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

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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 structure. 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 proportion of *Oceanospirillales* and *Bacillus* spp. in water and oyster larvae. Co-occurrence network analysis suggests that probiotic treatment had a systemic effect on bacterial community structure, mediated through select taxa associated with the probiotic target species.

# 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., 2015b). 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 (Gatesoupe, 1999; 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 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 the microbiome 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 Mathias Wegner, 2015; Pierce et al., 2016; Pierce and Ward, 2018). Additionally, the oyster microbiome is distinct from that of the surrounding water and is 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.

# Materials and Methods

## 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; Socha, 2008), 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 an OD550 measurement 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).

## Experimental Design and Sample Collection

Samples for microbiome analysis were collected during the hatchery trials reported in Sohn et al. (*in prep*). Eastern oysters (*Crassostrea virginica*) were spawned at the Blount Shellfish Hatchery at Roger Williams University (Bristol, RI, USA) 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 at ambient temperature (approximately 23 °C) and a salinity of 28 psu. Tanks were randomly assigned to treatments including no probiotics (control) and probiotic treatment with *B. pumilus* RI06-95. *B. pumilus* RI06-95 were administered daily at 104 CFU/mL to non-control tanks after being mixed with an algal feed. The microalgae strains used throughout the trial for feeding were *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea* (CCMP1324), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weisflogii* (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 UV-treated and filtered water prior restocking of the larvae.

Rearing water (1 – 2 L) was collected from each of the triplicate tanks during drain-down and filtered over 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 by swabbing a line of approximately 144 cm in length using with sterile cotton swabs. The cotton tips of the swabs were stored in RNAlater. Oyster larvae were collected on a 55 μm sieve after drain-down of tank water, resuspended in 5 L of seawater, and 10 ml of oyster larvae from each tank (about 150 – 1500 larvae) were 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 residual 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 samples were also collected from the inflow (water piped directly from the environment into the hatchery) and outflow pipes (UV-treated and filtered inflow water).

## 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) 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 Laboratories, USA) 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. 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 MO BIO vortex adaptor. Following extraction, DNA concentration was quantified with both a Nanodrop 2000 instrument and a Qubit Fluorometer (ThermoFisher Scientific, Wilmington, DE).

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). 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 1000 to generate 100 bp double strand reads with full overlap of the V6 region.

## 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), which is implemented in VAMPS, and annotated using SILVA.

## Statistical and Network Analysis

All descriptive and statistical analyses were performed in the R statistical computing environment with the *vegan* and *phyloseq* packages. Simpson’s diversity values were calculated for each sample at the order level using the *vegan* package Version 2.4-1 (Dixon, 2003). Non-metric dimensional analysis (NMDS) was used to determine the influence of time, probiotic treatment, or sample type on the bacterial communit*y* 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, max distance=0.5 (McMurdie and Holmes, 2013). 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 are more abundant in the control or probiotic-treated samples (0 to 3.6 times).

# Results

## 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, 24 tank biofilm swab, 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 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% ± 6%), *Bacteroidetes* (26% ± 10%), *Cyanobacteria* (12% ± 10%), *Actinobacteria* (5% ± 5%), and *Planctomycetes* (2% ± 1%) (Figure 1, bottom right). The larval samples were dominated by *Proteobacteria* (87% ± 12%) and the swab samples by *Proteobacteria* (68% ±17%), *Cyanobacteria* (19% ± 16%), and *Bacteroidetes* (8% ± 4%) (Figure 1, bottom left). Abundance of *Cyanobacteria* in swab samples was significantly higher than in water samples (p<0.001, Table S1). There was a significant enrichment in *Proteobacteria* in larval and swab samples, at the expense of *Bacteroidetes,* as compared to water samples (p<0.001, Table S1).

Overall, the bacterial community in rearing water was significantly more diverse than the communities in oyster larvae and biofilm swab samples (Simpson’s Diversity Index, p<0.001, Figure 2, Table S2), reflecting an enrichment in specific community members from the more diverse rearing water community in larvae and tank surfaces (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 (Figure 2, Figure S2). However, 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 diversity between control and treated samples at each time-point and sample type were detected (p=0.52).

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

## Effects of the Probiotic on the Selected Members of the Bacterial Community

Although control and probiotic-treated tanks showed no significant differences in diversity and structure of bacterial communities overall (Figure 3c), significant differences in the read abundance of several specific taxa were detected. In all trials, *Bacillus* spp. reads in the probiotic-treated water samples increased through time, and were significantly more abundant in samples from treated tanks than in the control tanks by the final sampling day on Trials 2 and 3 (p<0.05, Figure 4a, Table S6). These results suggest that the increase in reads corresponds to the added probiotic. The number of *Oceanospirillales* reads was also significantly higher at all time points (20-34% of reads) in probiotic-treated rearing water as compared to control water in Trial 3 (p<0.01, Figure 4b, Table S7). Over time, the abundance of *Oceanospirillales* reads in the water decreased by 41-62% depending on the trial (p<0.005, Figure 4b, Table S7).  No significant changes in read abundance of these two selected members of the bacterial community were detected in larval oysters or swabs (Trials 1 and 2; not shown).

Since *Vibrio* is a taxon that comprises a significant number of larval oyster pathogens (Elston et al., 1981; Le Roux et al., 2016; Richards et al., 2015a), we evaluated the effect of probiotic treatment on changes in *Vibrio* spp. diversity and abundance over time in the hatchery (Figures 5, S4, S5). Probiotic treatment led to a significant increase in vibrio diversity (as measured using the Simpson’s Index of diversity) in water samples collected on day 12 in Trial 1 (p< 0.05; Figure 5a, Table S8). However, no significant differences in abundance or diversity of *Vibrio* spp. between control and probiotic treated-tanks were detected for any of the sample types (Figure 5b, Table S9). When considering the effect of sample type, *Vibrio* read abundance was significantly lower in water samples than in swabs or oysters (all time points) and in swabs than in oysters (Day 12 only; p<0.001, Figure 5b, Table S9). When considering all time points, the diversity of *Vibrio* spp. was significantly higher in swab and oyster samples than in water samples (p<0.005, Table S8). An evaluation of the effect of time on *Vibrio* abundance and diversity showed a significant increase in diversity with swab and water samples (p<0.001), and a significant decrease in abundance in all sample types (Trial 1, p<0.05, Figure 5b, Tables S8, S9).

Since the V6 region of the 16S rRNA gene was deeply sequenced in Trial 3, we were able to perform an oligotyping analysis - a method that detects genetic variants within a taxon - of 1,727 *Vibrio* 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% ± 6%), probiotic tanks showed a mix of *V. alginolyticus* WW1 (31% ± 3%) and *Halovibrio* sp. 5F5 (31% ± 3%). By Day 12, the *Vibrio* composition in water in control tanks was dominated by *V. celticus* 5OM18 (75% ± 3%), while a mix of *V. orientalis* LK2HaP4 (51% ± 10%) and *V. celticus* 5OM18 (35% ± 8%) was detected in probiotic tanks.

## 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*, *Lentispherales*, *Acidithiobacillales*, *Chrococcales*, 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 and was most abundant in the treated samples, 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 these Orders of bacteria may have been directly associated. *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 acted through associated bacteria.

1. **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 the microbial community in an oyster hatchery through the rearing process; and b) the effect of probiotic treatment on that community. Despite the high spatial 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 that promotes the growth of potential pathogens to one that inhibits it.

Our results also 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).

Despite the high variability observed in these trials, our study observed clear differences in diversity and bacterial community structure between the rearing water, the tank biofilms (swabs), and the oyster larvae. In particular, oyster larvae selected for specific 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. Additionally, the microbiome of the rearing water changed significantly over time, specifically with an increase in *Actinobacteria* and a decrease in *Bacteroidetes*. *Proteobacteria* was, on average, the most abundant phylum in all samples (up to 87% in larvae), 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-Zárate and Olmos-Soto, 2006; Trabal Fernández et al., 2014). The other dominant phyla, including *Bacteroidetes, Cyanobacteria,* and *Actinobacteria*, showed variation in relative abundances based on sample type, day, and treatment.

Lower diversity indices in the larvae and tank biofilms (swabs) than the water indicates niche selection of larval and biofilm colonizers, particularly *Cyanobacteria* and *Proteobacteria*. It is likely that the oysters select their commensal bacteria from a diverse pool of bacteria in the rearing water and microalgal feed. Alternatively, it is possible that the commensal bacteria are choosing their ideal environment, and the oysters are “hot spots” for increased organic content in the rearing tanks. These hypotheses are consistent with outcomes of past studies that demonstrate interactions between microalgae, bacteria, and animals in aquaculture (Simons et al., 2018).  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). Studies have shown differential selection of microbes in eastern oysters based on size, nutrient availability, metabolites, and accompanying bacteria (Baldwin, 1995; Emmanuelle et al., 2009; Nevejan et al., 2016; Newell and Jordan, 1983). 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 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 oyster tanks.

Despite a significant improvement in larval survival with the addition of probiotics (Sohn et al. *in prep*), there was no effect on bacterial diversity or structure in any of the sample types, suggesting that the primary probiotic effect of *B. pumilus* RI06-95 is exerted directly on the larvae (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. The presence of the probiotic was confirmed with higher total *Bacillus* spp. read counts in the probiotic-treated water and increased abundance throughout the length of each trial, suggesting that the probiotic accumulates in larval oysters through time. 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 probiotic treatment (Geraylou et al., 2013; Gonçalves and Gallardo-Escárate, 2017). No such studies have been previously conducted in bivalves.

Amplification of other taxa in probiotic-treated samples compared to the control was observed, most notably in the *Oceanospirillales* order. This group of bacteria was consistently more abundant in probiotic-treated rearing water, while total reads also significantly decreased with time in all three trials. *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 affects the microbial community of the oyster host.

Previous research (Sohn et al., 2016) suggested that probiotic treatment with *B. pumilus* RI06-95 decreases levels of *Vibrio* spp. in the hatchery. 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 thegrowth of vibrios (Sohn et al., 2016; Vaseeharan and Ramasamy, 2003). In the current study, this trend was also observed in the reduced number of *Vibrio* 16S reads in treated tanks, but high variability and small sample sizes hindered statistically significant findings. Failure to detect a significant decrease in *Vibrio* reads in Trial 2 was most probably due to the low abundance of *Vibrios* in this trial. Trial 2 was conducted in January when colder temperatures lead to decreased environmental *Vibrio* presence (Costa Sobrinho et al., 2010). Interestingly, our research indicates that probiotic treatment leads to increased *Vibrio* diversity in rearing water through time. This increase in diversity signifies a likely decrease in the abundance of any specific pathogenic *Vibrio* spp., and therefore lower chances of a disease outbreak. Analysis of single base pair changes in 16S rRNA V6 hypervariable region allowed us to oligotype the *Vibrio* species in the water samples. In the probiotic treated tanks, this revealed a transition in the *Vibrio* community from a predominance of potentially pathogenic species (*Vibrio alginolyticus*, a virulent pathogen originally isolated from amphioxi (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 is often associated with adaptive functions (Mukhta et al., 2016; Tangl, 1983)). This trend further confirms that addition of *B. pumilus* RI06-95 causes 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 also 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). A network analysis of the rearing water from Trial 3 suggested that the probiotic effect on rearing water, and likely the larvae, is focused on selected bacteria that are dispersed throughout the bacterial community. This analysis showed a direct relationship 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 (Costa Sobrinho et al., 2010). Previous research and sequencing of the genome of *B. pumilus* RI06-95 show that mechanisms of probiotic action can include direct competition with *V. coralliilyticus*, biofilm formation, and water quality improvement (Hamblin et al., 2015; Karim et al., 2013). Competition between *B. pumilus* RI06-95 and its directly associated bacteria (including *Vibrionales*) could open niches in the oyster microbiome for advantageous microbes, in addition to providing immunoprotective benefits for the larval oysters.

The bacterial community dynamics observed in this study indicate a variety of interactions between the oysters, *Vibrio*, 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). These opportunistic vibriosmay be outcompeted by pre-colonization of other bacteria, such as probiotics, in the water, leading to a decrease in *Vibrio* abundance in both the larvae and biofilms over time (Beaz-Hidalgo et al., 2010a; Zhao et al., 2016, 2018). The co-occurrence network provides insight for future probiotic development and assessment. Various members of the microbial assemblage should differentially impact host health. Understanding how probiotics affect the entire microbiota may help in optimizing their benefits and preventing undesirable side-effects (Kesarcodi-Watson et al., 2008). For example, based on the results here, it is interesting to speculate how the co-occurrence of the probiont with an *Oceanospirillales* symbiont may amplify protection for the oyster larvae. Moreover, it appears that probiotic treatment can diversify *Vibrios* in the system over time, potentially decreasing an infective dose of pathogenic species and/or increasing competition with non-pathogenic strains. Elucidating such interactions will require more targeted 16S rRNA and metagenomic analyses to track specific species or within *Vibrio* populations over time.

The use of two different 16S amplicons, different extraction methods based on trial or sample type, and differing sequencing methods may have created biases in this study. Direct taxonomic classification of the complete amplicon sequence was used to minimize these biases, but preference for certain bacteria likely occurred based on the specific amplicon and database completeness (Tremblay et al., 2015). Future investigations of microbial response to probiotics within oyster hatcheries should include metagenomics and metatranscriptomics to perform functional analysis and identify potential processes and mechanisms involved in probiotic activity.

The relationships observed between the *Bacillus*, *Oceanospirillales*, and *Vibrio* taxa provide a basis for how probionts affect microbial communities in an oyster hatchery. Based on previous literature and this study, we hypothesize that the *Bacillus* probiotic inhibits pathogenic *Vibrios*, which allows beneficial *Oceanospirillales* to become more abundant. The larval oysters promote this symbiosis and probiotic effect by actively selecting microbes from the rearing water and algal feed. Additional research is needed to examine the specific interactions between *Oceanospirillales* symbionts, the *Bacillus* probiotic, *Vibrio* pathogens, and the oyster host.

# Conclusion

This study investigated the effects of time and probiotic treatment on bacterial communities in an oyster hatchery. Our results show that there is a strong effect of time on the microbiomes within larvae, on tank wall biofilms and in the rearing water, and that probiotic treatment leads to subtle changes in certain bacterial taxa, including an increase in *Oceanospirillales* in the rearing water and changes in the *Vibrio* community. These results provide evidence for how probiotics may influence bacterial communities in an oyster hatchery over temporal and spatial scales, leading to an overall improvement in larval health.

# 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.

# Author Contributions

RJS, SS, DRN, DCR, AFP, and MGC contributed conception and design of the study; RS and SS collected and prepared the samples for sequencing; RS performed the sequence analysis and wrote the first draft of the manuscript; All authors contributed to manuscript revision, read and approved the submitted version.

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