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

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**ABSTRACT – 350 words max**

Larval oysters in hatcheries are susceptible to diseases caused by marine bacterial pathogens, including *Vibrio* spp*.* The daily addition of probiotic *Bacillus pumilus* RI06-95 to water in rearing tanks leads to increased larval survival to challenge with *Vibrio coralliilyticus*. We propose that the presence of probiotics may change how the larvae respond to pathogens, regulate their environment, and recruit beneficial microbes. During three separate trials spanning the 2012-2015 growing seasons, larvae, tank biofilm, and rearing water samples were collected from control and probiotic-treated tanks in an oyster hatchery at timepoints between Day 0 (spawning) and Day 12. DNA was extracted from 0.22 μm water filters, larvae, and biofilm swabs, and then prepared for sequencing. All samples were analyzed using 16S rDNA sequencing of the V4 or V6 regions and direct taxonomic classification, in order to determine the microbial community. There were significant differences in microbial composition over time and between sample sources, but no major effect of probiotics on the overall rearing water bacterial communities (Bray-Curtis k=2, 95% confidence). However, probiotics increased niche selection of certain bacteria from the water in the oyster larvae, and a significantly higher proportion of *Oceanospirillales* spp. and *Bacillus* spp. was detected in the probiotic-treated rearing water. Co-occurrence network analysis suggests that the overall probiotic effect on the rearing water is mediated through select associated taxa. This research reveals interactions between probiont and microbial communities in oyster hatcheries, and how new probiotic formulations may be designed.

**INTRODUCTION – 2 pages**

Marine diseases caused by bacterial pathogens cause losses in aquaculture and wild populations of all commercial species, including shellfish and finfish (Groner et al., 2016; Lafferty et al., 2015). World aquaculture production alone 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, primarily by etiological agents from the genus *Vibrio* (Beaz-Hidalgo et al., 2010a; Dubert et al., 2017; Le Roux et al., 2016; Richards et al., 2015). These pathogenic *Vibrios* naturally found in the environment may lead to larval disease and contribute to a production bottleneck at the hatchery. In an effort to minimize disease in aquaculture, a healthy environment must be maintained, which includes optimum water quality, culture density, and filtration systems (Mckindsey et al., 2007).

An alternative method for the management of disease in aquaculture involves the use of probiotics, which are non-pathogenic microorganisms that are beneficial or harmless (Karim et al., 2013; Prado et al., 2010). Probiotics may increase the protection of larvae through a variety of broad mechanisms, including pathogen inhibition, nutrient competition, antimicrobial activity, and improvement of water quality (Gatesoupe, 1999; Kesarcodi-Watson et al., 2008, 2012). Previous studies have shown that the candidate probiotic bacteria used in this study, *Bacillus pumilus* RI06-95, significantly increases the survival of larval oysters in an *in vivo* challenge assay against the pathogen *Vibrio coralliilyticus* (Karim et al., 2013). Additionally, administration of this probiotic in a hatchery setting resulted in dramatic reductions in total *Vibrio* counts, compared to the control tanks (Sohn et al., 2016).

However, there is a lack of knowledge about the particular mechanisms of action involved in probiotic effects. There are also concerns about using probiotic bacteria to combat disease in aquaculture, as they will eventually disperse into the water and may later contribute to microbial diversity (Newaj-Fyzul et al., 2014). In especially poorly selected probiotics, microbial dysbiosis may occur and could ultimately impact the host health (Verschuere et al., 2000). As filter feeders, bivalves are especially susceptible to changes in microbial communities, since they can ingest many different microorganisms by filtering to gain nourishment from the water (Burge et al., 2016). Therefore, it is important to study the effects of probiotics not only on the health and protection of the host, but also the environment and microbial community as a whole.

Previous studies evaluating the microbiome of 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., 2016; Lokmer and Mathias Wegner, 2015; Pierce et al., 2016). Additionally, the oyster microbiome is distinct from the water and often dominated by *Proteobacteria*, *Cyanobacteria*, and *Firmicutes*. Three studies have evaluated the microbial communities in *Magallana/Crassostrea gigas* larviculture and found that the water microbiome changes throughout the year, there is little effect of rearing conditions, and the overall community is highly diverse and variable (Asmani et al., 2016; Powell et al., 2013; Trabal Fernández et al., 2014). Microbial analysis of juvenile oysters treated with *Streptomyces* showed an increase in species diversity and changes in the relative abundances of taxa, compared to the control (García Bernal et al., 2017). Bacteria in oysters serves as an indicator of health and function of the community (Le Roux et al., 2016) and may be a potential cause of variability between tanks. However, a description of the microbial communities present in a larval oyster hatchery and their potential mechanisms of response to probiotics has not been determined.

In this study, the structure and diversity of microbial communities in larval oysters, rearing water, and tank biofilms were analyzed in response to time and probiotic treatment. We hypothesized that the overall microbial community will change over time and when altered by the probiotic, may serve as a mechanism of protection for the oyster larvae. The changes in microbial communities observed with 16S rDNA amplicon sequencing of samples from three probiotic trials provide a foundation for aquaculture microbiome research and hypotheses for probiotic-pathogen interactions.

**MATERIALS and METHODS**

**Bacterial Strain**

The probiotic strain *Bacillus pumilus* RI06-95 was previously isolated from a marine sponge from the Narrow River in Rhode Island (Socha, 2008) and selected as a candidate probiotic by showing protection of eastern oyster (*C. virginica*) larvae against bacterial challenge *Vibrio coralliilyticus/tubiashii* RE22(Karim et al., 2013). The RI06-95 strain was cultured in yeast peptone with 3% NaCl (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). Additionally, serial dilution and spot plating on YP30 agar plates were used to determine the colony forming units (CFU) of actual bacterial suspension.

**Experimental Design and Sample Collection**

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). The day the larvae were spawned is defined as Day 0. Larvae (1 day old) were distributed and maintained in triplicate 120 L conical tanks per treatment. Tanks were randomly assigned to treatments including no probiotics (control) and candidate probiotic RI06-95. Cultured candidate probiotic RI06-95 at the dosage of 104 CFU/mL were administered to non-control tanks every day after being mixed with an algal feed. The microalgae strains used throughout the trial were *Chaetoceros muelleri* (CCMP1316), *Isochrysis galbana* (CCMP1323), *Tisochrysis lutea/Isochrysis* sp T-ISO (CCMP1324), *Pavlova pinguis* (CCMP609), *Pavlova lutheri* (CCMP1325), *Tetraselmis* sp. (CCMP892), and *Thalassiosira weisflogii* (CCMP1336). Tanks were drained every other day.

Rearing water (1 – 2 L) was collected from each of the triplicate tanks during drain-down and filtered through a 0.22 μm Sterivex filter (Millipore, Millford, MA, USA). The Sterivex filters were immediately stored frozen at -20 °C until DNA extraction. Biofilm swab samples were collected from inside of each tank surface by swabbing with sterile swabs (approximately 144 cm in length) and stored in RNAlater. Oyster larvae were passed through on a 55 μm sieve for drain-down water changes at the hatchery, 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 passed through a 40 μm nylon membrane and rinsed with filtered sterile seawater (FSSW) to reduce residual environmental bacteria. Swab and larvae samples were immediately flash frozen in liquid nitrogen and stored at -80 °C until DNA extraction. All sample types were collected from Trials 1 and 2, but only water samples were collected from Trial 3. Table 1 includes a summary of Trials, including collection dates and sample types.

**DNA Extraction, Amplification, and Sequencing**

Total microbial DNA from water samples was extracted from the Sterivex 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 microbial DNA from the swabs and oyster larvae were extracted using a PowerSoil DNA Isolation Kit (MoBio Laboratories, USA) with the following modifications. In brief, pooled oyster larvae were ground in a mortar with a sterile pestle and then placed into bead tubes for extraction. The cotton tops of the swabs were cut off 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, all DNA concentrations were 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). 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 overlapping reads.

**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 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 was separately normalized by maximum of total reads per 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 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 a variety of 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 microbial 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. T-test or ANOVA to confirm? What is appropriate?

A co-occurrence network was generated with normalized taxa counts at the Order level from water samples collected on the final timepoint in each trial (Trial 1: Day 12, Trial 2: Day 9, Trial 3: Day 12), to hypothesize relationships due to 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 104 taxa (nodes) and 440 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 2881166), and discrete shape and continuous color according to whether the taxa are more abundant in the control or probiotic-treated samples.

**RESULTS**

**Probiotics Protect Larvae Against *Vibrio* Challenge**

Previously published data shows…? Figure S1

Refer to (Sohn et al., 2016) (Modak et al., *in prep?*)

**Microbial Community Structure and Diversity Over Time**

A total of 19,201,424 quality controlled 16S rDNA gene amplicon sequences were analyzed from 42 rearing water, 31 tank biofilm swab, and 21 pooled larvae samples from three hatchery Trials. There was an average of 204,271 reads (961-1,117,380) from each of the 94 samples, which highly depended on the sequencing method and sample type (Figure 1, top). Direct taxonomical classification resulted in the detection of 168 orders in all samples. The most dominant phyla in the water community were *Proteobacteria* (52.89%, STD ± 6.32%), *Bacteroidetes* (25.72%, STD ± 9.84%), *Cyanobacteria* (12.25%, STD ± 9.80%), *Actinobacteria* (5.40%, STD ± 5.15%), and *Planctomycetes* (1.75%, STD ± 1.24%) (Figure 1, bottom). The larvae samples were dominated by *Proteobacteria* (87.33%, STD ±11.60%) and the swab samples by *Proteobacteria* (66.97%, STD ±14.07%), *Cyanobacteria* (19.73%, STD ±14.44%),), and *Bacteroidetes* (8.28%, STD ±3.59%). Simpson’s Diversity Index shows overall higher diversity in rearing water samples in Trial 3 (0.878-0.924), than Trials 1 (0.807-0.865) and 2 (0.795-0.908), likely due to differences in sequencing depth (Figure 2). There was no difference in overall alpha diversity over time in the rearing water, and the 3 replicate tanks per timepoint and treatment show high variability. The Simpson’s Diversity Index values for the oyster larvae and biofilm swab samples from Trials 1 and 2 do not show any significant trends, and there is very high variability within replicates.

The microbiomes of the water and oyster larvae samples were significantly different (Bray-Curtis, k=2, 95% confidence) in both Trial 1 and Trial 2 (Figure 3a). The swab biofilm microbiome was not significantly different from either the water or oyster larvae samples. The most abundant taxa in the oyster larvae and swab are similar to that of the water, with high variance in the larvae samples (Figure S3). Microbial 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 time has a major influence in the microbiomes in an oyster hatchery, despite the consistency in the inflow seawater microbial community.

**Effects of the Probiotic on the Microbial Community**

We were able to detect the administered *Bacillus* probiotic in the 16S rRNA microbial communities in all probiotic-treated samples. On the final sampling day, there was a higher abundance of *Bacillus* spp. reads in the probiotic-treated water samples (Trial 1: 16-112, Trial 2: 6-35, Trial 3: 504-2137) than in the control samples (Trial 1: 0-5, Trial 2: 0-1, Trial 3: 4-9) in all three Trials (Figure 4a). Additionally, the number of Bacillus spp. reads increased over time in the probiotic-treated tanks in Trial 3 (Day 5: 91-155 reads, Day 8: 244-258 reads, Day 12: 504-2137 reads). The number of *Oceanospirillales* reads was consistently more abundant in probiotic-treated rearing water compared to control water, and the abundance decreased over time in all Trials (Figure 4b). Although there were changes in certain taxa, the overall bacterial communities were not significantly different with probiotic treatment (Figure 3c).

**Changes in Vibrio spp. as a Result of Probiotics**

As a significant group of larval oyster pathogens, the changes in *Vibrio* spp. reads were evaluated. In Trial 1, the number of *Vibrio* spp. reads in the oyster larvae samples decreased over time in all tanks (Day 5: 8658-90954, Day 12: 101-266) and the diversity was not significantly different due to time or treatment (Figure 5, Figure S4). There were few *Vibrio* spp. reads detected in the biofilm swabs on Day 12, or any of the water samples. However, the diversity of the *Vibrio* spp. in the swab samples was the highest overall, and was higher on Day 12 (0.58-0.65) than Day 5 (0.51-0.53). *Vibrio* spp. diversity in the rearing water samples increased over time, and increased more in the treated than the control samples. This trend is further confirmed in the water samples from Trials 2 and 3 (Figure S5). Add in comparison with *Vibrio* spot counts? In Trial 3, oligotyping of *Vibrio* spp. in the water samples shows changes in the overall composition of the *Vibrio* community by time and treatment (Figure 6). On Day 5, the probiotic treated tanks are dominated by *Vibrio alginolyticus* WW1 and *Halovibrio* sp. 5F5, and the control tanks are dominated by *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.

**Microbial Relationships via Co-Occurrence Analysis**

Co-occurrence analysis of the 24 water samples from the final timepoint in each trial shows how each Order is changing relative to each other (edges), which Orders are most abundant in the system (node size), and how probiotic treatment affects their relative abundances (node color and shape) (Figure 7). The most abundant taxa (*Rhodobacterales, Micrococcales, Sphignobacteriales, Alteromonadales, Myxococcales*, and *Oceanospirillales*) are changing in a similar way, but have different occurrence ratios between control and treatment samples. The group of Orders more abundant in the control samples clusters together, including *Orbales, Halanaerobiales, Thermotogales,* and *Prochlorales*. Similarly, *Gaiellales, Spartobacteria*, and *Acidobacterales* are relatively more abundant in the treated samples and cluster together. *Bacillales*, our probiotic’s Order, is most abundant in the treated samples, and shares edges with Orders *Anaerolineales, Parvularculales, Oscillatoriales, Cytophagia;Order II, Chromatiales, Thiotrichales,* and *Xanthomonadales*. These taxa have comparable abundances in both treated and control samples, and occur in relatively low abundances similar to *Bacillales*. This indicates that the probiont does not directly alter the overall microbial community in the rearing water in an oyster hatchery.

**DISCUSSION – 4 pages**

Bacterial dynamics in aquaculture, and specifically an oyster hatchery, are a potential mechanism of disease protection, variability between tanks, and probiotic enrichment. Our study found that microbial community structure and diversity is different between rearing water, oyster larvae, and tank biofilm swabs. Additionally, the microbiome changes significantly over time in the rearing water, specifically with an increase in *Actinobacteria* and a decrease in *Bacteriodetes*. The effect of time has the strongest influence on the microbial community, and obscures the effects of treatment, trial, or sample type. *Proteobacteria* was highly abundant in all samples (up to 87% in larvae), consistent with previous studies where it is the largest and most diverse phylum in oyster-associated microbiota (Hernández-Zárate and Olmos-Soto, 2006; Trabal Fernández et al., 2014). The second-most abundant phylum in the rearing water, *Bacteroidetes*, are highly abundant in marine environments (Thomas et al., 2011).

Our results show variability within the replicate samples, especially the oyster larvae bacterial communities, consistent with past studies (King et al., 2012; Wegner et al., 2013). These differences within replicates may have been due to varying abundances of larvae, mortality, or differences in husbandry technique. Lower diversity indices in the larvae and tank biofilm swabs than the water indicates niche selection of larval and biofilm colonizers in the tank. The oysters are actively choosing their symbionts from a diverse pool of bacteria in the rearing water and algal feed.

Despite a dramatic improvement in larval survival with the addition of the probiotic, there was no global effect on microbial diversity or structure in any of the sample types. Previous studies of the impact of probiotics on microbiota in humans and fish show subtle changes of certain taxa, but no consistent effect on the diversity of the host’s microbial community (Laursen et al., 2017; Merrifield and Carnevali, 2014; Schmidt et al., 2017; Standen et al., 2015). The presence of the probiotic was confirmed with total *Bacillus* counts higher in the probiotic-treated water and increased abundance throughout the Trials, likely due to natural mortality 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 is consistently more abundant in probiotic-treated rearing water, and decreases 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). However, they are best known 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 abundance of *Oceanospirillales* 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. Additional research is needed to examine the specific interactions between *Oceanospirillales* symbionts, the *Bacillus* probiotic, and the oyster host. Probiotics also alter the niche selection of certain bacteria from the water, in the oyster larvae, but not consistently.

Changes in *Vibrio* reads in Trial 2 were not observed since the hatchery trial was conducted in January, when there is lower environmental *Vibrio* presence (Sobrinho et al., 2010). Characterization studies have shown that our probiotic species, *Bacillus subtilus*, reduces *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 small sample sizes. Overall, *Vibrios* are much more abundant in the larval oyster and biofilm swab samples in Trial 1, and the total number decreases over time. The diversity of *Vibrios* in the larval samples general decreases over time, but the swab *Vibrio* diversity increases. There are few *Vibrios* in the water samples, but with increasing diversity over time and increased diversity with probiotic treatment. This trend is also observed in Trial 3.

These dynamics may indicate a variety of interactions between the oysters, *Vibrio*, and the *Bacillus* probiotic. First, that 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 by its ability to determine the species or pathogenicity of the *Vibrio* populations in each sample.

The analysis of single base pair changes in *Vibrio* species in the water samples from the high-resolution sequencing performed in Trial 3 reveals succession of species that changes with probiotic treatment. Three major oligotypes were determined to be the following species: *Vibrio alginolyticus* WW1, a virulent pathogen originally isolated from amphioxus (Zou et al., 2016); *Vibrio celticus* 5OM18, a virulent anaerobic clam pathogen (Beaz-Hidalgo et al., 2010b); and *Vibrio orientalis*, a species that is often associated with adaptive functions (Mukhta et al., 2016; Tangl, 1983). The dominant oligotype in the control tanks evolved from *Vibrio alginolyticus* WW1 to *Vibrio orientalis* between Day 5 and Day 12. However, the major oligotype in the probiotic treated samples transitioned from *Vibrio alginolyticus* WW1 on Day 5 to *Vibrio orientalis* on Day 12.

Over time, the *Vibrio* community in the probiotic-treated rearing water transitioned from potentially pathogen-dominated to beneficial symbiont-dominated, while the control water remained dominated by pathogens. This trend further confirms that the addition of the *Bacillus* probiotic causes subtle changes in certain taxa in the hatchery system, including the *Vibrio* taxon. Although this data cannot confirm the activity of these bacteria in the rearing water, it provides a hypothesis for the mechanism of protection and changes in the microbial community by *Bacillus pumilus*.

Co-occurrence networks can help identify associations, patterns, roles, and inform hypotheses from 16S abundance data (Barberán et al., 2012). A network analysis of the rearing water from the final timepoint in all three Trials analysis suggests that the probiotic effect on the rearing water, and likely the larvae, is mediated through select associated bacteria. Analyses of samples from Trials 1 and 3 (July 2012 and June 2016) show a direct relationship between *Bacillales* with *Vibrionales*, when *Vibrionales* is more abundant in the environment and oysters, particularly with probiotic treatment (Sobrinho et al., 2010). However, there is no overall connection in *Bacillales* with *Vibrionales* since Trial 2 was conducted in January 2013, when there is a low abundance of *Vibrio* species in the environment.

*Bacillales* is directly associated with seven Orders: four *Proteobacteria* that are more abundant in control samples and three bacteria from various Orders that are more abundant in treated samples. *Oceanospirillales* is 5 edges away from *Bacillales* and shares an edge with the treatment-abundant *Flavobacteriales*, a common environmental bacteria taxa (Bernardet et al., 2015). 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).

Low temporal resolution and sample size in Trials 1 and 2 may have concealed additional trends that were only detected in Trial 3. In addition, standardized sampling timepoints across the three trials would have helped to clarify the chronological changes observed. A factor of the oyster hatchery that was not considered in this study is the algal feed and its associated bacteria. This live feed added to the tanks twice daily is an additional source of bacteria, pathogens, and nutrients that may have a significant impact on the hatchery microbial community (Borowitzka, 1997). Additional research should be conducted to elucidate the additional impact of the algae on the oyster hatchery microbiome.

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 reduce bias from dead bacteria in the system.

**CONCLUSION – 1 paragraph?**

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, but the effect of probiotic is limited to niche selection by certain taxa. Probiotics amplify *Oceanospirillales* in the rearing water, lead to the succession of species in the *Vibrio* community, and act through associated bacteria in the community. These results provide a basis for how probiotics may interact with microbial communities in an oyster hatchery over temporal and spatial scales.

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