation

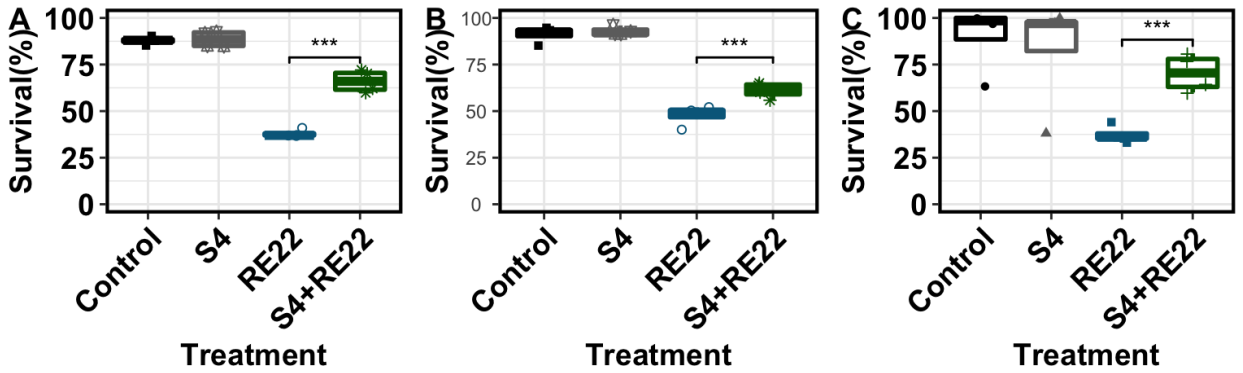

Figure II-6. Effect of S4 formulation treatment in the hatchery on the ability of larvae to survive RE22 challenge. Larvae from each of the hatchery tanks were transported to the laboratory at the end of the trial and exposed to a 24h challenge with RE22 before determining survival. Survival is expressed as % ± SD. Abbreviations: Control = no probiotic provided; S4 = S4 formulation; RE22 = *V. coralliilyticus* RE22. *** indicates statistical significance between the treatments connected by the labeled bracket (One Way ANOVA, $p < 0.05$

TableII-1. Hatchery trials performed in this study. Abbreviations: Control = no probiotic provided; S4 = *P. inhibens* S4 formulation added at 10^4 CFU/mL. VIMS = Virginia Institute of Marine Sciences, MOOK = Mook Sea Farms

Trial	Hatchery	Treatment Type	Tanks Per Treatment	Treatment Period (Days)	Trial Date
1	VIMS	Control (C), S4	C =3; S4=6	12	June 2019
2	VIMS	Control (C), S4	C =3; S4=3	6	July 2019
3	VIMS	Control (C), S4	C =4; S4=4	14	May 2020
4	VIMS	Control (C), S4	C =4; S4=4	7	June 2020
5	MOOK	Control (C), S4	C =1; S4=1 (Static, days 1-8); C=3, S4=3 (flowthrough, days 9-12)	12	January 2021
6	MOOK	Control (C), S4	C =4 ; S4=4	12	June 2021

Supplemental Table

Table S1. Effect of S4 formulation treatment in the hatchery on the ability of larvae to survive a laboratory bacterial challenge with the bacterial pathogen RE22. Data is expressed as average Relative Percent Survival plus/minus standard deviation (SD) (RPS, average % ± SD) of challenged oyster larvae from tanks exposed to probiotics in the hatchery relative to challenged oysters from tanks not exposed to probiotics in the hatchery. Abbreviations: S4 = S4 formulation, VIMS = Virginia Institute of Marine Sciences, MOOK = Mook Sea Farms.

Trial #	Hatchery	Treatment	Relative Percent Survival (RPS, % ± SD)
3	VIMS	S4	54 ± 11
4	VIMS	S4	74 ± 11
6	MOOK	S4	46 ± 19

CHAPTER III: Effect of probiotic treatment on bacterial microbiomes of larval eastern oysters, *Crassostrea virginica,* raised in different hatcheries

By

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Keywords: microbiome, 16S rRNA sequencing, oyster hatchery, probiotics, *Vibrio*, *Crassostrea virginica,* larvae

Abstract

Aquaculture of the eastern oyster (*Crassostrea virginica*) is a rapidly expanding and economically important industry. Probiotics are an alternative strategy for promoting the growth and prevention of diseases in aquaculture. A liquid formulation of marine bacterium *Phaeobacter inhibens* S4 (S4), known to protect eastern larval oysters against challenge with the bacterial pathogen *Vibrio coralliilyticus* RE22, has been developed for commercial use in shellfish hatcheries. This study investigated the effect of the S4 formulation on the microbial communities of oyster larvae by analyzing, using 16S rRNA amplicon sequencing of the V6 hypervariable region, the structure and diversity of larval bacterial communities in eight different trials performed in four different hatcheries. The daily addition of the S4 liquid formulation (10^4 CFU/ml) to *C. virginica* larvae culture tanks daily from day one post-spawning to day eight (veliger stage) caused significant changes in the structure of oyster larval bacterial communities but had no effect on bacterial alpha diversity. Larval bacterial communities significantly differed by hatchery, trial, and, to a lesser extent, probiotic treatment. Probiont S4 treatment led to changes in the relative abundances of selected taxa, including ASVs in the *Alteromonas, Pseudomonas*, and *Vibrionaceae,* suggesting species-specific effects of S4 on the larval bacterial community. A better understanding of the effects of probiont S4 on bacterial ecology in hatcheries could be applied to optimize probiont use in hatcheries, maximizing benefits for the commercial culture of eastern oyster larvae and preventing undesirable side effects.

Introduction

Aquaculture is an important industry, one of the essential sources of food and nutrition, and livelihoods for humans (Chumpol et al., 2017). Shellfish aquaculture occupies a vital position in the world economy and oyster production through aquaculture in the United States totaled 219 million dollars (USD) in 2018 (NOAA Fisheries, 2019). One of the major drawbacks in oyster production is disease outbreaks and mortality caused by infections, particularly from the genus *Vibrio*. These outbreaks affect aquaculture production and pose a health threat to both the oyster and the consumer (Dubert et al., 2017; King et al., 2019; Yeh et al., 2020). Therefore, there is the need to develop prophylactic and therapeutic methods of managing bacterial pathogens in the hatchery. Probiotics offer an alternative means to antibiotics by which pathogens can be reduced in aquaculture systems. These live microbial supplements include beneficial bacteria that improve host health and reduce diseases when administered alive and at adequate concentrations (FAO/WHO, 2006; Kesarcodi-Watson et al., 2012; Prado et al., 2010).

The marine bacterium *Phaeobacter inhibens* S4 (S4) is a gram-negative alpha-Proteobacterium in the Rhodobacter clade. Previous laboratory and hatchery experiments have shown the probiotic ability of S4 and characterized several of the mechanisms leading to protection of eastern oyster, *Crassostrea virginica,* against mortality from the bacterial pathogen *Vibrio coralliilyticus* RE22 (RE22) (Karim et al., 2013; Modak & Gomez-Chiarri, 2020; Sohn, 2016; Zhao et al., 2016, 2019). A liquid formulation of probiont S4 has been developed and tested in trials performed in a variety of shellfish hatcheries. These trials demonstrated that daily treatment of larvae in the hatchery with the S4 formulation consistently leads to protection against challenge with the pathogen RE22 (Takyi et al., Chapter 2 of this dissertation).

The bacterial microbiota performs a variety of beneficial functions to the host, such as providing nutrition, influencing immune responses, reducing or preventing detrimental microorganisms from proliferating and causing disease by creating competition for nutrients, reducing space for pathogen settlement, and producing antimicrobials (Castro et al., 2002; Gomez-Gil et al., 2000; Kesarcodi-Watson et al., 2012; Prado et al., 2010; Sanches-Fernandes et al., 2021; Schulze et al., 2006; Sonnenschein et al., 2021). Therefore, changes in the host microbiota can influence the health of their hosts (Ross et al., 2010; Le Roux et al., 2016). Probiotics can act by directly (e.g., by antibiosis) or indirectly (e.g., through immunomodulation) to target other members of the microbial community in a system, thus influencing host microbiomes and host-microbial interactions (Karim et al., 2013; Modak & Gomez-Chiarri, 2020; Sohn et al., 2016; Stevick et al., 2019).

The impacts of probiotics on marine microbes have been studied in various marine hosts, and some studies have shown that probiotics can alter the microbial community composition to promote host health (Sánchez et al., 2017). For example, juvenile Kumamoto oysters (*Crassostrea sikamea)* treated with *Streptomyces* N7 and RL8 showed increased species diversity and changes in the relative abundances of bacterial taxa, compared to control oysters (García Bernal et al., 2017). Restrepo et al. (2021) showed that the probiont *Vibrio diabolicus* ILI maintained a healthy microbial community in the shrimp gastrointestinal tract after being challenged with the AHPND pathogen. *Phaeobacter inhibens* DSM7151 caused changes in the microbiome structure of microalgae *Emiliania huxleyi* and minor changes in the community structure of the European flat oyster (Dittmann et al., 2019), while other work by Majzoub et al., (2019)

showed that *P. inhibens strain* 2.10 does not necessarily influence bacteria community assembly on microalgae *T. rotula*. Stevick et al. (2019) showed that adding the probiotic *Bacillus pumilus* RI106-95 to eastern oyster larvae resulted in subtle changes in the bacterial community of larvae and water.

Since bacterial communities associated with the host may have impacts on host function and probiotics can have broad or targeted effects on these microbial communities, it is important to assess the impact of delivery of probiont S4 to oyster larvae in the hatchery on bacterial communities associated with the larval host. This study characterizes: 1) the bacterial community of eastern oyster larvae grown in different hatcheries; and 2) how this bacterial community changes following daily treatment with probiont S4. Based on previous studies, we hypothesized that probiont S4 influences the bacterial community structure in oyster larvae by targeting particular community members. The research will provide knowledge on how the probiont may alter the larval oyster bacterial community, and determine if there is a potential for dysbiosis. It will also provide insights into mechanisms of action of probiont S4 in the hatchery environment, information that could be used in the future to optimize and facilitate probiotic use as a disease management tool in shellfish hatcheries.

Methods

Hatchery trials: Experimental Design and Sample Collection

Bacterial community structure was studied using larval samples collected from 6 hatchery trials (reported in Takyi et al. Chapter 2 of this dissertation) and two additional trials performed at the Blount Shellfish Hatchery at Roger William University (RWU,

Rhode Island), and the Matunuck Oyster hatchery (MAT, Rhode Island) (Table 1). Briefly, at VIMS, four independent trials (Trials 1- 4) were conducted using 60 L conical larval rearing tanks. Tanks (minimum of 3 per treatment) were randomly assigned to the following treatments: no probiotics (control) or S4 formulation (probiotic treatment). In Trial 5, conducted at Matunuck (MAT), larvae were raised in two 60 L conical tanks per treatment (control or S4). In Trial 6, conducted at MOOK, larvae were raised in two single 3000 L static tanks (one control, one treated with S4). In Trial 7 at MOOK, subsets of larvae produced in the 3000 L tanks were raised in 4 x15 L buckets per treatment (C, S4), to allow for replication. In Trial 8, conducted at RWU, two types of water treatment systems were tested: one system used UV-treated water (resident time of 6 days) and the other used non-treated water. For each of the water treatment groups (UV and non-UV), larvae were raised in triplicate 60 L conical tanks per treatment (C, S4).

In all trials, a formulation of probiont S4 (Takyi et al. Chapter 2) was added daily at a dose of 10^4 CFU/mL at the time of algal feeding from day 1 (24 hr after spawning) until the termination of the trial. Larval tanks were drained down every other day for size grading of larvae and maintenance of water quality (Helm et al., 2004). Oyster larvae were collected at selected dates (Table 1) on a 40 μm sieve after drain-down of tank water, placed into a sterile tube, and stored at −80°C until DNA extraction.

DNA Extraction, Amplification, and Sequencing

Bacterial DNA was extracted from the oyster larvae using the PowerSoil DNA Isolation Kit (Qiagen) following the manufacturer's protocol. DNA concentration was quantified with a Nanodrop 2000 instrument and a Qubit Fluorometer (Thermo Fisher

Scientific, Wilmington, DE, United States). 16S rRNA gene amplification was performed with 967F/1064R primers to amplify the V6 hypervariable region of the 16S rRNA gene (Stevick et al. 2019). PCR reactions were performed following Illumina's 16S Sequencing Library Preparation Protocol, and amplicons were sequenced using a 2x100 paired-end sequencing on an Illumina MiSeq at the Genomics and Sequencing Center at the University of Rhode Island. Negative and positive controls were included following the recommendations of the Earth Microbiome (Lin & Peddada, 2020).

Amplicon sequence analysis

Demultiplexed read pairs from the MiSeq runs were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software version 2019.7 (Bolyen et al., 2019). First, the raw reads were imported using the *qiime tools* import function, and the read quality was inspected for the selection of trimming parameters using the *qiime demux* summarize function. Next, the DADA2 plugin was used for quality filtering, merging, de-noising, and chimera detection. The *qiime dada2* denoise-paired function trimming the forward and reverse reads at 19 bp at the 5′ end (Callahan et al., 2016). This step led to generating a count table that maps the occurrence of ASVs in each sample. Next, the taxonomic assignment was performed with QIIME2's sklearn classifier mapping to the SILVA database release 132 (Quast et al., 2013). Based on the SILVA taxonomic assignments, mitochondrial and chloroplast sequences were removed from the ASV count table. The resulting ASV count table and taxonomy data were exported and analyzed in R version 4.0.2 (Martin, 2021). The taxonomic assignments were used to validate the identification of all the bacterial species in the ZymoBIOMICS Microbial Community Standard sample (positive control).

Statistical Analysis

Chao1 and Simpson alpha diversity indices were computed based on the ASV count table with the diversity function in the *vegan* package version 2.5-6 (Oksanen et al., 2020). Statistical significance testing of differences in bacterial richness and Simpson diversity index comparisons was evaluated using the non-parametric Kruskal–Wallis rank-sum test in R. Non-metric dimensional analysis (NMDS) was used to determine the influence of hatchery, location, trial, season, year, and probiotic treatment on the bacterial community composition implemented using the *vegan* package (Torondel et al., 2016; Oksanen et al., 2020). The Bray-Curtis dissimilarity metric was calculated with k=2 for max 50 iterations, and 95% confidence intervals (standard deviation) were plotted. Statistical testing of the beta-diversity was done using the *adonis2* test implemented in *vegan* (Warton et al., 2012; Oksanen et al., 2020). Additionally, relative percent abundances of specific taxa were extracted and plotted according to hatchery, trial and treatment. Differential abundance of taxa was evaluated among the trials and between treatments using the linear discriminant (LDA) effect size (LEFSe) analysis method to determine taxa that are significantly enriched in each trial, treated larvae and untreated controls (Segata et al., 2011; Lin & Peddada, 2020).

Results

Dataset overview

The impact of *P. inhibens* S4 on the bacterial communities of larvae was determined by sequencing 16S rRNA gene V6-region amplicons and analyzing their taxonomic composition and diversity. A total of 8,919,989 quality-controlled 16S rRNA

gene amplicon sequences from the V6 hypervariable region were obtained from 167 total DNA samples of pooled larval samples from eight trials carried out in four different hatcheries (Table III-1). The reads for each sample ranged between 12,883 – 168,084 reads and clustered into 3,357 ASVs, 30 Phyla, and 129 Orders. Rarefaction analysis was performed to determine if all the diversity present in the data had been sufficiently recovered. The rarefaction curves for each sample in the treatment and control showed saturation, the sequencing depth was enough to cover the bacterial diversity present in the larval samples (Figure III-S1). The sample with the lowest number of bacterial reads was at 10,000 reads.

Daily probiotic treatment in the hatchery had no significant impact on the diversity of the bacterial communities in oyster larvae

Alpha diversity analyses were performed on rarefied sequence reads (10,000 reads per sample) to determine whether treating the larvae with S4 formulation could alter bacterial richness and diversity at the ASV level in the oyster larvae. Bacterial richness calculated with the Chao1 index (measures the number of species in a bacterial community) ranged from 103 ± 29 ASVs to 266 ± 36 ASVs and bacterial diversity calculated with Simpson's diversity Index (a combined measure of the number of species present and the relative abundance of each species) ranged from 0.87 ± 0.10 to 0.94 ± 0.01. Results showed no significant difference in the bacterial richness and diversity between control and treated larvae in each trial (Figure III-1 one-way ANOVA followed by Tukey's pairwise tests with $p > 0.05$). However, bacterial richness and diversity significantly differed between trials (one-way ANOVA, $p < 0.05$).

Bacterial community structure in oyster larvae differed mainly by region and hatchery, but significant effects of trial, year, and treatment were also detected.

We used multivariate statistics to identify factors that influenced bacterial community composition in oyster larvae in hatcheries. Community structure analyses were based on the Bray Curtis dissimilarity index. Differences in β-diversity between larval samples were visualized using NMDS plots (Figure III-3) and analyzed using PERMANOVA (Table III-S1). Larval bacterial communities were analyzed separately for the effects of location/region (Figure III-2A), hatchery (Figure III-2B), trial (Figure III-2C), season (Figure III-2D), year (Figure III-2E), and probiotic treatment (Figure III-2F). The largest and most significant impact on the differences in the community was driven by location, hatchery, and trial, with hatchery showing the most variation (Figure III-3 A – C and Supplementary Figure III-S5). The effect of season, year, and probiotic treatment was significant but explained slight variation in larval bacterial communities when all trials are considered together, due to the large levels of variability between hatcheries and trials (Figure III-3 D – F, Supplementary Table III-S1). However, when examining each trial independently, there was a significant difference between the structures of larval oyster bacterial communities of untreated and S4-treated larvae, which was more evident in some of the trials (e.g. Trial 1; Supplementary Figure S4).

Bacterial communities in oyster larvae from different hatcheries were dominated by *Proteobacteria*

The taxonomy and relative abundances of bacterial taxa in the community composition were assessed at different taxonomic levels (Phylum and Order). The bacterial composition of the larvae at the phylum level primarily consisted of Proteobacteria (80%)

which dominated the bacterial communities in all trials, followed by Firmicutes (8%) and Bacteriodetes (2%) (Figure III- S4). At the Order level, out of 3,357ASVs, the majority of ASVs belonged to the 12 most abundant orders, which were *Alteromonadales* (24.8%), *Rhodobacterales* (18.7%), *Vibrionales* (12.8%), and *Oceanospirillales* (4.1%) (Figure III-3).

Venn diagrams (upset plots) were generated to identify and compare bacterial ASVs that were shared and unique between trials. Only 40 out of the 3,357 bacterial ASVs were shared between all larval samples (Figure III-4). These 40 shared ASVs were highly abundant, corresponding to 89% of the total reads retrieved across the larval samples from all trials. The 40 ASVs common between all samples were classified to the most abundant orders: *Rhodobacterales* (10 ASVs), *Alteromonadales* (11 ASVs), unclassified gammaproteobacteria (4 ASVs), and *Oceanospirillales* (3 ASVs). Although these 40 ASVs were present in all trials, their relative abundance differed between trials and treatments (Figure III-3), with some trials being dominated by *Alteromonadales* (e.g. Trial 7 at MOOK), others by *Vibrionales* (Trial 8 RWU-UV, Trial 1 S4), others by *Rhodobacterales* (Trail 8, RWU UV), and others by similar proportions of these orders. Unique ASVs to each trial ranged from 546 (Trial 3, VIMS; 16.3% of all ASVs and 43.7% of the ASVs detected in the trial) to 149 (Trial 2, VIMS; 4.4% of all ASVs and 30% of the ASVs detected in this trial), with most of these trial-unique ASVs being detected in very low abundance.

Probiotic treatment led to subtle changes in bacterial composition in oyster larvae

Given the significant difference in the community structure observed between the control and S4-treated larvae, a linear discriminant analysis effect size (LEfSe) was performed to identify ASVs that consistently (*i.e.* in all trials) differed in relative abundance between untreated and S4-treated larvae. Overall for all trials, 16 ASVs showed differential abundance between untreated controls and S4-treated larvae. Most of these ASVs were present in low abundance, except for ASV18 (*Alteromonas* spp.), which is part of the most abundant and common taxa found in most trials and showed the highest LDA score and relative percentage abundance (Figure III- 5). Ten ASVs were enriched in S4-treated larval samples and classified at the genera level as *Alteromonas, Pseudoalteromonas, Umboniibacter, Psychrobacter, Haemophilus, Flavobacteriaceae, Marinimicrobium* and *Reichenbachiella*. Six ASVs classified as *Bradymonadales,* Unknown Bacteria, *Roseimarinus, Sphingomonas, Marinibacterium* and *Pseudomonas* were decreased in S4-treated larvae relative to the untreated control (Figure III- 5).

Probiotic treatment did not consistently result in significant changes on the *Vibrio* community

Vibrio spp. are naturally found in coastal waters, and some of them can be pathogenic to larval oysters (Vezzulli et al., 2016). Previous research showed that S4 formulation treatment did not affect the concentration of culturable colony vibrios on selective media (Chapter 2 of this dissertation). Therefore, we assessed the effect of probiont S4 treatment on the diversity and abundance of *Vibrio* genera in larvae, given the potential pathogenicity of certain species within these taxa to bivalve larvae. Several (15) ASVs in the larval bacterial community were assigned to the order *Vibrionales*. No

significant difference in *Vibrio* diversity and relative percent abundance, ranging from 0.6% ± 0.4% (Trial 7) to 32.1% ± 15% (Trial 8) were observed between untreated control and S4-treated larvae in any of the trials (Figure III- 6). However, a significant difference in *Vibrio* spp. community structure between untreated and S4-treated was observed in Trial 1 (Figure III- 7), while slight and non-significant changes were observed in other trials (Supplementary Figure III- S7). The resolution of each *Vibrio* ASV to a specific species was not possible due to the short sequence length of the V6 region sequenced in this study.

The influence of UV water treatment on the bacterial community structure of oyster larvae and interactions with probiotic treatment

Hatcheries that rear larval oysters use different methods such as ultraviolet (UV) irradiation to treat water to reduce potential pathogens; however, this process is expensive and could also potentially eliminate beneficial microbes in the water, so implementation in each hatchery is variable (Brown and Russo, 1979). Therefore we assessed the effect of UV treatment on the microbiota of oyster larvae in the hatchery in one of the trials. Results show a significant difference in the larval alpha diversity between the control and treatment larvae in UV-treated water (one-way ANOVA, $p<0.05$) but no significant difference in larval alpha diversity between the control and S4 treatment in non-UV treated water (Figure III- 7; one-way ANOVA, $p>0.05$). The bacterial composition of the larvae significantly differed both by UV and probiotic treatment (adonis2 PERMANOVA). Taxa that significantly differentiated between UV and non-UV treatment included *Clostridiales,* which increased in abundance in non-UV raised larvae. In contrast, *Alteromonadales* and *Vibrionales* were significantly increased in abundance in the UV-treated water. Probiotic

treatment of larvae raised in UV and non-UV treated water led to an increased abundance of *Rhodobacterales* in both water types.

Discussion

Bacterial communities associated with eukaryotes have a significant impact on the health and function of their hosts, and investigating how microbiomes of organisms are affected by external and internal factors has become an area of interest (Lebeis et al., 2015; Lokmer & Mathias Wegner, 2015). Bacteria used as probiotics confer health benefits that may arise from their ability to affect the host's microbiota and help restore microbial balance and other benefits to the host (Maloy et al., 2007). Our extensive characterization of the bacterial communities of oyster larvae grown in 8 trials performed in 4 different hatcheries indicate that: 1) eastern oyster larvae showed diverse and variable bacterial communities that are dominated by Proteobacteria; 2) bacterial community structure in larvae was mainly determined by geographical region and hatchery, and, to a lower extent, by trial and the season/month/year in which the larvae were grown; 3) probiont S4 treatment impacted the larval bacterial community structure by altering the relative abundance of specific taxa in the community, mostly confined to low abundant taxa; 4) raising larvae in UV treated water resulted in a decreased abundance of specific bacterial taxa; and 5)S4 treatment of larvae raised in the UV treated water led to the increase abundance of specific taxa such as *Rhodobacterales* that were abundant in the larvae.

Our results show that *Proteobacteria* was the dominant phyla in the larval composition in all hatcheries. This is consistent with previous studies that showed that *Proteobacteria* makes up the largest and the most diverse Phylum in the oyster-associated microbiota and coastal waters (Arfken et al., 2021; Wang et al., 2021; Dittmann et al.,

2019; Hernández-Zárate & Olmos-Soto, 2006; Stevick et al., 2019; Trabal Fernández et al., 2014; Wegner et al., 2013; King et al., 2019; Pierce and Ward, 2019; Yeh et al., 2020).

Oyster larvae showed a wide range of variation in their bacterial composition, primarily due to region/geographical location (VA, RI, ME), hatchery/facility (differences were seen in the two hatcheries in RI), and, to a lesser extent, trial (variability was observed between trials performed in the same hatchery for VIMS and MOOK) and time (season/year) (Figure III-4, Table III-S1). Some potential factors that could be responsible for variability between the hatcheries include differences in bacterial composition of the incoming seawater, water filtration methods, feeding protocols, and larval handling methods, which all can contribute to the uniqueness of individual hatchery operations, directly or indirectly impacting the bacteria associated with the larvae. The temporal variability seen in trials performed at the same hatchery may be attributed to seasonal and yearly changes in community structure of the incoming water (Arfken et al., 2021; Asmani et al., 2016), environmental effects on the host-microbe interactions, and differences between trials on host genetics, and physiology/health status of the larvae (Sakowski, 2015; Schultz et al., 2003). Variability in the bacterial composition within tanks and between replicate tanks within treatments is consistent with past studies and is most probably driven by variability in husbandry occurring at the hatchery (e.g., differences in thoroughness in tank cleaning/rinsing, or introduction of microbes into individual tanks due to splashing or handling) or minor variations in environmental parameters due to tank positional effects (King et al., 2012; Stevick et al., 2019; Wegner et al., 2013).

Despite the high variability observed in the bacterial community between trials, 40 ASVs were shared across all the oyster larval samples. These 40 ASVs may be considered

part of a core larval microbiome. This is comparable with findings in adult *C. virginica,* where the gut and stomach core bacterial community members were limited to 5 and 44 OTUs (King et al., 2012). This core microbiome is relatively small as compared to the size of the bacterial community, which ranged between 149 and 546 ASVs depending on the trial. Nevertheless, while the number of core taxa were small, most of the 40 ASVs comprising the core represent a higher percentage in abundance in the bacterial communities. Most of the core taxa identified in this study are commonly found in the marine environment and have been previously identified in other oyster microbiome studies (Arfken et al., 2021; Fuhrman et al., 2006; Logares et al., 2014; Pierce & Ward, 2018). The most abundant core ASVs in larval eastern oysters belonged to the Orders *Rhodobacterales* and *Alteromonadales*, which form the majority of taxa in the community. The relatively higher abundance of these taxa in larvae may suggest a role in larval development and physiology and/or a particular ability of those taxa to colonize and survive in the host. Generally, it is known that *Rhodobacters* are indigenous to molluscan species and are rapid primary surface colonizers (Dang et al., 2008; Martens et al., 2006; Prado et al., 2009). They are abundant in phytoplankton cultures used in bivalve larvae feed (Nicolas et al., 2004), and others, specifically from the genera *Phaeobacter* have been isolated from the hatchery environment (D'Alvise et al., 2012; Grotkjær et al., 2016; Kesarcodi-Watson et al., 2012), and the inner surface of the shell of adult (for probiont S4; Karim et al., 2013) or juvenile oysters (for *Alliiroseovarius crassostreae,* the pathogen responsible of Juvenile or Roseovarius Oyster Disease; Boettcher et al., 2000; Gómez-León et al., 2008). An ability to colonize and survive in specific niches in the feed and/or in the oyster host (i.e*.,* phytoplankton feed and the oyster shell or the gut) may explain the

dominance of *Rhodobacteraceae* as a dominant family in the core larval bacterial communities. *Alteromonadales* have been reported to play a role in oyster larvae development and degrade the microalgae used to feed the larvae (Laroche et al., 2008). The other most abundant taxa shared by the larvae were *Oceanospirillales* and unclassified *Gammaproteobacteria*. *Oceanospirillales* are heterotrophs commonly associated with mollusks, mostly found in the gills of many bivalves, and are recognized for their ability to degrade organic compounds in the environment (Beinart et al., 2014; Costa et al., 2012; Jensen et al., 2010; Zurel et al., 2011). Other taxa include *Pseudomonadales*, *Vibrionales*, *Nitrosococcales*, *Gammaproteobacteria Incertae Sedis*.

Despite the variability in the larval bacterial communities between hatcheries, this study showed that probiont S4 subtly, but significantly, affected the larval microbiome. Treatment of larvae in the hatchery did not alter the richness and diversity of bacterial communities in any of the trials, which is consistent with previous studies that found no effect of other probionts, such as *P. inhibens* DSM17395 and *Bacillus pumilus* RI06-95, on bacterial diversity in their respective hosts, the flat adult oyster *O. edulis* (Dittmann et al. (2019) and *C. virginica* larvae (Stevick et al. 2019). As also seen with these other probionts used in aquaculture systems, treatment of larvae with probiont S4 led to subtle and targeted changes in bacterial communities in their hosts (Dittmann et al., 2019, 2020; Liu et al., 2015; Stevick et al. 2019; Boutin et al., 2013; Laursen et al., 2017; Merrifield & Carnevali, 2014; Schmidt et al., 2017; Standen et al., 2015). Results from abundant differential testing at the ASV level between the untreated control and treated larvae identified ASVs contributing to the differences in community composition. Considering all trials, two ASVs in the core microbiome in the genera *Alteromonas* significantly

increased in abundance in the S4 treated larvae compared to the control larvae, and *Pseudomonas* decreased in abundance in the treated larvae relative to the control. Targeted changes in these ASVs may contribute to the probiotic effect of S4 on oyster larvae. Previous studies have shown that some members of the *Alteromonas* genus produce compounds that control harmful algal blooms in aquaculture (Cho, 2012). *Alteromonas macleodii* has been demonstrated to protect oyster larvae against the pathogens *V. coralliilyticus* and *V. pectenicida* (Kesarcodi-Watson et al., 2012; Pathak et al., 2021; Schulze et al., 2006). Other *Alteromonas* have also been shown to play roles in oyster development and production of polysaccharides (Concórdio-Reis et al., 2021). *Pseudoalteromonas* have also been shown to be persistent in the hemolymph of adult oysters and likely contribute to defense against pathogenic infections (Vezzulli et al., 2018). The increase in abundance of these taxa in S4 treated larvae could potentially benefit the larvae. *Pseudomonas* (which decrease in relative abundance in response to S4 treatment) have been identified in this study and other studies as one of the predominant species in oysters (Pathak et al., 2021). However, many *Pseudomonas* species are considered opportunistic pathogens and have been reported to cause fish mortality in aquaculture (Altinok et al., 2006; Kusuda & Toyoshima, 1976; Miyazaki et al., 1984; Pathak et al., 2021). On the other hand, other species of *Pseudomonas* perform biodegradation of oil hydrocarbons (Looper et al., 2013; Pathak et al., 2021) and demonstrated probiotic activity to suppress fish bacterial pathogens in aquaculture (Liu et al., 2015). Further studies are needed to identify these species of *Alteromona*s and *Pseudomonas,* and elucidate their interactions with the probiotic S4 and the larval host.

Despite the well documented *in vitro* effect of probiont S4 and other *Phaeobacter* spp. characterized as probionts species on several pathogenic *Vibrio* spp., including *V. coralliilyticus, V. anguillarum,* and *V. parahaemolyticus* (Karim et al., 2013;D'Alvise et al., 2012), there was no consistent impact of S4 treatment on the diversity of *Vibrio* spp. in the hatchery. As described in a companion manuscript reporting the effect of S4 treatment in the hatchery on larval performance, S4 treatment did not affect the concentration of culturable colony vibrios on selective media (Takyi et al., Chapter 2). Although S4 did not affect the diversity or relative abundance of *Vibrio* species in the larval bacterial community, differences in *Vibrio* ASV composition were observed in treated larvae. The extent of this effect differed between trials, with the most significant effect being detected in Trial 1, the trial that had the longest duration of S4 treatment before larvae were sampled for microbiome analysis (12 days versus 6 – 8 as compared to all other trials). A previous study showed that oyster larval treatment with probiotic RI06-95 in the hatchery resulted in changes in the Vibrio-specific species in the community by day 12 of the trial, with probiotic treatment leading to a shift from potentially pathogenic to non-pathogenic species (Stevick et al., 2019). Unfortunately, *Vibrio* species in the bacterial community could not be characterized beyond the genus level in our study due to the limit of the length of sequencing fragments used in this study. Therefore more research is needed to identify the specific *Vibrio* species in the community using a more targeted approach using more informative genes.

Conclusion

The use of probiotics in aquaculture is a practical alternative to promote animal health and prevent disease. We characterized the effect of the S4 formulation on the

bacterial communities in the larvae. Our results show a strong impact of the hatchery, location, and trial on the bacterial communities of the larvae probiotic S4 treatment led to slight changes in specific bacteria taxa. This indicates that the effect of larval treatment in the hatchery with probiotics S4 may not cause any significant perturbations in the bacterial communities alongside the beneficial impact of the oyster larvae. This study significantly advanced our understanding of the role of probiotic S4 in oyster larvae-associated bacteria communities in the hatchery and revealed potential members that could be affected by probiotic S4.

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Figures and Tables

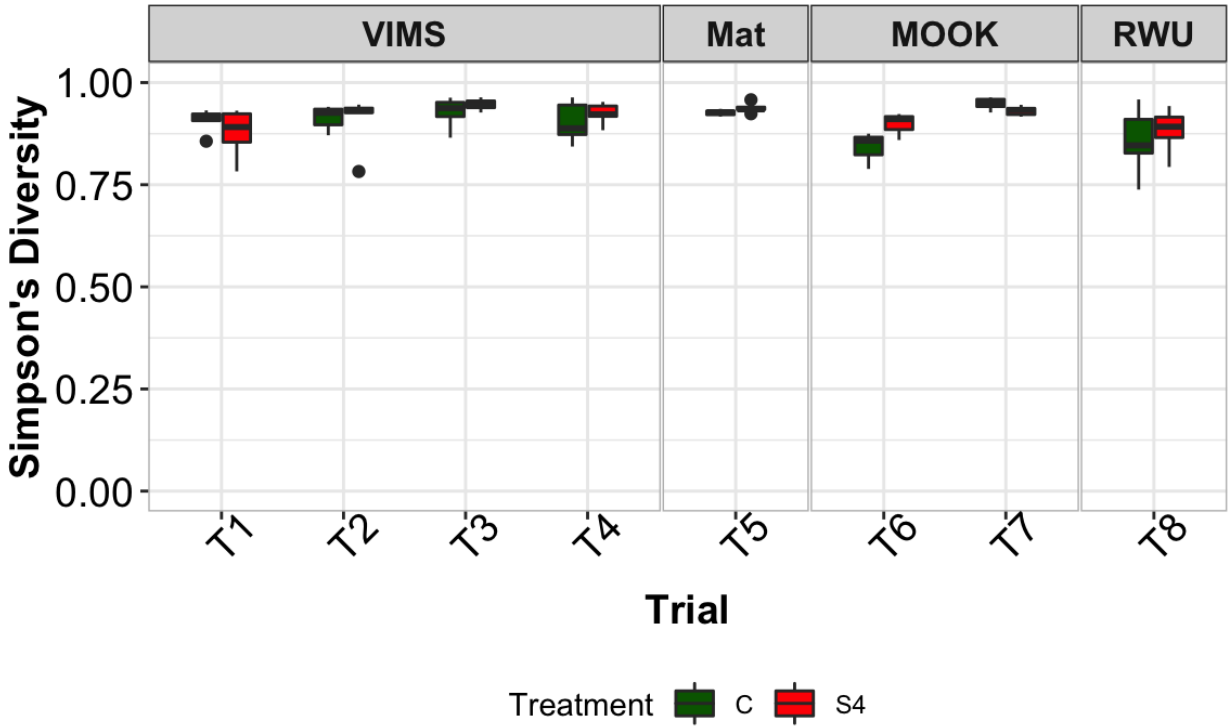

Figure III- 1.**Effect of probiotic treatment on bacterial diversity in oyster larvae**. Average and standard deviation of Simpson's index of the diversity of bacterial communities in samples of larvae from n = 2 - 4 tanks per treatment (C is control, untreated larvae; S4 is larvae treated with *P inhibens* S4 formulation) are shown for each trial. No significant differences in diversity were found between treatments within trials. Trial (T) names are designated T1-T8, VIMS: Virginia Institute of Marine Sciences; Mat = Matunuck; MOOK = Mook Sea Farms; RWU=Roger Williams University.

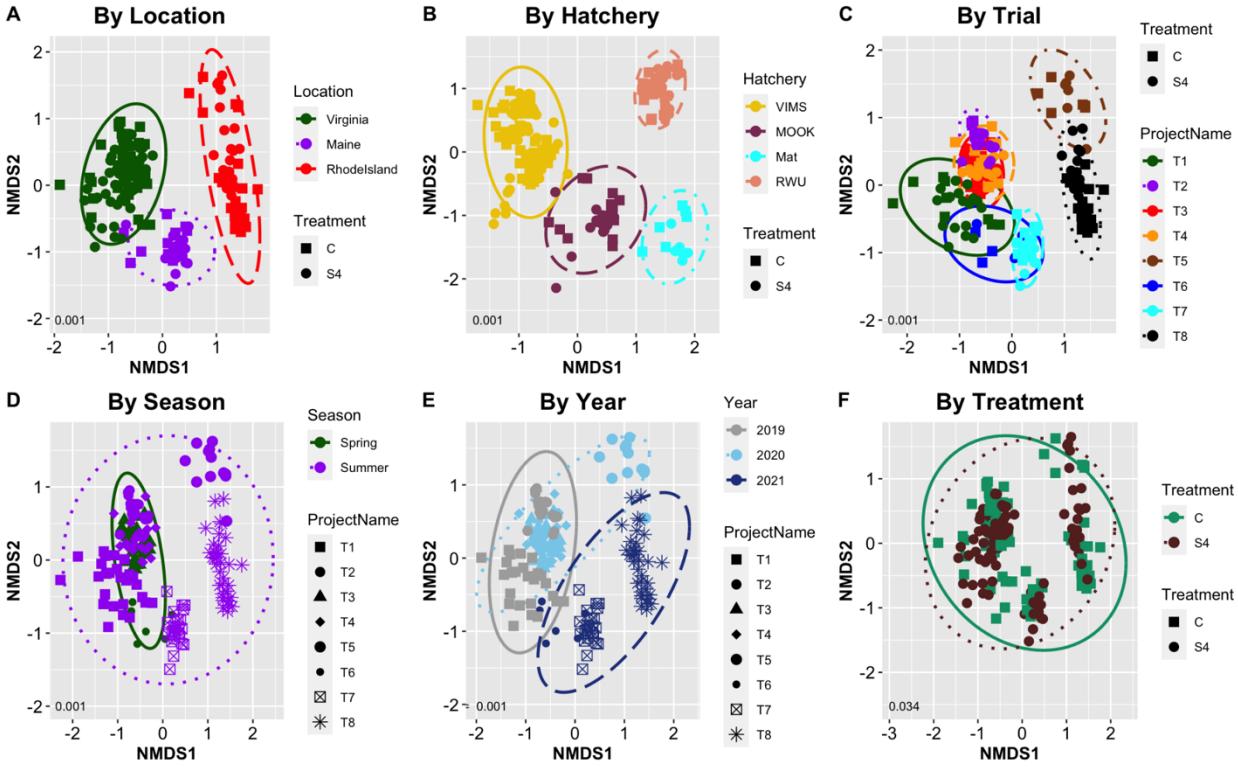

Figure III-2. Diversity in the structure of bacterial communities in larvae from different hatcheries. Non-metric multidimensional scaling (NMDS) visualization of Bray-Curtis distance (an index of beta-diversity) at the ASV level, grouped by (A) location, (B) hatchery, (C) Trial, (D) Season, (E) Year, (F) Treatment. Each dot represents the bacterial community in 3 larval pools from a single tank. Ellipse lines show the 95% confidence interval. P-values indicate the significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test (PERMANOVA). Larval oyster bacterial communities showed significant differences in composition due to location, hatchery. T1-T8: Trials 1 to 8. C: control, S4: treated daily with probiotic

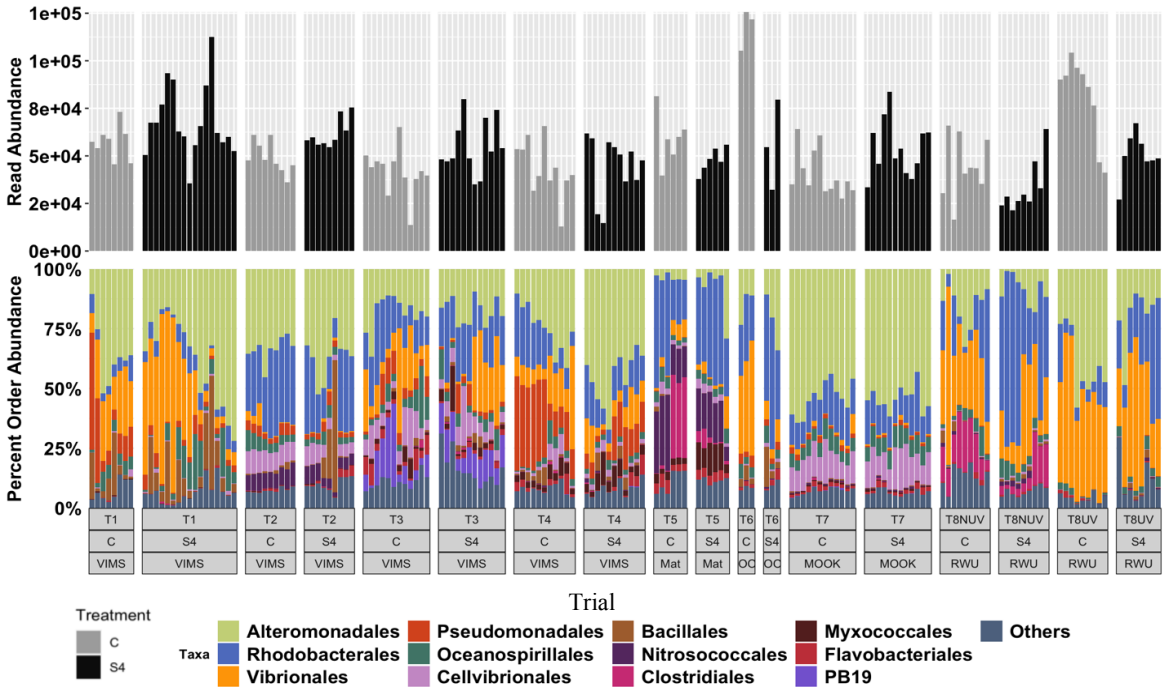

Figure III-3. Taxonomic composition of bacterial communities in oyster larvae from different hatcheries. Percent abundances of the 12 most abundant taxa (Order level) in oyster larvae from all trials and hatcheries based on 16S rRNA amplicon sequencing data. Each column represents a single larval pool, and 3 pools were collected from each tank, with the number of tanks per treatment ranging from 1 (Trial 6) to 4 (Trial 1). Trials are designated T1-T8. VIMS = Virginia Institute of Marine Sciences, Mat = Matunuck, MOOK = Mook Sea Farms, RWU=Roger Williams University. NUV=nonUV treated water, UV= UV treated water.

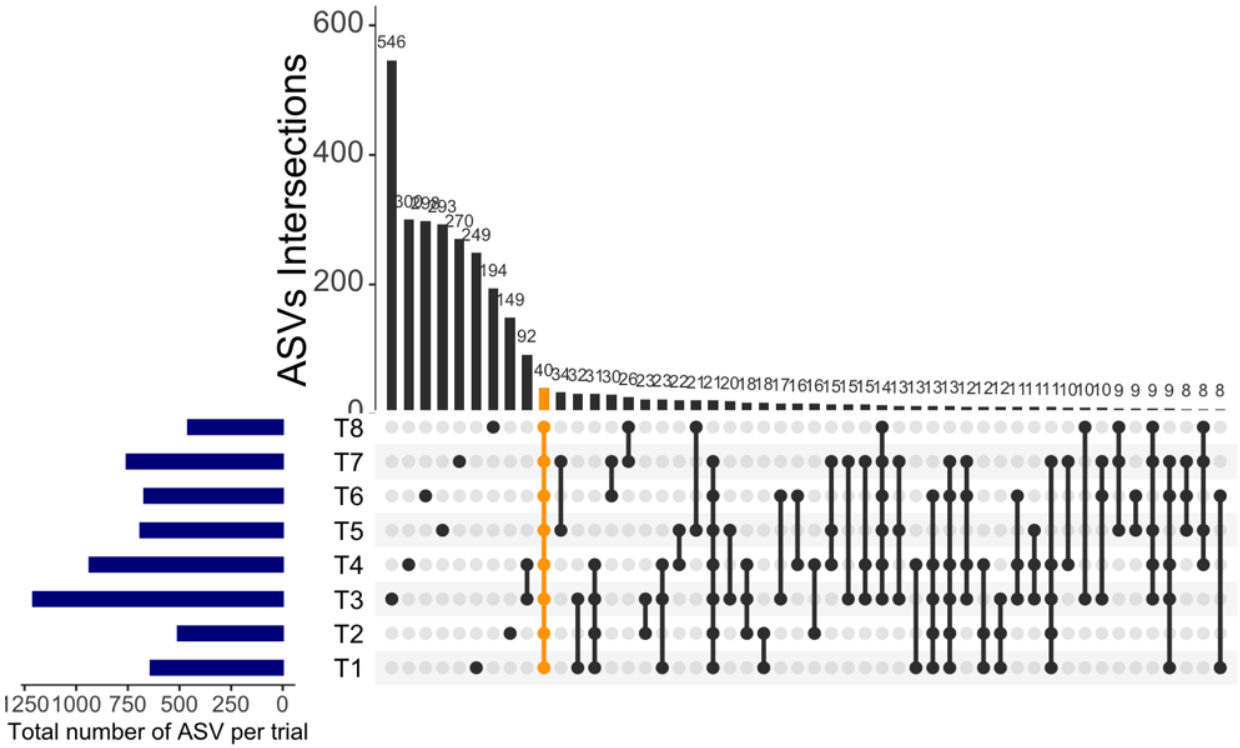

Figure III-4. Shared and unique bacterial ASVs in larvae from the between hatchery trials. The number of bacterial ASVs unique and shared between the oyster larvae from all trials is shown in this UpSet plot by vertical bars (top). The total number of ASVs found in each trial is shown on the horizontal bar graph on the left (in blue). Intersections denote comparisons between the trials. The highlighted bar and intersection (in orange) show the number of ASVs (40) shared between all trials.

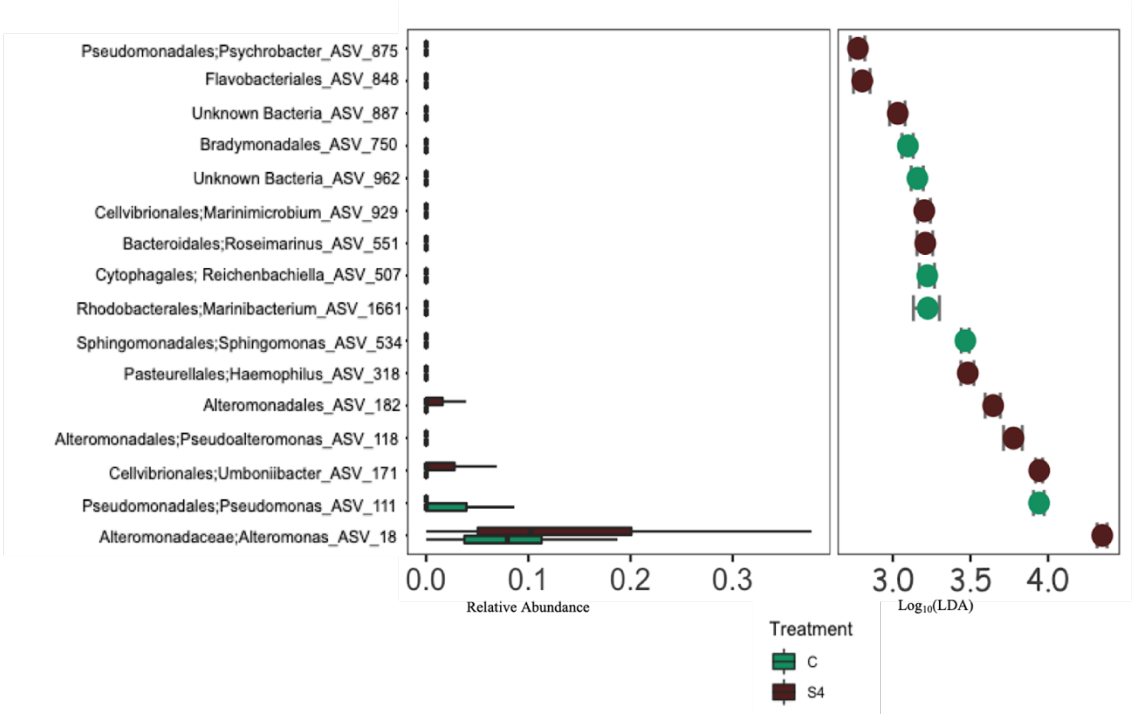

Figure III- 5. Bacterial ASVs showing significant differences in relative abundance between probiont S4-treated and untreated larvae. A linear discriminant analysis effect size (LEfSe) was conducted to determine the significant differences in the abundance of ASVs between controls and treated larvae in all trials. The x-axis shows the LDA scores, representing the degree of differences in the relative abundance of ASVs between control and treatment expressed in logarithmic scale. The y-axis shows ASVs whose relative abundance was affected by S4 treatment, colored by the treatment they are most enriched in (plot to the right). The plot to the left shows the relative abundances of the differentially abundant taxa between the control and treatment. ASV_18 and ASV_111 are commonly found in all trials.

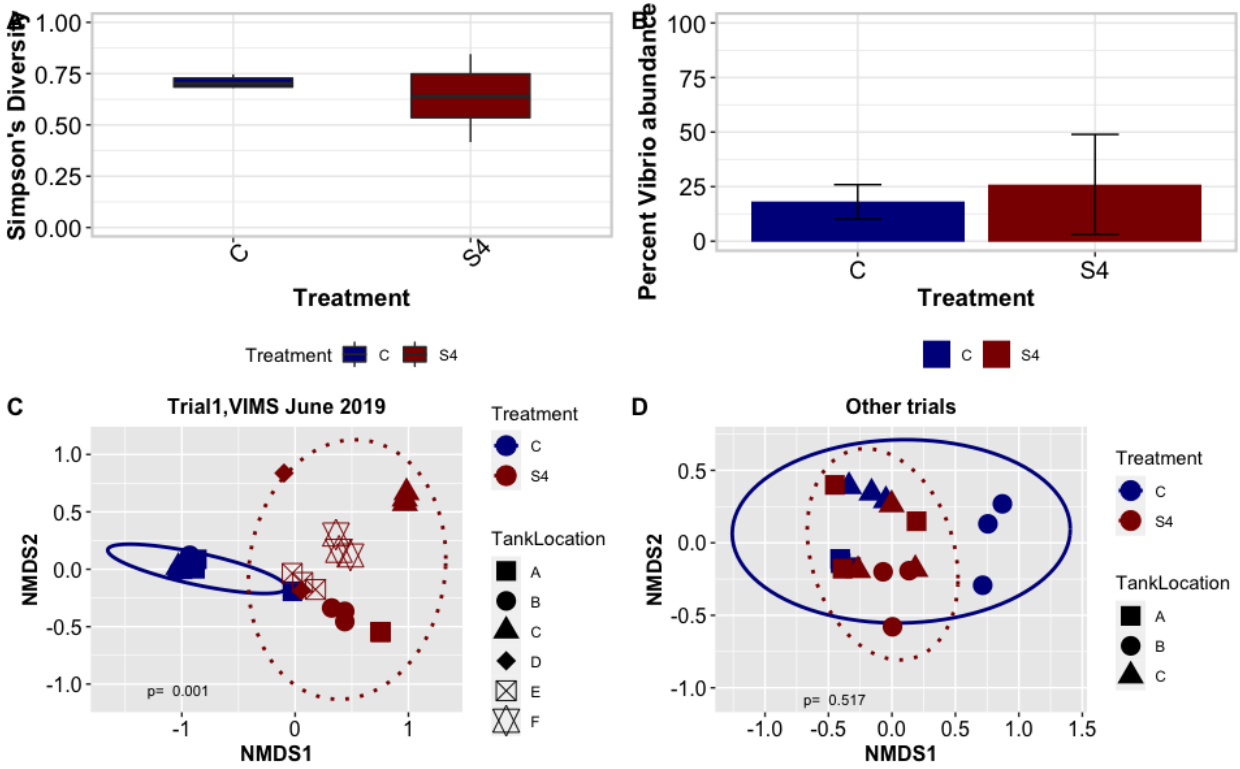

Figure III- 6. Probiotic treatment led to significant changes in *Vibrio* community structure only in Trial 1. Effect of S4 treatment on Simpson's Index of diversity for *Vibrionales* taxa (A, boxplots), *Vibrionales* relative percent, read abundance (B, bar graph), and bacterial community structure (C, NMDS plot) for trial 1. (D) NMDS plot shows the *Vibrio* ASV community structure for all other trials. There was no significant difference in diversity and relative abundance of vibrios between controls and treatment in all trials. However, there was a significant separation between the control and S4 treated shown by the NMDS in Trial 1. Note: Larvae in Trial 1 was collected on day 12 after spawning, while in all other trials it was collected on days 6 – 8

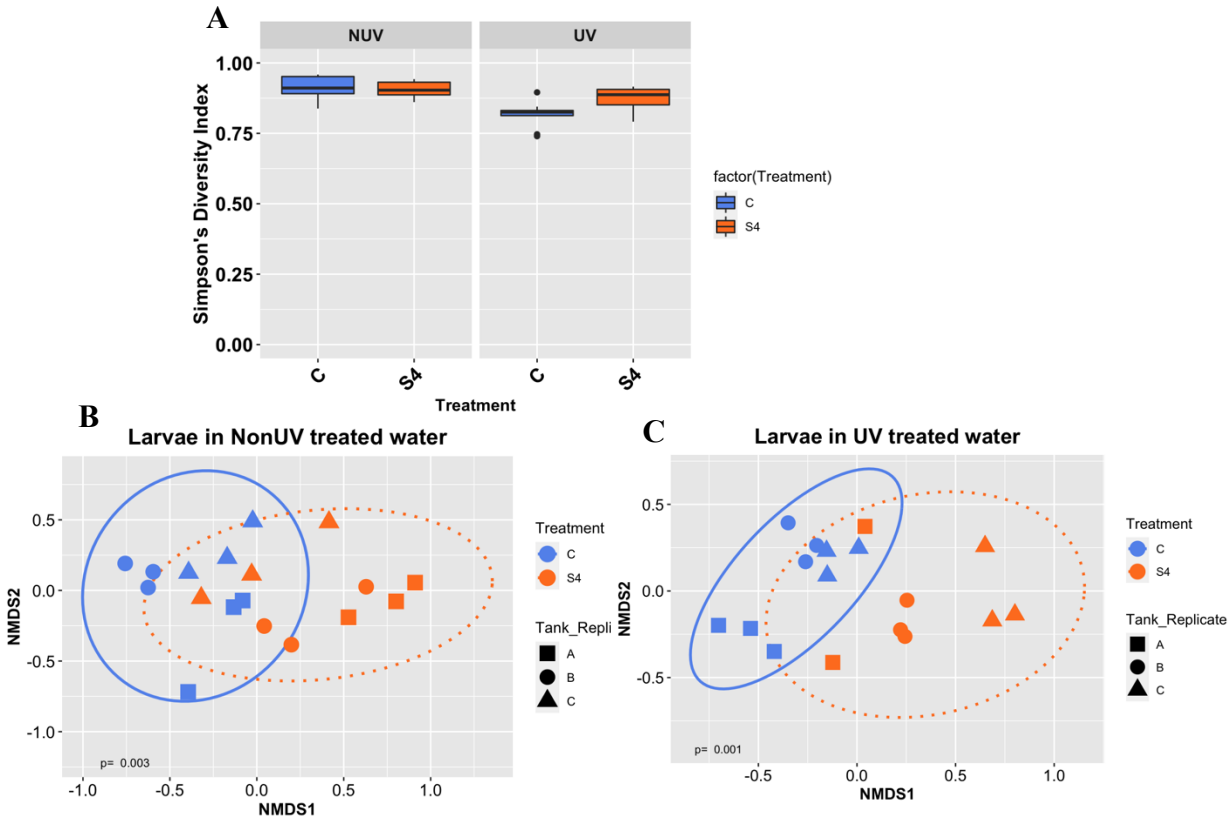

Figure III- 7. Probiotic treatment had an effect on larval bacterial alpha diversity raised in the UV treated and non-UV water from the hatchery. Simpson's Index of diversity (A, box plots), and bacterial community structure (NMDS plot). (B) for larvae raised in non-UV water, (C) larvae raised in UV water.

Table III- 1. Hatchery trial information. Water treatment indicates the type of filtration used to treat water at each hatchery and the nominal size of the filters, and if ultraviolet light (UV) was used or not. Treatments tested at each hatchery included C: control of untreated larvae (no probiotic provided) and S4: *P. inhibens* S4 formulation added daily from day 1 at 10^4 CFU/mL. Treatment period indicates the number of days for each trial. VIMS: Virginia Institute of Marine Sciences, MOOK: Mook Sea Farms hatchery, MAT: Matunuck hatchery, RWU: Blount Shellfish Hatchery, Roger Williams University.

Trial	Hatchery	Water treatment (filtration/UV)	Treatment groups	Tanks Per Treatment	Treatment Period (Days)	Trial Date
1	VIMS	Sand, Cartridge 1µm, UV	C, S4	C =3, S4=6	12	June 2019
2	VIMS	Sand, Cartridge 1µm, UV	C, S4	C =3, S4=3	6	July 2019
3	VIMS	Sand, Cartridge 1µm, UV	C, S4	C =4, S4=4	8	May 2020
4	VIMS	Sand, Cartridge 1µm, UV	C, S4	C =4, S4=4	7	June 2020
5	MAT	Cartridge 10 µm	C, S4	C=2, S4=2	7	June 2020
6	MOOK	Bag filtration, 5µm,1 µm	C, S4	C =1, S4=1	8	January 2021
7	MOOK	Bag filtration, 5µm,1 µm	C, S4	C =4, S4=4	8	June 2021
8	RWU (UV)	Cartridge 1µm, UV	C, S4	C =3, S4=3	8	July 2021
8	RWU (non-UV)	Cartridge 1µm, non- UV	C, S4	C =3, S4=3	8	July 2021

Supplemental Table and Figures

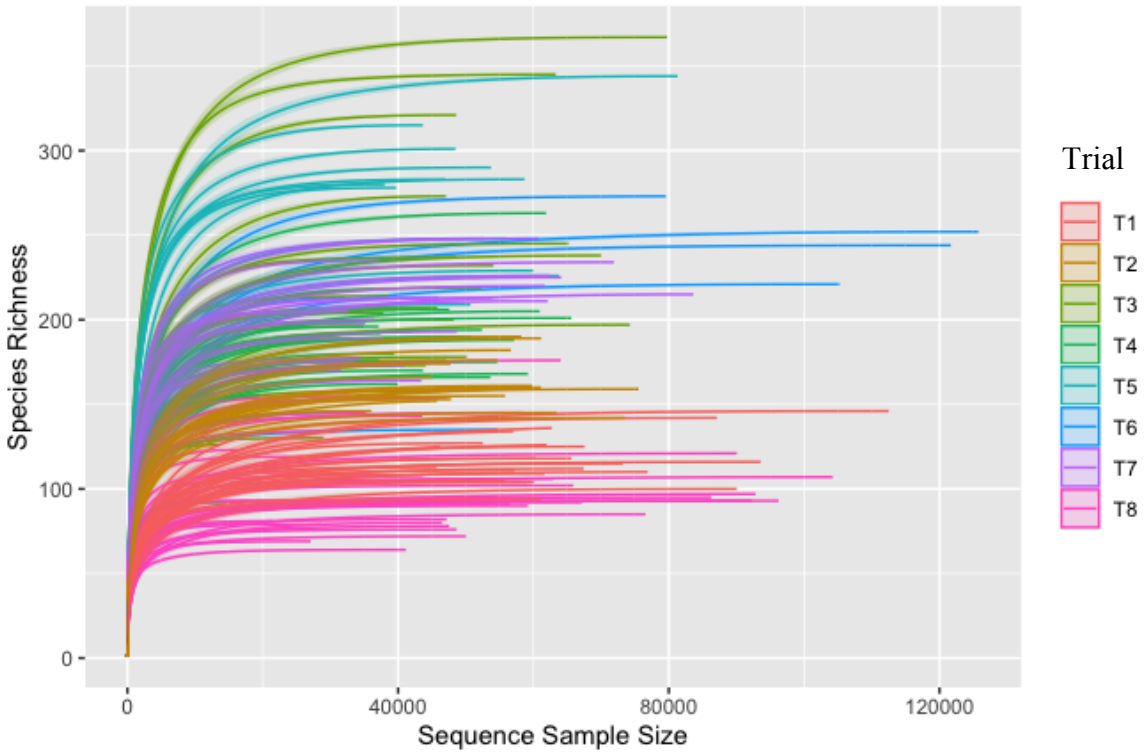

Figure III-S1. Rarefaction curve from all larval samples from all trials based on taxonomic classification at the ASV level.

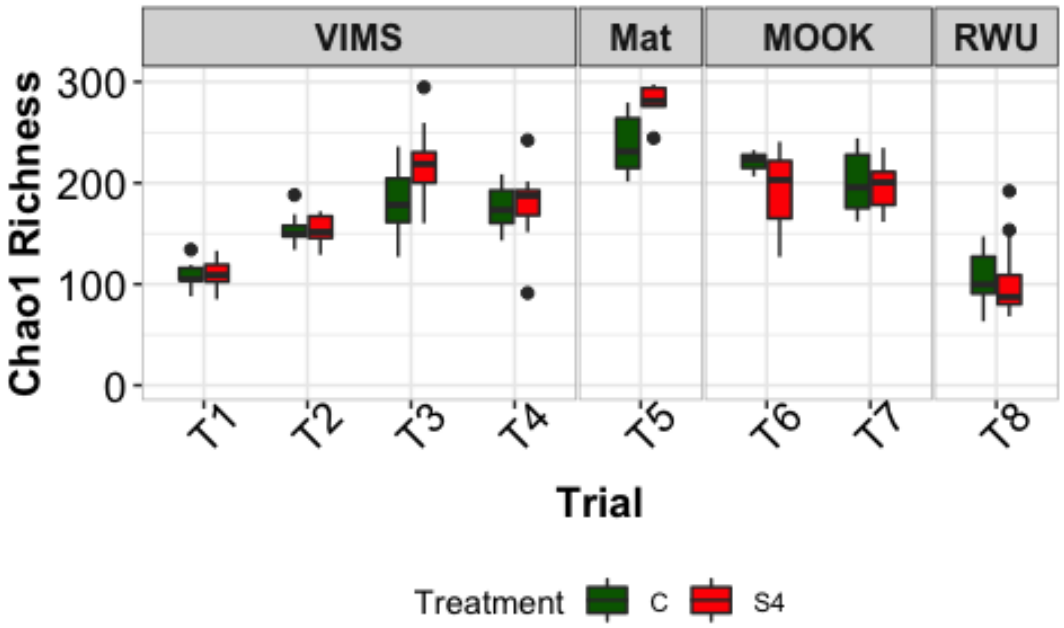

Figure III-S2. Effect of S4 treatment on bacterial richness in oyster larvae. Chao1 index measuring bacterial richness in the larvae between controls and treatment in each trial. No significant differences in diversity were found between treatments within trials. Trial(T) names are designated T1-T8. Abbreviations: VIMS = Virginia Institute of Marine Sciences, Mat = Matunuck, MOOK = Mook Sea Farms, RWU=Roger Williams University. C= Control(untreated larvae), S4=P inhibens S4 formulation (treated larvae).

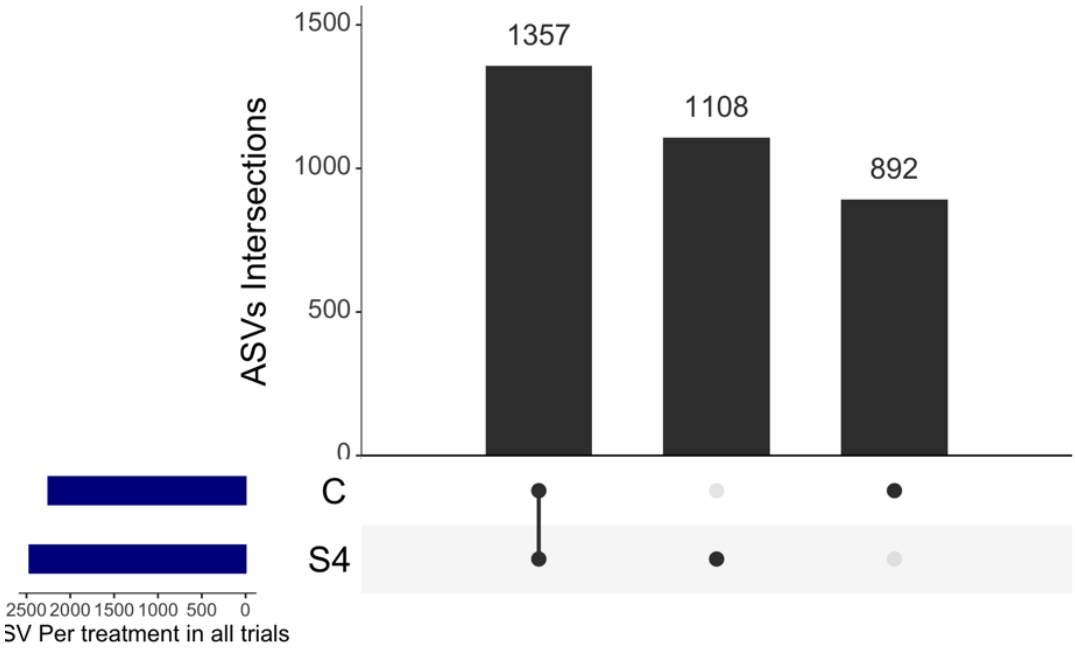

Figure III-S3. Shared and unique bacterial ASVs between treatments. Number of bacterial ASVs unique and shared between the controls and S4 treatment from all trials is shown on top of the bar(vertical bars). The total number of ASVs found in controls and S4 treatment is shown on the horizontal bar graph on the left(in blue). Intersections denote comparisons between the controls and S4 treatments.

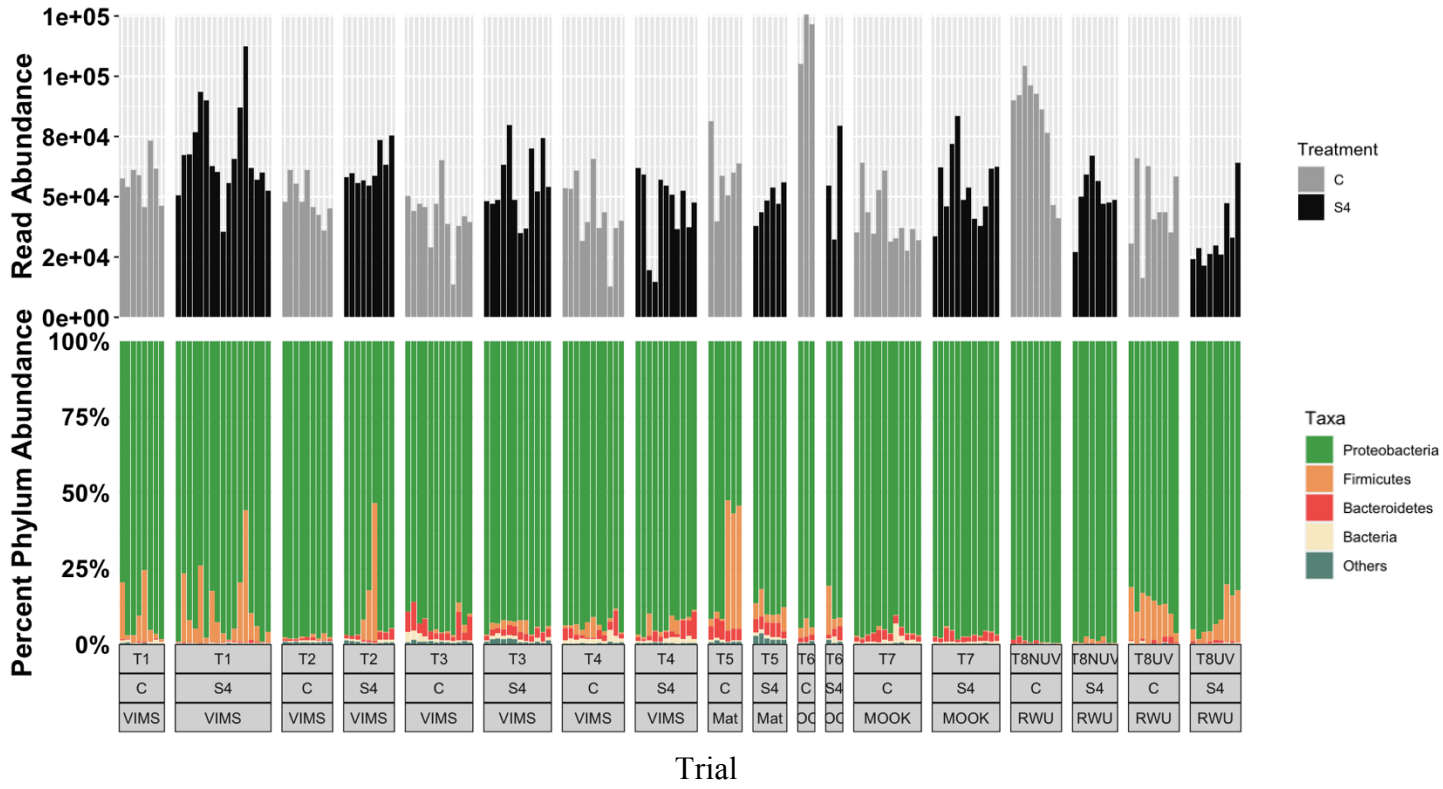

Figure III-S4. Taxonomic composition of bacterial in oyster larvae from different hatchery trials. Percent abundances of the 4 most abundant taxa (Phylum level) in oyster larvae from all trials and hatcheries based on 16S rRNA amplicon sequencing data. Trial names are designated T1-T8. Abbreviations: VIMS = Virginia Institute of Marine Sciences, Mat = Matunuck, MOOK = Mook Sea Farms, RWU=Roger Williams University. NUV=nonUV treated water, UV= UV treated water.

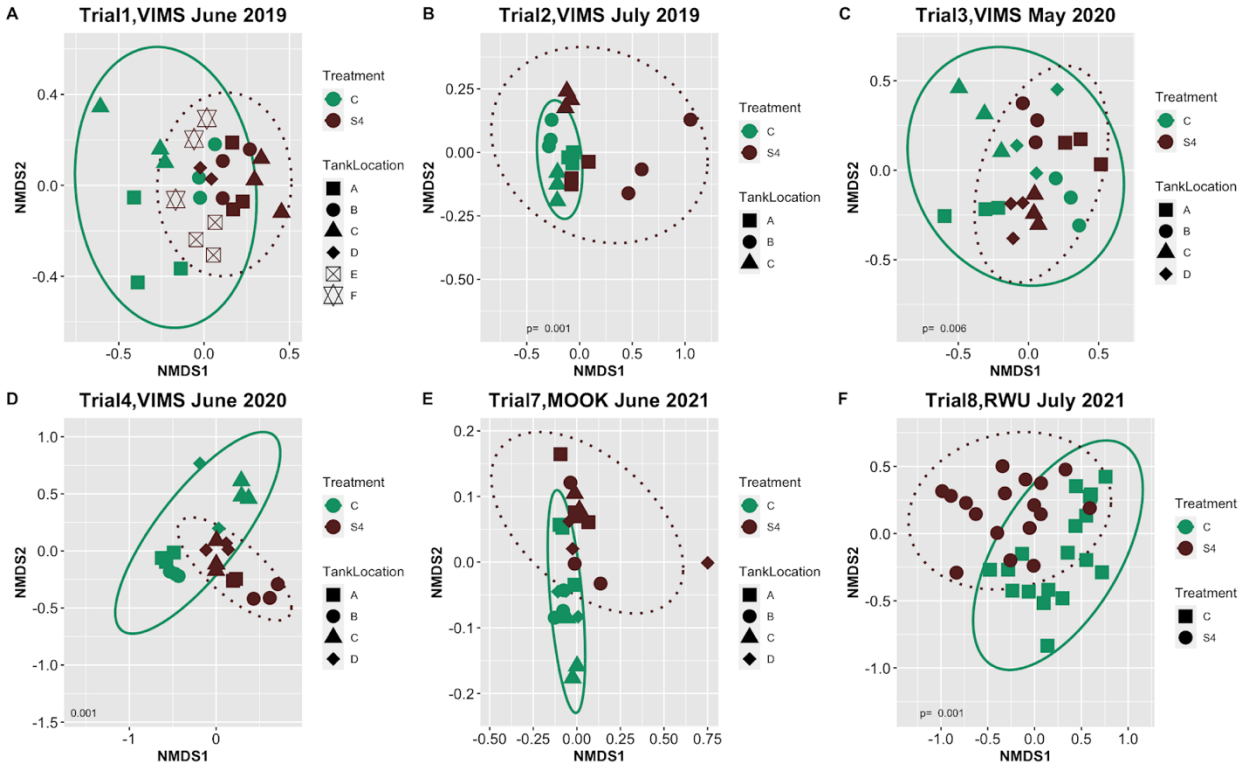

Figure III-S5. Diversity in the structure of bacterial communities in larvae between treatments (control – probiotic) from different trials. Non-metric multidimensional scaling (NMDS) visualization of Bray-Curtis distance (an index of beta-diversity) at the ASV level, grouped by (A) location, (B) hatchery, (C) Trial, (D) Season, (E) Year, (F) Treatment. Each dot represents the bacterial community in 3 larval pools from a single tank. Ellipse lines show the 95% confidence interval. P-values indicate the significance of grouping with adonis2 Permutational Multivariate Analysis of Variance Using Distance Matrices test (PERMANOVA). Larval oyster bacterial communities showed significant differences in composition due to location, hatchery. T1-T8: trials 1 to 8. C: control, S4: treated daily with probiotic. Note: trial5 and trial6 did not have enough replicate tanks for statistical significance testing

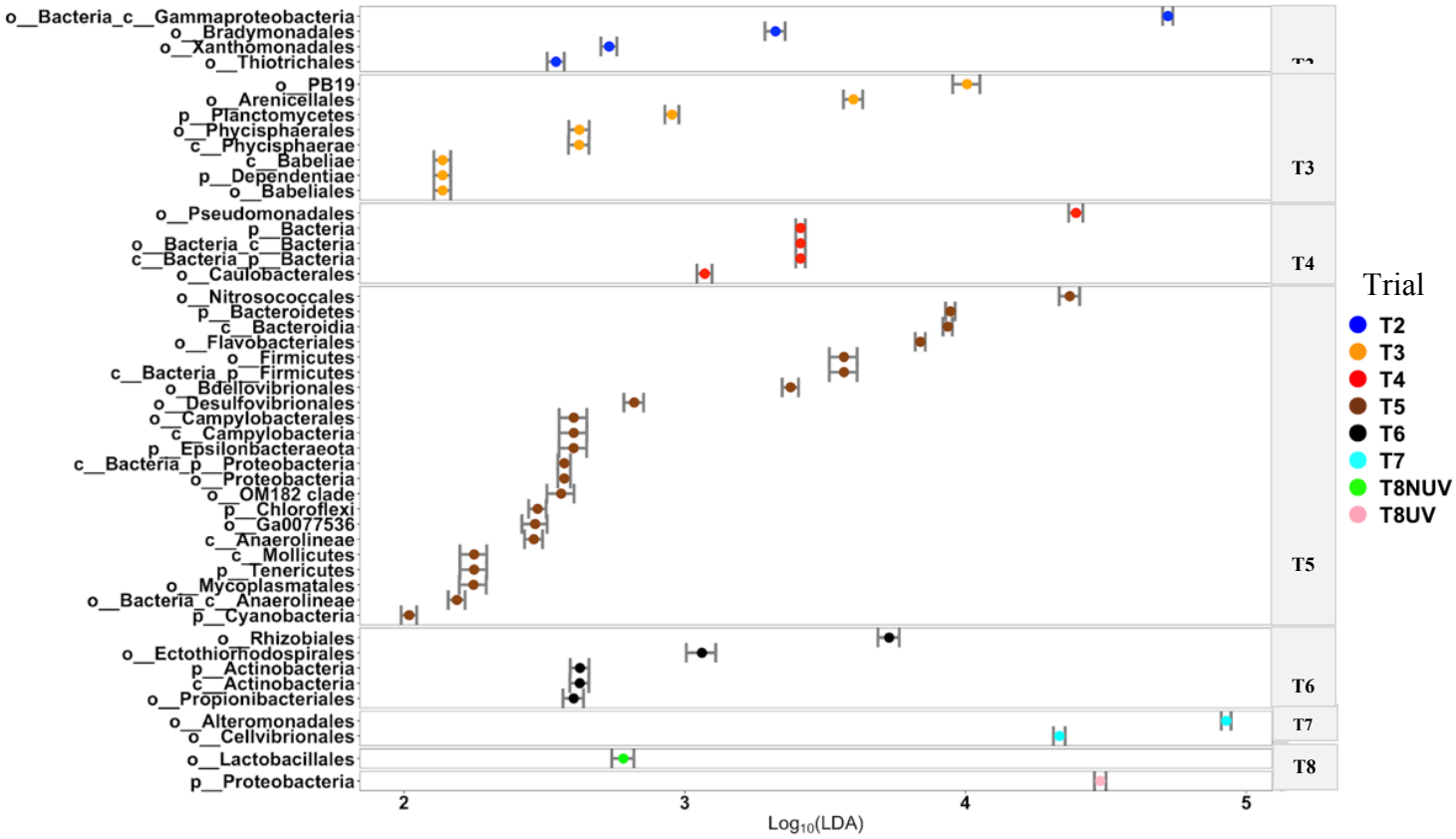

Figure III-S6. Bacterial Taxa showing significant differences in relative abundance between trials. Linear discriminant analysis (LDA) combined with effect size (LEfSe) used to identify bacteria taxa that differs between trials. The x-axis shows the LDA scores which represent the degree of differences in relative abundance of taxa between trials expressed in logarithmic scale. The y-axis shows the taxa that are differentially abundant, colored and separated by the trial they are most enriched in comparison with other trials. Gray bars to the right represent the name of each trial(T2-T8).Note: Trial1 did not show any taxa significantly different in comparison to all trials.

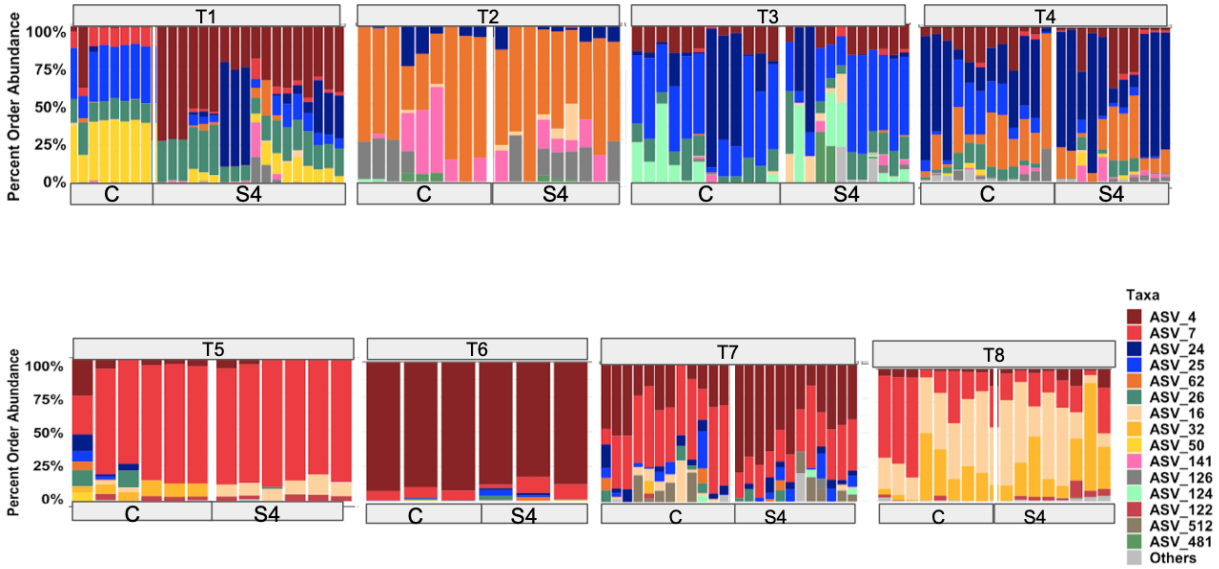

Figure III-S7. Taxonomic composition of *Vibrio* ASVs between control and treatment of oyster larvae. Percent abundances of the 15 most abundant *Vibrio* ASVs in oyster larvae from all trials based on 16S rRNA amplicon sequencing data.

Table III-S1. Permutational Multivariate Analysis of Variance Using Distance Matrices (adonis2) for Bray-Curtis beta-diversity (*k*=2) for each factor.

	DF	R2	F	P value
Location	2	0.32	38.79	0.001
Hatchery	3	0.49	51.3	0.001
Trial	8	0.64	35.774	0.001
Treatment	1	0.01	1.89	0.04
Year	1	0.15	29.97	0.001
Month	3	0.21	14.46	0.001
Season	1	0.06	10.94	0.001

CHAPTER IV: The relationship between microbial composition, larval performance, and environmental conditions in eastern oyster hatcheries

By

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Keywords: *Crassostrea virginica,* larvae, hatchery, 16S rRNA sequencing, environmental parameters

Abstract

Hatchery production of larvae is an integral component of oyster farming. Larvae are particularly vulnerable to environmental change, so understanding the interactive effects of factors on larval life is essential in determining larval performance in hatcheries. This study used previously reported data from seven trials at three hatcheries to examine the relationship between larval performance (survival and growth), bacterial community structure, and environmental conditions. The mean specific growth rate (SGR) of larvae across these trials ranged from 3.4% -11.3% and percentage survival ranged from 7.2 % to 99%. Temperature and salinity influenced larval growth, survival, and bacterial community composition associated with the larvae. The lowest growth and survival were observed at water temperatures above 27^{o}C and salinity above 29 psu. Pearson's correlation coefficient showed a strong positive correlation between bacterial community structure and temperature (p = 0.0001, r = 0.59) or salinity (p = 0.0001, r = 0.63) and a weaker, but significant relationship with pH (p = 0.02, r = 0.28). Bacterial community structure also showed a significant correlation with growth (p = 0.0001, r = 0.36) and survival (p = 0.002, r = 0.21). Several bacterial taxa whose abundance correlated with larval performance were identified. Most bacterial taxa whose relative abundance correlated with larval survival and growth, including *Alteromonas*, *Pseudoalteromonas, Vibrio, Sulfitobacter, Pseudomonas,* and *Cellvibrionales,* also showed correlations with temperature and salinity. These results are consistent with a complex interplay between microbial community composition, larval performance, and environmental conditions in the hatchery. Further characterization of species identified in this study as potentially associated with low or high larval performance in a variety of trials may provide for useful tools for hatchery management.

Introduction

Hatchery production of eastern oyster, *Crassostrea virginica,* seed has become increasingly important to fuel domestic aquaculture production on the Gulf and Atlantic coasts of the US. This plays an important role in enhancing wild fisheries and restoration efforts in many coastal bays and estuaries (Barton et al., 2015; Hornick & Plough, 2019), and meeting the increasing seafood market demands of oysters. Hatchery facilities adopt various well-developed techniques to produce bivalve larvae (e.g. Helm et al. 2004); however, periodic crashes or unexpected mortalities still occur in bivalve hatcheries worldwide (Gray et al., 2022; Jones, 2006). These problems are usually indicated by slow larval growth, larvae ceasing to swim and dropping out of the water column, or simply as mortalities. Larval mortality in the hatcheries could be due to several players such as predators, parasites, pathogens, competitors for food and space, low food quality and/or food quantity, host genetics, or poor water quality, including the presence of toxins and pollutants (Chávez-Villalba et al., 2008). These crashes are costly as they result in loss of revenue while wasting labor and other production resources.

Microbiomes play vital roles in the health and survival of their host (Apprill, 2017). It has been shown that early colonization of essential bacteria in the oyster larval microbiome may provide advantages to the oyster as it transitions into an adult, such as the development of a bivalve's gastrointestinal tract. On the other hand, the microbiome acts as a reservoir for primary or opportunistic pathogens such as *Vibrio* that can cause disease (Dupont et al., 2020; Richard et al., 2021; De Lorgeril et al., 2018). Larvae and juveniles are also susceptible to viral pathogens, for example, oyster Hemocyte Infection Virus in Pacific oysters, *Crassostrea gigas* (Renault and Novoa 2004). *C. virginica* juveniles are susceptible to Roseovarius Oyster Disease caused by *Roseovarius crassostreae* (Maloy et

al. 2007). Certain bacteria may also reduce or prevent detrimental microorganisms from proliferating and causing disease by creating competition for nutrients, reducing space for settlement, or producing antimicrobials (Schulze et al., 2006; Prado et al., 2010; Kesarcodi-Watson et al., 2012). Probiotics, for example, include beneficial bacteria that improve health or reduce disease and, when administered to bivalve larvae at early stages of development, have been shown to increase the survival of oysters, possibly through inhibition of pathogenic bacteria.

Environmental conditions such as temperature, salinity, pH, are known to directly affect species performance through impacts on metabolism and physiology (Lokmer et al., 2016a; Wegner et al., 2013, Brown et al., 2004; Lowe et al., 2017; Pusack et al., 2018; Rue- sink et al., 2015; La Peyre et al., 2013, Rybovich et al., 2016, Wang et al., 2017, Kirchman et al., 2004, Hill et al. 2012; Neulinger et al., 2009). In the hatchery, temperatures of 25 –27°C and salinities of 10 – 28 psu are known to support oyster larval growth and survival (Helm et al., 2004). Environmental conditions may also directly affect the microbial communities associated with the oyster host (Khan et al., 2018; King et al., 2012; Lokmer et al., 2016b; Pierce et al., 2016; Wendling et al., 2014). Timmins-Schiffman et al. (2021) identified that pH correlates with differences in the bacterial community and drives mortality events in a hatchery. Jiang et al. (2018) also demonstrated that salinity was the dominant factor influencing the composition and community structure of the bacterial population in a hypersaline lake. Rising ocean temperatures are known to increase the abundance of *Vibrio* spp., some of which are well-known pathogens of larval bivalves (Schmitt et al. 2012).

Despite the worth of studies and reviews on oyster development, growth, host-microbiome, and the effect of biological and environmental factors on oyster production, the interactions between oyster larval performance, environmental conditions, and host-associated microbiota is unexplored. Therefore, a knowledge gap remains in understanding the crosstalk between these components and how they impact larval performance in the hatchery. This study analyzed previously reported data collected from 7 different trials (Chapters 2 and 3 of this dissertation) to determine the relationship between larval performance, environmental parameters, and bacterial community structure in order to identify factors associated with larval performance in the hatchery. This study also identified bacterial species (identified as 16S rRNA gene amplicon sequence variants or ASVs) that were associated with larval performance in the hatchery and described the relationship between these ASVs and environmental conditions in the hatchery. Further research on the role of these bacterial species on larval performance may lead to the identification of markers used to forecast mortality events in the hatchery.

Methods

Hatchery trials: Experimental Design and Sample Collection

This research further analyzes data collected on larval performance (Chapter 2) and microbial community structure (Chapter 3) at seven trials in four different hatcheries. Trials were performed at the Aquaculture Breeding Center, Virginia Institute of Marine Sciences (VIMS, Gloucester, Virginia, USA) hatchery, Mook Sea Farms hatchery (MOOK, Walpole, Maine, USA), and Blount Shellfish Hatchery at Roger William University (RWU, Bristol, Rhode Island, USA) (Table 1). Briefly, adult eastern oysters were spawned at each of the hatcheries following standard procedures (Helm et

al., 2004). Larvae (1 day old) were distributed and maintained in replicate conical tanks per treatment (120 L for ABC-VIMS, and 60 L for MSF and RWU) and fed with live microalgae feed depending on the hatchery. Tanks were randomly assigned to following treatments: no probiotics (control), and candidate probiotic S4. In all trials, control tanks were fed with only algae feed and probiotic formulation of probiont S4 (Chapter 2) was added daily at a dose of 10^4 CFU/mL at algal feeding from day 1 (24 hr after spawning) until the termination of the trial. Larval tanks were drained every other day for size grading of larvae and maintenance of water quality (Helm et al., 2004). Oyster larvae were collected at selected dates (Table 1) on a 40 μm sieve after drain-down of tank water, placed into a sterile tube, and stored at −80°C until DNA extraction.

Dataset used for statistical analysis

Larval performance (growth and survival)

Data of larval survival and growth is from Chapter 2 of this dissertation. Larval specific growth rate at the end of each trial was calculated from the larval sizes using the formula: SGR (Specific Growth Rate (%)) = ((LnWt - LnWo)/t)x100, where LnWt = ln final body size (μm), LnWo = ln initial body size (μm), and t = feeding time (day) (Nimrat et al., 2011). Cumulative percentage survival of larvae at the end of trial was calculated. Data for larval performance is in Supplemental Table S1.

Environmental parameters in tanks

Environmental data was collected from each tank daily at the hatchery using a hand-held YSI instrument for temperature and salinity, and a hand-held pH meter (Oakton)

for pH. At MOOK, salinity was measured using a seawater monitoring system. Data is presented in supplementary Table S2.

Bacterial community structure in oyster larvae

Data used in this analysis is from Chapter 3 of this dissertation Briefly, bacterial DNA was extracted from the oyster larvae using the PowerSoil DNA Isolation Kit (Qiagen) following the manufacturer's protocol. PCR reactions were performed following Illumina's 16S Sequencing Library Preparation Protocol amplifying V6 hypervariable region of the 16SrRNA gene. The PCR products amplicons were sequenced using a 2x100 paired-end sequencing on an Illumina MiSeq at the Genomics and Sequencing Center at the University of Rhode Island. Demultiplexed read pairs were analyzed using the Quantitative Insights Into Microbial Ecology 2 (QIIME2) software version 2019.7 (Bolyen et al., 2019). Taxonomic assignment was performed with QIIME2's sklearn classifier mapping to the SILVA database release 132 (Quast et al., 2013). Based on the SILVA taxonomic assignments, mitochondrial and chloroplast sequences were removed from the ASV count table. The resulting ASV count table and taxonomy data were exported and analyzed in R version 4.0.2 (Martin, 2021). Relative abundances of technical replicates were merged to mean relative abundances for bacterial community analysis of alpha diversity and structure (Table 1).

Total Culturable Vibrios

Data used is from Chapter 2 of this dissertation and also in supplemental table S1. Total number of culturable *Vibrio* spp from larval oysters was determined using a plate count method on thiosulfate-citrate-bile salts-sucrose medium (TCBS, Difco).

Statistical Analysis

Using a linear model function in R version 4.0.2 (Martin, 2021), a polynomial regression analysis was used to relate larval growth and survival to each environmental parameter due to the non-linear relationship between the independent (environmental variables) and dependent variables (survival and growth). The correlation between larval performance (Table S1), environmental parameters (Table S2), and bacterial community richness was estimated using the '*breakaway*' package in R. Two methods were used to assess the relationship between bacterial community and environmental parameters and larval performance. First, a Mantel test (calculates the correlation coefficient between matrices based on two independent data sets) was performed with 10,000 permutations to compare the similarity matrix of environmental parameters, larval performance and the Bray-Curtis similarity matrix of bacterial community profiles (Mantel, 1967). Secondly, Principal Component Analysis (PCA) using *prcomp* (scale.=TRUE) and plotted using *autoplot*() within the *ggfortify* v0.4.7 package (Tang et al., 2016) was used to assess variation across the different trials. Spearman's rank correlation (measures the strength and direction of association between two ranked variables) were performed with 999 permutations using R statistical computing environment to determine which bacterial taxa associated with larval performance or environmental parameters in the hatchery. All data were standardized before analysis using log transformation.

Results

Larval survival, growth, environmental parameters, and microbial community structure were variable between trials, allowing for evaluation of the potential relationships between these parameters. Probiotic treatment had no significant effect on larval

performance in any of the trials (Chapter 2) and had a subtle impact on microbial community composition (Chapter 3), therefore this factor was not directly evaluated in this analysis.

Variation in larval performance in the hatchery was mostly explained by variation in temperature

This dissertation (Chapter 2; Chapter 3) and previous research (Sohn et al., 2016), observed levels of variability between tanks, within treatment and hatchery trials in larval survival, growth and culturable *Vibrio* spp. in water, tank surface, and larvae. This variability is likely to be influenced by variations in environmental parameters. A polynomial regression analysis was used to determine the relationship between environmental parameters (temperature, salinity, pH) and larval performance (survival, growth). Overall, water temperatures ranged from 24.2°C in June 2021 (Trial 7) to 28°C in July 2019 (Trial 2). Salinity varied from 14.1 psu in June 2019 (Trial 1) to 30 psu in June 2021 (Trial 7). pH varied from 8.0 (Trial 3) to 8.5 (Trial 3). Larval growth, expressed as a specific growth rate (SGR), ranged from 3.4% in Trial2 to 11.8% in Trial 3. Cumulative percentage survival of larvae ranged from 7.2 % in Trial 2 to 99% in Trial 4. A strong non-linear relationship between larval growth, survival, and temperature (survival: $r^2 = 0.83$; growth: $r^2 = 0.87$, $p < 0.05$ Figure IV-1) was observed. Salinity showed a weak non-linear relationship with survival and growth (Survival: $r^2 = 0.3$, $p < 0.05$; growth: $r^2 = 0.38$, $p < 0.05$). pH also showed a weak relationship with growth and survival (Survival: $r^2 = 0.21$; growth: $r^2 = 0.19$, $p < 0.05$). Based on the curves, the best larval performance was predicted to be at 25.5 – 26.5^{o}C and a salinity at 16 – 18 psu (Figure IV-1).

Larval bacterial community composition showed a significant relationship with both larval performance and environmental conditions in the hatchery

The relationship between larval bacterial community composition, larval performance, and environmental conditions in the hatchery was analyzed. No significant relationship was observed between bacterial community richness (Alpha diversity), environmental parameters, and larval performance (Figure IV-2). Pearson's correlation analysis using Mantel tests revealed significant associations between bacterial community structure (beta diversity), environmental conditions, and larval performance (Table 2). Temperature ($p = 0.0001$, $r = 0.59$) and salinity ($p = 0.0001$, $r = 0.63$) showed strong positive correlations with the bacterial community composition in larvae. Growth ($p = 0.0001$, r = 0.36), survival ($p = 0.002$, $r = 0.21$). Principal component analysis (PCA) also showed varying levels of covariation between bacterial community composition, environmental variables, larval growth, and survival (Figure IV-4). The variables included in the PCA which are composed of specific ASVs and environmental parameters accounted for 51% of the total variation. An arrow represents the variables that explain the variation, and the projection of any given variable along an axis shows the level where it is most abundant. PC1 explains 31.8% of the variation, and the largest variable loadings explained by PC1 are salinity (25%) and growth (25%). The variable loadings for PC2 were survival (22.2%), temperature (17%), and pH (16%). Trials 1, 3, and 4 were characterized by higher larval growth (ANOVA, $p < 0.05$ compared to all trials). Trial 2 was characterized by higher temperature and decreased larval survival and growth (ANOVA, $p < 0.05$ compared to all trials). Trials 6, 7 and 8 were characterized by higher salinity (ANOVA, $p < 0.05$ compared

to all trials) (Appendix A). This suggests that temperature and, to a lesser extent, salinity influenced both larval performance and bacterial community composition in the hatchery.

Identification of bacterial species whose abundance in larvae were associated with larval performance and/or environmental conditions in the hatchery

Following identification of a significant relationship between bacterial community composition and both larval performance and environmental conditions at the hatchery, the relationship between the relative abundance of the 45 most abundant bacterial taxa (ASVs) with either larval performance and/or environmental conditions at the hatchery was evaluated using Spearman rank correlation (Figure IV-5). A few selected (13) bacterial ASVs showed a positive correlation with both larval performance and temperature, including ASV_3, 5 and 18 (*Alteromonas*), ASV_20 (*Pseudoalteromonas*), ASV31 (*Rheinheimera*), ASV_44 (*Myxococcales*), ASV_51 (*Arenicellales*), ASV_11, ASV_42 (*Pseudomonas*), ASV_12 (*Sediminimonas*), and ASVs_24, 28 (*Vibrio*). ASVs showing a negative correlation with both larval performance and temperature included ASV_17 (*Cellvibrionales*), ASV_33 (*Sulfitobacter*), ASV_39 and 9 (*Gammaproteobacteria*), ASV_7 (*Vibrio*), and ASV_1 (*Pseudoalteromonas*). On the other hand, the abundance of ASV_5, 8, 11, 18, 24, 28, 42, 44, and 51 was positively correlated with larval performance but negatively associated with salinity while ASV_1, 7, 17, 36, 39, 49 were negatively associated with larval performance and positively associated with salinity (Figure IV-4, Figure IV-S1).

Very few ASVs showed significant correlations with larval performance but not environmental conditions at the hatchery. These included ASV_20 (*Pseudoalteromonas*),

ASV_25, 26 (*Vibrio*), ASV_47 (*Francisella*), which showed positive correlations with larval performance, and ASV_9 (*Gammaproteobacteria*), ASV_56 (Loktanella), which showed a negative correlation with larval performance (Figure IV-S1).

A few ASVs showed significant correlations with environmental conditions but not larval performance. ASVs whose abundance was positively correlated with salinity included ASV_10 (*Rhodobacterales*), ASV_29 (PB19), and those that were negatively correlated with salinity included ASV_19 (*Alteromonas*). The abundance of ASV_2 (*Yangia*) positively correlated with temperature and ASV_62 (*Vibrio*) negatively correlated with temperature(Figure IV-S1).

Discussion

Previous trials identified significant variability in eastern oyster larval performance (growth and survival) in the hatchery (Chapter 2). These variability in larval performance could be due to factors such as differences in quality and health status of larvae, the impact of various environmental and biological factors such as salinity, pH, temperature, and season at the hatchery, variability in the characteristics of different rearing systems, location of hatchery, and the effect of variability in the composition of microbial communities (Martínez Cruz et al., 2012). Multivariate analysis helps to evaluate several different parameters together to identify the impact of these different factors on a particular system (Besemer et al., 2005). The relationship between larval performance (survival and growth), bacterial community structure, and environmental parameters collected at different hatcheries shows that: 1) Temperature and salinity influenced larval growth, survival, and bacterial community composition; 2) Lower growth and survival were

observed at water temperatures above 27°C and salinity above 29 psu; 3) Most bacterial taxa whose relative abundance correlated with larval survival and growth also correlated with changes in temperature and salinity.

Consistent with previous research on eastern oyster larval biology, temperature and salinity showed an impact on larval growth and survival in this study, with temperature showing a stronger correlation. Previous studies have shown that salinity and temperature affect larval development, survival, growth, and mortality (Bhurtun et al., 2019; Lowe et al., 2017; Sehlinger, 2018), and that exposure to conditions outside a host optimal range can have negative consequences for their growth or survival (Heilmayer et al., 2008; Munroe et al., 2017; Rybovich et al., 2016). The environmental conditions observed in our study, however, were within the optimal ranges described for eastern oyster larvae (Helm et al., 2004) so it is likely that other biotic or abiotic factors that show associations with season – temperature and or salinity, such as such as nutrient availability or the presence of harmful algal blooms and pathogens, could be responsible for differences in larval growth and survival.

More importantly, our study identifies members of the bacterial community that are associated with differences in oyster larval performance. Studies have demonstrated that microbiota associated with larvae could relate to the health and performance in the hatchery (Sainz-Hernández and Maeda-Martinez, 2005; King et al., 2019; Apprill et al., 2017). Other studies show that survival of eastern (Gray et al., 2022) and Pacific (Timmins-Schiffman et al., 2021) oyster larval survival could be associated with specific bacterial ASVs. Species from the taxa *Alteromonas*, *Pseudoalteromonas, Bacillus* and *Vibrio* associated with larval performance in our study. These genera have been shown to

contribute to defense against pathogenic infections in other studies (García Bernal et al., 2017; Karim et al., 2013; Restrepo et al., 2021; Schulze et al., 2006). Several *Bacillus* spp. have been identified as a multi-strain probiotic which can increase resistance to diseases. Some species of *Vibrio*, for example *Vibrio diabolicus* and *Vibrio* OY15, have been shown to have antagonistic effects against different bacterial pathogens, including known *Vibrio* spp that are coral pathogens (Neulinger et al., 2009; Retrespo et al., 2021), while other species are known commensals or are associated with adaptive functions (Mukhta et al., 2016). Some contrasting trends were also observed, with different ASVs belonging to *Vibrio* and *Pseudoalteromonas* either positively or negatively correlating with larval survival and growth. Interestingly, we identified one bacterial ASV (ASV_18 (*Alteromonas*)) that was significantly more abundant in larvae treated with the probiotic *Phaeobacter inhibens* S4 as compared to non-treated larvae (Chapter 3. Further studies are needed to elucidate the interactions between species within *Alteromona*s genera and probiotic S4. One caveat to our study is the inability to identify most ASVs to the species level; however, future studies will consider this.

This study also identified ASVs associated with particular environmental conditions in the hatchery. It is well known that bacterial communities in the coastal environments where oysters are cultured change in response to seasonal environmental conditions, such as temperature (Kirchman et al., 2004), salinity, dissolved oxygen, and nutrients (Hill et al. 2012; Neulinger et al., 2009). The ASVs associated with temperature, salinity, growth, and survival are consistent with the fact that temperature and salinity can directly impact larval performance independent of the bacterial community and bacterial

community can also directly impact larval performance(Brown et al., 2004; Mackenzie et al., 2014; Lowe et al 2017; Pusack et al., 2018).

Conclusions

In conclusion, the study describes the relationship between bacterial community, environmental variables, and larval performance from different hatcheries and identifies bacterial ASVs associated with larval performance in the hatchery, as well as identified one particular ASVs associated with high larval performance that is also affected by probiont *Phaeobacter inhibens* S4 treatment. Future studies should focus on the interactions between *Alteromonas* genera and other taxa associated with larval performance and probiotic S4 using metagenomics and metatranscriptomics.

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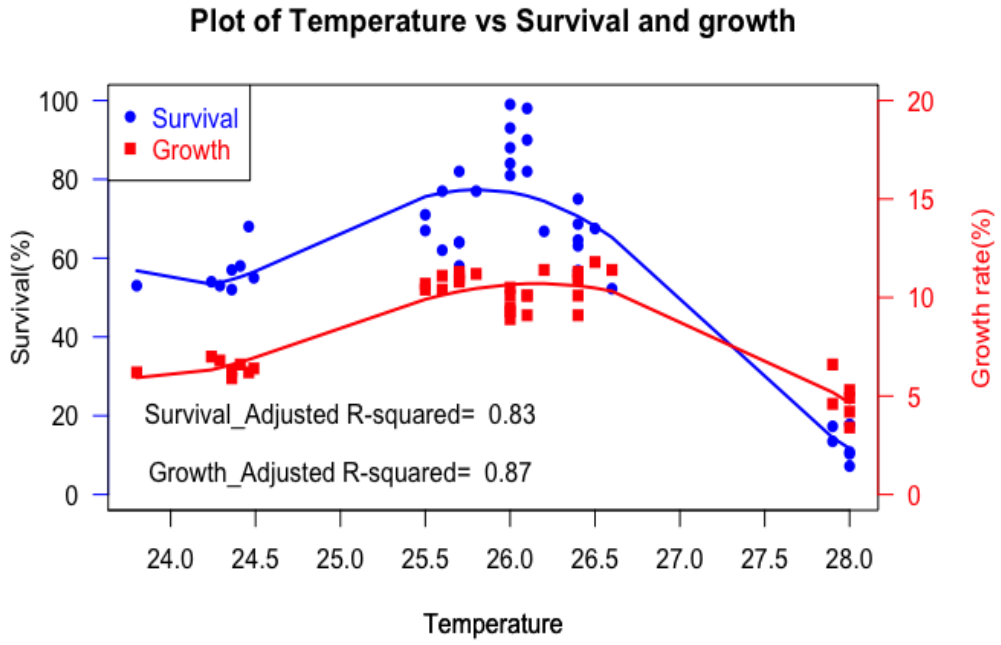
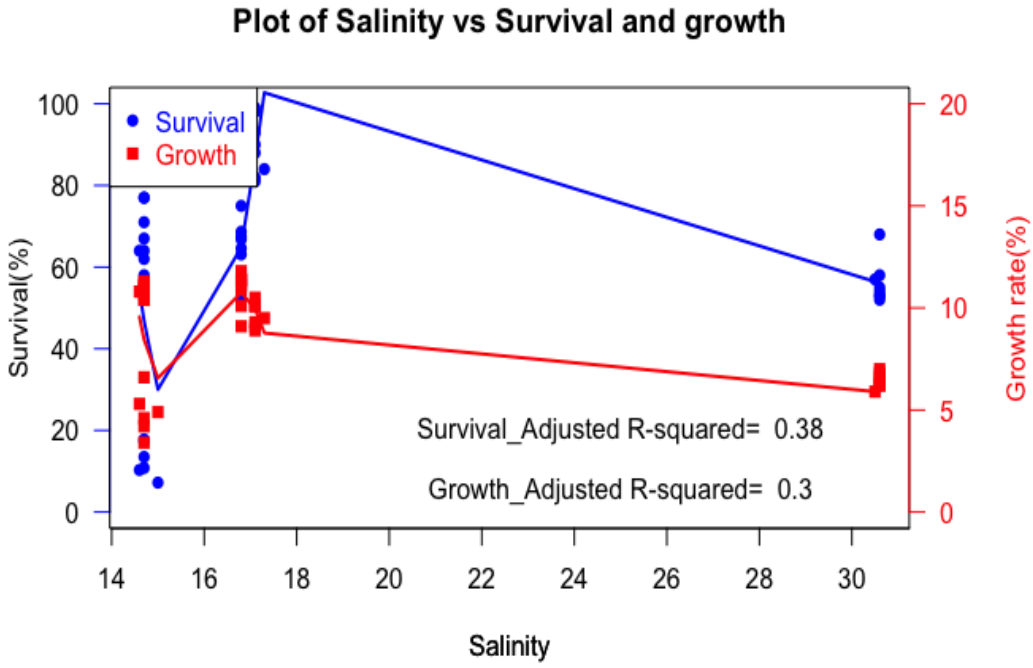
Tables and Figures





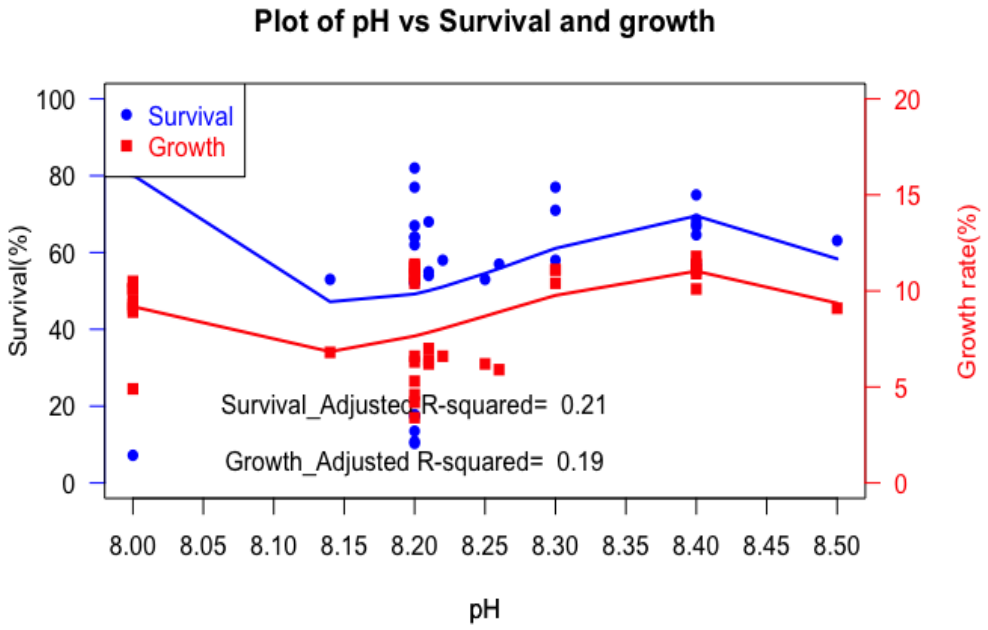


Figure IV-1. Polynomial regression of specific growth rate (SGR) and cumulative survival of larvae raised in the hatchery on the measurement of temperature (a), salinity (b) and pH (c).

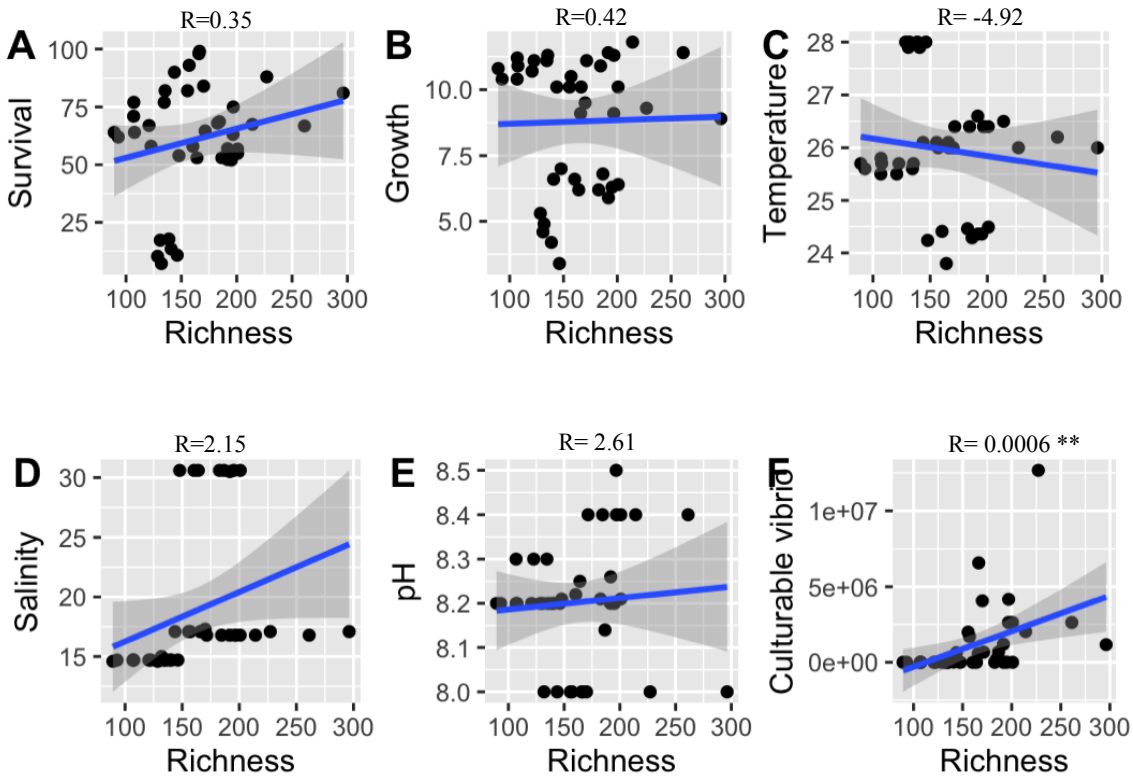


Figure IV-2. Relationship between bacterial community richness with environmental parameters (temperature, salinity, pH), larval performance (survival, growth), and culturable vibrios. Each scatter plot shows a different parameter on the y-axis versus richness (rarefied ASVs) on the x-axis. Points represent individual tank samples, and lines represent the regression lines (in blue) with standard errors (shaded in grey). Pearson's correlations (indicated on top of each plot) and significance indicated with asterisks (*) ($p < 0.05$).

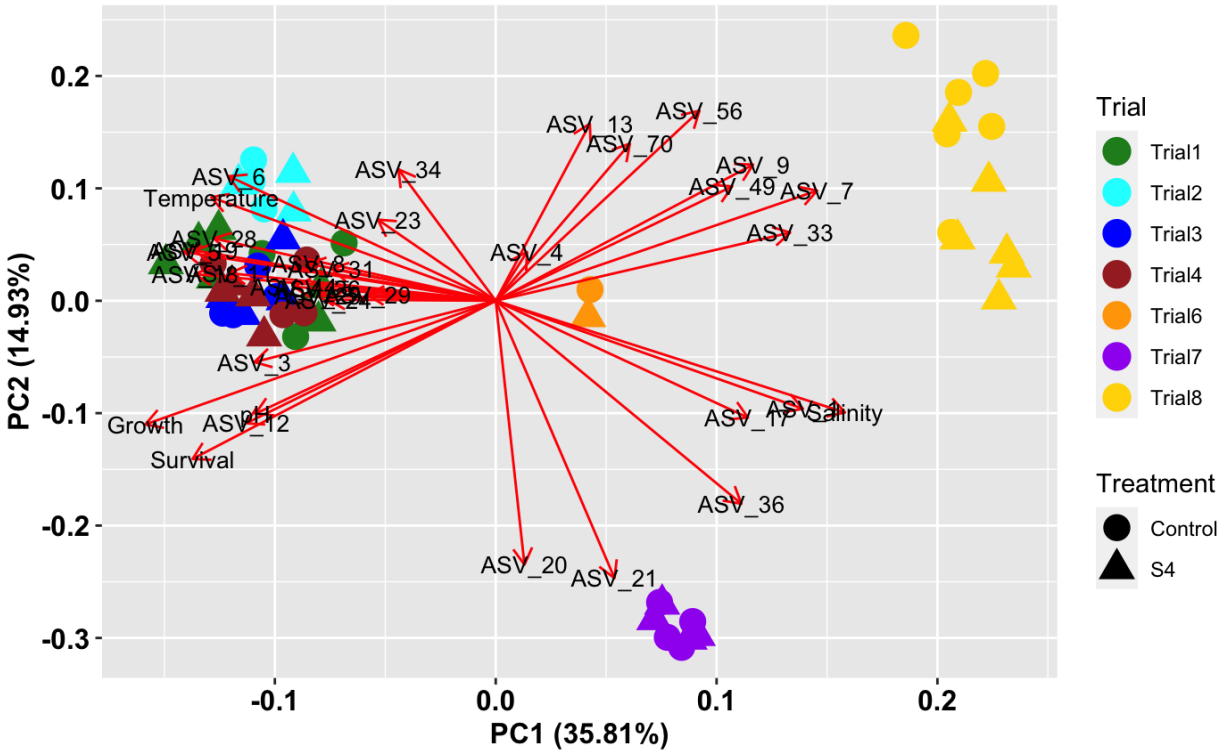


Figure IV-3. Relationship between bacterial species (ASVs), larval performance (growth and survival), and environmental parameters at the hatchery, as determined by Principal Component Analysis. Each symbol represents a tank. The arrows show variables contributing to the variation observed. The variance contributed by the first and second principal components (PC1 and PC2) is plotted along the horizontal and vertical axes. Pairs of variables with relatively large weights in the same direction represent positive correlations, and variables whose weights have opposite directions exhibit inverse correlations.

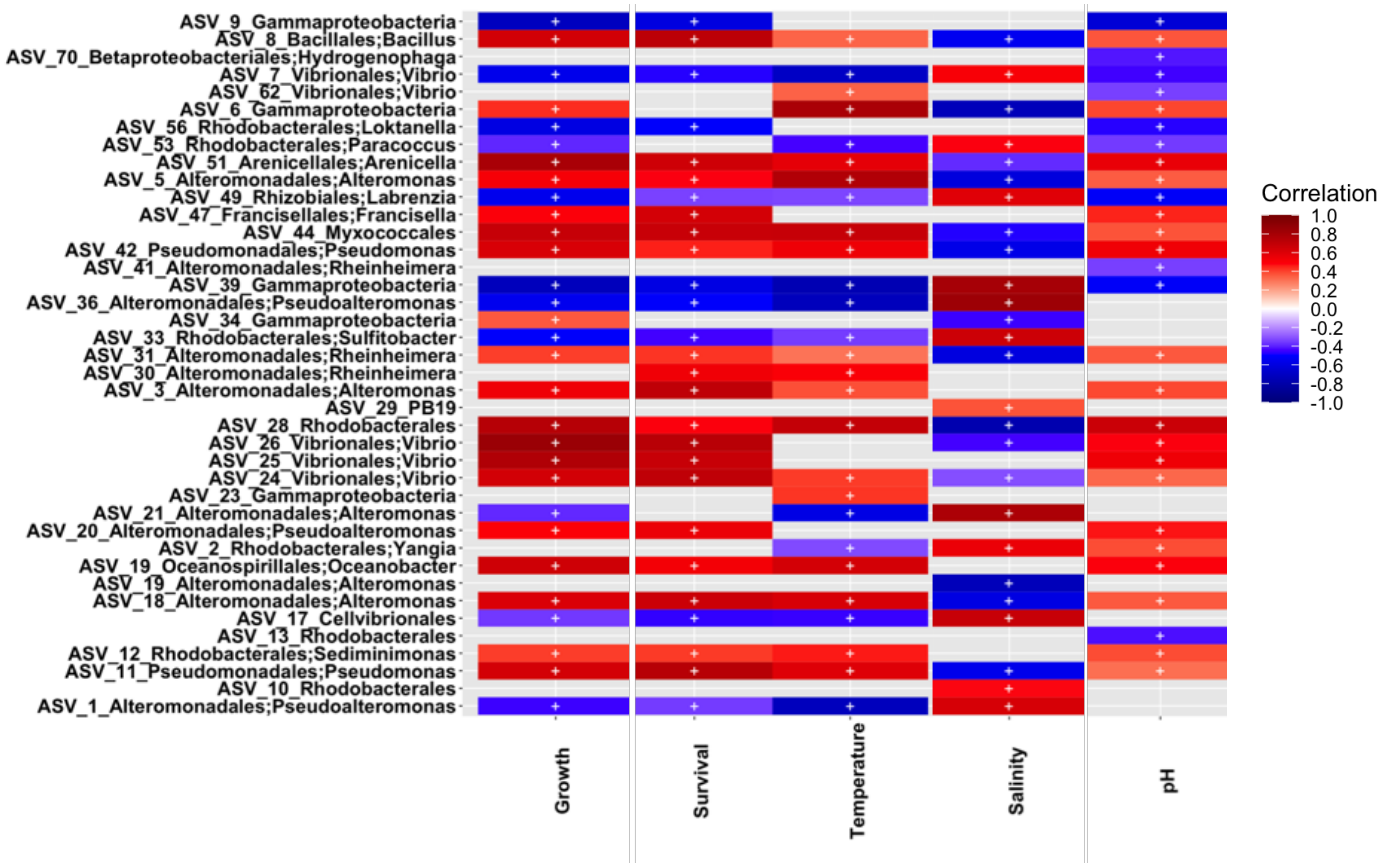


Figure IV-4. Spearman rank correlations between the 45 most abundant ASVs and environmental parameters. The correlations between the ASVs and environmental parameters are indicated by colors (red: positive; blue: negative). The color represents the effect size and direction of the correlation. Blue squares show positive changes in relative abundance, whereas red squares show negative correlations. The intensity of color correlates with the magnitude of the (log) fold change value. Significant correlations ($P < 0.05$) are indicated by '+'; Clustering of the rows and columns highlights groups of significantly correlated ASV and environmental variables.

TableIV-1. Summary of hatchery trials and data collected on larval performance and environmental parameters. VIMS: Virginia Institute of Marine Sciences, MOOK: Mook Sea Farms hatchery, RWU: Blount Shellfish Hatchery, Roger Williams University (from Takyi et al. in prep, Chapter II).

Hatchery	Period (Days)	Trial Date	Larval Performance	Environmental parameters	Microbial community
VIMS	12	June 2019	Survival; Growth	Temperature, pH, Salinity	Alpha Diversity /Beta Diversity
VIMS	6	July 2019	Survival; Growth	Temperature, pH, Salinity	
VIMS	8	May 2020	Survival; Growth	Temperature, pH, Salinity	
VIMS	7	June 2020	Survival; Growth	Temperature, pH, Salinity	
MOOK	8	January 2021	Survival; Growth	Temperature, pH, Salinity	
MOOK	8	June 2021	Survival; Growth	Temperature, pH, Salinity	
RWU	8	July 2021	-	Temperature, pH, Salinity	

Table IV- 2. Relationship between bacterial community structure and larval performance and environmental parameters as described using Mantel tests. The community distance matrix was based on Bray–Curtis distance, while environmental and larval performance were based on Euclidean distance. A permutation test with 999 permutations determined p-values. The r-value shows the strength of association between the variable and the community structure.

Variables compared with bacterial community structure	Mantel statistics (r)	P-value	Significance
Temperature	0.47	0.001	***
Salinity	0.68	0.001	***
Growth	0.44	0.001	***
Survival	0.56	0.002	**
pH	0.28	0.001	***
Total Culturable Vibrios	0.052	0.15	

Supplemental Table and Figures

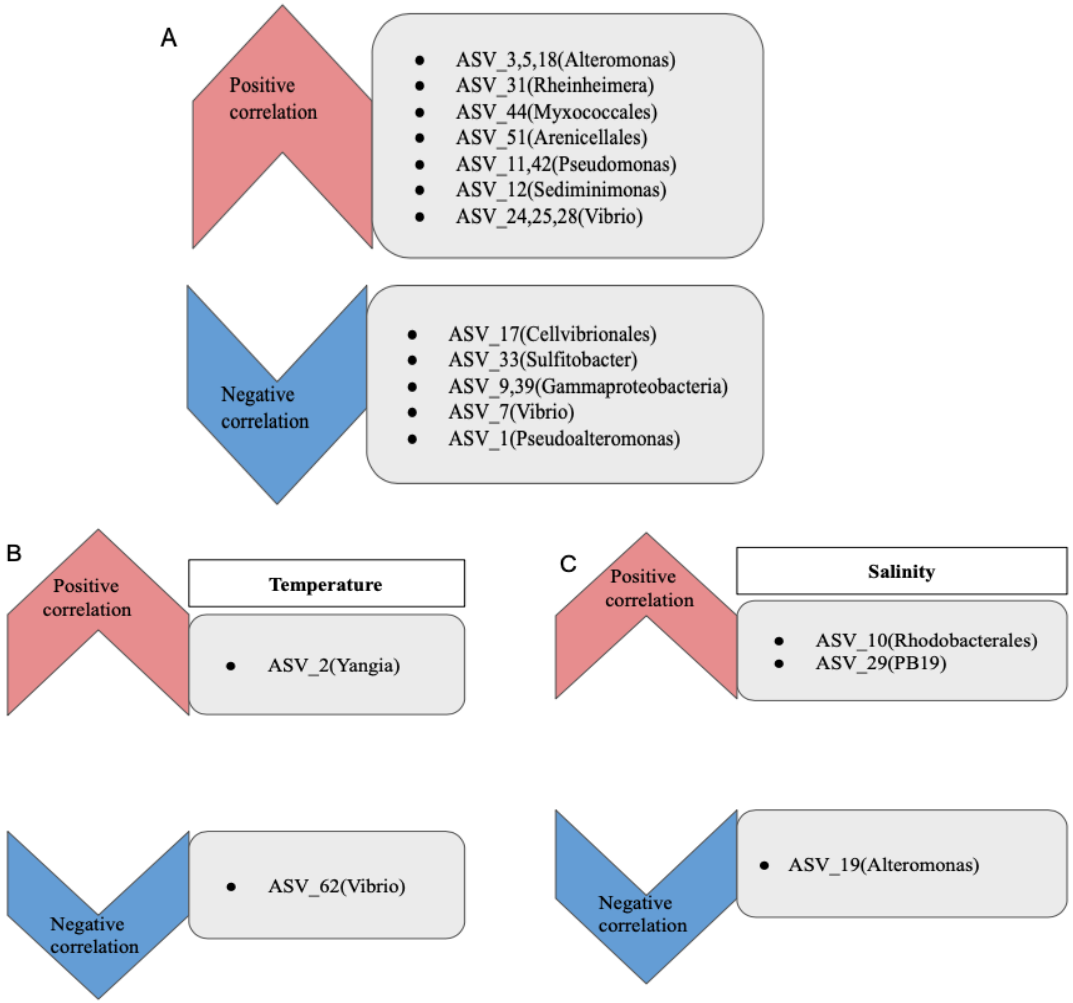


Figure IV-S1. Summary figure of the correlations between bacterial ASVs with larval performance and environmental parameters. (A) ASVs correlating with both larval performance and environmental parameters, (B) ASVs correlating with temperature, (C) ASVs correlating with salinity.

Table S1. Cumulative Percent Survival and Specific Growth Rate for each treatment for Control and S4 formulation. Treatments tested at each hatchery included C: control of untreated larvae (no probiotic provided) and S4: *P. inhibens* S4 formulation. VIMS: Virginia Institute of Marine Sciences, MOOK: Mook Sea Farms hatchery, RWU: Blount Shellfish Hatchery, Roger Williams University. (-): indicates data was not collected

Trial	**Hatchery**	**Treatment**	**Trial Date**	**Specific Growth Rate**	**Cumulative Survival**	**Culturable Vibrios**
Trial1	VIMS	Control	June_2019	6.9	67	0
Trial1	VIMS	Control	June_2019	8.2	64	0
Trial1	VIMS	Control	June_2019	8.8	71	0
Trial1	VIMS	S4	June_2019	8.5	77	0
Trial1	VIMS	S4	June_2019	8.2	62	0
Trial1	VIMS	S4	June_2019	8.2	64	0
Trial1	VIMS	S4	June_2019	9.1	82	0
Trial1	VIMS	S4	June_2019	8.5	77	0
Trial1	VIMS	S4	June_2019	8.5	58	0
Trial2	VIMS	Control	July_2019	4.9	7.2	31111
Trial2	VIMS	Control	July_2019	5.3	10.3	6667
Trial2	VIMS	Control	July_2019	4.6	17.3	0
Trial2	VIMS	S4	July_2019	3.4	10.8	30000
Trial2	VIMS	S4	July_2019	6.6	13.5	13333
Trial2	VIMS	S4	July_2019	4.2	17.8	63333
Trial3	VIMS	Control	May_2020	11.4	52.24	1200000
Trial3	VIMS	Control	May_2020	11.1	64.6	666667
Trial3	VIMS	Control	May_2020	9.1	63.11	2633333
Trial3	VIMS	Control	May_2020	10.9	68.65	200000
Trial3	VIMS	S4	May_2020	11.8	67.5	2033333

Trial3	VIMS	S4	May_2020	11.4	66.8	2633333
Trial3	VIMS	S4	May_2020	11.3	75	4166667
Trial3	VIMS	S4	May_2020	10.1	56.84	2633333
Trial4	VIMS	Control	June_2020	10.1	99	6566667
Trial4	VIMS	Control	June_2020	9.5	84	4066667
Trial4	VIMS	Control	June_2020	10.1	82	2000000
Trial4	VIMS	Control	June_2020	9.3	88	12666667
Trial4	VIMS	S4	June_2020	8.9	81	1166667
Trial4	VIMS	S4	June_2020	10.1	90	633333
Trial4	VIMS	S4	June_2020	9.1	98	566667
Trial4	VIMS	S4	June_2020	10.5	93	1700000
Trial6	MOOK	Control	January_2021	6.1	85.1	0
Trial6	MOOK	S4	January_2021	6.1	89.6	0
Trial7	MOOK	Control	June_2021	6.8	53	676667
Trial7	MOOK	Control	June_2021	6.3	52	4667
Trial7	MOOK	Control	June_2021	7.0	54	0
Trial7	MOOK	Control	June_2021	6.6	58	0
Trial7	MOOK	S4	June_2021	6.2	68	400
Trial7	MOOK	S4	June_2021	6.4	55	0
Trial7	MOOK	S4	June_2021	6.2	53	1233
Trial7	MOOK	S4	June_2021	5.9	57	0
Trial8	RWU	Control	July_2021	-	2	-
Trial8	RWU	Control	July_2021	-	3	-
Trial8	RWU	Control	July_2021	-	2	-
Trial8	RWU	S4	July_2021	-	6	-
Trial8	RWU	S4	July_2021	-	0	-

Trial8	RWU	S4	July_2021	-	1	-
Trial8	RWU	Control	July_2021	-	0	-
Trial8	RWU	Control	July_2021	-	0	-
Trial8	RWU	Control	July_2021	-	0	-
Trial8	RWU	S4	July_2021	-	0	-
Trial8	RWU	S4	July_2021	-	1	-
Trial8	RWU	S4	July_2021	-	1	-

Table S2. Environmental Parameter(temperature, salinity, pH) for each treatment for Control and S4 formulation.

Trial	**Hatchery**	**Treatment**	**Trial Date**	**Temperature**	**Salinity**	**pH**
Trial1	VIMS	Control	June_2019	25.5	14.7	8.2
Trial1	VIMS	Control	June_2019	25.7	14.7	8.2
Trial1	VIMS	Control	June_2019	25.5	14.7	8.3
Trial1	VIMS	S4	June_2019	25.8	14.7	8.2
Trial1	VIMS	S4	June_2019	25.6	14.7	8.2
Trial1	VIMS	S4	June_2019	25.7	14.6	8.2
Trial1	VIMS	S4	June_2019	25.7	14.7	8.2
Trial1	VIMS	S4	June_2019	25.6	14.7	8.3
Trial1	VIMS	S4	June_2019	25.7	14.7	8.3
Trial2	VIMS	Control	July_2019	28	15	8
Trial2	VIMS	Control	July_2019	28	14.6	8.2
Trial2	VIMS	Control	July_2019	27.9	14.7	8.2
Trial2	VIMS	S4	July_2019	28	14.7	8.2
Trial2	VIMS	S4	July_2019	27.9	14.7	8.2
Trial2	VIMS	S4	July_2019	28	14.7	8.2

Trial3	VIMS	Control	May_2020	26.6	16.8	8.2
Trial3	VIMS	Control	May_2020	26.4	16.8	8.4
Trial3	VIMS	Control	May_2020	26.4	16.8	8.5
Trial3	VIMS	Control	May_2020	26.4	16.8	8.4
Trial3	VIMS	S4	May_2020	26.5	16.8	8.4
Trial3	VIMS	S4	May_2020	26.2	16.8	8.4
Trial3	VIMS	S4	May_2020	26.4	16.8	8.4
Trial3	VIMS	S4	May_2020	26.4	16.8	8.4
Trial4	VIMS	Control	June_2020	26	17.1	8
Trial4	VIMS	Control	June_2020	26	17.3	8
Trial4	VIMS	Control	June_2020	26.1	17.1	8
Trial4	VIMS	Control	June_2020	26	17.1	8
Trial4	VIMS	S4	June_2020	26	17.1	8
Trial4	VIMS	S4	June_2020	26.1	17.1	8
Trial4	VIMS	S4	June_2020	26.1	17.1	8
Trial4	VIMS	S4	June_2020	26	17.1	8
Trial6	MOOK	Control	January_2021	24.8	29.4	8.2
Trial6	MOOK	S4	January_2021	24.7	29.4	8.1
Trial7	MOOK	Control	June_2021	24.29	30.6	8.14
Trial7	MOOK	Control	June_2021	24.36	30.6	8.2
Trial7	MOOK	Control	June_2021	24.24	30.6	8.21
Trial7	MOOK	Control	June_2021	24.41	30.6	8.22
Trial7	MOOK	S4	June_2021	24.46	30.6	8.21
Trial7	MOOK	S4	June_2021	24.49	30.6	8.21
Trial7	MOOK	S4	June_2021	23.8	30.6	8.25

Trial7	MOOK	S4	June_2021	24.36	30.5	8.26
Trial8	RWU	Control	July_2021	24.7	27.7	7.9
Trial8	RWU	Control	July_2021	24.7	27.8	8
Trial8	RWU	Control	July_2021	22.6	27.6	8
Trial8	RWU	S4	July_2021	24.5	27.9	7.8
Trial8	RWU	S4	July_2021	26.1	27.7	7.9
Trial8	RWU	S4	July_2021	24.9	27.7	7.9
Trial8	RWU	Control	July_2021	24.5	27.7	7.8
Trial8	RWU	Control	July_2021	24.5	28.1	7.9
Trial8	RWU	Control	July_2021	24.7	28.4	7.9
Trial8	RWU	S4	July_2021	24.6	28.4	7.9
Trial8	RWU	S4	July_2021	24.7	28.4	8
Trial8	RWU	S4	July_2021	24.7	28.5	7.9

CHAPTER V: Summary of Results

This dissertation explored the evaluation of probiotics in the eastern oyster (*Crassostrea virginica)* in hatcheries and their effect on oyster microbial communities. The study also explored the associations between larval performance in the hatcheries, the microbial communities, and environmental parameters collected from the hatcheries. The ultimate use of the probiotics in a hatchery setting would require easy use and stable formulation of the probiotics instead of time-consuming laboratory-grown probiotic cultures that are viable for only a short duration of time. This research demonstrated the effectiveness of the candidate probiotic strain, *P. inhibens* S4 formulation, for use as probiotics in aquaculture. The S4 formulation was shown to be safe, easy to handle, stable to use in the hatchery environment, and aid in vibriosis management in larviculture of *Crassostrea virginica*. Understanding the factors that could impact probiotic efficacy in the hatchery would immensely help optimize its usage in the hatchery.

It has been shown that microbes associated with their host can significantly impact the health and function of their hosts (Le Roux et al., 2016). Probiotics can affect host-microbe interactions; hence it is important to assess the impact of probiont S4 on microbial communities associated with the larval host and not only on the host. Our extensive characterization of the bacterial communities of oyster larvae grown in 9 trials performed in 4 different hatcheries during probiotic trials showed diverse and variable communities that *Proteobacteria* dominate. Bacterial community structure in larvae was mainly determined by geographical region and hatchery and, to a lower extent, by trial and the season/month/year in which the larvae were grown. The probiont S4 treatment influenced

the larval bacterial community structure by altering the relative abundance of specific bacterial taxa (ASVs) in the community, such as increasing the abundance of ASVs in the *Alteromonas* genera and decrease in ASVs in *Pseudomonas* genera. This study suggests species-specific effects of S4 on the larval bacterial communities in the hatchery.

Also, the study explored the relationship between oyster larval performance, environmental conditions, and microbial communities to help understand the interactions of these variables and their influences on larval performance in the hatchery, as hatcheries are known to experience larval crashes or unexpected mortalities periodically. Results suggest that the best larval performance was observed at 25^{o}C and 26^{o}C, salinity at 14 - 17psu, and a pH of 8.2-8.3units. A principal component analysis (PCA) showed correlations between bacterial community composition, environmental variables, and larval performance. Variabilities in larval performance in the hatcheries were influenced mainly by temperature and salinity and, more importantly, the bacterial community compositions associated with the larvae. Taxa associated with larval performance include *Alteromonas*, *Pseudoalteromonas*, *Bacillus*, and *Vibrio*. One interesting observation in our study is that taxa associated with changes in salinity and temperature were mainly different from taxa associated with larval performance, suggesting that temperature and salinity can directly impact larval performance independent of the bacterial community.

Overall, the results from this dissertation confirm the benefits of the use of probiotics. *P. inhibens* S4 formulation as a natural and environmentally safe solution in disease management of *C. virginica* larviculture. It also enhanced our understanding of the effects of probiont S4 on the bacterial ecology of larvae in hatcheries. Information from

this research will optimize probiont S4 formulation use in hatcheries, maximizing its benefits for the commercial culture of eastern oyster larvae and preventing undesirable side effects, thereby providing essential contributions to aquaculture fisheries and conservation efforts.

Some limitations to the results presented in this dissertation have been identified. For example, some bacterial reads could not be annotated from the taxonomy annotation databases and were classified as unknown bacteria from the 16S rRNA amplicon data. Also, the inability of these databases to accurately resolve bacterial taxa to species level. Another limitation was that the V6 variable region of the 16S gene that was sequenced does not allow for better resolution of taxa to species level. Future studies should incorporate a longer region of the 16S gene, perform metatranscriptomics and metagenomics analysis to determine the potential roles of these bacterial taxa in oyster larvae performance.

APPENDICES

Appendix A - Chapter 2

One -way ANOVA for formulation method on the viability of S4

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	3	1.224e+17	4.079e+16	29.79	0.000108	***
Residual	8	1.095e+16	1.369e+15			

One -way ANOVA for comparison of fresh and formulated S4

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	5	3.256	0.6512	29.67	2.33e-06	***
Residual	12	0.263	0.0219			

One-way ANOVA for larval survival to S4 formulation treatment in the hatchery

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	1	96	96	2.284	0.14	
Trial	4	21233	5308	125.864	2e-16	***
Treatment:Trial	4	7	2	0.038	0.997	
Residual	29	1384	48			

One-way ANOVA for larval growth to S4 formulation treatment in the hatchery

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	1	0.00	0.00	20.007	0.93	
Trial	4	219.73	54.93	113.780	2e-16	***
Treatment:Trial	4	1.33	0.33	0.688	0.606	
Residual	29	14	0.48			

One-way ANOVA for the levels of Total culturable Vibrios in oyster in each trial.

>Trial2

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value
Treatment	1	7.909e+08	790926091	1.726	0.259
Residual		1.833e+09	1.369e+15		

>Trial3

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value
Treatment	1	5.723e+12	5.723e+12	5.891	0.05
Residual	6	5.830e+12	9.716e+11		

>Trial4

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value
Treatment	1	5.636e+13	5.636e+13	5.209	0.06
Residual	6	6.492e+13	1.082e+13		

>Trial6

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value
Treatment	1	7.700e+10	7.700e+10	1.016	0.3
Residual	4	3.032e+11	7.579e+10		

One-way ANOVA for larval survival to experimental bacterial challenge

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	3	0.8588	0.28628	112.5	4.72e-09	***
Residual	12	0.0305	0.00254			

Appendix B - Chapter 3

Table1. Kruskal-Wallis Rank Sum Test for Simpson's Index of Diversity of relative percent reads by trial and treatment group.

	DF	**Chi-Squared**	**P value**
Treatment	1	0.24634	0.6197
Trial	7	62.739	4.276e-11

Table2. Kruskal-Wallis Rank Sum Test for Chao1 richness of Alpha diversity of relative percent reads by trial and treatment group.

	DF	**Chi-Squared**	**P value**
Treatment	1	0.06775	0.7946
Trial	7	128.25	2.2e-16

Table3. Permutational Multivariate Analysis of Variance Using Distance Matrices (adonis2) for Bray-Curtis beta-diversity (k=2) for each factor.

	DF	R2	F	P value
Location	2	0.32	38.79	0.001
Hatchery	3	0.49	51.3	0.001
Trial	8	0.64	35.774	0.001
Treatment	1	0.01	1.89	0.04
Year	1	0.15	29.97	0.001
Month	3	0.21	14.46	0.001
Season	1	0.06	10.94	0.001

Table 4. Kruskal-Wallis Rank Sum Test for Simpson's Index of Diversity of *Vibrionales* relative percent reads by Treatment and Trial.

	DF	**Chi-Squared**	**P value**
Treatment	1	0.30572	0.5803
Trial	7	41.982	5.243e-07

Appendix C - Chapter 4

Table 1.Results of One-Way ANOVA for environmental conditions measured each trial in the hatchery

>Temperature

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	1	0.00	12.188	0.009	0.9	
Trial	4	48.75	54.93	748.185	2e-16	***
Residual	33	0.54	0.016			

>Salinity

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	1	0.00	0.00	2.028	0.164	
Trial	4	1417.0	354.3	85008.238	2e-16	***
Residual	33	0.1	0.00			

>pH

ANOVA Table	DF	Sum Sq	Mean Sq	F-value	P -value	
Treatment	1	0.0056	0.00557	1.739	0.196	
Trial	4	0.6189	0.15473	48.323	2.41e-13	***
Residual	33	0.1057	0.00320			

Table 2. Spearman rank correlations between the 45 most abundant ASVs, environmental parameters and larval performance

	Bacterial taxa	Correlation	P-value
Growth	ASV_26_Vibrionales;*Vibrio*	0.85	1.5E-13
Salinity	ASV_36_Alteromonadales;*Pseudoalteromonas*	0.84	5.6E-13
Salinity	ASV_39_Gammaproteobacteria	0.80	3.8E-11
Growth	ASV_51_Arenicellales;*Arenicella*	0.79	1.1E-10
Temperature	ASV_6_Gammaproteobacteria	0.78	1.4E-10
Salinity	ASV_21_Alteromonadales;*Alteromonas*	0.78	1.6E-10
Salinity	ASV_28_Rhodobacterales	-0.78	2.5E-10
Growth	ASV_25_Vibrionales;*Vibrio*	0.76	8.1E-10
Temperature	ASV_5_Alteromonadales;*Alteromonas*	0.76	9.7E-10
Temperature	ASV_39_Gammaproteobacteria	-0.76	9.7E-10
Growth	ASV_28_Rhodobacterales	0.74	3.7E-09
Survival	ASV_11_Pseudomonadales;*Pseudomonas*	0.74	5.2E-09
Salinity	ASV_19_Alteromonadales;*Alteromonas*	-0.74	5.2E-09
Salinity	ASV_6_Gammaproteobacteria	-0.73	5.8E-09
Survival	ASV_26_Vibrionales;*Vibrio*	0.73	6.2E-09
Temperature	ASV_36_Alteromonadales;*Pseudoalteromonas*	-0.73	6.2E-09
Growth	ASV_39_Gammaproteobacteria	-0.73	6.3E-09
Temperature	ASV_1_Alteromonadales;*Pseudoalteromonas*	-0.72	9.3E-09
Survival	ASV_8_Bacillales;*Bacillus*	0.72	1.1E-08
Growth	ASV_9_Gammaproteobacteria	-0.72	1.5E-08
Survival	ASV_3_Alteromonadales;*Alteromonas*	0.71	2.6E-08
Survival	ASV_24_Vibrionales;*Vibrio*	0.71	2.7E-08
Temperature	ASV_7_Vibrionales;*Vibrio*	-0.70	5.5E-08
Temperature	ASV_28_Rhodobacterales	0.70	6.2E-08
Growth	ASV_44_Myxococcales	0.68	1.2E-07
Temperature	ASV_44_Myxococcales	0.68	1.2E-07
Salinity	ASV_17_Cellvibrionales	0.68	1.6E-07
Survival	ASV_25_Vibrionales;*Vibrio*	0.68	1.6E-07
Survival	ASV_44_Myxococcales	0.68	1.9E-07
pH	ASV_28_Rhodobacterales	0.67	2.6E-07

Survival	ASV_18_Alteromonadales;*Alteromonas*	0.66	3.6E-07
Salinity	ASV_33_Rhodobacterales;*Sulfitobacter*	0.66	4.5E-07
Survival	ASV_51_Arenicellales;*Arenicella*	0.65	6.7E-07
Growth	ASV_19_Oceanospirillales;*Oceanobacter*	0.65	7.4E-07
Growth	ASV_8_Bacillales;*Bacillus*	0.64	1.1E-06
Growth	ASV_24_Vibrionales;*Vibrio*	0.64	1.4E-06
Growth	ASV_11_Pseudomonadales;*Pseudomonas*	0.64	1.4E-06
pH	ASV_9_Gammaproteobacteria	-0.64	1.5E-06
Survival	ASV_47_Francisellales;*Francisella*	0.64	1.5E-06
Temperature	ASV_19_Oceanospirillales;*Oceanobacter*	0.63	1.6E-06
Salinity	ASV_1_Alteromonadales;*Pseudoalteromonas*	0.63	2.1E-06
Temperature	ASV_18_Alteromonadales;*Alteromonas*	0.62	2.9E-06
Growth	ASV_42_Pseudomonadales;*Pseudomonas*	0.62	3.1E-06
Growth	ASV_18_Alteromonadales;*Alteromonas*	0.61	5.3E-06
Salinity	ASV_5_Alteromonadales;*Alteromonas*	-0.61	5.6E-06
Survival	ASV_9_Gammaproteobacteria	-0.60	7.1E-06
Salinity	ASV_31_Alteromonadales;*Rheinheimera*	-0.60	7.1E-06
Temperature	ASV_11_Pseudomonadales;*Pseudomonas*	0.60	9.0E-06
Growth	ASV_56_Rhodobacterales;*Loktanella*	-0.60	1.0E-05
Salinity	ASV_18_Alteromonadales;*Alteromonas*	-0.59	1.1E-05
Survival	ASV_39_Gammaproteobacteria	-0.59	1.3E-05
Salinity	ASV_49_Rhizobiales;*Labrenzia*	0.59	1.3E-05
Temperature	ASV_21_Alteromonadales;*Alteromonas*	-0.59	1.5E-05
Temperature	ASV_51_Arenicellales;*Arenicella*	0.58	2.4E-05
Salinity	ASV_42_Pseudomonadales;*Pseudomonas*	-0.57	2.7E-05
Growth	ASV_7_Vibrionales;*Vibrio*	-0.57	3.4E-05
Salinity	ASV_11_Pseudomonadales;*Pseudomonas*	-0.56	3.9E-05
pH	ASV_51_Arenicellales;*Arenicella*	0.56	4.0E-05
Growth	ASV_49_Rhizobiales;*Labrenzia*	-0.56	5.0E-05
Survival	ASV_20_Alteromonadales;*Pseudoalteromonas*	0.55	5.4E-05
Growth	ASV_36_Alteromonadales;*Pseudoalteromonas*	-0.55	5.4E-05
Salinity	ASV_2_Rhodobacterales;*Yangia*	0.55	5.4E-05
Salinity	ASV_8_Bacillales;*Bacillus*	-0.55	6.4E-05

Temperature	ASV_42_Pseudomonadales;*Pseudomonas*	0.54	7.9E-05
pH	ASV_25_Vibrionales;*Vibrio*	0.54	8.3E-05
Growth	ASV_3_Alteromonadales;*Alteromonas*	0.54	9.0E-05
pH	ASV_42_Pseudomonadales;*Pseudomonas*	0.54	9.9E-05
Survival	ASV_30_Alteromonadales;*Rheinheimera*	0.54	1.1E-04
Survival	ASV_19_Oceanospirillales;*Oceanobacter*	0.53	1.4E-04
pH	ASV_49_Rhizobiales;*Labrenzia*	-0.53	1.5E-04
pH	ASV_39_Gammaproteobacteria	-0.52	1.8E-04
Growth	ASV_5_Alteromonadales;*Alteromonas*	0.52	2.0E-04
Growth	ASV_33_Rhodobacterales;*Sulfitobacter*	-0.51	2.3E-04
Survival	ASV_56_Rhodobacterales;*Loktanella*	-0.51	2.3E-04
Survival	ASV_36_Alteromonadales;*Pseudoalteromonas*	-0.51	2.5E-04
Salinity	ASV_7_Vibrionales;*Vibrio*	0.51	2.5E-04
Temperature	ASV_30_Alteromonadales*;Rheinheimera*	0.50	3.3E-04
Growth	ASV_20_Alteromonadales;*Pseudoalteromonas*	0.50	3.5E-04
Growth	ASV_47_Francisellales;*Francisella*	0.49	4.3E-04
pH	ASV_19_Oceanospirillales;*Oceanobacter*	0.49	4.9E-04
Survival	ASV_28_Rhodobacterales	0.49	5.5E-04
pH	ASV_26_Vibrionales;*Vibrio*	0.48	5.9E-04
Salinity	ASV_53_Rhodobacterales;*Paracoccus*	0.48	6.1E-04
Salinity	ASV_44_Myxococcales	-0.48	6.3E-04
Survival	ASV_5_Alteromonadales;*Alteromonas*	0.48	6.3E-04
Survival	ASV_7_Vibrionales;*Vibrio*	-0.48	6.9E-04
Salinity	ASV_10_Rhodobacterales	0.48	6.9E-04
pH	ASV_56_Rhodobacterales;*Loktanella*	-0.48	7.0E-04
pH	ASV_20_Alteromonadales;*Pseudoalteromonas*	0.47	8.9E-04
Survival	ASV_17_Cellvibrionales	-0.46	1.0E-03
Temperature	ASV_12_Rhodobacterales;*Sediminimonas*	0.46	1.0E-03
Temperature	ASV_17_Cellvibrionales	-0.46	1.3E-03
Survival	ASV_42_Pseudomonadales;*Pseudomonas*	0.46	1.3E-03
Salinity	ASV_34_Gammaproteobacteria	-0.45	1.4E-03
Growth	ASV_1_Alteromonadales;*Pseudoalteromonas*	-0.45	1.4E-03
pH	ASV_47_Francisellales;*Francisella*	0.45	1.4E-03

pH	ASV_7_Vibrionales;*Vibrio*	-0.45	1.7E-03
Survival	ASV_33_Rhodobacterales;*Sulfitobacter*	-0.44	1.8E-03
Salinity	ASV_26_Vibrionales;*Vibrio*	-0.44	1.8E-03
Growth	ASV_6_Gammaproteobacteria	0.44	2.1E-03
Temperature	ASV_53_Rhodobacterales;*Paracoccus*	-0.44	2.1E-03
pH	ASV_13_Rhodobacterales	-0.43	2.8E-03
Survival	ASV_31_Alteromonadales;*Rheinheimera*	0.43	2.9E-03
Temperature	ASV_23_Gammaproteobacteria	0.42	3.0E-03
pH	ASV_70_Betaproteobacteriales;*Hydrogenophaga*	-0.42	3.4E-03
Survival	ASV_12_Rhodobacterales;*Sediminimonas*	0.41	3.8E-03
Temperature	ASV_24_Vibrionales;*Vibrio*	0.41	3.8E-03
Growth	ASV_12_Rhodobacterales;*Sediminimonas*	0.41	4.4E-03
Growth	ASV_31_Alteromonadales;*Rheinheimera*	0.41	4.4E-03
pH	ASV_6_Gammaproteobacteria	0.39	6.4E-03
Growth	ASV_53_Rhodobacterales;*Paracoccus*	-0.39	6.5E-03
pH	ASV_3_Alteromonadales;*Alteromonas*	0.39	6.8E-03
Growth	ASV_21_Alteromonadales;*Alteromonas*	-0.39	7.0E-03
Salinity	ASV_51_Arenicellales;*Arenicella*	-0.39	7.3E-03
pH	ASV_12_Rhodobacterales;*Sediminimonas*	0.38	8.1E-03
pH	ASV_2_Rhodobacterales;*Yangia*	0.38	8.6E-03
Temperature	ASV_3_Alteromonadales;*Alteromonas*	0.38	9.5E-03
Salinity	ASV_29_PB19	0.37	1.1E-02
pH	ASV_44_Myxococcales	0.37	1.1E-02
pH	ASV_8_Bacillales;*Bacillus*	0.36	1.2E-02
Growth	ASV_17_Cellvibrionales	-0.36	1.4E-02
Growth	ASV_34_Gammaproteobacteria	0.36	1.4E-02
pH	ASV_31_Alteromonadales;*Rheinheimera*	0.36	1.4E-02
pH	ASV_53_Rhodobacterales;*Paracoccus*	-0.36	1.4E-02
pH	ASV_18_Alteromonadales;*Alteromonas*	0.36	1.4E-02
Survival	ASV_1_Alteromonadales;*Pseudoalteromonas*	-0.35	1.5E-02
Temperature	ASV_33_Rhodobacterales;*Sulfitobacter*	-0.35	1.5E-02
pH	ASV_5_Alteromonadales;*Alteromonas*	0.35	1.7E-02
pH	ASV_41_Alteromonadales;*Rheinheimera*	-0.35	1.7E-02

pH	ASV_62_Vibrionales;*Vibrio*	-0.35	1.7E-02
Survival	ASV_49_Rhizobiales;*Labrenzia*	-0.34	1.9E-02
Temperature	ASV_62_Vibrionales;*Vibrio*	0.34	2.1E-02
Temperature	ASV_49_Rhizobiales;*Labrenzia*	-0.33	2.2E-02
Temperature	ASV_8_Bacillales;*Bacillus*	0.33	2.2E-02
pH	ASV_24_Vibrionales;*Vibrio*	0.33	2.5E-02
Temperature	ASV_2_Rhodobacterales;*Yangia*	-0.32	2.6E-02
Salinity	ASV_24_Vibrionales;*Vibrio*	-0.32	2.9E-02
pH	ASV_11_Pseudomonadales;*Pseudomonas*	0.31	3.4E-02
Temperature	ASV_31_Alteromonadales;*Rheinheimera*	0.30	4.2E-02