

Bacterial Community Dynamics in an Oyster Hatchery in Response to Probiotic Treatment

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

Abstract

Larval oysters in hatcheries are susceptible to diseases caused by bacterial pathogens, including *Vibrio* spp. Previous studies have shown that daily addition of the probiotic *Bacillus pumilus* RI06-95 to water in rearing tanks increases larval survival when challenged with the pathogen *Vibrio coralliilyticus*. We propose that the presence of probiotics causes shifts in bacterial community structure in rearing tanks, leading to a net decrease in the relative abundance of potential pathogens. During three trials spanning the 2012-2015 hatchery seasons, larvae, tank biofilm, and rearing water samples were collected from control and probiotic-treated tanks in an oyster hatchery over a 12-day period after spawning. Samples were analyzed by 16S rRNA sequencing of the V4 or V6 regions followed by taxonomic classification, in order to determine bacterial community structures. There were significant differences in bacterial composition over time and between sample types, but no major effect of probiotics on the structure and diversity of bacterial communities (phylum level, Bray-Curtis $k=2$, 95% confidence). Probiotic treatment, however, led to a significantly higher abundance of *Oceanospirillales* and *Bacillus* spp. in water and oyster larvae. In the water, an increase in *Vibrio* spp. diversity in the absence of a net increase in read abundance suggests a likely decrease in the abundance of specific pathogenic *Vibrio* spp., and therefore lower chances of a disease outbreak. Co-occurrence network analysis also suggests that probiotic treatment had a systemic effect on targeted members of the bacterial community, leading to a net decrease in potentially pathogenic species.

1 Introduction

Diseases caused by bacterial pathogens result in losses in aquaculture and wild populations of commercially important shellfish and finfish (Groner et al., 2016; Lafferty et al., 2015; Pérez-Sánchez et al., 2018). World aquaculture production is valued at \$243.5 billion USD, and disease is a primary limiting factor on its growth and economic worth (FAO, 2018; Stentiford et al., 2012). Larval oysters are especially susceptible to disease, often by etiological agents from the genus *Vibrio* (Beaz-Hidalgo et al., 2010a; Dubert et al., 2017; King et al., 2018; Le Roux et al., 2016; Richards et al., 2015). Pathogenic *Vibrio* spp. are naturally occurring microbes in coastal waters, which makes them difficult to avoid. In an effort to maintain a healthy environment, hatcheries work towards optimum water quality by controlling larval culture density and the use of water treatment systems (Mckindsey et al., 2007; Pérez-Sánchez et al., 2018).

An alternative method for the management of disease in aquaculture involves the use of probiotics, microorganisms that provide health benefits to the host, including protection against bacterial pathogens. Probiotics exert their beneficial effects through a variety of mechanisms, including direct pathogen inhibition, competition for nutrients, secretion of antibacterial substances, and improvement of water quality (Kesarcodi-Watson et al., 2008, 2012; Prado et al., 2010). Previous studies have shown that treatment of larval oysters in the laboratory or the hatchery with the probiotic bacterium *Bacillus pumilus* RI06-95 significantly increases their survival when challenged with the pathogen *Vibrio coralliilyticus* (Karim et al., 2013; Sohn et al., 2016). Additionally, administration of this probiotic in a hatchery setting results in reductions in total *Vibrio* abundance in tank water and surfaces, compared to the control tanks (Sohn et al., 2016).

However, there is a lack of knowledge regarding the effects of probiotics on the systems in which they are used. There are concerns about using probiotic bacteria to combat disease in open aquaculture systems, as they will eventually disperse into the environment and may thus affect bacterial diversity in these systems (Newaj-Fyzul et al., 2014). Improper selection of probiotics may result in bacterial dysbiosis, which could ultimately impact host health (Verschuere et al., 2000). As filter feeders that process large volumes of seawater daily, bivalves are especially susceptible to changes in bacterial community composition in the water (Burge et al., 2016). Moreover, bacteria both contribute to and serve as indicators of oyster health and function of the microbial community (Le Roux et al., 2016) and likely mediate the effect(s) of probiotics on the host. Therefore, it is important to assess the effects of probiotics not only on the health and protection of the host, but also on the bacterial communities in the systems in which oysters are grown.

Previous studies of microbiomes in adult oysters have shown differences in microbiota according to tissue type, geographic location, season, and environmental conditions (Chauhan et al., 2014; King et al., 2012; Lokmer et al., 2016b; Lokmer and Wegner, 2015; Pierce et al., 2016; Pierce and Ward, 2018). Additionally, the oyster microbiomes are distinct from those of the surrounding water and are often dominated by *Proteobacteria*, *Cyanobacteria*, and *Firmicutes* (Lokmer et al., 2016a). Three independent microbiome studies of larval cultures of the Pacific oyster, *Crassostrea gigas* found that, even though the microbiome in the rearing water changes throughout the year, there is little effect from direct manipulation of rearing conditions themselves, including salinity and temperature (Asmani et al., 2016; Powell et al., 2013; Trabal Fernández et al., 2014). Microbiome studies of juvenile Kumamoto oysters treated with *Streptomyces* N7 and RL8 showed an increase in species diversity and changes in the relative abundances of taxa, compared to control oysters (García Bernal et al., 2017). However, the effect of probiotics on bacterial communities in an oyster hatchery has not yet been determined.

In this study, we analyzed the structure and diversity of bacterial communities in larval oysters, their rearing water, and in tank biofilms over a 12-day period following treatment with the probiotic *Bacillus pumilus* RI06-95. We hypothesized that probiotic treatment has a cascading effect on the bacterial community structure that alters the microbiomes of the rearing water, tank biofilms, and larvae, leading to a net decrease in potentially pathogenic species.

2 Materials and Methods

2.1 Bacterial Strain and Culture Conditions

The probiotic strain *Bacillus pumilus* RI06-95, previously isolated from a marine sponge from the Pettaquamscutt River in Rhode Island (Karim et al., 2013), was cultured in yeast peptone with 3% salt (mYP30) media (5 g L⁻¹ of peptone, 1 g L⁻¹ of yeast extract, 30 g L⁻¹ of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 170 rpm. The bacterial cell concentration was estimated by OD₅₅₀ measurements using a spectrophotometer (Synergy HT, BioTek, USA) and confirmed using serial dilution and spot plating on mYP30 agar plates to determine colony forming units (CFU).

2.2 Experimental Design and Sample Collection

Samples for microbiome analysis were collected during 3 hatchery trials performed at the Blount Shellfish Hatchery at Roger William University (Bristol, RI, USA) (Table 1). Eastern oysters (*Crassostrea virginica*) were spawned following standard procedures (Helm and Bourne, 2004). Spawning day is referred to as Day 0 throughout the manuscript. Larvae (1-day old) were distributed and maintained in static conditions in triplicate 120 L conical tanks for each treatment containing filtered and UV sterilized seawater at 21 - 23 °C and a salinity of 28 psu. Tanks were randomly assigned to treatments including no probiotics (control) and probiotic treatment with probiotic *B. pumilus* RI06-95. The probiotic was administered daily at 10⁴ CFU/mL to treatment tanks after being mixed with the microalgal feed. The microalgae strains used for feeding included *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea* (CCMP1324), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weissflogii* (CCMP1336). Experimental tanks were drained every other day to perform larval counts and grading. Tanks were washed thoroughly with a diluted bleach solution, rinsed, and replenished with filtered and UV-treated water prior to restocking the larvae.

Rearing water (volumes in Table 1) was collected from each of the triplicate tanks during drain-down and filtered through a 0.22 µm Sterivex filter (Millipore, Milford, MA, USA). The Sterivex filters were immediately frozen and stored at -80 °C until DNA extraction. Biofilm swab samples were collected from the surface inside of each tank after drain-down of the water by swabbing a line of approximately 144 cm in length with sterile cotton swabs. The cotton tips of the swabs were stored in RNAlater (Ambion, Inc., Foster City, CA, USA). Oyster larvae were collected on a 55 µm sieve after drain-down of tank water and resuspended in 5 L of seawater. 10 mL of oyster larvae (from each tank, about 150 – 1500 larvae) were then placed into a sterile tube. In the laboratory, oyster larvae were collected on a 40 µm nylon membrane and rinsed with filtered sterile seawater (FSSW) to reduce loosely attached environmental bacteria. Swab and larvae samples were flash frozen in liquid nitrogen and stored at -80 °C until DNA extraction. All sample types were collected during Trials 1 and 2, but only water samples were collected during Trial 3 (Table 1). In Trial 3, water (1 – 2 L) was also collected from the inflow (water piped directly from the environment) and outflow (water

collected after filtration and UV-treatment prior to reaching the hatchery tanks) and processed as described above for tank water.

2.3 DNA Extraction, Amplification, and Sequencing

Total DNA from water samples was extracted from the filters using the PowerWater Sterivex DNA Isolation Kit (MoBio Laboratories, USA) according to manufacturer recommendations (Trials 1 and 2) or Gentra Puregene Reagents (Qiagen, Hilden, Germany) with an added proteinase K-lytic enzyme digestion step (Sinigalliano et al., 2007; Trial 3). In addition, total bacterial DNA from the tank biofilm swabs and oyster larvae were extracted using the PowerSoil DNA Isolation Kit (MoBio) with slight modifications detailed below. In brief, frozen pooled oyster larvae were ground in a mortar with a sterile pestle and then placed into bead tubes for extraction (Qiagen). The RNAlater samples containing the cotton tops of the swabs were placed directly into bead tubes. Bead tubes were incubated at 65 °C for 10 min and then shaken horizontally at maximum speed for 10 min using the MoBio vortex adaptor. Following extraction, DNA concentration was quantified with both a Nanodrop 2000 instrument and a Qubit Fluorometer (ThermoFisher Scientific, Wilmington, DE, USA).

16S rRNA gene amplicon analysis was performed using 515F/806R primers to amplify the V4 region (Trials 1 and 2) or 967F/1064R primers to amplify the V6 region (Trial 3). The V4 region was used in Trials 1 and 2 for better taxonomic resolution of all sample types and the V6 region was used in Trial 3 for independent confirmation with greater sequencing depth. A two-step PCR reaction following Illumina's 16S Metagenomic Sequencing Library Preparation Protocol was performed on the samples from Trials 1 and 2 (Illumina Inc., San Diego, CA, USA). The PCR products were then analyzed with 250 bp paired-end sequencing to obtain fully overlapping reads on an Illumina MiSeq at the Genomics and Sequencing Center at the University of Rhode Island. The samples from Trial 3 were prepared with a 2-step fusion primer PCR amplification according to the protocols from the Keck Sequencing Center at the Marine Biological Laboratory (MBL) (<https://vamps.mbl.edu/resources/primers.php>). Paired-end sequencing was performed at the MBL on an Illumina HiSeq 2500 to generate 100 bp double strand reads with full overlap of the V6 region.

2.4 Processing of Sequencing Data

Sequences from Trials 1 and 2 were demultiplexed using FastQC v0.11.4 (Andrews, 2010), then merged and trimmed using Trimmomatic v0.32 (Bolger et al., 2014). All sequences shorter than 200 bp were removed from the dataset. Sequences from Trial 3 were demultiplexed and quality filtered following standard protocols at the MBL Bay Paul Center that remove reads where forward and reverse sequences do not match perfectly (Eren et al., 2013b). All sequences were uploaded to VAMPS (Visualization and Analysis of Microbial Population Structure) and classified directly using the GAST pipeline with the SILVA database, in order to compare between the three trials (Huse et al., 2014). The taxonomy data from each trial were separately normalized to the total reads of each sample and then exported as a matrix or BIOM file for analysis in R (Version 3.3.1). *Vibrio* spp. sequences in water samples from Trial 3 were processed through the oligotyping pipeline described in Eren et al. (2013a) as implemented in VAMPS, and annotated using SILVA.

2.5 Statistical and Network Analysis

All descriptive and statistical analyses were performed in the R statistical computing environment with the *vegan* and *phyloseq* packages (Dixon, 2003; McMurdie and Holmes, 2013). Simpson's diversity values were calculated for each sample at the order level using the *vegan* package Version

2.4-1. Non-metric dimensional analysis (NMDS) was used to determine the influence of time, probiotic treatment, or sample type on the bacterial community composition, based on methods by Torondel et al. (2016) and implemented using *vegan*. The Bray-Curtis dissimilarity metric was used with $k=2$ for 50 iterations and 95% confidence intervals were plotted. Additionally, relative abundances of specific taxa were extracted and plotted according to treatment and time, and analyzed using t-tests and ANOVAs in R.

A co-occurrence network was generated with normalized taxa counts at the Order level from water samples in Trial 3 ($n=18$) to determine hypothetical relationships resulting from each treatment. The `make_network()` command from the *phyloseq* package was used with the Bray-Curtis dissimilarity metric, $\text{max distance}=0.5$. The mean resulting relationship table including 123 taxa (nodes) and 670 relationships (edges) was exported to Cytoscape Version 3.6.0 for visualization and analysis (Shannon et al., 2003). Nodes were assigned continuous size attributes based on the number of total reads in all samples per taxa (2 to 2,720,021), and discrete shape and continuous color according to whether the taxa were more abundant in the control or probiotic-treated samples (0 to 3.6 times).

3 Results

3.1 Bacterial Structure and Diversity Over Time

A total of 18,103,647 quality-controlled 16S rRNA gene amplicon sequences were analyzed from 42 rearing water samples, 24 tank biofilm swabs, and 21 pooled larvae samples from three hatchery trials. There was an average of 208,087 reads for each of the 87 samples, ranging between 961-1,117,380 depending on the sequencing method and sample type (Figure 1, top). Direct taxonomical classification resulted in the detection of a total of 168 Orders across 29 Phyla in all samples. Overall, bacterial communities for each trial and sample type shared many of the most dominant phyla, although differences in relative abundance were seen between trials, time points, and sample types (Figure 1, bottom left). The most dominant phyla in the water community, averaged from all samples, were *Proteobacteria* ($53 \pm 6\%$), *Bacteroidetes* ($26 \pm 10\%$), *Cyanobacteria* ($12 \pm 10\%$), *Actinobacteria* ($5 \pm 5\%$), and *Planctomycetes* ($2 \pm 1\%$) (Figure 1, bottom right). The larval samples were dominated by *Proteobacteria* ($87 \pm 12\%$) and the swab samples by *Proteobacteria* ($68 \pm 17\%$), *Cyanobacteria* ($19 \pm 16\%$), and *Bacteroidetes* ($8 \pm 4\%$) (Figure 1, bottom left). Abundance of *Cyanobacteria* was significantly higher in swab than in water samples ($p<0.001$, Table S1). Larval and swab samples showed a significantly higher proportion of *Proteobacteria*, and lower abundance of *Bacteroidetes*, as compared to water samples ($p<0.001$, Table S1).

Overall, the bacterial communities in rearing water were significantly more diverse than the communities in oyster larvae and tank biofilm swab samples (Simpson's Diversity Index, $p<0.001$, Figure 2, Table S2), reflecting an enrichment in specific community members in larvae and tank surfaces from the more diverse rearing water community (Figure 1). Simpson's Diversity Index indicated significantly higher diversity in rearing water samples from Trial 3 (0.88-0.92), than from Trials 1 (0.81-0.87) and 2 (0.80-0.91) ($p<0.001$, Figure 2, Table S2), most probably due to the greater sequencing depth and different target 16S variable region in Trial 3 (Figure S1), but potentially also due to seasonal and yearly differences in bacterial composition of the rearing water source (Table 1). There was also high variability among replicate samples from each timepoint and treatment, especially in oyster larvae samples (Figure 2, Figure S2). Significant increases in bacterial diversity over time were detected in the oyster larvae and biofilm swabs in Trial 1 ($p<0.01$, Table S3), and in the rearing water in Trials 2 and 3 ($p<0.01$, Figure 2, Tables S4, S5). No significant differences in

210 Simpson's Diversity Index were detected between control and treated samples at any timepoints for
211 any of the sample types ($p=0.52$).

212 The bacterial community structures of the water and oyster larvae samples were significantly
213 different (Bray-Curtis, $k=2$, 95% confidence) in both Trial 1 and Trial 2. The community structure of
214 microbiomes in tank biofilms (swab samples) was not significantly different from the structure of
215 either the water or oyster larvae samples, suggesting an intermediate microbiome stage (Figure 3A).
216 Bacterial communities in the rearing water were significantly different between sampling timepoints
217 (Bray-Curtis, $k=2$, 95% confidence) in all three trials (Figure 3B). Moreover, the bacterial
218 community in samples of inflow and outflow seawater, which were collected on days 5, 8, and 12
219 during Trial 3, was distinct from that of the water in rearing tanks (Figure S3). These results suggest
220 that hatchery tanks containing oyster larvae have dynamically developing microbiomes, despite the
221 fact that they are all receiving the same inflow seawater. There was no significant effect of treatment
222 on the beta-diversity in water samples from all time points (Figure 3C).

223 3.2 Effects of the Probiotic on the Selected Members of the Bacterial Community

224 Although control and probiotic-treated tanks showed no significant differences in diversity and
225 structure of bacterial communities overall (Figure 3C), significant differences in the read abundance
226 of several specific taxa were detected. In all trials, *Bacillus* spp. reads in the probiotic-treated water
227 samples increased through time, and were significantly more abundant in samples from treated tanks
228 than in the control tanks by the final sampling day on trials 2 and 3 ($p<0.05$, Figure 4A, Table S6).
229 These results suggest that the increase in reads corresponded to the added probiotic. The number of
230 *Oceanospirillales* reads was also significantly higher by 20-34% at all time points in probiotic-
231 treated rearing water as compared to control water in Trial 3 ($p<0.01$, Figure 4B, Table S7). The
232 abundance of *Oceanospirillales* reads in the water significantly decreased over time by 41-62%
233 (depending on the trial) ($p<0.005$, Figure 4B, Table S7). No significant changes in read abundance
234 of these two selected members of the bacterial community were detected in larval oysters or swabs,
235 but abundance was low in these sample types (Trials 1 and 2; not shown).

236 *Vibrio* is a taxon that comprises a significant number of larval oyster pathogens (Elston et al., 1981,
237 2008; Le Roux et al., 2016; Richards et al., 2015), therefore we evaluated the effect of probiotic
238 treatment on changes in *Vibrio* spp. diversity and abundance over time during each of the hatchery
239 trials (Figures 5, S4, S5). Probiotic treatment led to a significant increase in *Vibrio* diversity (as
240 measured using the Simpson's Index of diversity) in water samples collected on day 12 in Trial 1 ($p<$
241 0.05 ; Figure 5A, Table S8). However, no significant differences in abundance of *Vibrio* spp. between
242 control and probiotic treated-tanks were detected for any of the sample types (Figure 5B, Table S9).
243 When considering the effect of sample type, *Vibrio* read abundance was significantly lower in water
244 samples than in swabs or oysters (all time points) and in swabs than in oysters (Day 12 only;
245 $p<0.001$, Figure 5B, Table S9). When considering data from all timepoints together, the diversity of
246 *Vibrio* spp. was significantly higher in swab and oyster samples than in water samples ($p<0.005$,
247 Figure 5A, Table S8). An evaluation of the effect of time on *Vibrio* abundance and diversity showed
248 a significant increase in the diversity of *Vibrio* spp. in swab and water samples (Trial 1, $p<0.001$,
249 Figure 5A, Table S9), and a significant decrease in abundance in all sample types (Trial 1, $p<0.05$,
250 Figure 5B, Tables S8, S9).

251 Since the V6 region of the 16S rRNA gene was deeply sequenced in Trial 3, we were able to perform
252 an oligotyping analysis - a method that detects genetic variants within a taxon - of the 1,727 *Vibrio*
253 reads in the 18 water samples. Changes in the overall composition of the *Vibrio* community over time

and by treatment were observed by oligotyping (Figure 6). On Day 5, while the *Vibrio* community in control tanks was dominated by an oligotype most closely related to *V. alginolyticus* WW1 ($64 \pm 6\%$), probiotic tanks showed a mix of *V. alginolyticus* WW1 ($31 \pm 3\%$) and *Halovibrio* sp. 5F5 ($31 \pm 3\%$). By Day 12, the *Vibrio* composition in water in control tanks was dominated by *V. celticus* 5OM18 ($75 \pm 3\%$), while a mix of *V. orientalis* LK2HaP4 ($51 \pm 10\%$) and *V. celticus* 5OM18 ($35 \pm 8\%$) was detected in probiotic tanks.

3.3 Bacterial Relationships with Co-Occurrence Analysis

A co-occurrence analysis of members of the bacterial community (Figure 7) in the 18 water samples from Trial 3 was performed to illustrate: a) how abundance of each Order changed relative to others (edge connections); b) which Orders were most abundant in the system (node size); and c) how probiotic treatment affected their relative abundances (node color and shape). The most abundant taxa (*Rhodobacterales*, *Micrococcales*, *Sphingobacteriales*, *Flavobacteriales*, *Deferribacterales*, and *Oceanospirillales*) changed in similar fashion, but had different occurrence ratios between control and treatment samples. Orders that were more abundant in the treatment samples than in control samples included *Oceanospirillales*, *Caulobacterales*, *Lentisphaerales*, *Acidithiobacillales*, *Chroococcales*, and *Bacillales*. These nodes were scattered throughout the network and did not share direct edges, but were within 3-5 edges of each other.

Bacillales, the Order to which the probiotic used in these experiments belongs, was shown to be most directly associated in the network with four other Orders that changed in abundance between control and treatment samples: *Chromatiales*, *Xanthomonadales*, *Cytophagia* Order II, and *Vibrionales*. This direct connection between *Bacillales* and *Vibrionales* in the network indicated that the probiotic may have directly affected members of *Vibrionales*. *Oceanospirillales* was placed in the network 5 edges away from *Bacillales*, sharing an edge with the treatment-abundant *Flavobacteriales*, a common environmental bacteria taxon (Bernardet et al., 2015). This network suggests that the probiotic did not directly alter the overall bacterial community in the rearing water in an oyster hatchery, but targeted specific members of the community.

4 Discussion

A better understanding of bacterial community dynamics in aquaculture systems is critical for optimizing disease management strategies such as probiotic treatment. This study characterized: a) changes in microbial communities in an oyster hatchery through the rearing process; and b) the effect of probiotic treatment on those communities. To our knowledge, this is the first study to characterize the effects of probiotics on microbiomes in a bivalve hatchery. Despite the high spatial (by sample type and replicate tank) and temporal variability in bacterial composition at the hatchery detected in this research, results support the hypothesis that probiotic treatment leads to shifts in the microbial community in the hatchery from a state promoting the growth of potential pathogens to one that inhibits it.

Our results showed high variability in bacterial composition between replicate samples within trials and between trials, especially among the bacterial communities of oyster larvae. Variability between the 3 trials, conducted in July, January, and June in different years, is consistent with natural seasonal variation in microbial communities in Narragansett Bay (Staroscik and Smith, 2004). High variability in microbial communities in oysters from a single location is consistent with past studies, and is most probably driven by genetic and environmental effects on host-microbe interactions (King et al., 2012;

Wegner et al., 2013). Moreover, variability between replicates (tanks within the hatchery) and between trials, may have been due to inevitable variance in husbandry and handling techniques at the hatchery (Elston et al., 1981, 2008).

Despite the high variability observed in these trials, our study observed clear differences in diversity and bacterial community structure between the rearing water, the biofilms on tank surfaces (swabs), and the oyster larvae. In particular, oyster larvae microbiomes were a subset of taxa present in the water and in biofilms, including *Firmicutes* and *Proteobacteria*, while tank biofilms showed a diversity and composition state that was intermediate between water and larvae. Lower diversity indices in the larvae and tank biofilms (swabs) than the water indicates niche selection of larval and biofilm colonizers, particularly *Cyanobacteria* in tank biofilms and *Proteobacteria* in oyster larvae. The dominance of *Proteobacteria* in the system, the most abundant phylum in all samples (up to 87% in larvae), is consistent with previous studies where it was shown to make up the largest and most diverse phylum in oyster-associated microbiota (Dittmann et al., 2018; Hernández-Zárte and Olmos-Soto, 2006; Trabal Fernández et al., 2014). Bacteria are an essential component of aquaculture nutrition, as a source of both nutrients and growth factors for the microalgae, and as food for the larvae (Kamiyama, 2004; Natrah et al., 2014; Nevejan et al., 2016). Factors such as size, nutrient availability, metabolites, and accompanying bacteria lead to differential ingestion of algae and associated microbes in eastern oysters (Baldwin, 1995; Nevejan et al., 2016; Newell and Jordan, 1983; Pales Espinosa et al., 2009). Interestingly, strong temporal changes were seen in the structure of microbial communities of oyster larvae, tank surface biofilms, and/or rearing water in each of the trials. Considering the short duration of the trials (less than 15 days), this indicates that temporal changes in microbial communities in the tanks may be driven by developmental and health changes in the oyster larvae, since it is unlikely that these major changes are due to transient changes in the microbial composition of incoming water (as observed in Trial 3). More research is needed to evaluate the role of oyster-microbial interactions on the dynamics of microbial communities in rearing tanks in hatcheries.

There was no effect on bacterial community diversity or structure in any of the sample types, suggesting that the primary probiotic effect of *B. pumilus* RI06-95 is exerted directly on larval health (e.g. by modulation of the immune system) and/or that it is mediated by subtle, targeted changes in the oyster microbiomes that are obscured by larger temporal effects and/or by homogenization of large pools of larvae from each tank. The presence of the probiotic was confirmed with higher total *Bacillus* spp. read counts in the probiotic-treated water and increased abundance throughout the duration of each trial, suggesting that the probiotic accumulates in the larvae through time (tanks were scrubbed and water changed every other day). Previous studies of the impact of probiotics on microbiota in humans and fish also showed subtle changes of certain taxa, but no consistent effect on the diversity of the host's bacterial community (Boutin et al., 2013; Laursen et al., 2017; Merrifield and Carnevali, 2014; Schmidt et al., 2017; Standen et al., 2015). However, other studies report dramatic changes in fish intestinal microbiomes as a result of prebiotic treatment (Geraylou et al., 2013; Gonçalves and Gallardo-Escárate, 2017).

In addition to *Bacillus* spp., significant amplification of taxa was observed in probiotic-treated water samples compared to the control samples, most notably in the *Oceanospirillales* order. *Oceanospirillales* are heterotrophs commonly associated with mollusks and are found in the gills of many bivalves (Beinart et al., 2014; Costa et al., 2012; Jensen et al., 2010; Zurel et al., 2011). Additionally, they are recognized for their ability to degrade organic compounds in the environment and their abundance in oil plume microbial communities (Dubinsky et al., 2013; Hazen et al., 2010). These observations indicate that *Oceanospirillales* may confer a beneficial effect to the oyster host

and contribute to the mechanism of oyster larval protection by the *B. pumilus* RI06-95 probiotic. Additionally, this suggests that the presence of *B. pumilus* RI06-95 has targeted effects on specific members of the microbial community in larval tanks in the hatchery.

Previous research showed that probiotic treatment with *B. pumilus* RI06-95 decreases levels of *Vibrio* spp. in the hatchery (Sohn et al., 2016). This may be due to the production of antimicrobial secondary metabolites produced by *B. pumilus* RI06-95, as well as other *Bacillus* spp., that inhibit the growth of vibrios (Karim et al., 2013; Sohn et al., 2016; Vaseeharan and Ramasamy, 2003). In the current study, a similar trend (as determined by a reduction in read abundance) was observed in treated tanks, but high variability and small sample sizes may have hindered detecting statistically significant differences. Moreover, failure to detect a significant decrease in *Vibrio* reads in Trial 2 (performed in January) was most probably due to the low abundance of *Vibrio* spp. in this trial, which is consistent with low levels of these species in coastal waters of the North Atlantic during winter (Staroscik and Smith, 2004). Interestingly, our research indicates that probiotic treatment leads to increased *Vibrio* diversity in rearing water through time. This increase in diversity in the absence of a net increase in read abundance signifies a likely decrease in the abundance of specific pathogenic *Vibrio* spp., and therefore lower chances of a disease outbreak. Moreover, rRNA oligotyping of the *Vibrio* species in the water samples revealed a transition in the *Vibrio* community in probiotic-treated tanks from a predominance of potentially pathogenic species (*Vibrio alginolyticus*, a virulent pathogen originally isolated from amphioxius (Zou et al., 2016) and *Vibrio celticus*, a virulent anaerobic clam pathogen (Beaz-Hidalgo et al., 2010b)) to a predominance of a likely non-pathogenic species (*Vibrio orientalis*, a species that has been associated with adaptive functions (Mukhta et al., 2016; Tangl, 1983)). This trend further confirms that addition of *B. pumilus* RI06-95 causes targeted changes in certain taxa, especially *Vibrios*, which is highly relevant for decreasing infective doses and, consequently, disease dynamics (Chauhan and Singh, 2018).

This interpretation is consistent with results from the co-occurrence network analysis, a tool used to identify associations, patterns, roles, and inform hypotheses from 16S abundance data (Barberán et al., 2012). This analysis suggests a negative association between *Bacillales* with *Vibrionales* in the trials performed in summer months (Trials 1 and 3), when *Vibrionales* are more abundant in the environment and oysters. Previous research and sequencing of the genome of *B. pumilus* RI06-95 show that potential mechanisms of probiotic action can include direct competition with other species and biofilm formation (Hamblin et al., 2015; Karim et al., 2013). Competition between *B. pumilus* RI06-95 and other bacteria (including *Vibrionales*) could open niches in the oyster microbiome for potentially beneficial microbes.

In summary, the bacterial community dynamics observed in this study indicate a variety of interactions between larval oysters and specific members of the microbiome, such as *Vibrio* spp. and the *Bacillus* probiotic. First, *Vibrio* spp., as well as other Proteobacteria, appear to be particularly capable of colonizing and surviving within oyster larvae (Romalde et al., 2014). As seen in other probiotic species, these opportunistic *Vibrios* may be outcompeted by pre-colonization of other bacteria in the system, leading to a decrease in *Vibrio* abundance and/or an increase in diversity over time (Beaz-Hidalgo et al., 2010a; Zhao et al., 2016, 2018). We hypothesize that inhibition of *Vibrio* spp. by probiotic *B. pumilus* RI06-95 may allow for potentially beneficial *Oceanospirillales* to become more abundant in the system. Additional research is needed to examine the specific interactions between *Oceanospirillales* symbionts, the *Bacillus* probiotic, *Vibrio* pathogens, and the oyster host. Elucidating such interactions will require more targeted 16S rRNA and functional metagenomic analyses to track specific species over time, as well as functional studies using *in vitro* and *in vivo* competition experiments.

389

390 5 Conclusion

391 This study investigated the effects of time and probiotic treatment on bacterial communities in an
 392 oyster hatchery. Understanding how probiotic treatment affect microbiota in aquaculture systems
 393 may help in optimizing their benefits and preventing undesirable side-effects (Kesarcodi-Watson et
 394 al., 2008). Our results show that there is a strong effect of time on the microbiomes within oyster
 395 larvae, on tank walls and in the rearing water, and that probiotic treatment leads to subtle changes in
 396 certain bacterial taxa, including an increase in *Oceanospirillales* in the rearing water and changes in
 397 the *Vibrio* community. These results inform how probiotics may influence bacterial communities in
 398 an oyster hatchery over temporal and spatial scales, leading to an overall improvement in larval
 399 health.

6 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

7 Author Contributions

RJS, SS, DRN, DCR, AFP, and MGC contributed conception and design of the study; KT, RS, and KML contributed to performance of the hatchery trials; RJS and SS collected and prepared the samples for sequencing; RJS performed the sequence analysis and wrote the manuscript; all authors contributed to manuscript revision, read and approved the submitted version.

8 Funding

This work was supported by NSF Graduate Research Fellowship 1244657 to RJS, USDA AFRI 2016-67016-24905 and USDA NRAC 2258-Z55106 to DCR, DRN, and MGC. This material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, the Genomics and Sequencing Center, supported in part by the National Science Foundation EPSCoR Cooperative Agreements 0554548, EPS-1004057, and OIA-1655221.

9 Acknowledgments

We would like to thank the RWU Blount Shellfish Hatchery, the URI Genomics and Sequencing Center, and the MBL Keck Sequencing Center. We gratefully acknowledge several undergraduate students at URI and RWU for their assistance during this study.

10 Data Availability

The raw sequences generated for this study can be found in the NCBI Short Read Archive under BioProject no. PRJNA518081. In addition, further public analysis and exploration of Trial 3 data are possible on the VAMPS website (<https://vamps.mbl.edu/>) using software under the project name AFP_RWU1_Bv6.

11 References

Andrews, S. (2010). FASTQC. A quality control tool for high throughput sequence data. 2010. <http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>.

Asmani, K., Petton, B., Le Grand, J., Mounier, J., Robert, R., and Nicolas, J. L. (2016). Establishment of microbiota in larval culture of Pacific oyster, *Crassostrea gigas*. *Aquaculture* 464, 434–444. doi:10.1016/j.aquaculture.2016.07.020.

- 434 Baldwin, B. S. (1995). Selective particle ingestion by oyster larvae (*Crassostrea virginica*) feeding
435 on natural seston and cultured algae. *Mar. Biol.* 123, 95–107. doi:10.1007/BF00350328.
- 436 Barberán, A., Bates, S. T., Casamayor, E. O., and Fierer, N. (2012). Using network analysis to
437 explore co-occurrence patterns in soil microbial communities. *ISME J.* 6, 343–351.
438 doi:10.1038/ismej.2011.119.
- 439 Beaz-Hidalgo, R., Balboa, S., Romalde, J. L., and Figueras, M. J. (2010a). Diversity and
440 pathogenecity of *Vibrio* species in cultured bivalve molluscs. *Environ. Microbiol. Rep.* 2, 34–
441 43. doi:10.1111/j.1758-2229.2010.00135.x.
- 442 Beaz-Hidalgo, R., Diéguez, A. L., Cleenwerck, I., Balboa, S., Doce, A., de Vos, P., et al. (2010b).
443 *Vibrio celticus* sp. nov., a new *Vibrio* species belonging to the *Splendidus* clade with pathogenic
444 potential for clams. *Syst. Appl. Microbiol.* 33, 311–315. doi:10.1016/j.syapm.2010.06.007.
- 445 Beinart, R. A., Nyholm, S. V., Dubilier, N., and Girguis, P. R. (2014). Intracellular Oceanospirillales
446 inhabit the gills of the hydrothermal vent snail *Alviniconcha* with chemosynthetic, ??-
447 Proteobacterial symbionts. *Environ. Microbiol. Rep.* 6, 656–664. doi:10.1111/1758-2229.12183.
- 448 Bernardet, J.-F., Bernardet, and Jean-François (2015). “*Flavobacteriales* ord. nov.,” in *Bergey’s*
449 *Manual of Systematics of Archaea and Bacteria* (Chichester, UK: John Wiley & Sons, Ltd), 1–
450 2. doi:10.1002/9781118960608.obm00033.
- 451 Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina
452 sequence data. *Bioinformatics* 30, 2114–2120. doi:10.1093/bioinformatics/btu170.
- 453 Boutin, S., Audet, C., and Derome, N. (2013). Probiotic treatment by indigenous bacteria decreases
454 mortality without disturbing the natural microbiota of *Salvelinus fontinalis*. *Can. J. Microbiol.*
455 59, 662–70. doi:10.1139/cjm-2013-0443.
- 456 Burge, C. A., Closek, C. J., Friedman, C. S., Groner, M. L., Jenkins, C. M., Shore-Maggio, A., et al.
457 (2016). The Use of Filter-feeders to Manage Disease in a Changing World. in *Integrative and*
458 *Comparative Biology* (Oxford University Press), 573–587. doi:10.1093/icb/icw048.
- 459 Chauhan, A., and Singh, R. (2018). Probiotics in aquaculture: a promising emerging alternative
460 approach. *Symbiosis*, 1–15. doi:10.1007/s13199-018-0580-1.
- 461 Chauhan, A., Wafula, D., Lewis, D. E., and Pathak, A. (2014). Metagenomic Assessment of the
462 Eastern Oyster-Associated Microbiota. *Genome Announc.* 2, e01083-14-e01083-14.
463 doi:10.1128/genomeA.01083-14.
- 464 Costa, P. M., Carreira, S., Lobo, J., and Costa, M. H. (2012). Molecular detection of prokaryote and
465 protozoan parasites in the commercial bivalve *Ruditapes decussatus* from southern Portugal.
466 *Aquaculture* 370–371, 61–67. doi:10.1016/j.aquaculture.2012.10.006.
- 467 Dittmann, K., Sonnenschein, E. C., Egan, S., Gram, L., and Bentzon-Tilia, M. (2018). Impact of
468 *Phaeobacter inhibens* on marine eukaryote associated microbial communities. *Appl. Environ.*
469 *Microbiol.* Submitted. doi:10.1111/1758-2229.12698.
- 470 Dixon, P. (2003). VEGAN, a package of R functions for community ecology. *J. Veg. Sci.* 14, 927–

- 471 930. doi:10.1111/j.1654-1103.2003.tb02228.x.
- 472 Dubert, J., Barja, J. L., and Romalde, J. L. (2017). New insights into pathogenic vibrios affecting
473 bivalves in hatcheries: Present and future prospects. *Front. Microbiol.* 8, 762.
474 doi:10.3389/fmicb.2017.00762.
- 475 Dubinsky, E. A., Conrad, M. E., Chakraborty, R., Bill, M., Borglin, S. E., Hollibaugh, J. T., et al.
476 (2013). Succession of hydrocarbon-degrading bacteria in the aftermath of the deepwater horizon
477 oil spill in the gulf of Mexico. *Environ. Sci. Technol.* 47, 10860–10867. doi:10.1021/es401676y.
- 478 Elston, R., Hasegawa, H., Humphrey, K., Polyak, I., and Häse, C. (2008). Re-emergence of *Vibrio*
479 *tubiaschii* in bivalve shellfish aquaculture: severity, environmental drivers, geographic extent and
480 management. *Dis. Aquat. Organ.* 82, 119–134. doi:10.3354/dao01982.
- 481 Elston, R., Leibovitz, L., Relyea, D., and Zatlá, J. (1981). Diagnosis of vibriosis in a commercial
482 oyster hatchery epizootic: Diagnostic tools and management features. *Aquaculture* 24, 53–62.
483 doi:10.1016/0044-8486(81)90043-0.
- 484 Eren, A. M., Maignien, L., Sul, W. J., Murphy, L. G., Grim, S. L., Morrison, H. G., et al. (2013a).
485 Oligotyping: Differentiating between closely related microbial taxa using 16S rRNA gene data.
486 *Methods Ecol. Evol.* 4, 1111–1119. doi:10.1111/2041-210X.12114.
- 487 Eren, A. M., Vineis, J. H., Morrison, H. G., and Sogin, M. L. (2013b). A Filtering Method to
488 Generate High Quality Short Reads Using Illumina Paired-End Technology. *PLoS One* 8,
489 e66643. doi:10.1371/journal.pone.0066643.
- 490 FAO (2018). The State of World Fisheries and Aquaculture 2018 - Meeting the sustainable
491 development goals. Available at: www.fao.org/publications.
- 492 García Bernal, M., Trabal Fernández, N., Saucedo Lastra, P. E., Medina Marrero, R., and Mazón-
493 Suástegui, J. M. (2017). Streptomyces effect on the bacterial microbiota associated to
494 *Crassostrea sikamea* oyster. *J. Appl. Microbiol.* 122, 601–614. doi:10.1111/jam.13382.
- 495 Geraylou, Z., Souffreau, C., Rurangwa, E., De Meester, L., Courtin, C. M., Delcour, J. A., et al.
496 (2013). Effects of dietary arabinosyl-oligosaccharides (AXOS) and endogenous probiotics on
497 the growth performance, non-specific immunity and gut microbiota of juvenile Siberian
498 sturgeon (*Acipenser baerii*). *Fish Shellfish Immunol.* 35, 766–775.
499 doi:10.1016/j.fsi.2013.06.014.
- 500 Gonçalves, A. T., and Gallardo-Escárate, C. (2017). Microbiome dynamic modulation through
501 functional diets based on pre- and probiotics (mannan-oligosaccharides and *Saccharomyces*
502 *cerevisiae*) in juvenile rainbow trout (*Oncorhynchus mykiss*). *J. Appl. Microbiol.* 122, 1333–
503 1347. doi:10.1111/jam.13437.
- 504 Groner, M. L., Maynard, J., Breyta, R., Carnegie, R. B., Dobson, A., Friedman, C. S., et al. (2016).
505 Managing marine disease emergencies in an era of rapid change. *Philos. Trans. R. Soc. B Biol.*
506 *Sci.* 371, 20150364. doi:10.1098/rstb.2015.0364.
- 507 Hamblin, M., Spinard, E., Gomez-Chiarri, M., Nelson, D. R., and Rowley, D. C. (2015). Draft
508 Genome Sequence of the Shellfish Larval Probiotic *Bacillus pumilus* RI06-95. *Genome*

- 509 *Announc.* 3, e00858-15. doi:10.1128/genomeA.00858-15.
- 510 Hazen, T. C., Dubinsky, E. A., DeSantis, T. Z., Andersen, G. L., Piceno, Y. M., Singh, N., et al.
511 (2010). Deep-sea oil plume enriches indigenous oil-degrading bacteria. *Science* 330, 204–8.
512 doi:10.1126/science.1195979.
- 513 Helm, M., and Bourne, N. (2004). *Hatchery culture of bivalves. A practical manual.*
514 doi:10.15713/ins.mmj.3.
- 515 Hernández-Zárate, G., and Olmos-Soto, J. (2006). Identification of bacterial diversity in the oyster
516 *Crassostrea gigas* by fluorescent in situ hybridization and polymerase chain reaction. *J. Appl.*
517 *Microbiol.* 100, 664–672. doi:10.1111/j.1365-2672.2005.02800.x.
- 518 Huse, S. M., Mark Welch, D. B., Voorhis, A., Shipunova, A., Morrison, H. G., Eren, A. M., et al.
519 (2014). VAMPS: A website for visualization and analysis of microbial population structures.
520 *BMC Bioinformatics* 15, 41. doi:10.1186/1471-2105-15-41.
- 521 Jensen, S., Duperron, S., Birkeland, N.-K., and Hovland, M. (2010). Intracellular *Oceanospirillales*
522 bacteria inhabit gills of *Acesta* bivalves. *FEMS Microbiol. Ecol.* 74, 523–533.
523 doi:10.1111/j.1574-6941.2010.00981.x.
- 524 Kamiyama, T. (2004). The microbial loop in a eutrophic bay and its contribution to bivalve
525 aquaculture. *Fish. Res.*, 41–50. doi:10.3892/or.2013.2813.
- 526 Karim, M., Zhao, W., Rowley, D., Nelson, D., and Gomez-Chiarri, M. (2013). Probiotic Strains for
527 Shellfish Aquaculture: Protection of Eastern Oyster, *Crassostrea virginica* , Larvae and
528 Juveniles Against Bacterial Challenge. *J. Shellfish Res.* 32, 401–408. doi:10.2983/035.032.0220.
- 529 Kesarcodi-Watson, A., Kaspar, H., Lategan, M. J., and Gibson, L. (2008). Probiotics in aquaculture:
530 The need, principles and mechanisms of action and screening processes. *Aquaculture* 274, 1–14.
531 doi:10.1016/j.aquaculture.2007.11.019.
- 532 Kesarcodi-Watson, A., Miner, P., Nicolas, J. L., and Robert, R. (2012). Protective effect of four
533 potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster
534 (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). *Aquaculture* 344–
535 349, 29–34. doi:10.1016/j.aquaculture.2012.02.029.
- 536 King, G. M., Judd, C., Kuske, C. R., and Smith, C. (2012). Analysis of Stomach and Gut
537 Microbiomes of the Eastern Oyster (*Crassostrea virginica*) from Coastal Louisiana, USA. *PLoS*
538 *One* 7. doi:10.1371/journal.pone.0051475.
- 539 King, W. L., Jenkins, C., Seymour, J. R., and Labbate, M. (2018). Oyster disease in a changing
540 environment: Decrypting the link between pathogen, microbiome and environment. *Mar.*
541 *Environ. Res.* doi:10.1016/J.MARENRES.2018.11.007.
- 542 Lafferty, K. D., Harvell, C. D., Conrad, J. M., Friedman, C. S., Kent, M. L., Kuris, A. M., et al.
543 (2015). Infectious Diseases Affect Marine Fisheries and Aquaculture Economics. *Ann. Rev.*
544 *Mar. Sci.* 7, 471–496. doi:10.1146/annurev-marine-010814-015646.
- 545 Laursen, M. F., Laursen, R. P., Larnkjær, A., Michaelsen, K. F., Bahl, M. I., and Licht, T. R. (2017).

- 546 Administration of two probiotic strains during early childhood does not affect the endogenous
547 gut microbiota composition despite probiotic proliferation. *BMC Microbiol.* 17, 175.
548 doi:10.1186/s12866-017-1090-7.
- 549 Le Roux, F., Wegner, K. M., and Polz, M. F. (2016). Oysters and Vibrios as a Model for Disease
550 Dynamics in Wild Animals. *Trends Microbiol.* 24, 568–580. doi:10.1016/j.tim.2016.03.006.
- 551 Lokmer, A., Goedknecht, M. A., Thielges, D. W., Fiorentino, D., Kuenzel, S., Baines, J. F., et al.
552 (2016a). Spatial and temporal dynamics of pacific oyster hemolymph microbiota across multiple
553 scales. *Front. Microbiol.* 7, 1367. doi:10.3389/fmicb.2016.01367.
- 554 Lokmer, A., Kuenzel, S., Baines, J. F., and Wegner, K. M. (2016b). The role of tissue-specific
555 microbiota in initial establishment success of Pacific oysters. *Environ. Microbiol.* 18, 970–987.
556 doi:10.1111/1462-2920.13163.
- 557 Lokmer, A., and Wegner, K. M. (2015). Hemolymph microbiome of Pacific oysters in response to
558 temperature, temperature stress and infection. *ISME J.* 9, 670–682. doi:10.1038/ismej.2014.160.
- 559 Mckindsey, C. W., Landry, T., Beirn, F. X. O., and Davies, I. A. N. M. (2007). Bivalve Aquaculture
560 and Exotic Species : a Review of Ecological Considerations and Management Issues. *J. Shellfish*
561 *Res.* 26, 281–294. doi:10.2983/0730-8000(2007)26[281:BAAESA]2.0.CO;2.
- 562 McMurdie, P. J., and Holmes, S. (2013). Phyloseq: An R Package for Reproducible Interactive
563 Analysis and Graphics of Microbiome Census Data. *PLoS One* 8, e61217.
564 doi:10.1371/journal.pone.0061217.
- 565 Merrifield, D. L., and Carnevali, O. (2014). Probiotic Modulation of the Gut Microbiota of Fish.
566 *Aquac. Nutr.* 588, 185–222. doi:10.1002/9781118897263.ch8.
- 567 Mukhta, S., Mehnaz, S., Mirza, M. S., Mirza, B., and Malik, K. A. (2016). Accession No. LT221239,
568 *Vibrio orientalis* partial 16S rRNA gene, isolate LK2HaP4 - Nucleotide - NCBI. Available at:
569 <https://www.ncbi.nlm.nih.gov/nuccore/LT221239.1> [Accessed December 19, 2017].
- 570 Natrah, F. M. I., Bossier, P., Sorgeloos, P., Yusoff, F. M., and Defoirdt, T. (2014). Significance of
571 microalgal-bacterial interactions for aquaculture. *Rev. Aquac.* 6, 48–61. doi:10.1111/raq.12024.
- 572 Nevejan, N., De Schryver, P., Wille, M., Dierckens, K., Baruah, K., and Van Stappen, G. (2016).
573 Bacteria as food in aquaculture: Do they make a difference? *Rev. Aquac.* 10, 180–212.
574 doi:10.1111/raq.12155.
- 575 Newaj-Fyzul, A., Al-Harbi, A. H., and Austin, B. (2014). Review: Developments in the use of
576 probiotics for disease control in aquaculture. *Aquaculture* 431, 1–11.
577 doi:10.1016/j.aquaculture.2013.08.026.
- 578 Newell, R. I., and Jordan, S. J. (1983). Preferential ingestion of organic material by the American
579 oyster *Crassostrea virginica*. *Mar. Ecol. - Prog. Ser.* 13, 47–53. doi:10.3354/meps013047.
- 580 Pales Espinosa, E., Mickael, P., Evan Ward, J., Shumway, S. E., and Bassem, A. (2009). Lectins
581 associated with the feeding organs of the oyster *Crassostrea virginica* can mediate particle
582 selection. *Biol. Bull.* 217, 130–141. doi:217/2/130 [pii].

- 583 Pérez-Sánchez, T., Mora-Sánchez, B., and Balcázar, J. L. (2018). Biological Approaches for Disease
584 Control in Aquaculture: Advantages, Limitations and Challenges. *Trends Microbiol.* 26, 896–
585 903. doi:10.1016/j.tim.2018.05.002.
- 586 Pierce, M. L., and Ward, J. E. (2018). Microbial Ecology of the Bivalvia, with an Emphasis on the
587 Family *Ostreidae*. *J. Shellfish Res.* 37, 793–806. doi:10.2983/035.037.0410.
- 588 Pierce, M. L., Ward, J. E., Holohan, B. A., Zhao, X., and Hicks, R. E. (2016). The influence of site
589 and season on the gut and pallial fluid microbial communities of the eastern oyster, *Crassostrea*
590 *virginica* (Bivalvia, Ostreidae): community-level physiological profiling and genetic structure.
591 *Hydrobiologia* 765, 97–113. doi:10.1007/s10750-015-2405-z.
- 592 Powell, S. M., Chapman, C. C., Bermudes, M., and Tamplin, M. L. (2013). Dynamics of Seawater
593 Bacterial Communities in a Shellfish Hatchery. *Microb. Ecol.* 66, 245–256.
594 doi:10.1007/s00248-013-0183-6.
- 595 Prado, S., Romalde, J. L., and Barja, J. L. (2010). Review of probiotics for use in bivalve hatcheries.
596 *Vet. Microbiol.* 145, 187–197. doi:10.1016/j.vetmic.2010.08.021.
- 597 Richards, G. P., Watson, M. A., Needleman, D. S., Church, K. M., and Häse, C. C. (2015).
598 Mortalities of Eastern And Pacific oyster larvae caused by the pathogens *Vibrio coralliilyticus*
599 and *Vibrio tubiashii*. *Appl. Environ. Microbiol.* 81, 292–297. doi:10.1128/AEM.02930-14.
- 600 Romalde, J. L., Diéguez, A. L., Lasa, A., and Balboa, S. (2014). New *Vibrio* species associated to
601 molluscan microbiota: A review. *Front. Microbiol.* 4, 413. doi:10.3389/fmicb.2013.00413.
- 602 Schmidt, V., Gomez-Chiarri, M., Roy, C., Smith, K., and Amaral-Zettler, L. (2017). Subtle
603 Microbiome Manipulation Using Probiotics Reduces Antibiotic-Associated Mortality in Fish.
604 *mSystems* 2, e00133-17. doi:10.1128/mSystems.00133-17.
- 605 Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape:
606 A software Environment for integrated models of biomolecular interaction networks. *Genome*
607 *Res.* 13, 2498–2504. doi:10.1101/gr.1239303.
- 608 Sinigalliano, C. D., Gidley, M. L., Shibata, T., Whitman, D., Dixon, T. H., Laws, E., et al. (2007).
609 Impacts of Hurricanes Katrina and Rita on the microbial landscape of the New Orleans area.
610 *Proc. Natl. Acad. Sci. U. S. A.* 104, 9029–9034. doi:10.1073/pnas.0610552104.
- 611 Sohn, S., Lundgren, K. M., Tammi, K., Karim, M., Smolowitz, R., Nelson, D. R., et al. (2016).
612 Probiotic Strains for Disease Management in Hatchery Larviculture of the Eastern Oyster
613 *Crassostrea virginica*. *J. Shellfish Res.* 35, 307–317. doi:10.2983/035.035.0205.
- 614 Standen, B. . T., Rodiles, A., Peggs, D. L., Davies, S. J., Santos, G. A., and Merrifield, D. L. (2015).
615 Modulation of the intestinal microbiota and morphology of tilapia, *Oreochromis niloticus*,
616 following the application of a multi-species probiotic. *Appl. Microbiol. Biotechnol.* 99, 8403–
617 8417. doi:10.1007/s00253-015-6702-2.
- 618 Staroscik, A. M., and Smith, D. C. (2004). Seasonal patterns in bacterioplankton abundance and
619 production in Narragansett Bay, Rhode Island, USA. *Aquat. Microb. Ecol.* 35, 275–282.

- 620 Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., et al. (2012).
621 Disease will limit future food supply from the global crustacean fishery and aquaculture sectors.
622 *J. Invertebr. Pathol.* 110, 141–157. doi:10.1016/j.jip.2012.03.013.
- 623 Tangl, J. S. (1983). *Vibrio orientalis*. *Current* 8, 95–100. doi:10.1007/BF01566965.
- 624 Torondel, B., Ensink, J. H. J., Gundogdu, O., Ijaz, U. Z., Parkhill, J., Abdelahi, F., et al. (2016).
625 Assessment of the influence of intrinsic environmental and geographical factors on the bacterial
626 ecology of pit latrines. *Microb. Biotechnol.* 9, 209–223. doi:10.1111/1751-7915.12334.
- 627 Trabal Fernández, N., Mazón-Suástegui, J. M., Vázquez-Juárez, R., Ascencio-Valle, F., and Romero,
628 J. (2014). Changes in the composition and diversity of the bacterial microbiota associated with
629 oysters (*Crassostrea corteziensis*, *Crassostrea gigas* and *Crassostrea sikamea*) during
630 commercial production. *FEMS Microbiol. Ecol.* 88, 69–83. doi:10.1111/1574-6941.12270.
- 631 Vaseeharan, B., and Ramasamy, P. (2003). Control of pathogenic *Vibrio* spp. by *Bacillus subtilis*
632 BT23, a possible probiotic treatment for black tiger shrimp *Penaeus monodon*. *Lett. Appl.*
633 *Microbiol.* 36, 83–87. doi:10.1046/j.1472-765X.2003.01255.x.
- 634 Verschuere, L., Rombaut, G., Sorgeloos, P., and Verstraete, W. (2000). Probiotic Bacteria as
635 Biological Control Agents in Aquaculture. *Microbiol. Mol. Biol. Rev.* 64, 655–671.
636 doi:10.1128/MMBR.64.4.655-671.2000.
- 637 Wegner, K., Volkenborn, N., Peter, H., and Eiler, A. (2013). Disturbance induced decoupling
638 between host genetics and composition of the associated microbiome. *BMC Microbiol.* 13, 252.
639 doi:10.1186/1471-2180-13-252.
- 640 Zhao, W., Dao, C., Karim, M., Gomez-Chiarri, M., Rowley, D., and Nelson, D. R. (2016).
641 Contributions of tropodithietic acid and biofilm formation to the probiotic activity of
642 *Phaeobacter inhibens*. *BMC Microbiol.* 16, 1. doi:10.1186/s12866-015-0617-z.
- 643 Zhao, W., Yuan, T., Piva, C., Spinard, E. J., Schutttert, C., Rowley, D. C., et al. (2018). The probiotic
644 bacterium, *Phaeobacter inhibens*, down-regulates virulence factor transcription in the shellfish
645 pathogen, *Vibrio coralliilyticus*, by N -acyl homoserine lactone production. *Appl. Environ.*
646 *Microbiol.* doi:10.1128/AEM.01545-18.
- 647 Zou, Y., Ma, C., Zhang, Y., Du, Z., You, F., Tan, X., et al. (2016). Isolation and characterization of
648 *Vibrio alginolyticus* from cultured amphioxus *Branchiostoma belcheri* tsingtauense. *Biol.* 71,
649 757–762. doi:10.1515/biolog-2016-0102.
- 650 Zurel, D., Benayahu, Y., Or, A., Kovacs, A., and Gophna, U. (2011). Composition and dynamics of
651 the gill microbiota of an invasive Indo-Pacific oyster in the eastern Mediterranean Sea. *Environ.*
652 *Microbiol.* 13, 1467–1476. doi:10.1111/j.1462-2920.2011.02448.x.
- 653

12 Tables

Table 1. Summary of probiotic trial information and sequencing data. W=water, S=swab, O=oysters.

| | Trial 1 | Trial 2 | Trial 3 |
|--|-------------------------------------|-------------------------------------|--|
| Sample Types | Water, Swabs, Oysters | Water, Swabs, Oysters | Water |
| Sampling Days (0=spawn) | W:1,12 / OS:5,12 | W:1,9 / OS:6,9 | W: 5,8,12 |
| Volumne water Filtered | 410-750 mL | 7-10 mL | 1300-1700 mL |
| Trial Dates | July 11-23, 2012 | Jan 9-18, 2013 | June 3-15, 2016 |
| Bacterial reads from 12 water samples | 1.3 million | 1.8 million | 5.7 million |
| Methods | MoBio extraction MiSeq, 2x250 PE | MoBio extraction MiSeq, 2x250 PE | Puregene extraction HiSeq, 2x100 PE |
| 16S region | V4 | V4 | V6 |

13 Figure Legends

Figure 1. Percent abundances of the 12 most abundant phyla in oyster larvae, biofilm swab, and rearing water samples from all 3 Trials based on 16S rDNA amplicon sequencing data (bottom). The total abundance of quality filtered sequencing reads is shown in the bar graph (top). The 12 dominant phyla include *Actinobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Deferribacteres*, *Firmicutes*, *Fusobacteria*, *Lentisphaerae*, *Planctomycetes*, *Proteobacteria*, *Spirochaetae*, *Verrucomicrobia*, and Unknown. Note: there are no treated oyster larvae samples from Trial 2, Day 6.

Figure 2. Simpson's index of diversity of bacterial communities by sample (larvae, swab, water) and trial (n=3 tanks). No significant differences in diversity were found between control (light blue) and treatment (dark red) within each sample type and trial. Bacterial community diversity significantly increased over time in larvae, swab, and water samples from Trial 1, and water samples from Trial 3. Diversity in water was significantly higher in Trial 3 than Trials 1 and 2. Note: there are no treated oyster larvae samples from Trial 2, Day 6.

Figure 3. NMDS plot visualization of Bray-Curtis beta-diversity (k=2) at the Order level by (A) sample type, (B) sampling day, and (C) treatment. The ellipse lines show the 95% confidence interval. (A) The different types of samples are indicated by colors (Oyster=dashed red, Swab=dashdot green, Water=dotted blue) and the days are indicated by symbols (Timepoint 1=circle, Timepoint 2=triangle). The water and swab communities were significantly distinct from each other in both trials. (B) The sampling timepoints are indicated by colors (1=longdash yellow, 5=shortdash red, 8=dashdot purple, 9=solid green, 12=dotted blue) and the treatment group is indicated by symbols (control=circle, probiotic treatment=triangle). The water community was significantly different between timepoints. (C) The treatment group is indicated by colors (control=light blue dashed, probiotic treatment=dark red dotted) and sampling timepoints are indicated by symbols. No significant differences in community structure in water from control and probiotic-treated tanks was detected when samples from all time points were analyzed together.

682 **Figure 4.** Effect of probiotic treatment on abundance of (a) *Bacillus* and (b) *Oceanospirillales* in
 683 water. Number of reads in treated (dark red) and control (light blue) samples (n=3 tanks per
 684 treatment) are represented for each sampling day and trial. (a) *Bacillus* abundance was significantly
 685 higher in the treated than the control water after 5 days of treatment, and (b) *Oceanospirillales* were
 686 consistently more abundant in probiotic-treated tank rearing water, and decreased with time.
 687 **Significance:** *p<0.05, **p<0.01, ***p<0.001

688 **Figure 5.** Effect of treatment, time, and samples type on Simpson's Index of Diversity for *Vibrio* (a,
 689 boxplots) and Total *Vibrio* read abundance (b, bar graph). Representative data from Trial 1 (n=3
 690 tanks per treatment). Note different scales for (b). **Significance:** *p<0.05, **p<0.01, ***p<0.001

691 **Figure 6.** *Vibrio* spp. oligotypes in Control (CON) and Treatment (T) water samples on Days 5, 8,
 692 and 12 from Trial 3. These 8 oligotypes were generated from changes in positions 23 and 37 in a total
 693 of 1727 sequences, represented with the 2 letter abbreviations in the legend. The taxonomy of the 4
 694 most abundant oligotypes is shown. *Vibrio* oligotypes showed differences in succession of species
 695 over time between control and treatment rearing water.

696 **Figure 7.** Co-occurrence network analysis based on Bray-Curtis dissimilarity metric (max distance
 697 =0.5, Order level) for water samples from Trial 3 (n=3 tanks per treatment and day, total of 18). Taxa
 698 that change in the same way share an edge; nodes that have edges occur in the same proportions and
 699 in the same samples. Darker blue circle nodes indicate taxa that occur in the Control significantly
 700 more than Treated water samples. White nodes have equal occurrence in treated and control water
 701 samples. Darker red diamond nodes indicated taxa that occurs in the Treated significantly more than
 702 Control water samples.