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Utilization of Probiotics to Manage Epizootic Shell Disease in American Lobsters, *Homarus americanus*

Melissa Hoffman
University of Rhode Island, melissahoffman@uri.edu

UTILIZATION OF PROBIOTICS TO MANAGE EPIZOOTIC SHELL DISEASE IN AMERICAN LOBSTERS, HOMARUS AMERICANUS

BY

MELISSA HOFFMAN

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MELISSA HOFFMAN

APPROVED:

Thesis Committee:

Major Professor Marta Gomez-Chiarri

Kathleen Castro

David C. Rowley

Nasser H. Zawia
DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

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Abstract

Epizootic shell disease (ESD) in the American lobster, *Homarus americanus*, has a major impact on the southern New England lobster industry, yet there are no practical tools for managing the disease. The goal of this study was to identify bacterial probiotics that could be used to manage ESD in wild lobster populations. Candidate bacterial isolates (n = 24) were previously isolated from lobsters in Narragansett Bay and identified as having probiotic characteristics against ESD-associated bacteria *Thalassobius* sp. and *Aquimarina macrocephali,* or the fish pathogen *Vibrio anguillarum*. Healthy lobster post-larvae were exposed to five of the candidate strains isolated from lobsters and a probiotic bacterial strain isolated from the Eastern oyster, *Crassostrea virginica (Phaeobacter inhibens* S4*)*. After several weeks of treatment, there were no significant differences in molting frequency, mortality, or growth of treated lobsters when compared with the control, indicating the candidate probiotics do not adversely affect lobster post-larvae. The effect of selected candidate probiotics (n = 3) on progression of ESD in adult lobsters was tested for three months. Frequent molting due to high disease severity confounded long-term effects of the treatments, and no significant differences were seen in mortality, molting, growth, or disease progression. These results highlight the challenges involved in the development of tools for the management of a chronic disease with a poorly understood etiology. Future research should focus on a better understanding of microbe-microbe-host interactions in ESD, and the effect of environmental conditions on these interactions.

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Dedication

I dedicate this thesis to my mom and dad, Martha Wells Hoffman and Ben Hoffman, who nurtured my curiosity in animals of the weird and slimy variety. Without them encouraging my love of all things oceanic from a young age, I would not have written this thesis. Thank you for your unwavering love, support, and confidence in me.

Preface

The following thesis has been prepared in manuscript format according to the guidelines of the Graduate School of the University of Rhode Island.

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CHAPTER I

Utilization of probiotics to manage epizootic shell disease in the American lobster, *Homarus americanus*

Melissa Hoffman[1]*, Kathy Castro[1], Grace Underwood[2], Hilary Ranson[3], Mitch Hatzipetro[1], Barbara Somers[1], David Rowley[3], David R. Nelson[2], Marta Gomez-Chiarri[1]

[1]Department of Fisheries, Animal, and Veterinary Sciences, University of Rhode Island, Kingston, RI
[2]Department of Cell and Molecular Biology, University of Rhode Island, Kingston, RI
[3]Department of Biomedical and Pharmaceutical Sciences, University of Rhode Island, Kingston, RI

Abstract

Epizootic shell disease (ESD) in the American lobster, *Homarus americanus*, has a major impact on the southern New England lobster industry, yet there are no practical tools for managing the disease. The goal of this study was to identify bacterial probiotics that could be used to manage ESD in wild lobster populations. Candidate bacterial isolates (n = 24) were previously isolated from lobsters in Narragansett Bay and identified as having probiotic characteristics against ESD-associated bacteria *Thalassobius* sp. and *Aquimarina macrocephali,* or the fish pathogen *Vibrio anguillarum*. Healthy lobster post-larvae were exposed to five of the candidate strains isolated from lobsters and a probiotic bacterial strain isolated from the Eastern oyster, *Crassostrea virginica (Phaeobacter inhibens* S4*)*. After several weeks of treatment, there were no significant differences in molting frequency, mortality, or growth of treated lobsters when compared with the control, indicating the candidate probiotics do not adversely affect lobster post-larvae. The effect of selected candidate probiotics (n = 3) on progression of ESD in adult lobsters was tested for three months. Frequent molting due to high disease severity confounded long-term effects of the treatments, and no significant differences were seen in mortality, molting, growth, or disease progression. These results highlight the challenges involved in the development of tools for the management of a chronic disease with a poorly understood etiology. Future research should focus on a better understanding of microbe-microbe-host interactions in ESD, and the effect of environmental conditions on these interactions.

Introduction

Heavy economic and cultural dependence on American lobsters (*Homarus americanus*) make coastal communities and stakeholders vulnerable in the face of disease outbreak. In 2016, roughly 159 million pounds of lobster were harvested in the U.S., worth nearly $670 million (NMFS 2018). Strong cultural ties with lobsters encourage tourism, further increasing their economic importance (Steneck et al. 2011). While southern New England makes up a small portion of this industry, this stock has been the most susceptible to an emergent disease in lobsters, known as epizootic shell disease (ESD).

In 1996, ESD was reported in lobsters in Rhode Island (Castro and Angell 2000). Since this onset, annual lobster catches in Rhode Island have reduced significantly, with annual landings falling in value from $19 million in the 1990's to around $12 million in 2015 (NMFS 2016). It was estimated in 2012 that 10-30% of lobsters in Rhode Island had ESD (Castro and Somers 2012). This proportion of disease prevalence was even higher in egg-bearing female lobsters, where prevalence was reported at 60-80% (Castro and Somers 2012). While ESD prevalence remains low in the Gulf of Maine, the mean prevalence has more than doubled in the past 8 years from 0.5% to 1.2% (Reardon et al. in press).

ESD is characterized by unsightly, melanized lesions that degrade the lobster's carapace. These lesions significantly decrease the lobster's market value, and can lead to negative impacts on lobster health, sometimes resulting in death (Smolowitz et al. 2005; Hoenig et al. 2017). Although the etiology of ESD is not well understood, outbreaks are likely influenced by a number of factors including compromised condition

of the host, presence of putative pathogens in the ecosystem, and environmental stress (Tlusty et al. 2007; Gomez-Chiarri and Cobb 2012; Shields 2013, Barris et al. in press). Comparative analysis of microbial communities between ESD-infected and healthy lobsters suggest the disease is polymicrobial, and two species of bacteria, *Thalassobius* sp. and *Aquimarina macrocephali* (formerly designated *Aquimarina 'homari'*), may be involved in the progression of ESD lesions (Chistoserdov et al. 2005; Quinn et al. 2012). Additionally, there is evidence of a transition to lower bacterial diversity in and around the lesions of laboratory-reared lobsters with shell disease, potentially initiated by dysbiosis in the microbial community on the shell (Feinman et al. 2017).

While the decrease in lobster populations in southern New England is not solely due to ESD, high rates of ESD have been linked to a decrease in overall larval supply and subsequent population declines in Rhode Island (Wahle et al. 2009). It is hypothesized that ESD triggers molting in lobsters as a defense mechanism, so lobsters can get rid of the diseased shell before the disease negatively impacts the lobster (Laufer et al. 2005). Consistent with this hypothesis, significantly high levels of the ecdysteroid receptor (a gene associated with molting) have been reported in lobsters with ESD, which may be triggering them to molt more frequently (Smolowitz et al. 2005; Castro and Somers 2012; Tarrant et al. 2012). If ovigerous females molt too early in an effort to rid themselves of the disease, this would result in the loss of their entire clutch of eggs. Alternatively, lobsters can die from ESD if the lesions are so severe that ulceration of the epidermis occurs and prevent the lobster from molting successfully (Smolowitz et al. 2005). Mark-recapture studies on lobsters in the southern New England stock estimate that lobsters with moderate to severe ESD have a survival rate of only 30%

when compared to healthy lobsters (Hoenig et al. 2017). High mortality in diseased lobsters, and especially in ovigerous females, could explain population declines and the poor larval recruitment observed in southern New England (Hoenig et al. 2017).

Despite characterization of the bacterial community on ESD-associated lesions (Meres et al. 2012; Feinman et al. 2017), Koch's Postulates have not been fulfilled for ESD (Gomez-Chiarri and Cobb 2012). Koch's Postulates specify that in order to identify the causative agent of an infectious disease, four criteria must be met: the pathogen must be present in all cases of disease; the pathogen can be isolated from the diseased host and grown in pure culture; the pure culture must cause disease when inoculated into a new healthy host; and finally, the pathogen must be reisolated from the new host and shown to be the same pathogen (Fredricks and Relman 1996). The absence of a laboratory model that fulfills Koch's Postulates makes ESD a particularly difficult disease to study, and leaves those who rely on lobsters for their livelihood with more questions than answers. Quinn and colleagues (2012) discovered that abrading the epicuticle of lobsters and then exposing them to *A. macrocephali* alone, or a suite of *A. macrocephali, Thalassobius* sp., and *Pseudoalteromonas gracilis* resulted in lesion formation. However, non-abraded lobsters with the same treatments did not form lesions, indicating that these bacteria may be opportunistically colonizing lesions and furthering their development rather than initiating disease (Quinn et al. 2012).

Currently, there are no applied management tools for controlling or mitigating ESD in wild lobster populations. Since transmission and onset of the disease is not understood, typical disease mitigation strategies cannot be proven effective in the southern New England system. When fishermen encounter a diseased lobster, they only

have a few options: remove the lobster from the population; throw the lobster back in the water after catching; or sell the lobster for processed meat at a significantly reduced price. Additionally, lobsters lack an adaptive immune system that is characteristic of vertebrates, rendering traditional vaccines aiding in disease prevention an unviable option (Vazquez et al., 2009). We propose that probiotics could offer an alternative, novel tool for disease management. Probiotics are naturally occurring microorganisms that can provide health benefits to the host. While often thought of as food supplements, probiotics can also be added to water for aquatic organisms. These microorganisms have already shown disease protection in many other invertebrates and fish in aquaculture farm settings (Verschuere et al. 2000; Karim et al., 2013; Wu et al., 2014; Zhao et al., 2016; Safari and Paolucci, 2017).

Probiotic bacteria may confer benefits to the host in a number of ways, including competition with pathogenic bacteria, or modulating the immune system of the host (Perez-Sanchez et al. 2014; Zhao et al. 2016). These mechanisms of action can give organisms an advantage when fighting disease (Newaj-Fyzul et al. 2014). Probiotics are a favorable alternative to other treatments, like antibiotics, because probiotics do not select for antibiotic-resistant bacteria. While the application of probiotics in a wild fishery is more challenging than administration in an aquaculture farm, probiotics could offer a promising approach for ESD treatment if they can be incorporated in currently used lobster fishing practices.

Due to the high prevalence of ESD in southern New England and expansion within the Maine lobster fishery, fishermen need tools to sustain the fishery long-term and avoid economic consequences. Previous work in our collaborative, interdisciplinary

probiotic group at the University of Rhode Island (URI) has identified 24 bacterial strains from the shells of healthy-appearing lobsters in Narragansett Bay in 2016 that exhibit probiotic characteristics (Underwood 2018). These strains were tested against putative ESD pathogens, *Thalassobius* sp. and *Aquimarina macrocephali,* to identify features characteristic of probiotic bacteria, including the ability to: 1) inhibit the growth of putative ESD pathogens; 2) form strong biofilms; and 3) compete with putative pathogens in co-incubation experiments (Zhao et al. 2016). Other criteria considered when selecting probiotics included: 4) the ease in which the bacterial strains can be cultured; 5) if the strain is safe to use on the host; 6) ability of the strain to enhance physiological processes such as growth; or 7) ability of the strain to slow the progression of disease (Perez-Sanchez et al. 2014).

Based on our understanding of how candidate probiotics isolated from lobsters interacted with putative ESD pathogens *in vitro* (Underwood 2018), we tested the effect of treatment with candidate bacterial strains on lobsters *in vivo*. This involved examining the safety of candidate probiotic strains on post-larval lobsters and testing the effect of selected candidate probiotics on the progression of ESD lesions in adult lobsters. Determining the efficacy of probiotics in slowing or stopping the progression of ESD can help establish probiotics as a potential disease mitigation tool for ESD, or diseases similar to ESD.

Methods

1) Bacterial Strains

Isolation, growth, and characterization of potential probiotics was conducted by Grace Underwood and Hilary Ranson at the University of Rhode Island (Underwood 2018). These strains (Table 1) were cryogenically preserved for further characterization and testing for *in vivo* trials. A probiotic isolate known to protect larval oysters against challenge with bacterial pathogens, *Phaeobacter inhibens* S4 (Karim et al. 2013) was included as control.

Table 1. Candidate probiotics used in this research.

Isolate ID	**16s Sequencing Identification**	**ZOI (mm) against *Thalassobius* sp.**	**ZOI (mm) against *Aquimarina macrocephali***	**Biofilm (OD 580)**
B*	*Bacillus sp.*	2	4.33	2.88
L210*	*Loktanella maritima*	2	0	3.71
L211	*Loktanella maritima*	1.7	0	3.71
P01	*Pseudoalteromonas sp.*	4.3	0	3.17
P11	*Pseudoalteromonas sp.*	0	2.2	3.32
P14*	*Pseudoalteromonas sp.*	2.5	0	3.24
P18	*Pseudoalteromonas sp.*	0	1.6	2.89
P21	*Pseudoalteromonas sp.*	1.17	0	2.18
P22	*Pseudoalteromonas sp.*	1.83	0	3.71
S4*	*Phaeobacter inhibens*	1	1.4	3.89

* Spontaneous streptomycin-resistant (Sm^r) strains were developed in previous research and were used in PL Experiment 3 (Underwood 2018).

Bacterial Growth Conditions

Bacterial species routinely grown included *A. macrocephali* I32.4, *Thalassobius* sp. I31.1 (Chistoserdov et al. 2005), and the selected potential probiotic isolates (Table 1; Underwood 2018). If available, spontaneous streptomycin-resistant (Sm^r) strains developed in previous research were used in PL experiment 3. This included: *L. maritima* 06-YPC210 Sm^r, *Pseudoalteromonas* sp. 03-YP014 Sm^r, and *Bacillus* sp. 06-YP001 Sm^r (Underwood 2018), and *P. inhibens* S4Sm^r (Zhao et al. 2016). Bacterial isolates were grown at room temperature (23°C) on YP30IOS (10 g peptone, 2 g yeast, 60 g of Instant Ocean Salt™ and 30 g agar, dissolved in 1 L deionized water, pH 7.0; with or without 100 μg/mL Sm depending on the isolate) agar plates or in YP30IOS liquid media in 50 mL polypropylene culture tubes with shaking (150 rpm).

2) Post-larval (PL) lobster probiotics screening

In order to detect any adverse effects of the candidate probiotic bacterial strains on survival and health, lobsters at the post-larval stage, a more vulnerable and easier to handle life stage than adults, were exposed to candidate probiotics (Table 1). Three experiments were performed, in which conditions to test the candidates were progressively optimized.

PL Experiment 1

Developmental stage three and four lobster post-larvae (PL) were obtained from the Mount Desert Oceanarium & Lobster Hatchery in Bar Harbor, Maine and brought to the East Farm Fisheries Center at URI on September 14, 2016. Plastic 750 mL

containers were filled with artificial seawater (ASW; 35 psu, 16°C) and 3 x 30 mL medicine cups were secured in each of the 750 mL containers. Small holes had been punched into the medicine cups using a syringe needle (25G) to ensure water flow and one PL was placed in each medicine cup. PLs were left to acclimate for two days.

Four randomly assigned 750 mL containers were used for each treatment, for a total of 12 PLs per treatment, 3 lobsters per container. There was one control group and seven experimental treatment groups (B, L210, L211, P14, P21, P22, and S4; Table 1). Experimental treatments were administered at a concentration of 10^4 colony forming units (CFU) per mL of seawater via addition to their container by pipette (85-125 μL per well, dependent on bacterial stock concentration), two days after acclimation began. Five days later, any PLs that died were replaced with extra PLs and a second treatment was administered. Twenty-four hours after the second probiotic treatment, all PLs were exposed to putative pathogen, *Thalassobius* sp., at a concentration of 10^4 CFU/mL. PLs were then left in their containers for another 15 days, for a total of 20 experimental days. Each day, mortality (number of PLs dead), molting (number of PLs that molted), salinity (psu), and temperature (°C) were monitored. PLs were fed frozen *Artemia* sp. (Sally's Frozen Brine Shrimp™, San Francisco Bay Brand, Newark, CA) daily and debris was removed from containers 1-2 hours after feeding. Water changes occurred every four days, with half of the total volume in the 750 mL holding container being exchanged. Temperature during this experiment was maintained at 16°C. At the end of the experiment, photographs were taken of PLs.

PL Experiment 2

Stage four and five PLs were obtained on March 30th, 2017 from the Sound School, a vocational aquaculture high school in New Haven, Connecticut, and brought to the Center for Marine Life Sciences at URI (Narragansett, RI). PLs were acclimated in a fifteen-gallon aquarium tank with ASW (30 psu, 18°C) for 24 h, and were then placed in six-well plates. Six-well plates were used instead of a shared container, like in PL experiment 1, to limit effects PLs would have on each other and to increase statistical power. Each well contained one PL and 10 mL of artificial seawater. PLs were left to acclimate for three days. There were two 6-well plates per treatment, for a total of 12 PLs per treatment. There was one control group, which received no treatment, and 12 experimental treatment groups (Table 1). Full water changes were administered daily using a serological pipette roughly 1 h after PLs were fed frozen *Artemia* sp. Lids were removed from the 6-well plates to better promote oxygen transfer into the water and the plates were held at room temperature (18-20°C).

Experimental treatments were administered at a concentration of 10^4 CFU/mL via addition to their container by pipette (12-20 μL per container, dependent on bacterial stock concentration) once a week for two weeks, with treatments starting on April 3, 2017. Each day, mortality (number of PLs dead), molting (number of PLs that molted), salinity (psu), and temperature (°C) was monitored. The experiment was halted after two weeks when cumulative mortality reached almost 70%. PLs were photographed at the beginning and end of the experiment under a dissecting microscope to look for lesion-like marking or spots using Lumenera Infinity-1 microscope camera and Infinity Capture software (Lumenera Corporation, Ottowa, ON).

PL Experiment 3

Experimental Design and husbandry

Stage seven and eight lobster post-larvae were obtained from the Sound School (New Haven, CT), and transported to the Center for Marine Life Sciences at URI on September 13, 2017. During transportation, PLs were held in ambient seawater from the Sound School and an ice pack. Upon arrival, PLs were transferred into individual 250 mL glass culture dishes (Carolina Biological Supply Co., Burlington, NC) with seawater (29-31 psu salinity) from Narragansett Bay filtered through 2μm pore-size filter and kept between 14-18°C. The containers were kept in a water bath at (target temperature of 16°C) to maintain temperature stability. All containers were wrapped in black electrical tape so that PLs would not be able to see each other in order to reduce potential stress due to their aggressive behavior. Containers were randomly assigned a candidate probiotic and a unique identifier, with ten PLs per treatment. There were six different experimental treatments treatment (BSm^r, $L210Sm^r$, L211, $P14Sm^r$, and $S4Sm^r$, and AT, a mix of *A. macrocephali*, or *Thalassobius* sp.; Table 1) and one control group which received no treatment. PLs acclimatized in the lab for seven days were photographed under a dissecting microscope (15x magnification) using a Lumenera Infinity-1 microscope camera. Images were used to measure PL size using Infinity Capture imaging software and count lesion-like markings or spots on their shell. Additional pictures were taken of all PLs (including dead) on days 25 and 40 of the experiment.

On day 1, PLs were exposed to their designated probiotic treatment at a concentration of 10^4 CFU/mL of seawater via addition to their container by pipette (150-

250 μL per container, dependent on bacterial stock concentration). PLs were exposed to their treatment for 24 h, at which point water was partially changed (150 out of 250 mL of seawater). PLs were treated once a week for 25 days and then twice a week for an additional 15 days. One day before treatments were administered, PLs were given a full (250 mL) water change. Mortality and molting occurrence were recorded daily. PLs were fed daily with frozen *Artemia* sp. Water in the holding containers was partially changed each day about 1 h after feeding, with 150 of 250 mL (16°C, 29-31 psu salinity) seawater being exchanged, so that excess food was removed. Before water changes, three randomly selected PL containers were chosen each day for testing of the following water quality parameters: ammonia (ppm), nitrate (ppm), and pH using the API Saltwater Master Test Kit (Mars Fishcare North America, Inc.), dissolved oxygen (DO, mg/L) using a Milwaukee MW600 portable DO meter (Milwaukee Instruments, Rocky Mount, NC), and salinity (psu) using a refractometer. Temperature loggers (HOBO, Water Temp Pro v2, Onset Computer Corporation, Bourne, MA) were deployed in the water baths to record ambient water temperature every 10 min. Additionally, dissolved ammonium levels (μmol) were measured in all experimental containers on day 35 after the start of treatments (two days after a probiotic treatment) and on day 40 (4 days after a probiotic treatment). Dissolved ammonium concentration was measured using standard colorimetric techniques (Solorzano 1969) in an Orion Aquamate 7000 VIS Spectrophotometer©. Dissolved oxygen was also measured at the end of the experiment (day 40). Surviving PLs were photographed and stored in 30 mL 95% ethanol.

Bacterial Sampling and Evaluation of Colony Forming Units (CFU):

Samples of water and biofilm on the surfaces of the incubation containers from Experiment 3 were collected from the PL containers to: 1) determine the ability of probiotics to persist after treatment; and 2) investigate effect of probiotic treatment on bacterial load. This sampling was performed only for streptomycin-resistant isolates (Table 1), to allow us to differentiate between probiotic counts (determined on Sm media) versus total bacterial counts in the containers (determined on media without Sm). Water and biofilm samples were taken immediately after probiotics were administered (day 0) and on days 1, 3, and 6 after probiotics treatment during the first week of the experiment, from 3 randomly selected containers. Biofilm samples from all containers were also taken on the last day of the experiment (day 40, Table 2). For water samples, approximately 1.5 mL of water was removed from PL holding containers and placed in a micro centrifuge tube. For the first four collection time points, three samples were taken from each treatment. For biofilm samples, a sterile polyester-tipped applicator was used to swab the entire circumference of PL containers. The applicator was then vigorously stirred in 1 mL of ASW for 20 seconds. Serial 1:10 dilutions up to 10^{-6} were performed for each sample, and all dilutions were spot plated in triplicate (10 μL/spot) on to YP30 agar plates (to measure total bacterial load) and onto YP30Sm agar with antibiotic (streptomycin, 200μg/mL). The plates were incubated at room temperature (18-20°C) for at least 2 days or until colonies formed. After incubation, all colonies were counted, counts for each dilution were averaged, and the average CFU/mL was calculated for each treatment. The morphology of colonies on YP30Sm plates was evaluated to confirm it corresponded to the candidate probiotic.

Table 2. Sampling timeline for water and swab samples for determination of bacterial concentrations (CFU/mL). Sampling started before probiotic treatments started (day 0), and ended after 40 days when the experiment was terminated. All water and swab samples were collected as described in the methods for every time point.

CFU Sampling Timeline						
Date, Time	9/21/17, 7:00am	9/21/17, 5:00pm	9/22/17, 4:00pm	9/24/17, 4:00pm	9/27/17, 4:30pm	11/1/17, 9:00am
# Day	-	0	1	3	6	40
Number of containers sampled for Sm^R bacterial concentrations in water	3	3	3	3	3	0
Number of containers sampled for total bacterial concentrations in water	3	0	1	3	1	0
Number of containers sampled for Sm^R bacterial concentrations in surfaces	0	0	3	0	3	[Control, BSm^R, $S4Sm^R$, L211, AT] = 7 $P14Sm^R$ = 6 $L210Sm^R$ = 5
Number of containers sampled for total bacterial concentrations in surfaces	0	0	1	0	1	
Treatments Sampled	Control, BSm^R, $S4Sm^R$, $P14Sm^R$, $L210Sm^R$					Control, BSm^R, $S4Sm^R$, $P14Sm^R$, $L210Sm^R$

3) Diseased Adult Lobster Probiotic Testing

In order to test the ability of the candidate probiotics to slow ESD progression, one laboratory trial was run with sublegal-sized lobsters from Narragansett Bay, RI with ESD. Lobsters averaged 82.5 mm (± 2.1 mm SD) carapace length and included 34 males and 6 females. Lobstermen from the RI Lobstermen's Association collected the adult lobsters for this trial from outside of Fort Wetherill in Narragansett Bay. Lobsters were

transported to the Graduate School of Oceanography at URI in the Center for Marine Life Sciences, where each was placed in an individual 57 L tank with flowing seawater (roughly 300-500 mL/min) from Narragansett Bay, RI and maintained at a temperature of 16°C and 29-31 psu salinity. The trial took place starting in October of 2016 and ending in January of 2017, since this is typically after lobsters have molted and ESD condition was expected to be less severe (Castro and Somers 2012).

Candidate probiotics for treatments were selected based on a combination of the *in vitro* data (Underwood 2018) and performance in PL screening experiments as determined. The following criteria were used in the selection process: ease in which the candidate probiotic isolate can be cultured in the lab; ability to inhibit the growth of pathogens (ZOI) and form biofilms; and lack of toxicity or potential decrease in mortality of PLs during exposure experiments as compared to non-treated PLs. Experimental treatments included one control group which received no treatment (n = 10 lobsters) and three experimental groups each tested with either B, P14, or S4 (Table 1, n = 10 lobsters per treatment). Tanks were assigned to treatments randomly. Prior to treatment, water flow to the tanks was halted (15 min) to ensure adequate residence time for the probiotic treatments. Probiotics were added to lobster tanks twice a week at a concentration of 10^4 CFU/mL, and left to incubate in their tanks for 15 minutes, at which point water flow was resumed.

Mortality (number of dead individuals), molting (number of individuals that molted), and tank water salinity (psu) and temperature (°C) were measured at least 3 times a week. Lobsters were photographed (using Olympus Stylus TG-4 camera) and measured at the beginning and end of the trial to document and analyze changes in

percent cover of lesions on the lobsters over time. The camera was placed at a fixed distance for photographs and 7 pictures were taken for each lobster at the beginning and end (dorsal carapace, right lateral carapace, left lateral carapace, dorsal abdomen, right lateral abdomen, left lateral abdomen, dorsal claws).

Data Analysis

Survival and molting occurrence curves were analyzed using Prism 6.0 (GraphPad Software, La Jolla, CA) to determine differences in mortality across treatments over time using a Log-rank Mantel-Cox statistical test. For molting occurrence curves, PLs that died during the experiment were censored. Growth (change in carapace length from start to end of experiment) was analyzed using a one-way ANOVA in R Studio. The proportion of PLs with lesions in each treatment was determined and analyzed using a chi-square test in R Studio. The effect of treatment on changes in the amount of bacteria (CFU/mL) in water and biofilm samples over time was analyzed using a two-way ANOVA with pairwise multiple comparison post-hoc test (Tukey) in Prism 6.

For the adult lobster trial, the percent change in lesion cover was measured for each lobster and placed in a binary category of ESD condition being “severe” or “not severe.” When an individual had more than 50% of their body covered in lesions, they were characterized as having severe ESD (Tarrant et al. 2012). A series of chi-square tests were run in R Studio to investigate if mortality, molting, or disease severity differ significantly based on treatment.

Results

PL Experiment 1

Over the course of the 20-day experiment, there were no significant differences in survival or molting occurrence between treatments (Fig. 1). At the end of the experiment, pinpoint, circular melanized lesion-like markings on the lobster's shells were observed (Figs. 2, 3). While there were no significant differences between treatments, the proportion of PLs with lesions was higher in the L210 treatment (Fig. 2). Since lobster appearance was not documented at the beginning of the experiment, it is uncertain whether these lesions developed during the experiment as an effect of treatment. The addition of *Thalassobius* sp. on day 6 of the experiment did not have an effect on PLs survival, molting or presence of lesions.

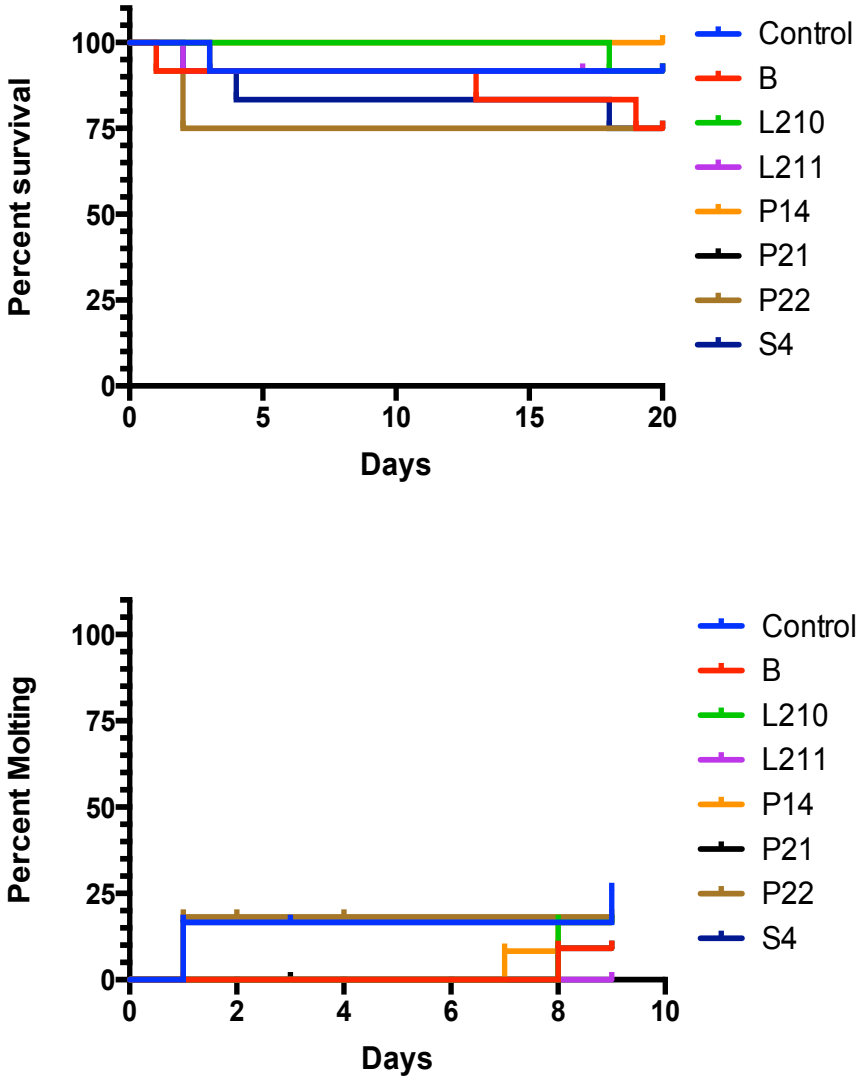

Figure 1. Effect of candidate probiotic treatment on percent survival and percent molting occurrence of lobster post-larvae (PL) over 20 days during Experiment 1. At time 0, probiotics were administered to PLs and one day later they were challenged with *Thalassobius* sp. No significant differences in survival or molting between treatments were observed (survival Log-rank test p=0.42, molting Log-rank test p=0.74).

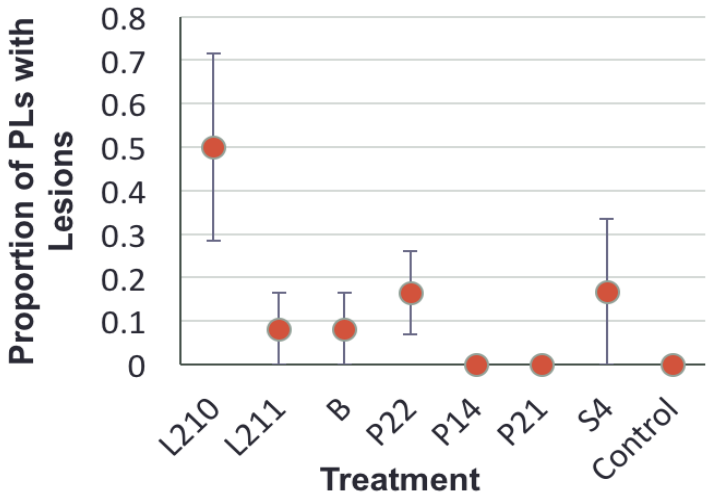

Figure 2. Effect of candidate probiotic treatment on mean proportion of lobster post-larvae with lesions after 15 days during PL Experiment 1. Averages were taken from the proportion of lobsters in each treatment (3 per beaker, 4 replicates) with lesions. No statistical differences were observed between groups (chi-square p=0.43).

Figure 3. Photograph of a representative lobster post-larvae with potential shell disease lesions. This PL was treated with probiotic candidate *Bacillus* sp. for 20 days in PL Experiment 1.

PL Experiment 2

This experiment was terminated 15 days after start of treatment due to heavy mortality (69% of all PLs died). Significant differences in survival were observed between control and L210 treatments, which showed the highest mortality (Log-rank test p=<0.0001, Fig. 4). Water temperature during this experiment ranged from 19-20°C. At this time point, percent survival was lowest in the control and L210 (0 and 8.3% survival, respectively) treatments when compared with the rest of the treatments (ranging from 25-75% survival). There were no significant differences in molting between treatments (Fig. 4), and lesions were not observed on PLs at either the start or end of the experiment.

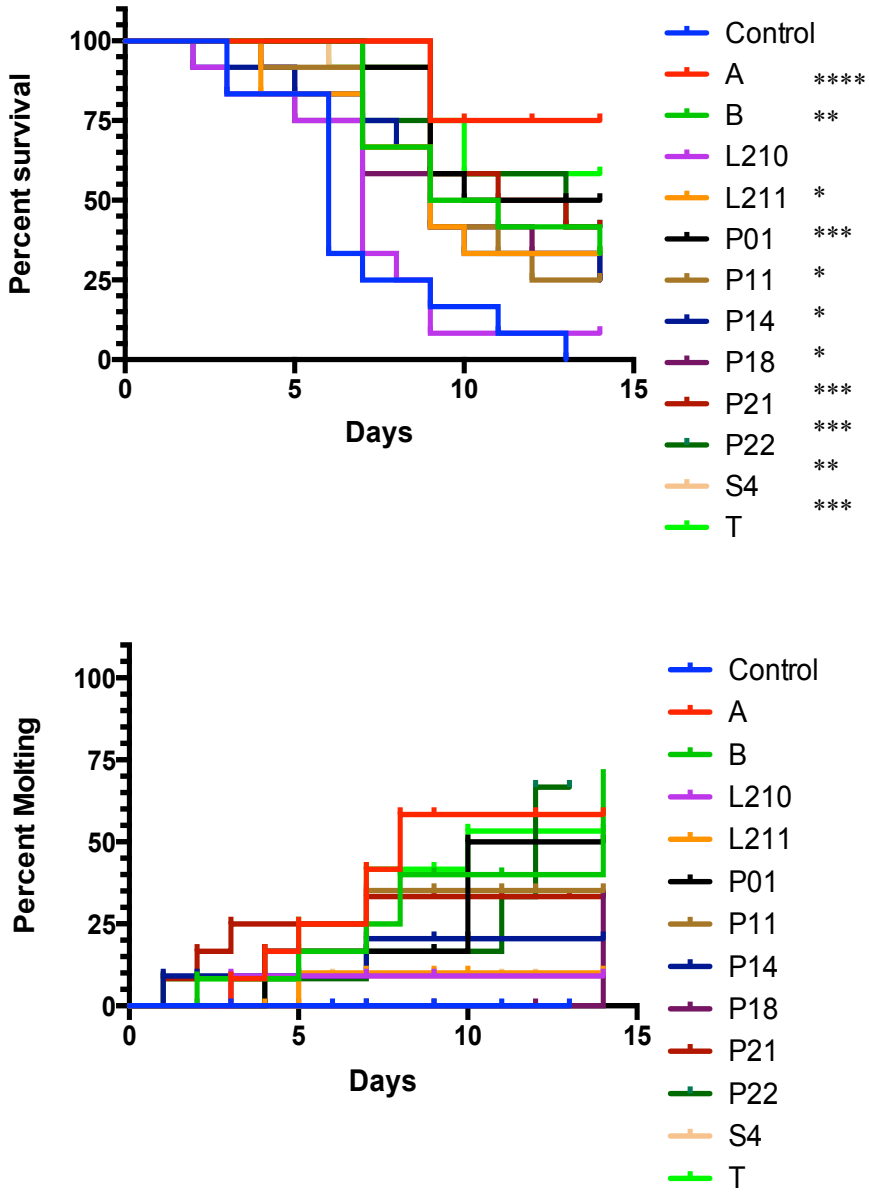

Figure 4. Effect of candidate probiotic treatment on percent survival (top) and percent molting occurrence (bottom) of lobster post-larvae over 14 days during PL Experiment 2. At time 0, probiotics were administered to PLs. Significantly higher mortality was observed in the L210 and control treatments (Survival Log-rank test p=<0.0001, Molting Log-rank test p=0.10) than in other treatments. Asterisks denote the level of significance (p-value) when compared to the control.

PL Experiment 3

Survival, Molting, Growth, and Lesions:

Throughout the 40-day experiment there were no significant differences in survival between treatments (Fig. 5). Cumulative mortality levels remained below 50% in all of the treatments. Treatment with a mix of *A. macrocephali* and *Thalassobius* sp., (AT) did not have a significant effect on PLs compared to other treatments, including the control. On day 34 of the experiment there was a mortality event where 12 PLs died (0-30% mortality in each treatment) (Fig. 5). There was no significant difference in molting occurrence between treatments (Fig. 5). There were also no significant differences in growth of the lobsters that molted and survived the entire experiment (Fig. 6). Two individuals from different treatments (L211 and P14Sm^{r}) developed lesions during the experiment (Fig. 7), and both died (on days 26 and 33, respectively).

Water Quality Parameters:

Average temperature ranged from 14-18°C and salinity ranged from 30-33 psu during the 40-day experiment (Fig. 8). Temperature fluctuations were a result of the water cooling system at the Center for Marine Life Science and changes in temperature in the source water from Narragansett Bay. Fluctuations were also observed in dissolved oxygen, nitrate, and pH, with ideal water quality parameters being observed after full water changes. Nitrate concentrations remained low until day 31, after which nitrate concentrations periodically spiked until the end of the experiment (Fig. 18). There were no significant differences between treatments in ammonium or dissolved oxygen measurements taken at the end of the experiment (Figs. 9, 10).

Effect of probiotic on bacterial levels:

Significantly higher levels of some of the candidate probiotics could be detected in samples of water (as determined by counts of streptomycin resistant bacteria) from the PL containers on day 0 (right after the probiotic treatment was administered; $S4Sm^r$ and BSm^r was significantly higher than $P14Sm^r$) and day 1 (24 hours after treatment; S4 was significantly higher than all treatments) (Two-way ANOVA, p=<0.05; Fig. 11 and Table 4). Significant differences in probiotic concentration between treatments on day 0, right after addition of candidate probiotics to the containers, were probably due to differences in the relationship between CFU and OD580 (the parameter used to estimate bacterial concentration in the stocks) between candidate probiotic isolates. There was a significant interaction between time and probiotic concentration (Two-way ANOVA, p=<0.05; Table 4). By day 3 and 6 (after full water changes in PL containers occurred) there was no statistical difference between treatments, but Sm^r bacteria could be detected at low levels in water samples from all treatments, including the control, on day 6 (Fig. 11).

Neither treatment nor time had a significant effect on probiotic bacterial concentration on the water-immersed surfaces of PL containers (Two-way ANOVA effect of treatment p=0.07, time p=0.09, time x treatment interaction p=0.12; Fig. 12 and Table 6). Post-hoc pair-wise comparisons of treatments within day 1 (24 hours after treatments were administered), however, showed that $S4Sm^r$ had a significantly higher biofilm concentration ($1.64x10^5$ CFU/ml) when compared with BSm^r ($2.67x10^4$ CFU/ml) (Two-way ANOVA, p=<0.05, Tables 5 and 6). By days 6 and 40, there was no statistical difference between treatments, but Sm^r bacteria could still be detected at

low levels in water samples from all treatments, including the control (Fig. 12, Tables 5 and 6). Colony morphologies of the control samples were variable between different PL containers and the day they were sampled.

Treatment did not have a significant effect on total bacterial load (as measured on YP30 agar plates) in water samples 3 days after probiotics were administered (One-way ANOVA p=0.34; Fig. 13) or in biofilm samples taken from PL container surfaces on day 40 (One-way ANOVA p=0.10; Fig. 14).

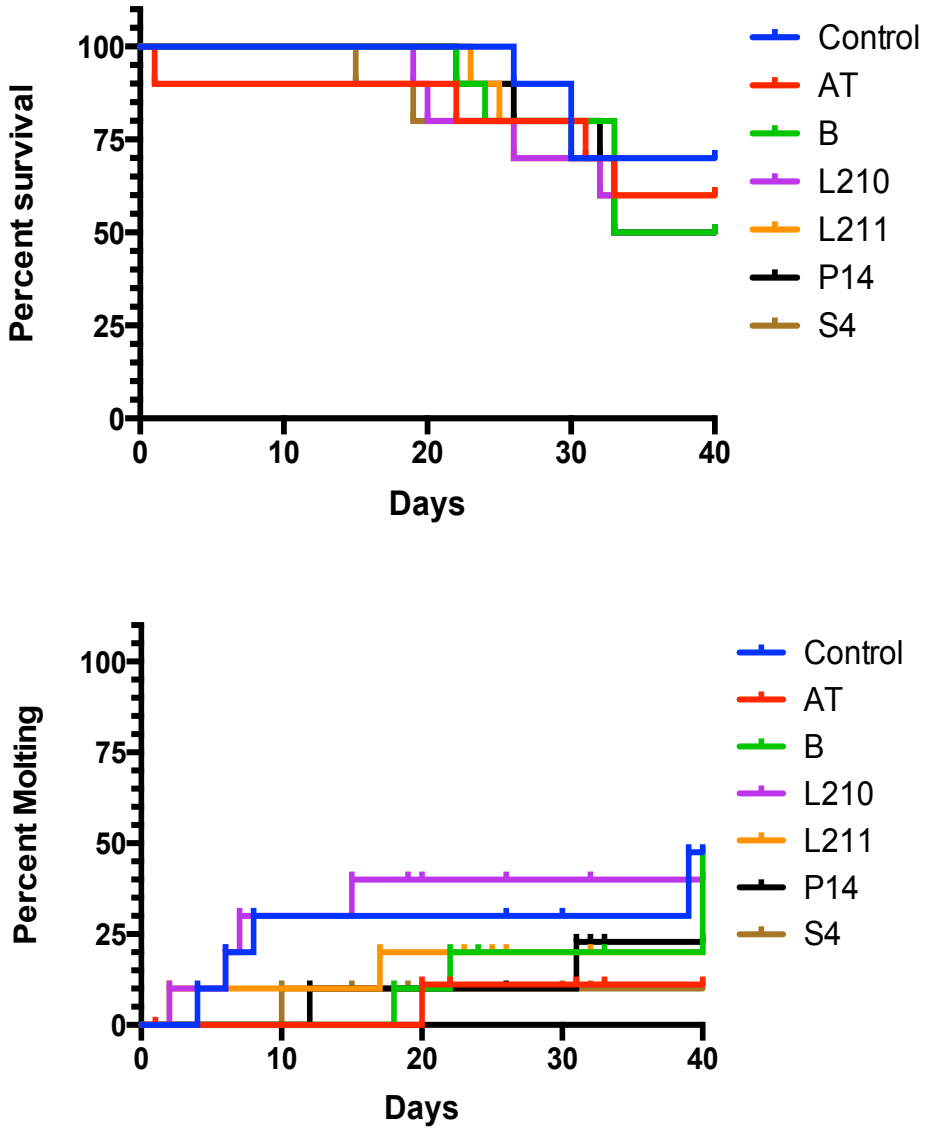

Figure 5. Effect of candidate probiotic treatment on percent survival and percent molting occurrence of lobster post-larvae over the course of the 40-day experiment during PL Experiment 3 (Log-rank Mantel-Cox test for survival p=0.98, and for molting p=0.42).

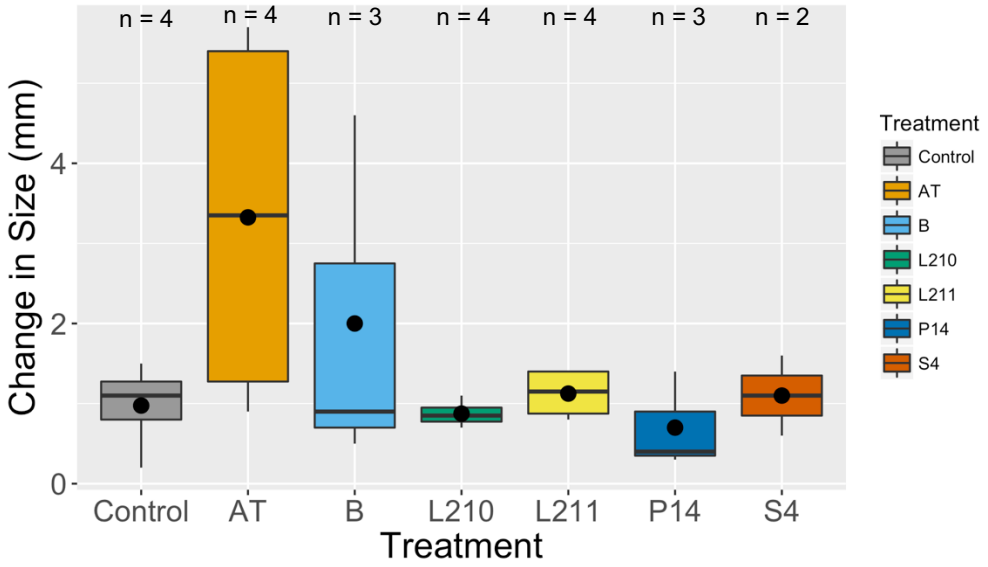

Figure 6. Effect of candidate probiotic treatment on average growth (mm) of lobster post-larvae that both molted and survived throughout PL Experiment 3. (One-way ANOVA p=0.17). Large black dots represent the mean, boxes represent upper and lower quartile, black line within the box represents the median, and whiskers indicate minimum and maximum values.

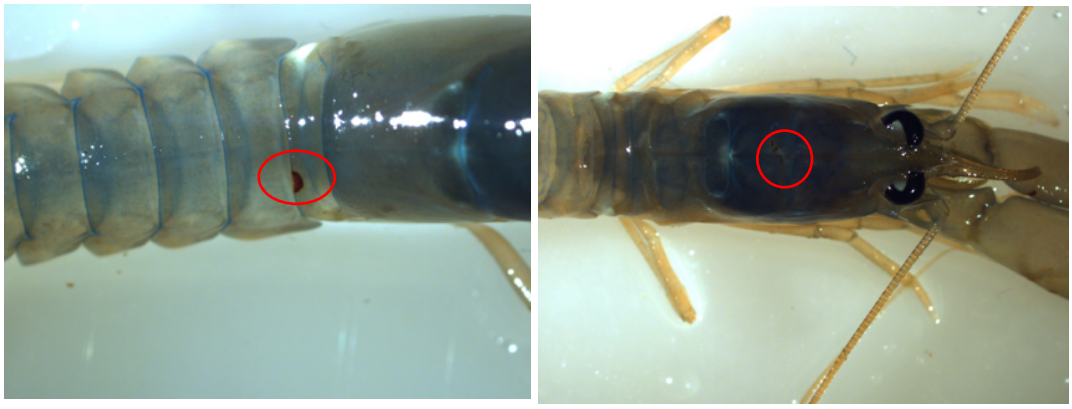

Figure 7. Photographs of lobster post-larvae that developed lesions in PL Experiment 3. These individuals were from the L211 (left) and P14 treatment (right). Both individuals died before the end of the experiment on days 26 and 33, respectively.

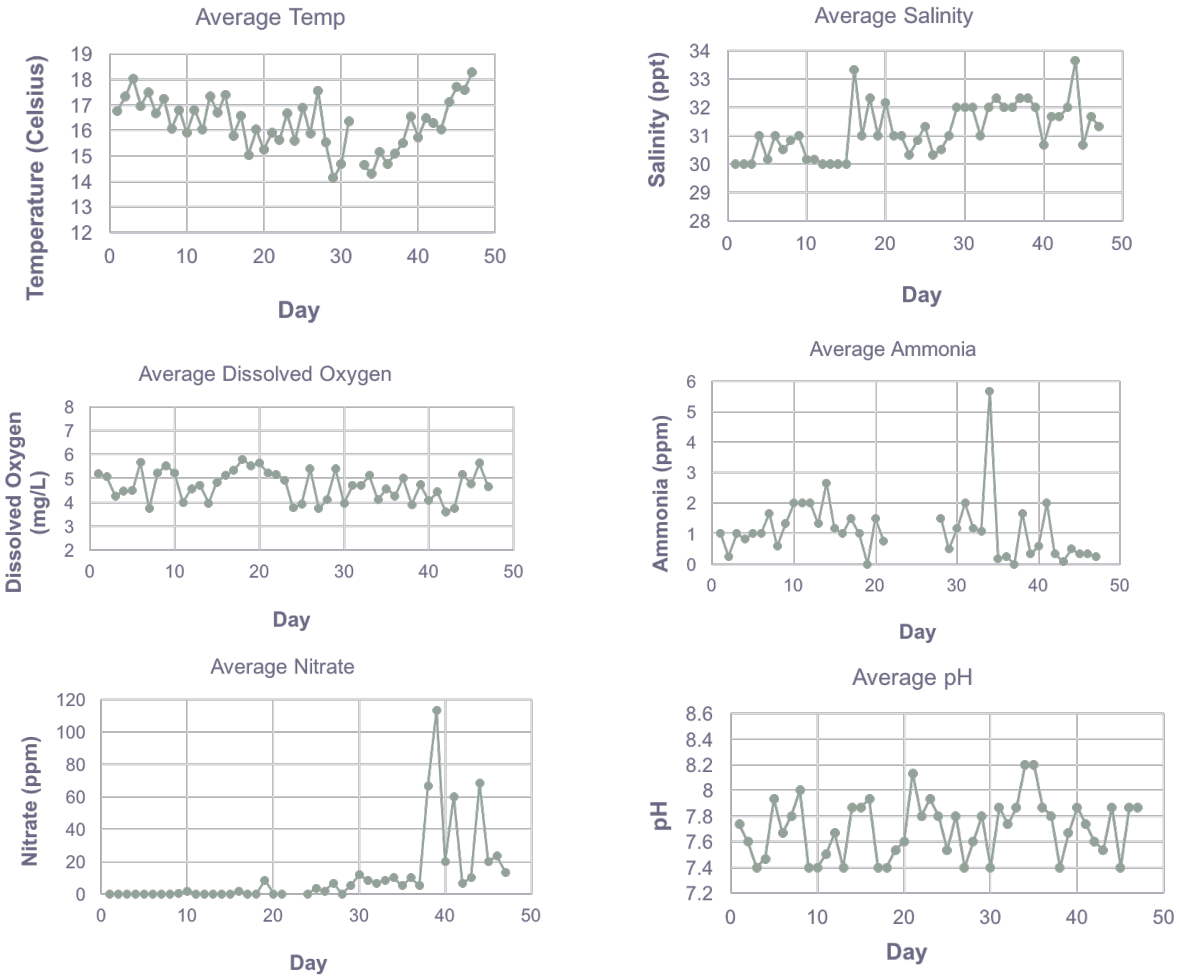

Figure 8. Representative water quality conditions in lobster post-larvae dishes during PL Experiment 3. Each day, three PL dishes were chosen at random to test water quality parameters.

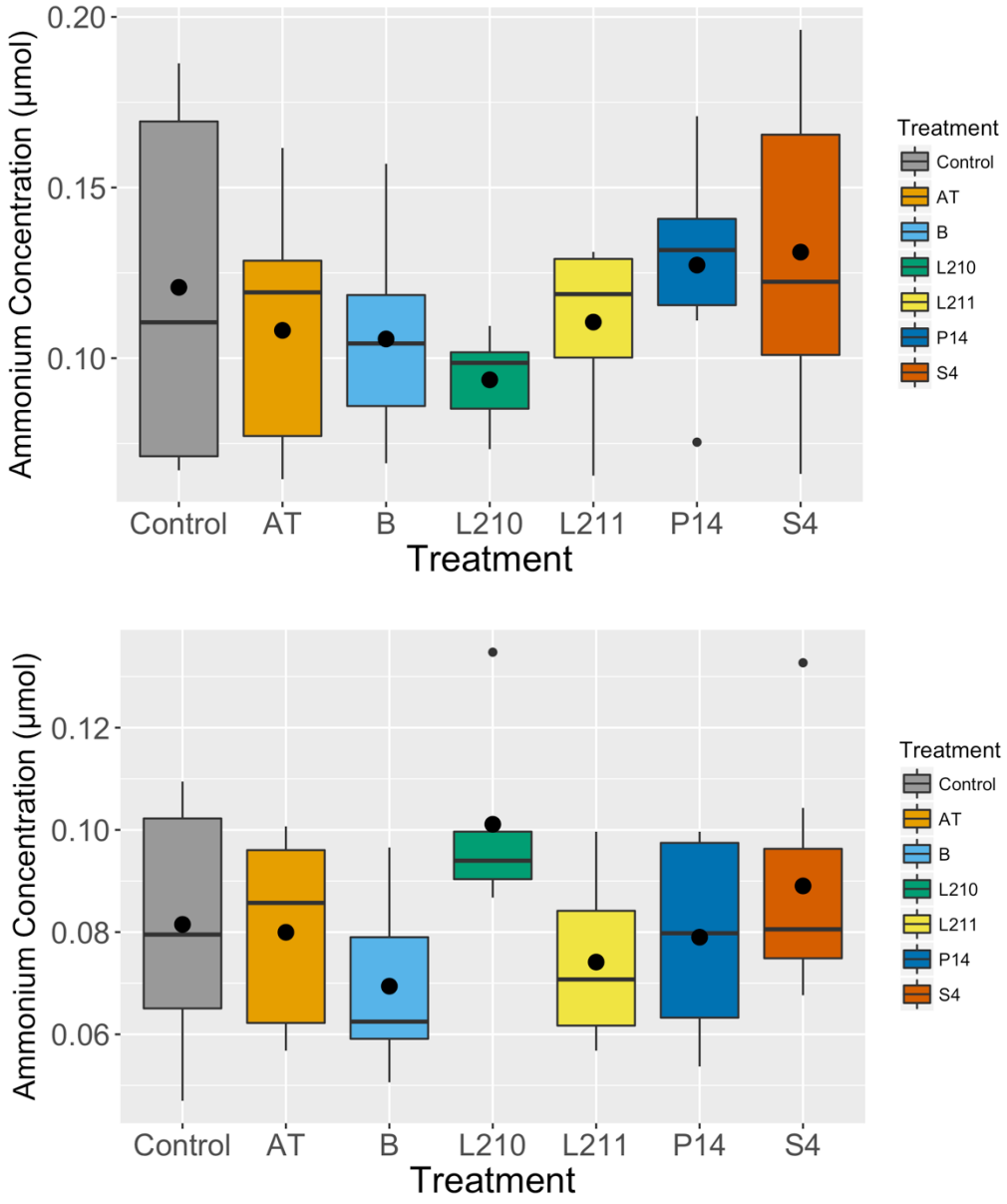

Figure 9. Effect of candidate probiotic treatment on ammonium levels (µM) at two time points at the end of experiment during PL Experiment 3. The top figure is from samples taken on day 35 of the experiment, 2 days after probiotic treatment. The bottom figure is from samples on day 40 of the experiment, 4 days after probiotic treatment (One-way ANOVA for day 35, p=0.60; One-way ANOVA for day 40, p=0.19). Large black dots represent the mean, boxes represent upper and lower quartile, black line within the box represents the median, and whiskers indicate minimum and maximum values.

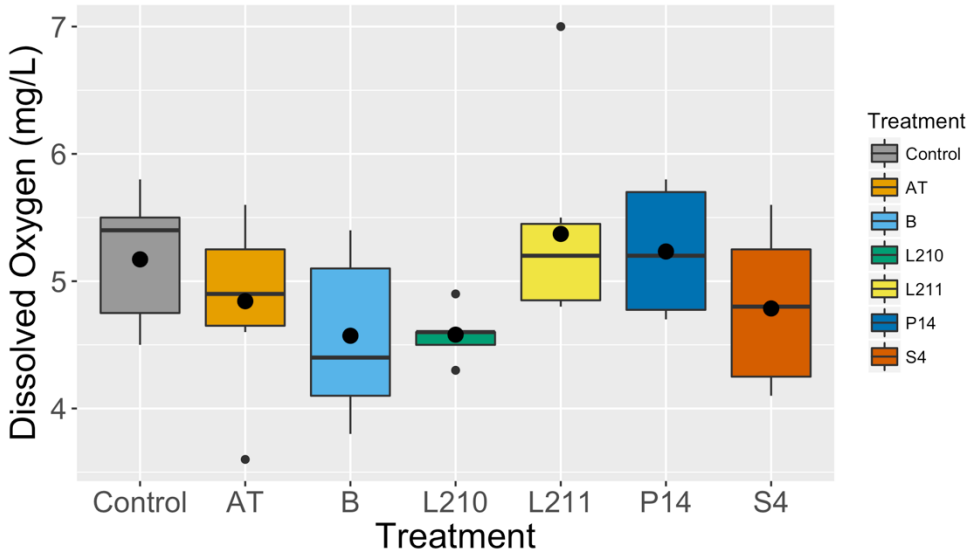

Figure 10. Effect of candidate probiotic treatment on dissolved oxygen (mg/L) in lobster post-larvae dishes on the last day of the experiment during PL Experiment 3. DO was recorded on day 40 of the experiment, 4 days after a probiotic treatment. Sample sizes ranged from 5-7 containers (One-way ANOVA p=0.10). Large black dots represent the mean, boxes represent upper and lower quartile, black line within the box represents the median, and whiskers indicate minimum and maximum values.

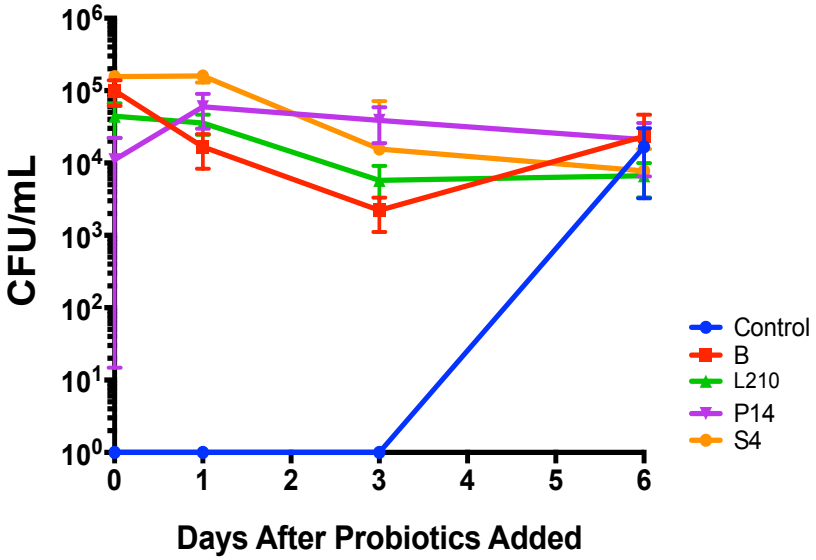

Figure 11. Effect of candidate probiotic treatment on average Sm^R bacterial concentration (CFU/mL, as determined on YP30Sm plates) from the water in lobster post-larvae dishes during the first week of PL Experiment 3. Error bars represent standard error. There were significant differences between treatments on day 0 and 1 (Two-way ANOVA p=<0.0001).

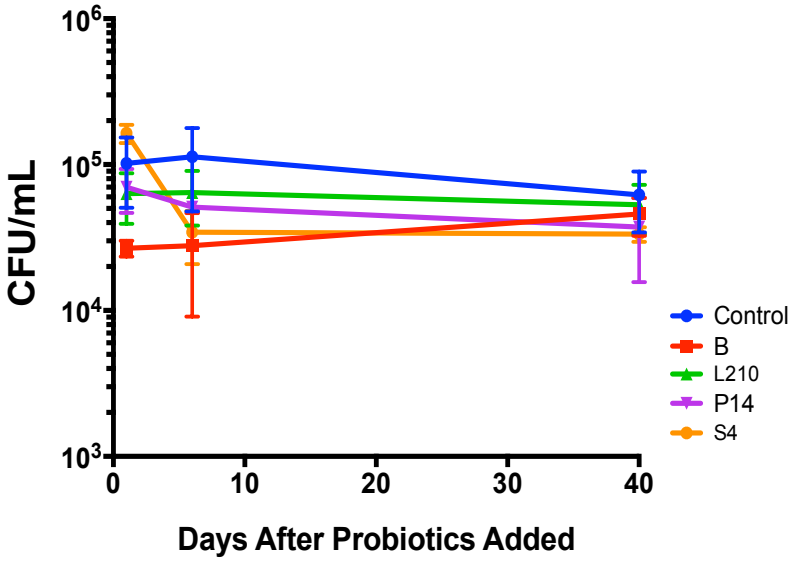

Figure 12. Effect of candidate probiotic treatment on average Sm^R concentration (CFU/mL, as determined on YP30Sm plates) on the surface of containers for PL Experiment 3. Error bars represent standard error. There were no significant differences between treatments over time (Two-way ANOVA p=<0.09), except on day 1, B was significantly lower than S4.

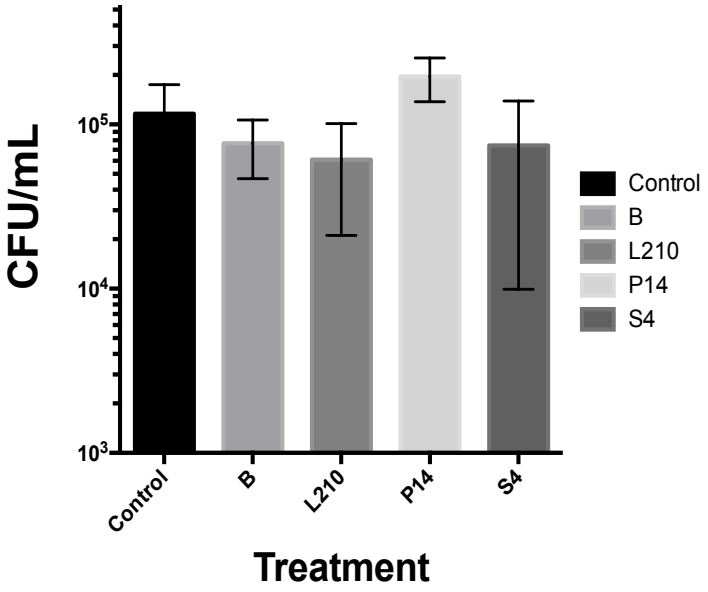

Figure 13. Effect of candidate probiotic treatment on average total bacterial concentration (in CFU/mL, YP30 media) from water samples collected from lobster post-larvae containers 3 days after probiotics were added for PL Experiment 3. Levels of Sm^R bacteria, as detected on YP30Sm agar plates, were subtracted from each of the treatments. Error bars show standard error and there were no significant differences between treatments (One-way ANOVA p=0.40).

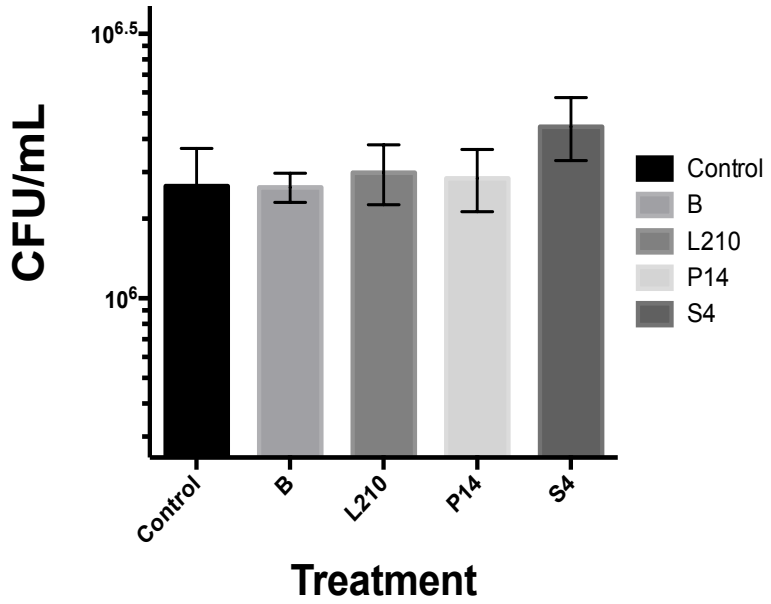

Figure 14. Effect of candidate probiotic treatment on average total bacterial concentration (in CFU/mL, YP30 media) from biofilm samples collected from the surfaces of lobster post-larvae containers on day 40 of PL Experiment 3. Levels of Sm^R bacteria, as detected on YP30Sm agar plates, were subtracted from treatments. Error bars show standard error (One-way ANOVA p=0.55).

Adult Lobster Experiment

Due to the high prevalence of ESD in RI (Castro and Somers, 2012) most (about 50%) of the lobsters collected had moderate ESD at the start of the experiment, with lesions affecting roughly 20-60% of the carapace surface. Mortality throughout the experiment was very low, with only 2 individuals dying in total, one of which died on the last day of the experiment. In both cases, mortality resulted from an unsuccessful molt most likely induced due to the severity of ESD lesions. Throughout the experiment, 27 of the 40 lobsters molted, and no statistically significant differences between treatments were detected (Log-rank test p=0.69; Fig. 15). Molting showed a significant interaction with disease severity (Chi-square p= 0.00059), indicating this was the main factor associated with molting, rather than treatment (Fig. 16). The number of lobsters characterized as having severe ESD at the beginning of the experiment was 5-6 individuals per treatment (out of a total of 10). At the end of the experiment or time of molt (whichever came first), the severity of ESD either stayed the same or worsened for all individuals (Fig. 17). For lobsters that did molt, there was no significant difference in growth between treatments (One-way ANOVA p=0.82; Fig. 18). None of the treatments had a visible impact on lesion severity; however, no individuals displayed signs of ESD again after molting.

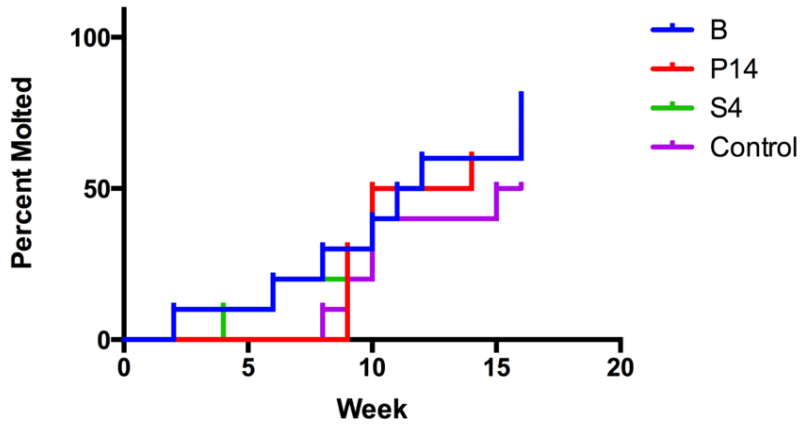

Figure 15. Effect of candidate probiotic treatment on percent molting occurrence of adult lobsters over 16 weeks. No statistical differences in molting were observed between treatments (Log-rank test p=0.69, Prism 6).

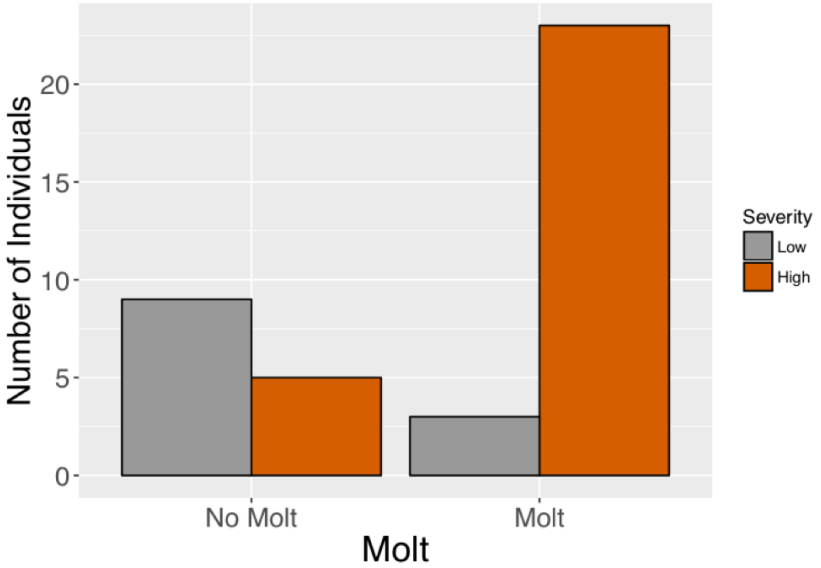

Figure 16. Relationship between molting occurrence and ESD severity for adult lobsters over 16 weeks. "High Severity" indicates lesions covered >50% of carapace. There was a significant relationship between severity of ESD and molting occurrence (Chi-square p= 0.00059, R Studio).

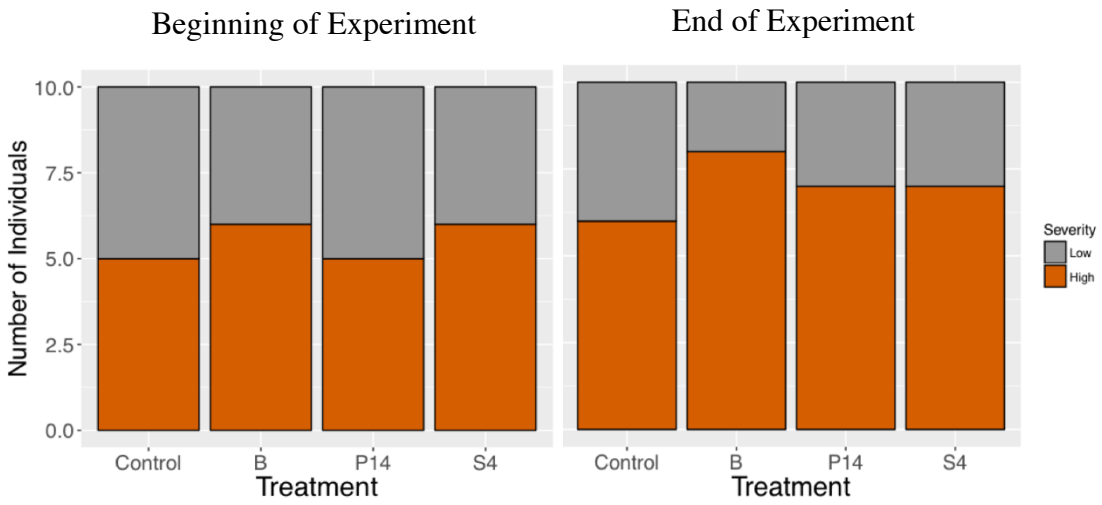

Figure 17. Effect of candidate probiotic treatment on progression of ESD from beginning to end of the adult lobster experiment. Individuals in each treatment were characterized as "high" or "low" severity at the beginning and end of the experiment (or at the time the lobster molted). "High Severity" indicates lesions covered >50% of carapace. There were no significant differences between treatments and ESD worsened for all treatments (Beginning of experiment Chi square p=0.93, End of experiment Chi square p=0.81).

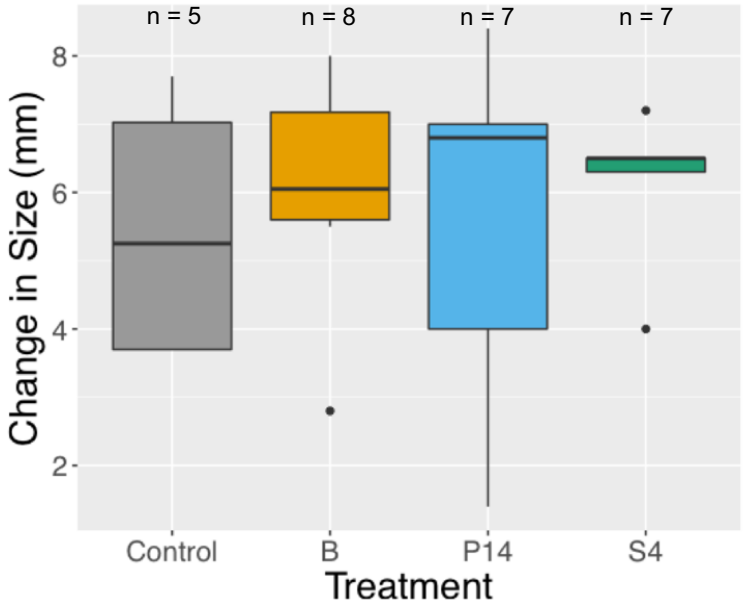

Figure 18. Effect of probiotic treatment on growth (mm) of lobsters that molted during the adult lobster experiment. There were no statistical differences in growth between treatments (One-way ANOVA p=0.82). Boxes represent upper and lower quartile, black line within the box represents the median, and whiskers indicate minimum and maximum values.

Discussion

The aim of this research was to 1) develop methods for *in vivo* screening of candidate probiotics against ESD in American lobsters; and 2) test the effect of these candidate probiotics on the progression of ESD in adult lobsters. Three different PL screening assays were performed to determine the best method for rapid screening of several candidate probiotics on live lobsters. While probiotic screenings have been conducted in other juvenile crustacean species for disease control applications, such as the spiny lobster, *Panulirus ornatus*, (Nguyen et al. 2014), crayfish (Safari and Paolucci 2017), and various shrimp species (Hai and Fotedar 2010; Kumar et al. 2016), these experiments have never been conducted for *Homarus americanus* in the context of ESD. Based on these screening experiments, several potential probiotics were selected for testing on adult lobsters with ESD. We found that: 1) potential probiotic isolates did not have adverse effects on lobsters at the post-larval (PL) stage; 2) several of the probiotic candidates may protect lobster PL from environmental stress; 3) there were no visual improvements to ESD lesions in an experiment performed with adult lobsters that had moderate to severe ESD before treatment.

Our collaborative probiotic group at URI was able to select, identify, and test seven different candidate probiotic strains that were isolated from lobsters. One of these strains, *P. inhibens* S4, was isolated from the eastern oyster, *Crassostrea virginica*, and has proven disease protection in the larvae of hatchery-reared oysters (Karim et al. 2013). Each of the candidate probiotics displayed growth inhibition against *A. macrocephali* and/or *Thalassobius* sp., two putative ESD pathogens isolated from lobsters with lesions (Chistoserdov et al. 2005). The role of these putative pathogens in

ESD progression, however, is not clear (Meres et al. 2012; Quinn et al. 2012; Feinman et al. 2017), and this is a limitation for the selection of possible probionts. Our assumption was that the antibiotic activity against these ESD-associated bacteria, combined with the ability to form strong biofilms on lobster shells, would indicate the likelihood of being protective against ESD.

Screening probiotic isolates on PLs allowed for pre-selection of the candidate probiotics with the highest potential for probiotic activity to be tested in labor-intensive adult lobster trials, and could be used as a model for future probiotic discovery strategy. We encountered several challenges in the development of screening protocols using lobster PLs, based on the need to keep PLs in individual containers due to their cannibalistic nature. In PL experiment 1, the design allowed for maintenance of water quality, but did not allow for high statistical power since three PLs were in each container. This concern was addressed in PL experiment 2 where PLs were kept in separate wells of 6-well plates, but the water temperatures that reached 20°C, which is known to be stressful to juvenile and larval lobsters (Waller et al. 2016), was likely detrimental to PL survival. The small volume of seawater in 6-well plates also could have led to poor water quality conditions such as low dissolved oxygen and high ammonia. In PL experiment 3, PLs were kept in larger glass bowls with frequent water changes and kept at a relatively constant temperature. This method was the most successful in providing a static system yet keeping physiological stress low as measured by mortality.

Despite differences in experimental design, we can use consistent findings from all three PL screening experiments to infer the effect of treatment of potential probiotics

on lobsters at a vulnerable, early life stage. The PL screening assays indicated that most of the candidate probiotic treatments did not have statistically significant toxic effects on individuals. Survival was low in PL experiment 2, likely due to adverse environmental conditions, most probably temperature stress. Interestingly, several of the probiotic isolates significantly enhanced PL survival as compared to the control group in these conditions. In larviculture of the blue swimming crab, *Portunus pelagicus*, a probiotic water additive was shown to be effective at improving water quality and enhancing survival when challenged with a pathogen (Dad Talpur et al. 2013). An effect of treatment on water quality parameters (dissolved oxygen, ammonium), however, was not seen in PL experiment 3. Further research is needed to confirm the ability of candidate probiotic treatment to increase PL survival under conditions of environmental stress, as well as determine the mechanisms involved in this protection. These could include, for example, the potential of probiotics to influence water quality parameters such as nitrate, ammonia, and pH or to protect PLs against opportunistic infections triggered by stressful conditions (Verschuere et al. 2000; Hai and Fotedar 2010; Dad Talpur et al. 2013).

Molting occurrence was not influenced by treatment in any of the experiments conducted in this study. Subsequently, probiotic treatments did not have significant effects on growth of lobsters that did molt during the experiments. This finding is inconsistent with evidence of enhanced growth from certain probiotic dietary application in *Homarus gammarus* larvae (Daniels et al. 2010) and *Panaeus monodon* (Boonthai et al. 2011), and with probiotic water additive application in freshwater crayfish (Dash et al. 2016). However, Middlemiss and colleagues (2015) found that

adding *Bacillus* sp. to the water of larval and post-larval *H. gammarus* had no significant impacts on growth or weight (Middlemiss et al. 2015). In the future, larger sample sizes and longer trials could help more rigorously determine treatment impacts of probiotics on growth.

Combined evidence from these experiments indicated that *Loktanella maritima* L210 should not be further considered as a probiotic candidate. This evidence included the higher (although not significant) incidence of lesions on PLs in experiment 1 and the fact that L210 was the only strain that did not show protective effects on survival under severe environmental stress in experiment 2. Additionally, bacteria from the genus Loktanella have been observed in cultures from ESD lesions and have been observed around lesions of laboratory-reared lobsters (Feinman et al. 2017)

The goal of measuring bacterial concentrations in water and biofilm samples from PL containers in experiment 3 was to determine if the candidate probiotics persist in the water, form biofilms on the PLs' containers, and/or impact overall bacterial load. Of the six bacterial treatments tested in this trial, four of them were streptomycin-resistant strains, which allowed measurement of their concentration by plating onto Sm-inoculated YP30 media. This experiment had several limitations that should be considered when interpreting the data. Contrary to expectations, colonies were present on YP30Sm plates from water samples collected from control (no probiotic treatment) containers on day 6 and biofilm samples collected on all days. There are some potential explanations for this observation: a) the presence of streptomycin resistant bacteria naturally occurring in the experimental system; b) contamination of some control containers with probiotic treatments due to spray or experimental errors on

delivering treatments; and/or c) contamination of the media used in sample dilution prior to spot plating. In order to distinguish between these potential explanations, colony appearance was also considered when counting colonies. Colony morphology of the biofilm control samples were variable between days and containers, indicating that it is unlikely that the media used for serial dilutions was contaminated, since the same media was used for water samples, which showed no streptomycin resistant bacterial growth from most control samples. Based on the data available, it is not possible to distinguish between the other two alternatives, but further work would be required to identify the colonies present on the plates. Another caveat of the experiment was variation in probiotic concentrations between treatments when added to PL containers, likely due to the inaccuracy of optical density measurements for determining of bacterial concentrations, which prevented accurate estimation of differences in bacterial loads between treatments. This variability could also be due to artifacts in the sampling (e.g. probiotic isolates were not homogenously distributed in the containers before bacterial sampling).

Despite these caveats, significantly higher levels of S4 and B were observed in water from the containers one day after treatment as compared to control treatments, suggesting that these strains can persist in the system for at least one day after treatment. Interestingly, although B showed comparable levels to S4 in water on day 1 after initial treatment, the containers treated with S4 showed significantly higher levels of bacteria in the glass surfaces than B. This is consistent with *in vitro* data showing that S4 is a stronger biofilm former than B (Underwood 2018) and previous research showing that biofilm formation contributes to the probiotic ability of S4 in larval oysters (Zhao et al.

2016). *P. inhibens* S4 and other isolates from the Roseobacter clade, have been shown to protect various aquatic animals from disease (D 'alvise et al. 2012; Karim et al. 2013; Prol García et al. 2014). Despite being an oyster bacterial isolate, S4 was one of the only strains that showed a zone of inhibition against both lobster putative pathogens, *A. macrocephali* and *Thalassobius* sp. These results, in conjunction with existing evidence of S4 protection against disease in oysters (Karim et al. 2013; Zhao et al. 2016) warrant further study of this particular isolate.

Probiotics had no measurable effect on overall bacterial load in the PL containers, as measured by CFU/mL. Conserved 16S ribosomal RNA sequencing, an approach commonly used to characterize microbiomes (Quinn et al. 2012; Closek et al. 2014; Arfken et al. 2017), of water and biofilm samples during PL assays could better elucidate how the microbial communities change over time and between treatments. Sequencing and quantification of bacteria through qPCR would also give a more accurate representation of how the candidate probiotics persist in the water. Further experiments looking at biofilm formation of candidate probiotics on lobster shells should be performed. Examining the success of probiotic colonization on shells could help us understand how successful probiotics could be competing with putative pathogens in ESD lesions (Verschuere et al. 2000; Zhao et al. 2016).

Based on the results from the PL screening assays and the results from the *in vitro* testing of antimicrobial activity and the ability of form biofilms, we decided to test S4, B, and P14 on adult lobsters that had ESD. After 3 months of treatments performed twice a week, the probiotics did not have a measureable effect on halting ESD progression. Our results were confounded by the fact that most lobsters molted at

different points throughout the experiment, making long-term tracking of ESD progression challenging. It was surprising that so many lobsters molted, considering the time of year (October-January) is not during the normal molting period for Rhode Island lobsters (Castro and Angell 2000; Castro and Somers 2012). Our experiments show that molting was related to the severity of ESD lesions, a result consistent with previous observations that molting behavior is altered in ESD lobsters, and may be a strategy used by lobsters to prevent the development of systemic infections (Smolowitz et al. 2005; Castro et al. 2006). It is also possible that the disease was too severe for the candidate probiotics to have any sort of therapeutic effect, or for us to detect an effect. Carapace erosions cannot be repaired, so if shell cuticles were already damaged, it would be difficult to measure improvements in progression of the disease. Additionally, bacterial communities in the lesions may have been too established and persistent for probiotics to be effective. Considering the lack of an experimental model of ESD in which the disease can be induced after treatment with candidate probiotics, lobsters with beginning stages of ESD should be used in the future to more effectively track disease progression. Different types of probiotic treatment delivery should also be considered, such as the filters saturated with candidate bacteria used in a study investigating the role of putative pathogens in lesion development (Quinn et al. 2012).

It may also be important to consider the probiotic isolates in this study in the context of other *H. americanus* diseases, or other crustacean diseases in general – in both fishery and aquaculture settings. For example, probiotics could be tested against *H. americanus* diseases with a better laboratory model, such as the bacterial *Vibrio fluvalis* infection (Beale et al. 2008) or Gaffkemia (Clark et al. 2013). Given the

importance of crustacean fisheries and aquaculture for global protein sources (Bondad-Reantaso et al. 2012), it is imperative to better understand their diseases and how to manage them.

Investigating the use of probiotic treatments in a wild fishery has not yet been researched to our knowledge. Implementing such technology is challenging, considering the scope of the disease in southern New England lobster populations (Hoenig et al. 2017), and that the etiology of ESD is not well understood. The current body of literature for probiotic treatments in diseased marine organisms generally applies to hatchery-based aquaculture systems rather than wild marine populations. More research must be done in order to make recommendations about how to best apply probiotics to the lobster fishery, or if probiotics will even be a viable option. Our goal was to investigate the use of probiotic treatments by placing diseased lobsters in a "probiotic bath." Lobstermen in RI expressed that they would be willing to keep a reasonably-sized probiotic bath on their fishing vessel, place diseased lobsters in this bath for a pre-determined amount of time, and then return lobsters back into the ocean. Our experiments were designed and conducted with this application model in mind, and we explored various probiotic screening methods to try and determine which candidate probiotic would be best-suited for application in the fishery.

The current application of probiotics in this study did not prove to be effective in slowing or stopping the progression of ESD. However, we have developed a model for screening candidate probiotics on PLs. An adult lobster experiment such as that described here can verify if candidates chosen from screening assays are effective. Further research determining the ability of probiotic isolates to form biofilms on lobster

carapaces may help inform application in this context. Experiments examining biofilm formation on sterile fragments of lobster shell (Underwood 2018) could also be used as an effective screening tool, and could potentially be used instead of using live PLs. Focusing on microbe-microbe interactions in future experiments will not only help us better understand the probiotics mechanism of action, but could also provide more information about how the bacteria from ESD lesions interact with potentially beneficial bacteria.

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Additional Tables

Table 3. Effect of candidate probiotic treatment on average Sm^R bacterial concentration (CFU/mL, as determined on YP30Sm plates) in water from lobster post-larvae dishes during the first week of PL Experiment 3.

Days	Control			B			L210			P14			S4		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
0	0.00E+00	0.00E+00	3	1.00E+05	6.67E+04	3	4.44E+04	3.85E+04	3	1.11E+04	1.92E+04	3	1.56E+05	1.92E+04	3
1	2.22E+03	3.85E+03	3	1.67E+04	1.45E+04	3	3.56E+04	1.92E+04	3	6.00E+04	5.24E+04	3	1.60E+05	5.24E+04	3
3	0.00E+00	0.00E+00	3	2.22E+03	1.92E+03	3	5.77E+03	5.77E+03	3	3.89E+04	3.47E+04	3	1.56E+04	9.68E+04	3
6	1.67E+04	2.33E+04	3	2.33E+04	4.04E+04	3	6.67E+03	5.77E+03	3	2.11E+04	2.52E+04	3	7.78E+03	1.35E+04	3

Table 4. Two-way ANOVA showing effect of probiotic treatment on average Sm^R bacterial concentration (CFU/mL, as determined on SmYP30 plates) in water from lobster post-larvae dishes during the first week of PL Experiment 3 (Prism 6).

Probiotic Concentration in Water			
Two-way ANOVA	P-value	Significant?	Summary
Interaction	0.0008	Yes	***
Time	0.0004	Yes	***
Treatment	< 0.0001	Yes	****
Day 0			
Control vs. B	<0.05	Yes	*
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	<0.05	Yes	****
B vs. L210	>0.05	No	ns
B vs. P14	<0.05	Yes	*
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	<0.05	Yes	**
P14 vs. S4	<0.05	Yes	***
Day 1			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	<0.05	Yes	****
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns

B vs. S4	<0.05	Yes	***
L210 vs. P14	>0.05	No	ns
L210 vs. S4	<0.05	Yes	**
P14 vs. S4	<0.05	Yes	*
Day 3			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns
Day 6			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns

Table 5. Effect of candidate probiotic treatment on average Sm^R concentration (CFU/mL, as determined on YP30Sm plates) on the surface of containers for PL Experiment 3.

Days	Control			B			L210			P14			S4		
	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N	Mean	SD	N
1	1.02E+05	8.91E+04	3	2.67E+04	5.77E+03	3	6.33E+04	4.16E+04	3	7.00E+04	4.04E+04	3	1.64E+05	4.04E+04	3
6	1.13E+05	1.13E+05	3	2.78E+04	3.24E+04	3	6.44E+04	4.53E+04	3	5.11E+04	5.09E+03	3	3.44E+04	2.36E+04	3
40	6.19E+04	7.30E+04	7	4.58E+04	3.52E+04	7	5.30E+04	4.38E+04	5	3.73E+04	5.30E+04	6	3.34E+04	1.02E+04	7

Table 6. Two-way ANOVA showing effect of probiotic treatment on average Sm^R concentration (CFU/mL, as determined on YP30Sm plates) on the surface of containers for PL Experiment 3 (Prism 6).

Probiotic Concentration in Water			
Two-way ANOVA	P-value	Significant?	Summary
Interaction	0.1236	No	ns
Time	0.0699	No	ns
Treatment	0.0864	No	ns
Day 0			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	<0.05	Yes	*
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns
Day 1			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns

Day 3			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns
Day 6			
Control vs. B	>0.05	No	ns
Control vs. L210	>0.05	No	ns
Control vs. P14	>0.05	No	ns
Control vs. S4	>0.05	No	ns
B vs. L210	>0.05	No	ns
B vs. P14	>0.05	No	ns
B vs. S4	>0.05	No	ns
L210 vs. P14	>0.05	No	ns
L210 vs. S4	>0.05	No	ns
P14 vs. S4	>0.05	No	ns