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 the recruitment of microbes that are beneficial to larvae. 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 following spawning. Samples were analyzed by 16S rDNA 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 (Bray-Curtis k=2, 95% confidence). Probiotic treatment, however, led to a significantly higher proportion of *Oceanospirillales* spp. and *Bacillus* spp and a decrease in XXXX? in water and oyster larva. Co-occurrence network analysis suggests that probiotic treatments have a cascading effect on bacterial community structures, mediated through select taxa associated with the probiotic target species.

# Introduction

Diseases caused by bacterial pathogens cause losses in aquaculture and wild populations of commercially important shellfish and finfish (Groner et al., 2016; Lafferty et al., 2015). World aquaculture production is valued at $1.57 trillion USD, and disease is a primary limiting factor on its growth and economic worth (FAO, 2015; 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; 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).

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 against challenge with the pathogen *Vibrio coralliilyticus* (Karim et al., 2013; Sohn et al., 2016)*.* Additionally, administration of this probiotic in a hatchery setting resulted in dramatic 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 mechanisms by which probiotics exert their effects. There are also concerns about using probiotic bacteria to combat disease in aquaculture, as they will eventually disperse into the water and may thus affect bacterial diversity in nature (Newaj-Fyzul et al., 2014). Improper selection of probiotics may result in bacterial dysbiosis, which could ultimately impact the 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 in oysters serve as an indicator of health and function of the oyster 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, 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). 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 of rearing conditions themselves (Asmani et al., 2016; Powell et al., 2013; Trabal Fernández et al., 2014). Microbiome studies of juvenile Kumamoto oysters challenged (or spiked?) with *Streptomyces* 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 each of the rearing water, tank biofilms, and larvae microbiomes.

# Materials and Methods

## Bacterial Strain

The probiotic strain *Bacillus pumilus* RI06-95, previously isolated from a marine sponge from the Narrow River in Rhode Island (Karim et al., 2013; Socha, 2008), was cultured in yeast peptone with 3% salt (YP30) media (5 g L-1 of peptone, 1 g L-1 of yeast extract, 30 g L-1 of ocean salt (Red Sea Salt, Ohio, USA)) at 28 °C with shaking at 170 rpm. The bacterial cell concentration was estimated by a OD550 measurement using a spectrophotometer (Synergy HT, BioTek, USA) and confirmed using serial dilution and spot plating on YP30 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 William University (Bristol, RI, USA) following standard procedures (Helm et al., 2004). Spawning 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 room temperature (approximately 23 °C) and a salinity of 20psu. 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), and *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 clean UV-filtered and sterilized 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, Milxford, 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 liters 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).

## 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 swabs and oyster larvae were extracted using a 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 less <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, Maignien, et al. (2013), 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. Overall 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 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 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. The most dominant phyla in the water community were *Proteobacteria* (52.9% ± 6.3%), *Bacteroidetes* (25.7% ± 9.8%), *Cyanobacteria* (12.3% ± 9.8%), *Actinobacteria* (5.4% ± 5.2%), and *Planctomycetes* (1.8% ± 1.2%) (Figure 1, bottom right). The larval samples were dominated by *Proteobacteria* (87.3% ± 11.6%) and the swab samples by *Proteobacteria* (67.5% ±16.6%), *Cyanobacteria* (18.9% ± 15.9%), and *Bacteroidetes* (8.4% ± 3.8%) (Figure 1, bottom left). There is a significant enrichment in *Proteobacteria* in larval and swab samples, at the expense of *Bacteroidetes,* as compared to water samples.

Simpson’s Diversity Index indicated significantly higher diversity in rearing water samples from Trial 3 (0.878-0.924), than from Trials 1 (0.807-0.865) and 2 (0.795-0.908) (Figure 2), most probably due to the greater sequencing depth and different 16S variable region in Trial 3 (Figure S1). There was no significant difference between control and treated samples at each time-point and sample type (T-test, p>0.05). However, there were significant increases in bacterial diversity over time in the rearing water in Trials 2 and 3 (T-test, p<0.05), and the oyster larvae and biofilm swabs in Trial 1 (T-test, p<0.001). The bacterial community in rearing water was significantly more diverse than the community in oyster larvae and biofilm swab samples (T-test, p<0.05), reflecting an enrichment in specific community members from the more diverse rearing water community in larvae and tank surfaces (Figure 1).

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). 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.

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

control and probiotic-treated tanksshowed no significant differences in diversity and structure of bacterial communities overall , 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 (Figure 4a, T-test p<0.05). These results suggest that those reads correspond to the added probiotic. This increase in *Bacillus* spp. reads at the final timepoint is due to natural mortality in larvae over time, with a consistent daily dosage of the probiotic. The number of *Oceanospirillales* reads was also significantly higher at all time points (20-34% of reads) in probiotic-treated rearing water compared to control water in Trial 3 (Figure 4b, T-test p<0.05). SAY SOMETHING HERE ABOUT HOW THEY ARE PRETTY ABUNDANT (IN ALL SAMPLES OR JUST WATER?) The abundance of *Oceanospirillales* reads decreased by 41-62% over time in all Trials (Figure 4b, T-test p<0.05). MENTION HERE IF THE SAME PATTERN WAS OBSERVED IN OTHER SAMPLE TYPES (OR THAT NO SIGNIFICANCE DIFFERENCE BETWEEN TREATMENTS WAS SEEN – OR THAT YOU DIDN’T ANALYZE BECAUSE OF LACK OF ENOUGH READS TO SEE DIFFERENCE?)

Since *Vibrio* spp. 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 changes in *Vibrio* spp. diversity and abundance over time in the hatchery. Overall, no significant differences in abundance of vibrios between control and probiotic treated-tanks were detected for any of the sample types or trials. However, a significant effect of treatment was observed in vibrio diversity (as measured using the Simpson’s Index of diversity) in water samples collected on day 12 in Trial 1 (Figure 5a). This trend was also detected in water samples from Trials 2 and 3 (Figure S4).

The abundance of *Vibrio* spp. in oyster larvae, biofilm swabs, and rearing water samples significantly decreased over time in Trial 1 (Figure 5b, Figure S3, T-test, p<0.05). *Vibrio* spp. reads were significantly more abundant in the larvae, than in the biofilm swabs on Day 12 and all of the water samples. No significant differences in Simpson’s Index of Diversity was detected between treatments in the larvae or biofilm samples (Figure 5a). The diversity of the *Vibrio* spp. was higher in swab and oyster samples, and significantly increased from Day 5 to Day 12 (T-test, p<0.001).

Since the V6 region of the 16S rRNA gene was amplified in Trial 3, we were able to perform an oligotyping analysis - a method that detects genetic variants within a taxon - of *Vibrio* spp. reads on this data (only water samples were collected). Changes in the overall composition of the *Vibrio* community over time and by treatment were observed by oligotyping (Figure 6). On Day 5, the probiotic treated tanks were dominated by oligotypes closely related to *Vibrio alginolyticus* WW1 and *Halovibrio* sp. 5F5, and the control tanks were dominated by the oligotype *Vibrio alginolyticus* WW1. By Day 12, *Vibrio alginolyticus* WW1 is succeeded by *Vibrio celticus* 5OM18 in the probiotic treated tanks and *Vibrio orientalis* LK2HaP4 in the control 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 length); 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 significantly more abundant in the control samples than in treatment samples include *Oceanospirillales*, *Caulobacterales*, *Lentispherales*, *Acidithiobacillales*, *Chrococcales*, and Bacillales. These nodes scattered throughout the network and did not share direct edges, but are 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 change in abundance between control and treatment samples: *Chromatiales, Xanthomonadales, Cytophagia* Order II, and *Vibrionales*. This direct connection between *Bacillales* and *Vibrionales* indicates that these Orders of bacteria may have been directly associated. *Oceanospirillales* is located in the network 5 edges away from *Bacillales,* sharing an edge with the treatment-abundant *Flavobacteriales*, a common environmental bacteria taxa (Bernardet et al., 2015). This network indicates 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**

Manipulation of bacterial community structures in aquaculture systems is a potential mechanism for prevention of disease among the commercial crop grown in these systems. Moreover, study of these microbial communities informs potential actions such as probiotic treatment. This information can then be used to optimize disease management strategies. Our study established that bacterial community structure in an oyster hatchery differed between rearing water, oyster larvae, and tank biofilm swabs. Additionally, the microbiome changed significantly over time in the rearing water, specifically with an increase in *Actinobacteria* and a decrease in *Bacteroidetes*. The strong effect of time initially obscured the overall effects of treatment, trial, or sample type. *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 (Hernández-Zárate and Olmos-Soto, 2006; Trabal Fernández et al., 2014). The other phyla, however, showed variation in relative abundances based on sample type, day, and treatment, including *Bacteroidetes, Cyanobacteria,* and *Actinobacteria*.

Our results show variability within the replicate samples, especially among the bacterial communities of oyster larvae, consistent with past studies (King et al., 2012; Wegner et al., 2013) and with variability in larval performance in replicate tanks (Sohn et al. *in prep*). These differences within replicates may have been due to inevitable variance in husbandry and handling technique at the hatchery. Lower diversity indices in the larvae and tank biofilm swabs than the water indicates niche selection of larval and biofilm colonizers in the tank (Figure 2, Figure S2). It is likely that the oysters select their symbionts from a diverse pool of bacteria in the rearing water and microalgal feed. This hypothesis is consistent with outcomes of past studies that suggest interactions between microalgae, bacteria, and animals in aquaculture. Bacteria are an essential component of aquaculture nutrition, as food for both the microalgae and the larvae, since they can effectively filter bacteria directly from the rearing water (Kamiyama, 2004; Natrah et al., 2014; Nevejan et al., 2016). Studies have shown differential selection of microbes in Eastern oysters based on size, chemistry, and accompanying bacteria (Baldwin, 1995; Emmanuelle et al., 2009; Nevejan et al., 2016; Newell and Jordan, 1983). The consequences of this bacterial selection in the larvae is observed in the relative abundances and diversity of bacterial taxa and differs over time with the presence of the probiotic. More effort is needed to elucidate the mechanisms of differential bacterial selection in aquaculture in the presence of probiotics.

Despite a significant improvement in larval survival with the addition of probiotics (Sohn et al. *in prep*), there was no global effect on bacterial diversity or structure in any of the sample types, suggesting that the probiotic effect 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 microbiome. 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 microbiomes as a result of probiotic treatment (Geraylou et al., 2013; Gonçalves and Gallardo-Escárate, 2017).The presence of the probiotic was confirmed with higher total *Bacillus* spp. read counts in the probiotic-treated water and increased abundance throughout the Trials, likely due to natural mortality and therefore decreased grazing in the tanks.

Amplification of certain taxa in 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, and 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). The observed increase in *Oceanospirillales* abundance with probiotic treatment indicates that this group of bacteria may respond to the *Bacillus* as a symbiont or mechanism of protection for the larval oysters.

Previous research (Sohn et al. 2016) suggested that probiotic treatment in the hatchery potentially decreases levels of *Vibrio* spp. in the hatchery. Characterization studies have shown that our probiotic species, *Bacillus pumilus*, as well as other *Bacillus* spp, inhibit *in vitro* growth of *Vibrios* (Sohn et al., 2016; Vaseeharan and Ramasamy, 2003). This trend is also observed in the number of *Vibrio* reads in our 16S study but is not significant due to high variability and small sample sizes. Moreover, 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, which was conducted in January, when there is lower environmental *Vibrio* presence (Costa Sobrinho et al., 2010). Interestingly, our research indicates that probiotic treatment leads to increased diversity in water and swabs through time. Moreover, the analysis of single base pair changes in *Vibrio* species in the water samples from the high-resolution sequencing performed in Trial 3 revealed that, over time, the *Vibrio* community in the probiotic-treated rearing water transitioned from a predominance in potentially pathogenic species, similar to *Vibrio alginolyticus* WW1, a virulent pathogen originally isolated from amphioxus (Zou et al., 2016) and *Vibrio celticus* 5OM18, a virulent anaerobic clam pathogen (Beaz-Hidalgo et al., 2010b) to a predominance in non-pathogenic species similar to *Vibrio orientalis*, a species that is often associated with adaptive functions (Mukhta et al., 2016; Tangl, 1983) and *Vibrio celticus* 5OM18, a virulent anaerobic clam pathogen (Beaz-Hidalgo et al., 2010b). This trend further confirms that the addition of the *Bacillus* probiotic causes subtle changes in certain taxa in the hatchery system, especially the *Vibrio* taxon.

This interpretation is also consistent with the 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 Trial 3 suggested that the probiotic effect on the 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 *Bacillus pumilus* RI06-95 show that mechanisms of probiotic action include direct competition with *Vibrio coralliilyticus*, biofilm formation, and water quality improvement (Hamblin et al., 2015; Karim et al., 2013). Competition between the *Bacillus* probiotic 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 co-occurrence network provides insight for future probiotic development, co-culturing of probiotics, and how a candidate probiotic is assessed. Perhaps probiotics need to be evaluated by their effect on the system as a whole to quantify and optimize their effect (Kesarcodi-Watson et al., 2008). For example, analyzing the microbiome during *in vivo* assays and challenges would ensure that the entire bacterial community is present and considered. Co-culturing *Bacillus* with an *Oceanospirillales* symbiont may result in amplified protection for the oysters. The bacterial community dynamics observed in this study indicate a variety of interactions between the oysters, *Vibrio*, and the *Bacillus* probiotic. First, the larvae are concentrating certain *Vibrio* bacteria in high abundances, regardless of probiotic treatment, as known molluscan symbionts (Romalde et al., 2014). These opportunistic *Vibrio* species may be outcompeted by other symbiotic bacteria in the water over time, leading to a decrease in abundance in both the larvae and biofilms (Beaz-Hidalgo et al., 2010a). Lastly, although the growth of *Vibrios* in the water decreases with probiotic treatment, this may result in the diversification of *Vibrios* over time. Unfortunately, this 16S rDNA study is limited in its ability to determine the species or pathogenicity of the *Vibrio* populations in each sample.

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.

Inconsistent 16S amplicons, extraction methods based on trial or sample type, and differing sequencing methods were used within this study, creating potential biases. Direct taxonomic classification was used to minimize these biases, but preference for certain bacteria likely occurred based on the method used and database completeness (Tremblay et al., 2015). Future investigations of the oyster hatchery’s microbial response to probiotics should include metagenomics and metatranscriptomics to perform functional analysis and identify processes involved in probiotic activity.

# Conclusion

This study investigated the effects of sample type, time, and probiotics on bacterial communities in an oyster hatchery. Our results show that there is a strong effect of time and sample type on the overall microbiome, and that the effect of probiotic leads to subtle changes in the community focused on certain taxa, including an increase in *Oceanospirillales* in the rearing water and changes in the *Vibrio* community. These results provide a basis for how probiotics may interact with bacterial communities in an oyster hatchery over temporal and spatial scales.

# 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

DN, DR, RS, SS, 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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