ENVIRONMENTAL EFFECTS ON THE MICROBIAL COMMUNITY OF HOMARUS AMERICANUS AFFLICTED WITH EPIZOOTIC SHELL DISEASE

by

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LIST OF ABBREVIATIONS

Epizootic Shell Disease..... ESD
Principal Coordinate Analysis ..... PCoA
Linear discriminant analysis Effect Size ..... LEfSe
Linear Discriminate Analysis ..... LDA
Length-heterogeneity Polymerase Chain reaction ..... LH-PCR
Multi-Tag Pyrosequencing ..... MTPS
Quantitative Insights into Microbial Ecology..... QIIME
Operational Taxonomic Units..... OTU
Kruskal-Wallis ..... KW

ABSTRACT

ENVIRONMENTAL EFFECTS ON THE MICROBIAL COMMUNITY OF HOMARUS AMERICANUS AFFLICTED WITH EPIZOOTIC SHELL DISEASE

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The American Lobster, *Homarus americanus*, is a commonly harvested shellfish on the North-East coast of the United States whose populations are currently threatened by epizootic shell disease (ESD). The main sign of the disease is the appearance of lesions on the lobster's shell and has a high mortality rate.

The exact etiology of ESD has not been fully established, but one proposed infectious agent is the bacterium *Aquimarina homaria*. This chitinoclastic bacterium is found in high abundance in lesions of diseased lobsters. A study done in the Buzzards Bay region also revealed a higher prevalence of the disease in contrast to the Outer Cape Cod region. The Buzzards Bay region has a higher average temperature than the surrounding region suggesting that temperature may play a possible role in *Homarus americanus'* succeptibility to ESD.

The current study evaluates bacterial dysbiosis, or imbalance of bacterial population, as a possible contributor of ESD by examining bacterial populations on both

healthy and diseased lobsters kept at various temperatures for two months. Polymerase Chain reaction (PCR) was conducted on lobster shell samples to amplify the 16S rRNA sequences from bacterial communities. The sequence data were then analyzed using Quantitative Insights into Microbial Ecology (QIIME) software, the Ribosomal Database Bayesian Classifier (RDP11), and Linear discriminant analysis Effect Size (LEfSe). We report a difference in the compositions of bacterial populations between healthy and diseased lobster shells as well as the lesion. In addition, we show that temperature of the environment influences bacterial populations in both healthy and diseased lobsters.

INTRODUCTION

The American Lobster, *Homarus americanus*, inhabits the Atlantic coast of North America from Newfoundland to North Carolina (FAO Fisheries & Aquaculture - Species Fact Sheets - Homarus americanus). Due to its prevalence, it has become an iconic species and one of the most important commercial shellfish species in the world. In 2014 alone, 66,801.9 metric tons of lobster were harvested, representing a $500 million industry.

However, this lucrative industry is currently in decline due to a disease known as epizootic shell disease (ESD). Caused by bacteria that degrade the lobster's carapace, or exoskeleton, ESD creates mild-to-deep lesions that can protrude into the endocuticle and discolor the lobster's meat (Smolowitz, Chistoserdov, & Hsu, 2005). These lesions and associated discoloration makes the once profitable lobsters unmarketable. Since 1996, the disease has spread to Maine. About 30 percent of all the lobsters harvested off the coast of South New England have been infected, costing the market millions of dollars (Castro, Factor, Angell, & Landers, 2006). Therefore, lobster fishermen are beginning to back out of the industry and significant decrease in landings has been reported. Fishermen and scientists are growing increasingly concerned that the disease will continue to spread rapidly, thwarting the recovery of the region's lobster stock and destroying the overall success of the industry.

Lobsters molt, or shed their shell, as they grow. This allows them to shed off the bacteria and lesions caused by mild cases of ESD. However, if the infection spreads quickly, it can prevent the lobster from molting and ultimately kill the animal. The mortality rate is particularly high for female lobsters because egg-bearing females do not molt. Therefore, the disease can spread unchecked throughout a gravid female's body and lead to death. Sometimes the female's body successfully fights against the disease with an early molt. However, this can cause female lobsters to lose their eggs, thus, contributing to the decline in the number of larvae in the population. (Laufer, Demir, & Biggers, 2005).

Microbial Species Possibly Connected to ESD

Despite the amount of research efforts made since its documentation in 1996, the etiology of ESD has yet to be fully defined. Recent studies have associated the disease with a change in the microbial community on the afflicted lobster's shell. Diseased lobsters have 2 to 4 orders of magnitude more bacteria on their ESD-infected lesions than on their healthy, ESD-free regions (Chistoserdov, Smolowitz, Mirasol, & Hsu, 2005). However, these bacteria are not the common pathogens typically associated other lobster or crustacean shell diseases. Chitonoclastic bacteria, common shell-eating microbes, were only a fraction of the bacteria found in diseased lobsters inhabiting Buzzard Bay, Massachusetts (Chistoserdov, Smolowitz, Mirasol, & Hsu, 2005). Instead, *Aquimarina homaria* and *Thalassobius* sp. have been identified as the dominant microbes associated with ESD (Chistoserdov, Quinn, Gubbala, & Smolowitz, 2012). Aquimarina species have

also been found to be present on both healthy and diseased lobsters but are marginally more abundant in diseased animals (Meres et al., 2012). Other bacteria associated with lesions include Flavobacteriaceae such as *Tenacibaculum*, *Polaribacter*, *Maribacter*, and *Cellulophaga* (Chistoserdov, Quinn, Gubbala, & Smolowitz, 2012).

To discern the direct effect of these unique bacterial species on lobster shells, Quinn et al. applied *Aquimarina homaria*, *Thalassobius* sp.I31.1, and *Pseudoalteromonas gracilis* to healthy carapaces of juvenile lobster (2012). They found that both *A. homaria* and *Thalassobius* sp. are capable of colonizing and developing lesions in abraded carapaces.

The Effect of Temperature on Bacterial Communities of ESD Lesions

Environmental factors have been examined as a potential cause for the change in microbial communities of infected lobsters. One study found that the incidence of ESD was higher in the Buzzards Bay region of Massachusetts compared to the Gulf of Maine and the Outer Cape Cod region (Glenn & Pugh, 2006). This could potentially be the result of different temperature as Buzzards Bay was reported to have a higher average water temperature than the other regions during the time of the study. This difference in temperature could potentially contribute to the proliferation of ESD bacteria and determine their growth patterns.

Bacteria in the Lobster's Hemolymph

Unlike vertebrae species, crustaceans do not have a sterile hemolyph. Their hemolymph is inhabited by various bacteria that are kept in check by antimicrobial peptides and lectins (Wang & Wang, 2015). For example, healthy crab hemolymph has been found to contain live *Vibrio* sp. in varying amounts depending on environmental conditions such as temperature (Welsh & Sizemore, 1985). Analysis of the hemolymph by other studies also showed that the bacterial groups from lesion sites in *Homarus americanus* were different than non-diseased lobsters (Quinn, Smolowitz, & Chistoserdov, 2013). Instead of various Bacteroidetes, the hemolymphs of diseased lobsters were dominated by Pelomonas aquatica, and a Novosphingobium sp., while Sediminibacterium sp. appeared only in the lesion and not in the healthy cuticle (Quinn, Smolowitz, & Chistoserdov, 2013). This could potentially mean that ESD could be associated with the dysbiosis of the lobster's hemolymph, rather than just physical lesions of the lobster's cuticle.

Objectives of the Study

In this study, we sought to determine the correlation between epizoodic shell disease and bacterial dysbiosis in *Homarus americanus*. We catalogued the specific taxa of bacteria associated with ESD by comparing the shells of diseased lobsters to that of their healthy counterparts. Then, the study determined the abiotic and biotic factors that led to a change in these microbial communities. In particular, we have attempted to understand the relationship between ESD and warmer temperatures. Finally, we also

studied the correlation between the microbial communities inhabiting the hemolymph and the development of ESD.

If ESD develops in association with specific bacterial communities, then we should be able to differentiate the distribution of bacterial groups that appear between healthy and diseased samples. Furthermore, if dysbiosis is related to higher temperatures, and bacterial dysbiosis is a factor of the disease, bacterial community populations of healthy and diseased lobster shells should differ significantly across a range of temperatures. Finally, if ESD is systemic, rather than lesion-limited disease, than bacterial communities in other compartments of the lobster's body, such as the hemolymph, should also be altered in the composition of their bacterial groups when comparing healthy lobsters to a diseased ones.

If the bacterial compositions remain similar across any of the tested environmental conditions, either dysbiosis is not correlated with ESD or the chosen environmental factors may not be related to ESD, or altering the bacterial environment.

METHODS

The study included healthy and diseased lobsters taken from Massachusetts in the area west of Vineyard Sound. After collection, the lobsters were shipped in Styrofoam coolers with wet newspapers and ice packs to the Virginia Institute of Marine Sceince (VIMS). After arrival at VIMS, lobsters were kept in individual 10-gallon aquaria with plastic lids, activated Carbon Whisper filter, aeration, and coarse coral substrate in artificial sea water (marine mix) at 33 psu. To simulate different temperature environments, all lobsters were kept in a 6°C cold room in individual tanks. Some tanks were then kept in large water baths of either 12° or 18°C to provide higher temperatures. All animals were fed *Leiostomus xanthurus* and squid twice a week with leftovers removed 24 hours after feeding.

After being exposed to the above water temperatures regime for ten days, the lobsters' shells and hemolymph were sampled. Scrapings were taken from each healthy lobster using a sterilized razor blade or a #10 scalpel and then stored in 95% ethanol. Diseased lobsters were scraped twice – once from their ESD-infected area and another from a seemingly healthy region of the same animal. To collect the hemolymph, lobsters were first swabbed with 95% ethanol at the juncture of the basis and ischium of the 5^{th} pereopod. A 27-gauge needle was then used to draw hemolymph samples into a 1-ml tuberculin syringe. Samples were stored in 95% ethanol until NextGen sequencing was

performed. Each of these steps was conducted once at the start of the experiment and again three months later for a second sampling.

Next Gen-Sequencing

Bacteria from each sample were extracted using MP Biomedicals Fast DNA Spin Kit for Soil to obtain purified DNA samples and then analyzed by high throughput Multitag NextGen sequencing (Gillevet, Sikaroodi, Keshavarzian, & Mutlu, 2010). Extracted DNA products were subjected to Length-heterogeneity Polymerase Chain reaction (LH-PCR), which amplifies variable regions V1 and V2 with 27F and 355R bacterial primers and separates the product using a ABI 3130 XL fluorescent sequencer to check the quality and reproducibility of the amplification (Suzuki et al. 1998). Multi-tag sequencing (MTS) was then used to analyze the samples by creating fusion primers with 16S rRNA primers, a 7-base barcode, and emulsion PCR adaptors (Gillevet, Sikaroodi, Keshavarzian, & Mutlu, 2010). The DNA was then amplified with the primers, run through emulsion PCR, and sequenced on an Ion Torrent PGM.

Data Analysis

The python package, Quantitative Insights into Microbial Ecology (QIIME, 2010), was used to analyze the sequences and compare the microbial community (Caporaso et al.). QIIME filtered the sequences, picked operational taxonomic units (OTU) from the sequences, assigned taxonomies to the sequences using the RDP11 database, and created phylogenies for the OTUs. QIIME was used to generated an OTU table that was then used to create a phylogenetic tree to investigate a beta diversity plot using the UniFrac metric (Lozupone & Knight, 2005). Linear discriminant analysis effect

size (LEfSe) was used with the OTUs assigned with taxonomic data from QIIME to characterize the significant differences between classes (Lozupone, Lladser, Knights, Stombaugh, & Knight, 2011). LEfSe analysis provided comparisons to the relative bacterial populations between distinct classes or categories. For the study, the classes consisted of scrapings from healthy lobster shells (HEALTHY), scrapings of healthy areas on diseased lobsters (HEALTHY on DISEASED), and scrapings from diseased areas on diseased lobsters (DISEASED). Scrapings were collected from animals held at different temperatures. In addition hemolymph samples were taken from lobsters classified as healthy or diseased. Lastly, the Kruskal-Wallis (KW) rank test was used to calculate significant differences between relative bacterial abundance with respect to classifications.

RESULTS

Baseline Analysis of Microbial Communities on Shells of HEALTHY Lobsters and Lobsters affected by Epizootic Shell Disease

Samples from *Homarus americanus* collected from the first time point and held at 12ºC were compared by health status. Bacterial communities on the shells of healthy lobster shells, on healthy shells from diseased lobsters and on diseased shell were compared using PCoA to determine clustering between individual samples.

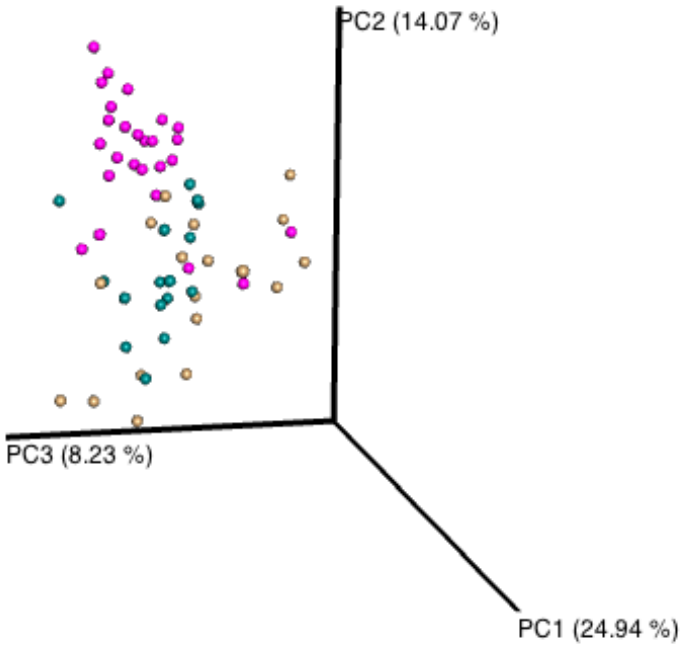

Figure 1. PCoA of healthy, lesion, and seemingly healthy samples on diseased lobsters. Purple= Diseased Samples, White = Healthy samples, Green = Healthy samples on diseased lobsters. Each axis represents an eigenvector representing the variance of the data.

Figure 1 shows that there was a variation among different microbiomes of healthy lobster shells. Biomes in healthy lobster shells were spread out and more variable. However, the microbiomes found on ESD-infected lesions were more uniform and clustered together. In contrast, samples from seemingly healthy areas of diseased lobsters showed less variance than the healthy lobsters. However, these samples were generally more similar to healthy lobster samples than the diseased regions from which they came from.

Bacterial samples from lobsters kept in 12ºC water were grouped together based on the environment they came from. The average relative abundance of bacterial groups was used to create a histogram at the Class level.

Figure 2 indicates healthy lobsters had almost three times as much Bacteroidia than both the ESD-infected lesions and seemingly heathy areas of diseased lobsters. In comparison, bacteria on both healthy and seemingly healthy samples of diseased lobsters had more Acidimicrobiia than in the diseased lesion samples. Saprospirae were also found to be twice as abundant in both the healthy and seemingly healthy samples as opposed to the diseased lesions. However, seemingly healthy samples of diseased lobsters were almost twice as high in Epsilonproteobacteria as the other two samples. Lastly, the ESD-infected lesion samples had >10% more Flavobacteria than both the completely healthy and seemingly healthy samples of diseased lobsters.

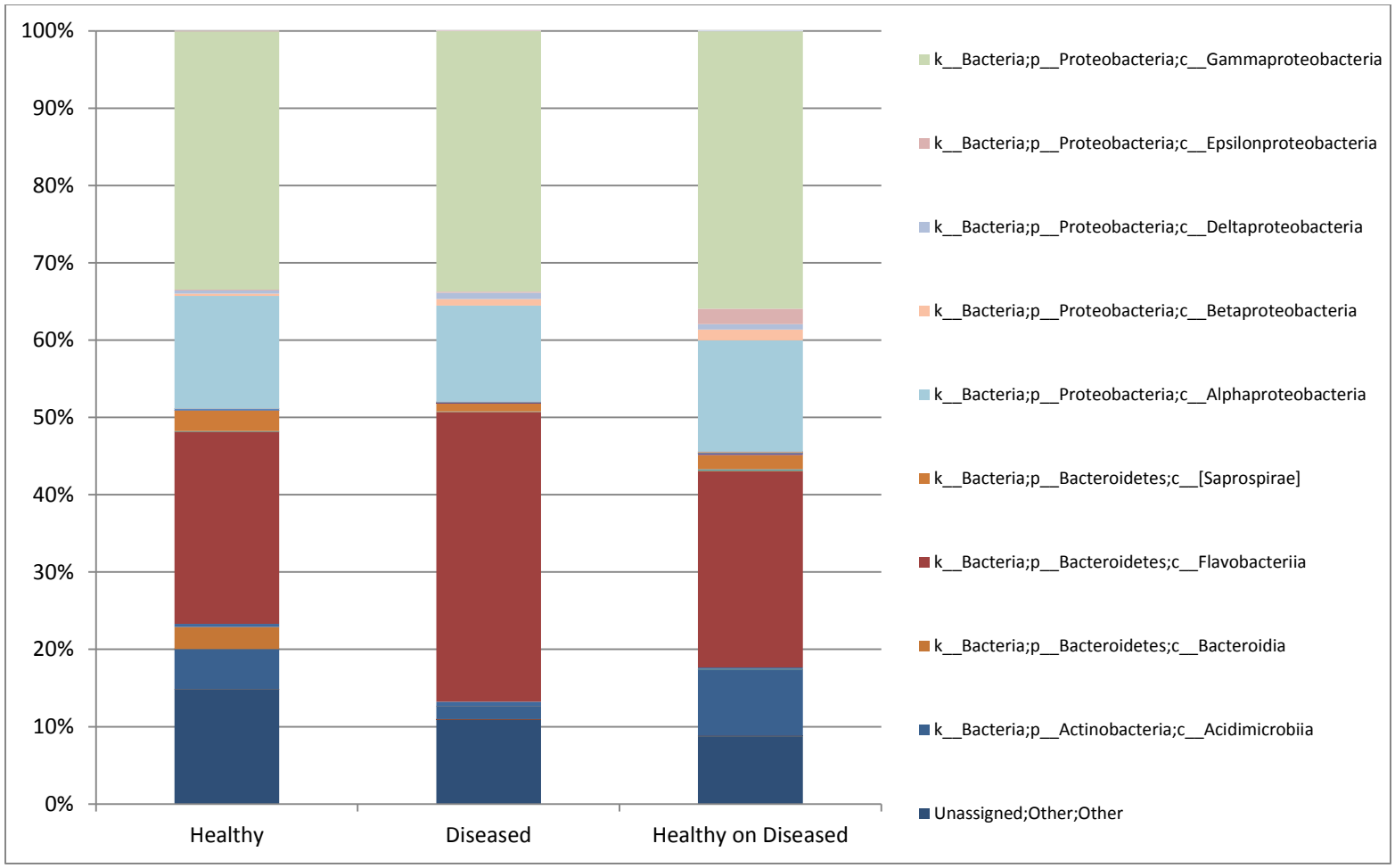

Figure 2. Histogram comparison of healthy and ESD infected *Homarus americanus*
Histogram representing the bacterial composition of healthy, seemingly healthy samples on diseased lobsters, and lesion samples of *Homarus americanus* kept in 12ºC water at the first sampling period. Bacteria identified down to the Class level.

Comparison of the Microbiomes from Healthy and Diseased Lobster Shells

Bacterial samples grouped together into healthy lobster shells, ESD lesions, and non-lesion areas on ESD infected lobsters kept in 12ºC water were compared against the other groups using LEfSe to determine statistically significant differences between the groups. Samples were distinguished down to the Genus level when possible.

LEfSe analysis showed a large difference of bacterial species found on healthy samples when compared to diseased ones (Appendix 1). From this analysis, 29 bacterial groups such as Aq*uimarina*, *Tenacibaculum*, and *Octadecabacter* were more prevalent on diseased lobsters than their healthy counterparts. Mainly bacteria from the phylum Bacteriodetes and Proteobacteria appeared in greater relative abundance on diseased samples, but there were certain Bacteriodetes, Proteobacteria, and Actinobacteria that were more dominant on healthy shells. These included Rhodobacteraceae, *Ulvibacter*, and *Roseovarius*. Additionally, 40 bacterial groups including Flavobacterium and Leucothrix were more abundant in the healthy samples.

When ESD-infected lesions were compared to the seemingly healthy regions of diseased lobsters (Appendix 2), the infected lesions had relatively more Aquimarina, Octadecbacter, and Tenacibaclum as well as other Bacteroidetes and Proteobacteria. In comparison, the healthy regions had many different bacteria from the phylum Actinobacteria, Proteobacteria and Bacteroidetes.

In general, scrapings from seemingly healthy lesions of diseased lobsters were more similar to scrapings from entirely healthy lobsters than the diseased areas of the

shell they came from (Appendix 3). However, all the bacteria that were relatively more common in the healthy lobsters were also more common when the healthy lobsters were compared to the diseased samples. A few of the bacteria that were more prevalent in healthy samples from diseased lobsters were also more prevalent in the diseased samples when compared to healthy samples from healthy lobsters.

The Effect of Temperature on Microbial Communities

The bacterial composition of healthy and ESD-infected *Homarus americanus* kept in 6ºC, 12ºC, and 18ºC water were compared against each other using LEfSe, PCoA, and Histograms to analyze temperature-dependent effects on both the bacterial environment of healthy lobsters and diseased lobsters. The results from the second sample set are also included to show the lobsters' further acclimation to their tank's water temperature.

Healthy lobsters showed a lot of variation in composition of microbial communities depending on the temperature of the water that they were housed in. Healthy lobsters kept in 12ºC and 6ºC had similar microbial populations with 9 taxa differing amongst them (Appendix 4). However, both varied largely from microbial populations of lobsters kept in 18ºC water. Lobsters in 6ºC water tended to have more Proteobacteria, Actinobacteria, and Cyanobacteria, while lobster in 18ºC water had more Bacteroidetes and certain Proteobacteria (Appendix 5). Healthy lobsters in 12ºC water had more Firmicutes and some groups of Proteobacteria and Bacteriodetes in their community than lobsters in 18ºC, who had larger populations of other groups of Proteobacteria and Bacteriodetes (Appendix 6).

The microbial populations of the diseased lobsters housed in 18ºC and those that lived in 6ºC water were similar to each other but had a few significantly different bacterial groups. (Appendix 7). When both 6ºC and 18ºC samples were compared to the microbial communities of lobsters kept 12ºC, we detected a large variation in the populations (Appendix 8, 9). In particular, *Aquimarina* was more abundant in lobsters housed at 12ºC than those housed at 18ºC.

When comparing healthy and diseased lobsters, the bacteria seemed to be different at every temperature level. The most significant finding was that *Aquimarina* were more dominant in diseased lobsters at every temperature compared to their healthy counterparts (Appendix 1, 10, 11). Bacterial populations between many taxa differed between healthy and diseased shells. Some of the taxa more prevalent on diseased shells than healthy shells included *Bdellovibrio, Fulvivirga,* Kiloniellaceae, Flammeovirgaceae JTB248, *and Nannocystis* at all three temperatures.

In general, there were few bacteria taxa lost from the healthy state when compared to the disease state that were similar in all 3 temperatures. These include *Methylomonas* and Altermonadeles HTCC2188. The 6ºC lobsters seemed to suffer less loss of bacterial populations, as well as gained less taxa in the transition from healthy to diseased state. The healthy 6ºC lobsters also seemed to cluster away from other healthy samples based on PCoA (Appendix 12), while the diseased samples were all clustered closely together at each temperature level (Appendix 13).

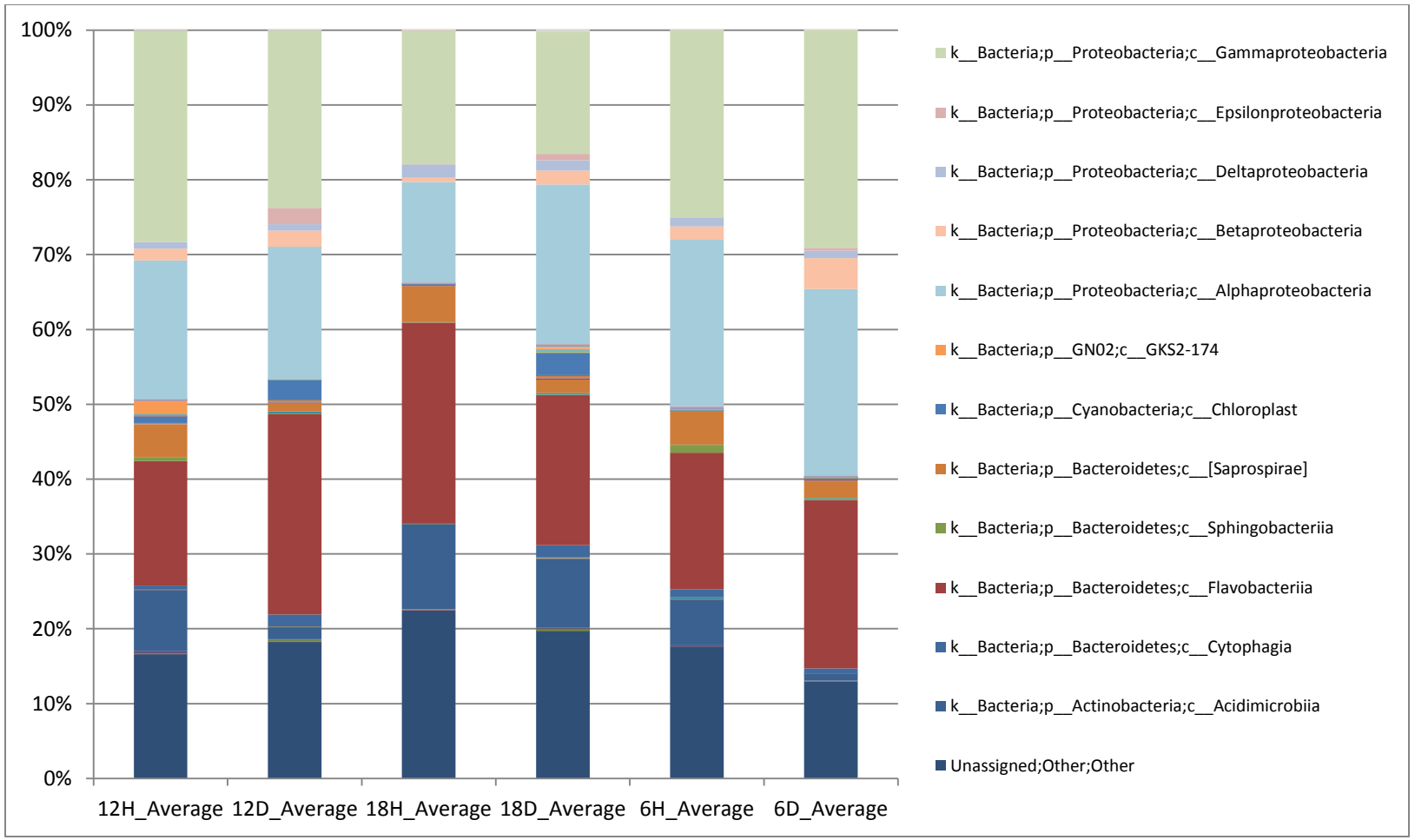

Figure 3 Temperature Histogram
Histogram representing the bacterial composition of healthy and lesion samples of *Homarus americanus* kept in 6ºC, 12ºC, and 18ºC water at the second sampling point. Bacteria identified down to the class level.

When breaking down the comparisons in the second sample set, there were also different types of bacteria that varied between healthy and diseased lobsters based on their base water temperature. Healthy lobsters housed in 12ºC and 18ºC temperatures had slightly more Gammaproteobacteria and Cytophagia and less Epsilonproteobacteria and Chloroplast than their diseased counterparts (Figure 3).However, healthy lobsters that were placed in 12ºC and 6ºC water had less Flavobacteriia than diseased lobsters at the same temperature

At the second sampling point, PCoA of lesion samples were all highly varied, but clustered based on temperature group. In the lobsters that were kept in 6ºC water, the bacteria clustering was highly variable, while 12ºC and 18ºC tempered lobsters were clustered with only a few outliers (Appendix 14).

In the first sample set, *Polaribacter* and Vibrionacea were more abundant in diseased lesions of lobsters kept at 6ºC. However, in the second sample period, it was much more abundant in lobsters at 12ºC. *Pararacoccus* was also more abundant in the first sample time point in diseased lobsters at 12ºC compared to those at 6ºC, but this was reversed after the second sampling (Appendix 15). The bacteria from the lesions of diseased lobsters kept at 6ºC also differed in certain Bacteroidetes and Proteobacteria than the infected lesions of diseased lobsters at 12ºC (Appendix 15). In addition, the lesions in lobsters kept at 12ºC had more Actinobacteria than lesions of diseased lobsters in 6ºC water.

When comparing the bacteria from the lesion samples of diseased lobsters kept at 6ºC and those kept at 18ºC (Appendix 16), it was found that they differed in certain

Bacteroidetes and Proteobacteria between the two sampling points. In addition, the samples from the lesions of lobsters housed in 18ºC water, had more Fusobacteria, Acidobacteria, and Actinobacteria as opposed to the ones in 6ºC water. In comparison to the bacteria from the lesions of diseased lobsters kept at 18ºC, the bacteria from lesions of diseased lobsters at 12ºC differed in certain Bacteroidetes and Proteobacteria (Appendix 17).

When analyzing healthy samples during the second sampling point, the bacteria of healthy lobsters kept at 6ºC had less Bacteroidetes and Proteobacteria than the shells of healthy lobsters at 12ºC (Appendix 18). Rhizobiales was the only bacteria that were relatively more abundant in the shell of lobsters kept at 12ºC compared to 6ºC.

Bacterial Environment of the Hemolymph

In addition to carapace samples, hemolymph samples were also collected. In order to discern other effects ESD had on *Homarus americanus*, healthy and infected hemolymph samples were compared to each other using Histograms, LEfSe, and PCoA.

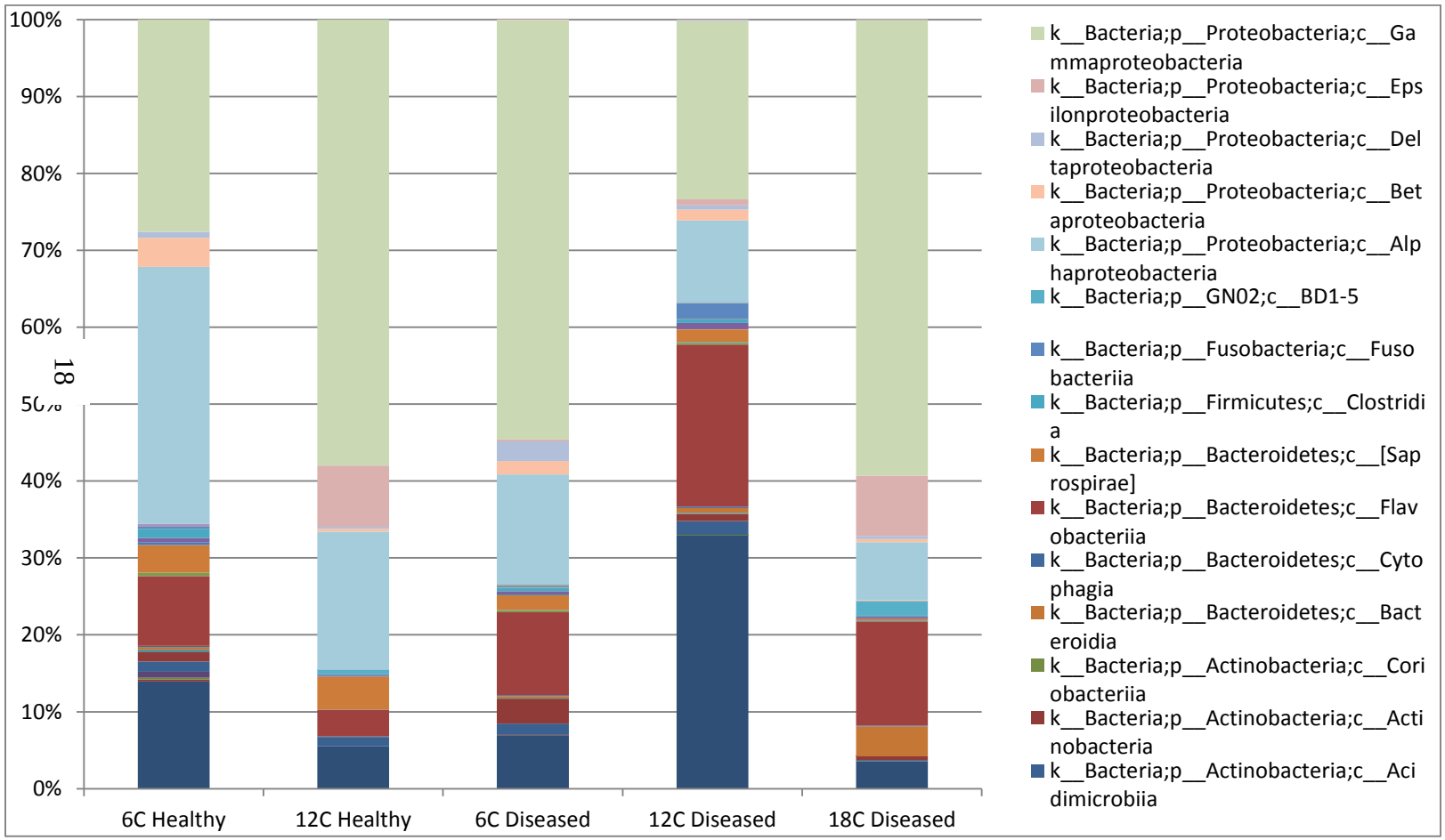

Figure 4 Hemolymph Histogram
Histogram representing the bacterial composition of healthy and diseased hemolymph samples of *Homarus americanus* kept in 6ºC, 12ºC, and 18ºC water at the second sampling point. Bacteria identified down to the class level.

Hemolymph samples at all environments had varied bacterial populations. Healthy Hemolymph from lobsters at 12ºC had more than twice as much Gammaproteobacteria and more than 7% more Epsilonproteobacteria (Figure 4). The healthy 6ºC hemolymph samples had almost twice as much Alphaproteobacteria and more than twice the Flavobacteria as the healthy bacteria in 12ºC hemolymph. Among the diseased hemolymph, 12ºC lobsters had less than half the Gammaproteobacteria of both 6ºC and 18ºC, while having almost twice as much Flavobacteria. Diseased hemolymph from 6ºC had about 3% Actinobacteria which was only similar to 6ºC healthy hemolymph. Healthy hemolymphs from 12ºC and diseased hemolymphs from 18ºC both had a high percentage of Epsilonproteobacteria which other experimental conditions did not share. In general, healthy hemolymph had more Aplhaproteobacteria than diseased samples and less Flavobacteria.

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PCoA of healthy and diseased hemolymph samples (Appendix 19) showed that the healthy were clustered together in the first sampling while the diseased were not clustered. In the second sampling period though, the PCoA of diseased hemolymph samples did not show significant clustering (Appendix 20).

In hemolymph from diseased Lobsters at 18ºC, only Tenacibaculum and Thalassomonas were relatively more abundant than in healthy hemolymph (Appendix 21). Several bacteria from phylum Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were also more prevalent in healthy lobster hemolymph as opposed to diseased lobster hemolymph.

There were more significant bacteria groups in diseased lobsters kept in the higher temperature tanks than the colder water tanks. 18ºC tempered lobsters also showed a lot more Firmicutes than those kept in 6ºC water. However, Proteobacteria differed in every temperature level.

The diseased 12ºC lobsters only had a few differences in bacterial populations (Proteobacteria, Firmicutres, Bacterodetes, and Actinobacteria) than their counterparts kept in 6ºC water (Appendix 22). However, when compared to diseased lobsters in 18ºC water (Appendix 23), there were only seven bacterial groups that differed at the phylum level. Throughout every temperature though, there was a difference in concentration of Firmicutes and Proteobacteria (Appendix 22, 23, 24).

DISCUSSION

The study found a relationship between microbial communities and epizootic shell disease. There were many groups of bacteria that were found to be different among the samples of healthy lobsters, ESD-infected lesions on diseased lobsters, and the seemingly healthy areas of diseased lobsters. These groups of bacteria also differed depending on the temperature that each lobster lived in for three months, as well as internal make-up. Overall, the study provided evidence that microbial community populations differ depending on disease state and temperature.

Bacteria previously Linked to ESD

The microbial populations were found to be related to the prevalence of ESD, as there was a shift in relative abundance of bacterial taxa between healthy and diseased lobsters. Similar to previous studies, *Aquimarina homaria* was found predominately in the ESD-infected lesions of diseased lobsters (Chistoserdov, Quinn, Gubbala, & Smolowitz, 2012), (Meres et al., 2012). It was also found more commonly on the seemingly healthy areas of diseased lobsters in comparison to completely healthy lobsters. Although *Aquimarina homaria* as well as *Tenacibaculum* and *Octadecabacter* were also found on healthy lobsters, the increased presence of it on diseased lobsters is significant. This suggests that ESD is associated with the microbial population of *Homarus americanus*'s shell.

However, this doesn't necessarily mean that these bacteria cause the lesions or ESD and this result could be explained by a general dysbiosis of the bacterial groups or one of the other bacteria that were altered. Aquimarina could simply be an opportunistic pathogen, or a type of bacteria that arises in a diseased or degraded environment. Therefore, Aquimarina could be a result of ESD and its lesions rather than a cause of ESD. In this case, loss of one of the bacteria in the healthy shell that contributes to the health of the lobster could be just as important as the presence of a dominant pathogen. Therefore, further research needs to be conducted to determine the role of Aquimarina in ESD. Is Aquimarina the culprit and source of ESD, or is it a side effect of a negatively altered environment?

There were also other bacteria that were observed more on diseased lobsters than healthy lobsters. *Tenacibaculum, Bdellovibrio, Nannocysti, JTB248, Fulvivirga,* and certain *Flammeovirgaceae*, as these bacteria were seen in more abundance on diseased lobsters than healthy lobsters at all temperature environments in the first sampling period. This shows that regardless of climate, these bacteria are linked to ESD.

Not only did ESD-infected lesions have a different microbial makeup than healthy lobsters, but the seemingly healthy regions of diseased lobsters also differed from completely healthy samples. This finding ultimately questions the etiology of ESD. ESD has generally been defined by the appearance of a lesion. If the microbial environment of the entire shell of a diseased lobster is completely different from a healthy lobster shell, then this might suggest that ESD effects on other parts of the lobster's body than the

infected lesion. Further research should be conducted to determine ESD's effects on other areas of the lobster, rather than just the lesion sites.

Effect of Temperature on Microbial Communities of Lobster Shells

Water temperature was found to have an important impact on the dynamics of bacteria living on a lobster's carapace, as well as the presence of ESD. Taxa generally differed amongst healthy and diseased lobsters depending on the controlled temperature habitat that they resided in for three months.

A significant finding from the study was that higher temperatures seemed ideal for ESD development. This directly supports previous research conducted on the relationship between temperature and ESD. (Glenn & Pugh, 2006). More lobsters kept at 18 ºC developed ESD symptoms than in the other 2 temperatures. Lobsters kept in 18C water produced a faster progression of lesion development than lower temperatures (Barris et al., 2018). Since lobsters can live up to water temperatures of 25C, it could be assumed that warmer temperatures between 18ºC and 25C could be the ideal conditions for ESD-bacteria growth (Lawto and Lavalli, 1995). However, this would need to be researched further to determine the results of lobsters living in temperatures over 18ºC.

Compared to higher temperatures, it was found that healthy lobsters kept in the colder 6ºC water, had more diversity in the bacteria population found on their shells than healthy lobsters in 12ºC water. This is a general ecological response where there is a decrease in community taxa to an environmental perturbation such as a temperature shift. Specifically, this could actually be a buffer for the lobsters to prevent them from developing ESD. In colder temperatures, the lobsters may have had a biofilm which

would be more resistant to ESD. The diversity may be conferring an immunity to the cause of ESD, or generating a more stable environment to resist changes that may lead to ESD.

It should be noted that the lobsters keep at 12ºC and 18ºC were kept in two rows, with the top row allowing access to more light. This could have affected the dynamics of the bacteria population and potentially explain the presence of more photosynthetic bacteria on lobsters in the upper row.

ESD-Associated Changes In The Composition Of Microbial Communities Residing Within The Hemolymph

Previous research has not shown a link between ESD and the hemolymph. Instead, it suggests that the overall makeup of hemolymph in lobsters varied largely between healthy and diseased lobsters. However, LEfse comparison of healthy and diseased Hemolymph showed that there was a significant difference in the presence of *Tenacibaculum* in the diseased hemolymph than the healthy. This result was also similar to both 18ºC and 12ºC lesion samples compared to their healthy counterparts. The only temperature that did not replicate those results was the 6ºC sample.

In this study, hemolymph was shown to vary depending on the water temperature the lobster was living in. Healthy lobsters in particular had different hemolymph from each other. For example, Firmicutes appeared largely in healthy lobsters stored in 18ºC, less so in 6ºC, and was almost non-existent in 12ºC. This could potentially show that Firmicutes appear in outlying temperatures. Specifically, when the lobsters were introduced to water different from 10-12ºC, populations of Firmicutes arose. This could

provide evidence that there may be a primary shift in the hemolymph environment in response to temperature which induces respiratory compensated metabolic acidosis in the hemolymph altering its pH (Qadri et al., 2007). It is important to note though, that there is a possibility of contamination with the lobster hemolymph samples, as bacteria may have been collected from the initial puncture of the syringe, however, such contamination would be marginal as no culturing was performed after the collection.

SUMMARY AND CONCLUSION

This study set out to understand the microbial communities of lobsters with epizootic shell disease. There was evidence of an altered bacterial community when comparing ESD samples to healthy samples, supporting the theory of bacterial Dysbiosis in ESD.

The second objective of the study was to specifically determine the types of bacteria associated with this rapidly spreading disease. *Aquimarina* is seen to be associated with ESD in all environments tested (the different temperatures). It is also seen in greater relative abundance on areas of the shell that appeared to be seemingly healthy on diseased lobsters. *Tencibaculum* may also play a role in the hemolymph, and was seen in more abundance in 12ºC and 18ºC lesion samples.

The third objective of this research was to determine the role that temperature played in the development of ESD. Temperature not only played a role in shaping the healthy bacterial community, but it also affected how the bacterial composition looked in the diseased state.

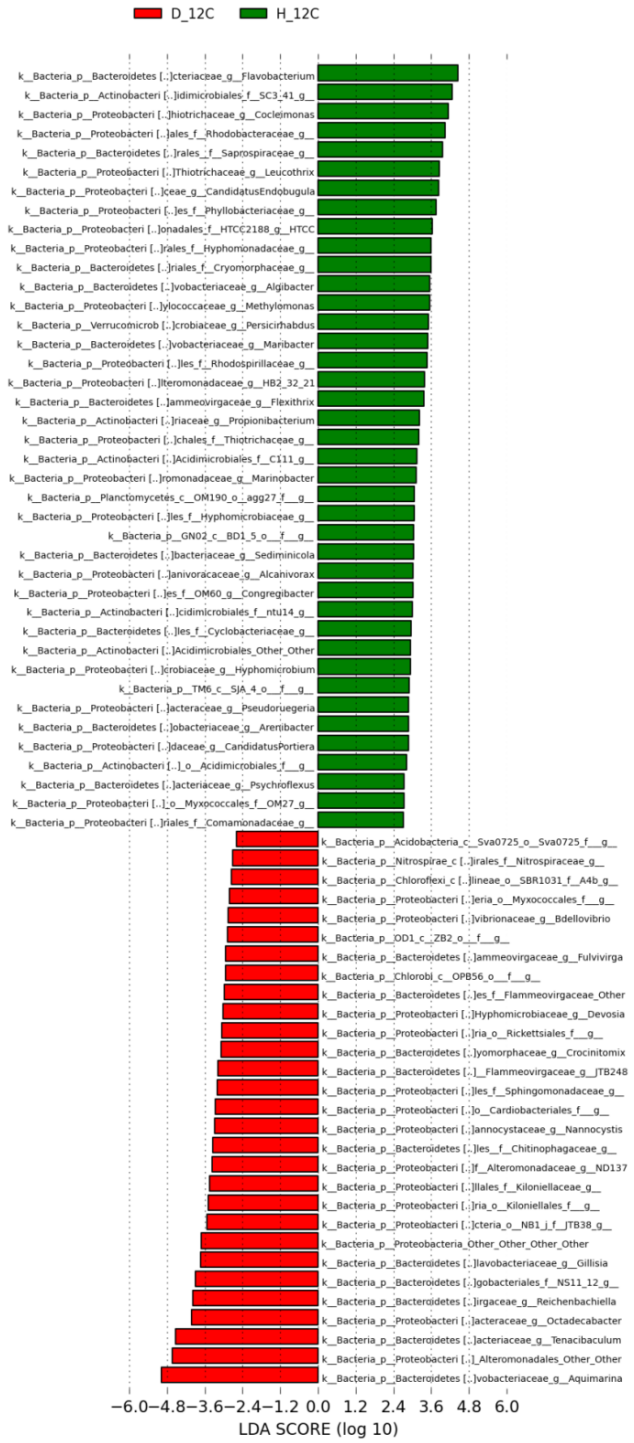
Lastly, the research looked at the link between a lobster's hemolymph and the bacteria associated with ESD. The hemolymph bacterial community was shown to look different among the healthy and diseased state. They also varied largely in between different temperature environments. This suggests the possibility that the mirocial

dysbiosis seen in ESD is a systemic shift and changes in the hemolymph may contribute to the etiology of the disease.

Further Research

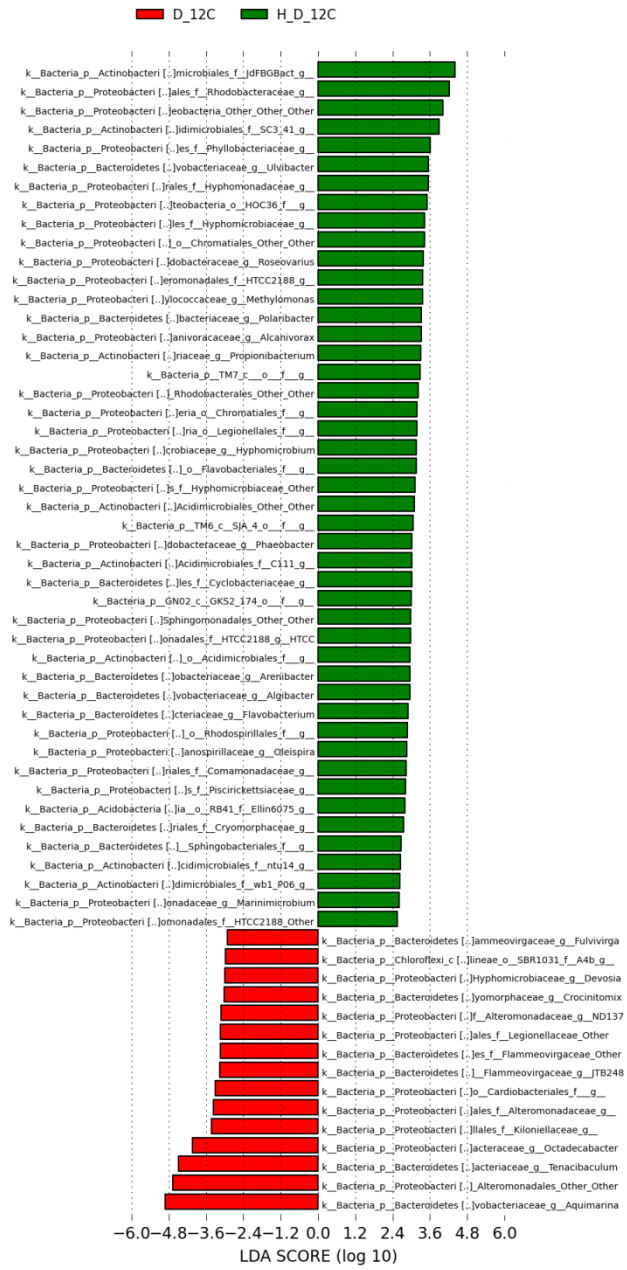
This study successfully reached its objectives. In particular, we have demonstrated significant connections between the composition of the microbial communities of American lobsters and the presence of ESD. However, it also revealed areas of interest that could be researched further. One of these is related to the etiology of the disease. The current study showed that ESD is associated with *Aquimarina*. However, further research would need to be done determine if these bacteria is a causative agent of ESD or the result of an altered bacterial environment, i.e. an opportunistic invader. Also, since the seemingly healthy samples of diseased lobsters varied from the sample of completely healthy lobsters, the research suggests that bacteria are not confined to the specific lesion sites. Therefore, another study could determine if the disease is only affecting the lobster's shell, or if it's affecting the lobster's entire internal microbiome in the hemolymph. Lastly, it would be interesting to study other factors that may be correlated or associated with the development of ESD such as size, age, and sex of the lobster. These latter variates will be addressed in a subsequent publication.

APPENDIX



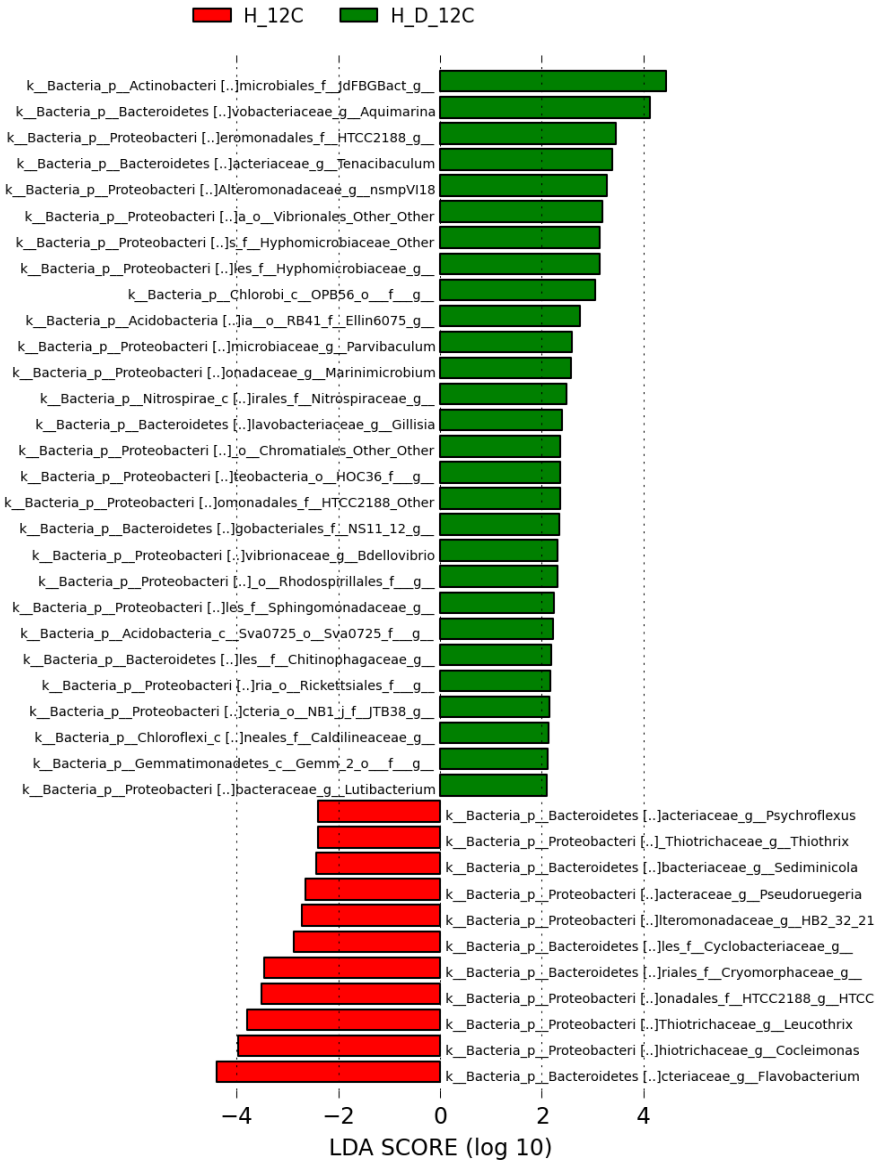
Appendix 1. LEfSe comparison of healthy and lesion samples of *Homarus americanus* kept in 12ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in the healthy samples in comparison to lesion samples. The red bar represents

the LDA scores of significantly different bacteria in the lesion samples compared to healthy samples.



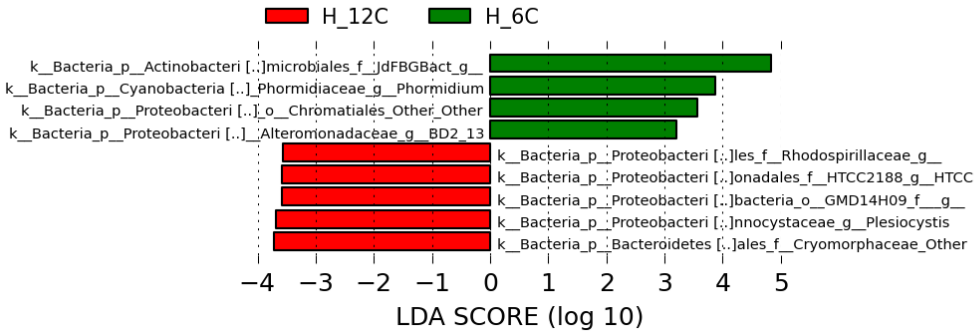
Appendix 2. LEfSe comparison seemingly healthy samples on diseased lobsters of and lesion samples of *Homarus americanus* kept in 12ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in the seemingly healthy samples on diseased lobsters in comparison to lesion samples. The red bar

represents the LDA scores of significantly different bacteria in the lesion samples compared to seemingly healthy samples on diseased lobster samples.

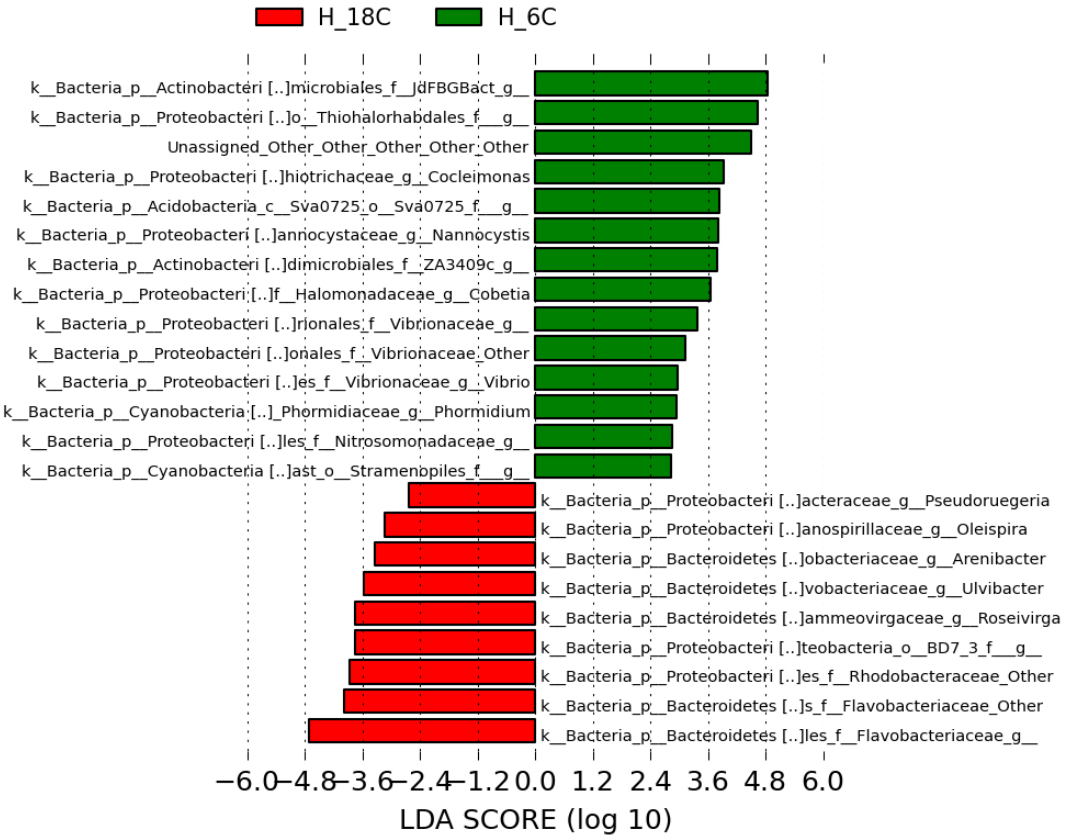


Appendix 3. LEfSe comparison of seemingly healthy samples on diseased lobsters and healthy samples of *Homarus americanus* kept in 12ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in the seemingly healthy samples on diseased lobsters in comparison to healthy samples. The red bar

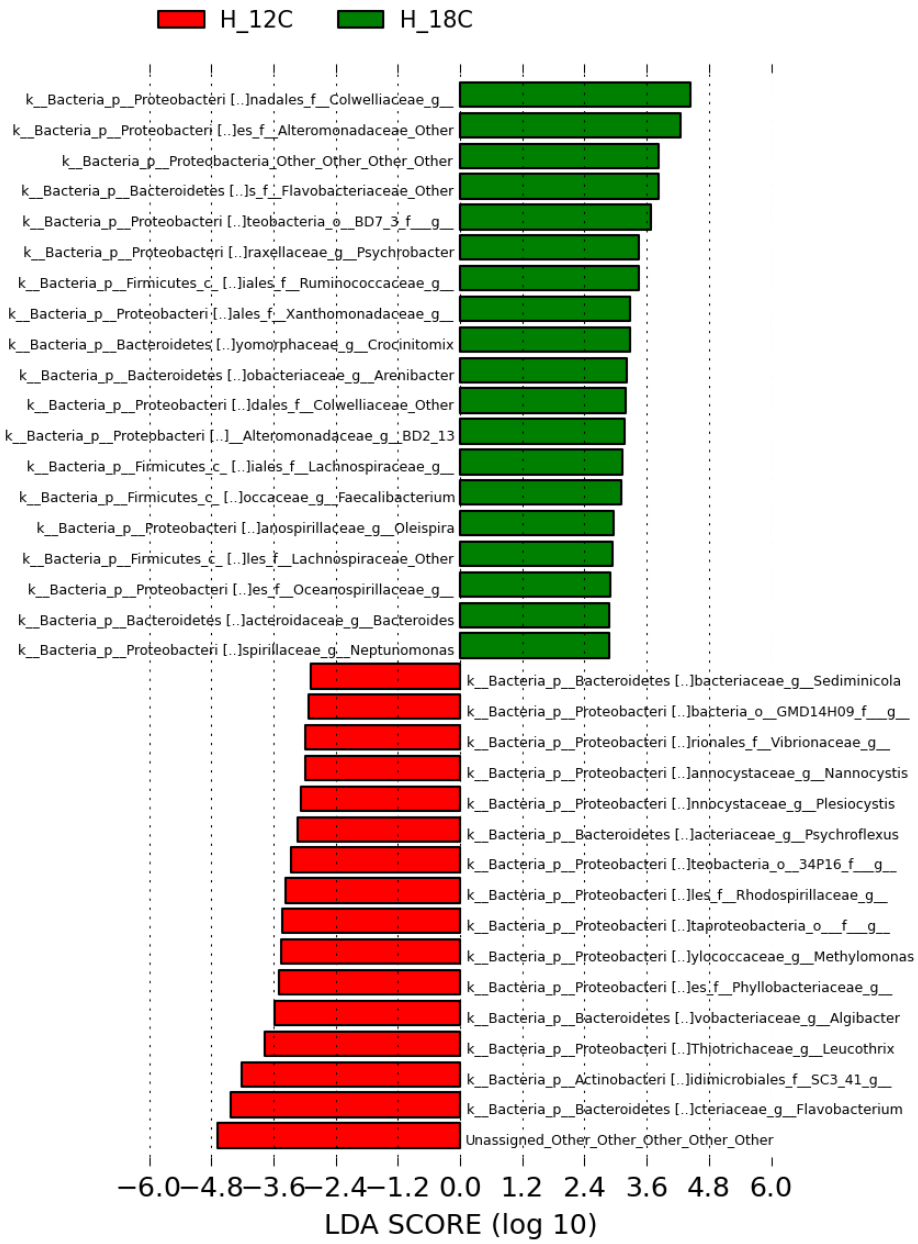
represents the LDA scores of significantly different bacteria in the healthy samples compared to seemingly healthy samples on diseased lobster samples.



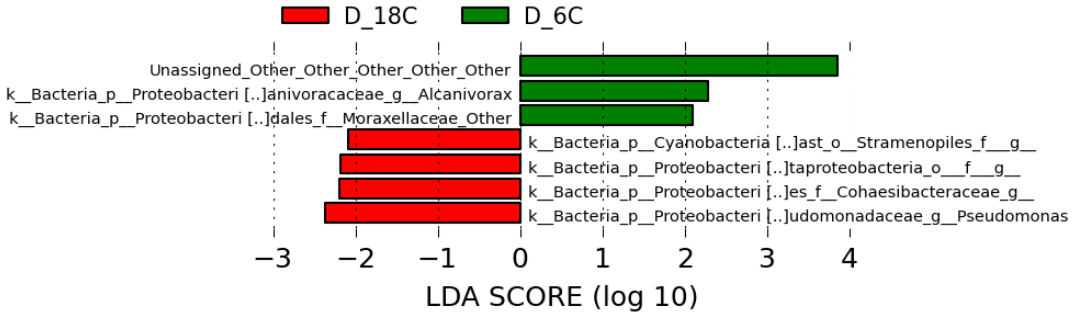
Appendix 4 LEfSe comparison of healthy samples of *Homarus americanus* kept in 12ºC and 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in healthy samples from the 6ºC environment in comparison healthy samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in healthy samples from the 12ºC environment compared to healthy samples from the 6ºC environment.



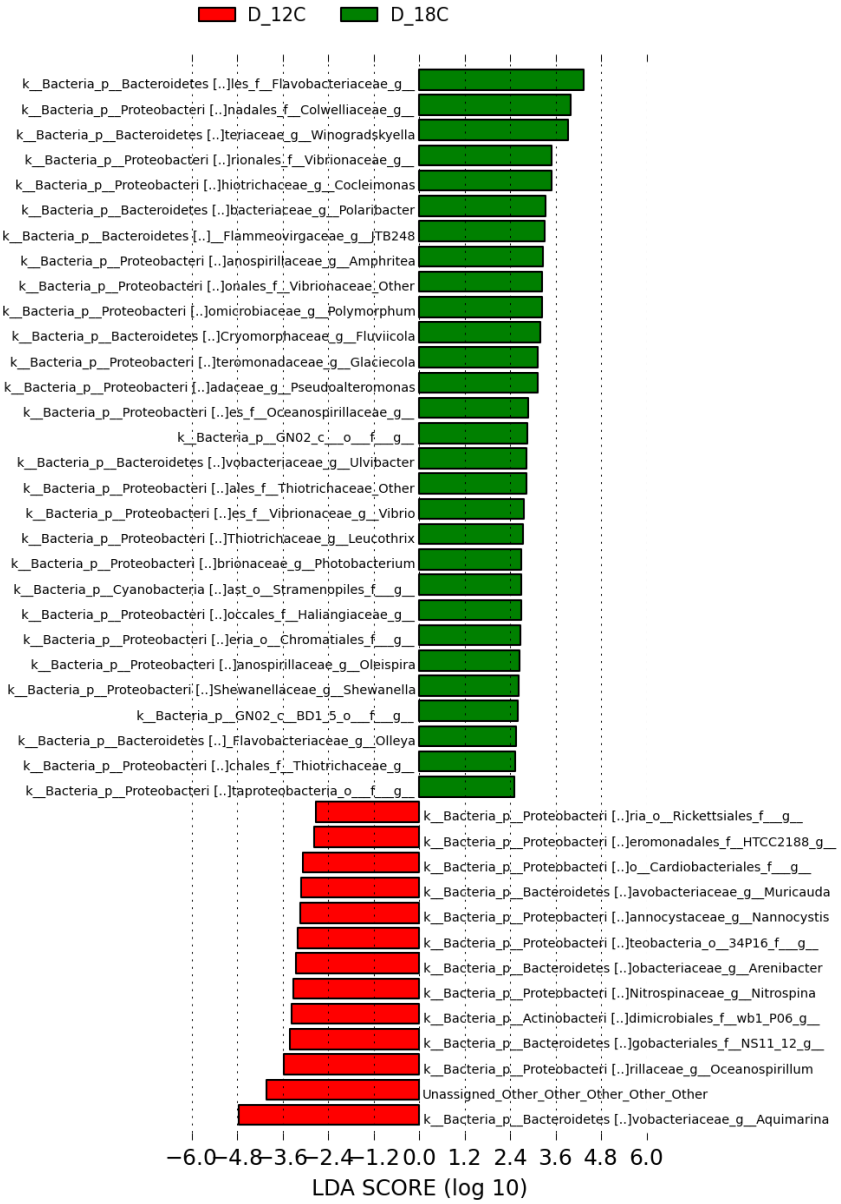
Appendix 5. LEfSe comparison of healthy samples of *Homarus americanus* kept in 18ºC and 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in healthy samples from the 6ºC environment in comparison healthy samples from the 18ºC environment. The red bar represents the LDA scores of significantly different bacteria in healthy samples from the 18ºC environment compared to healthy samples from the 6ºC environment.



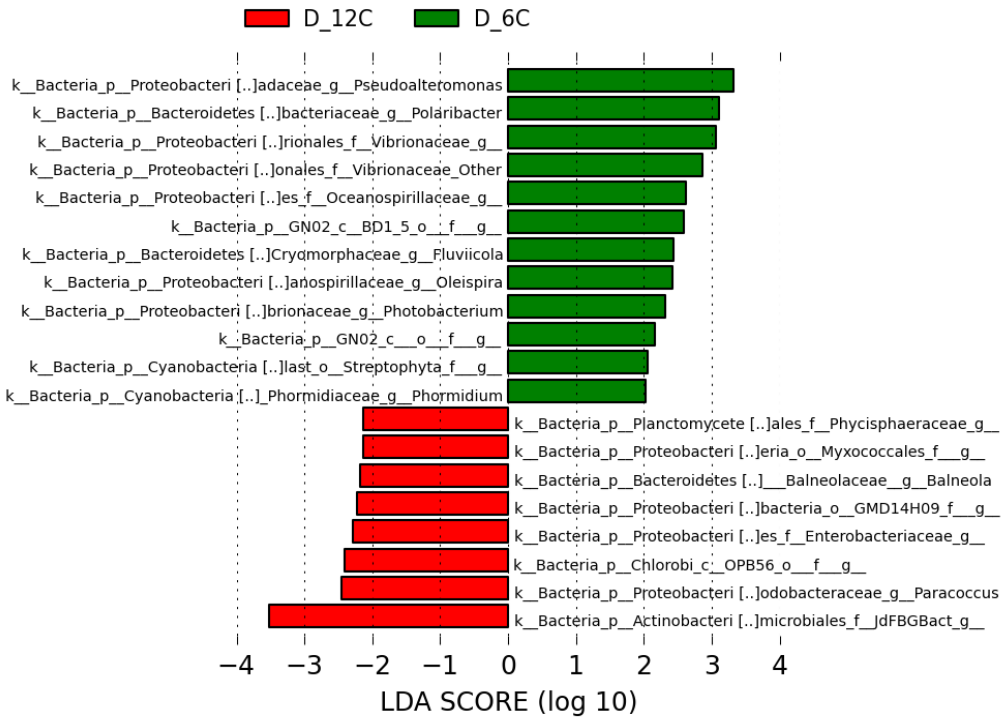
Appendix 6. LEfSe comparison of healthy samples of *Homarus americanus* kept in 12ºC and 18ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in healthy samples from the 18ºC environment in comparison healthy samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in healthy samples from the 12ºC environment compared to healthy samples from the 18ºC environment.



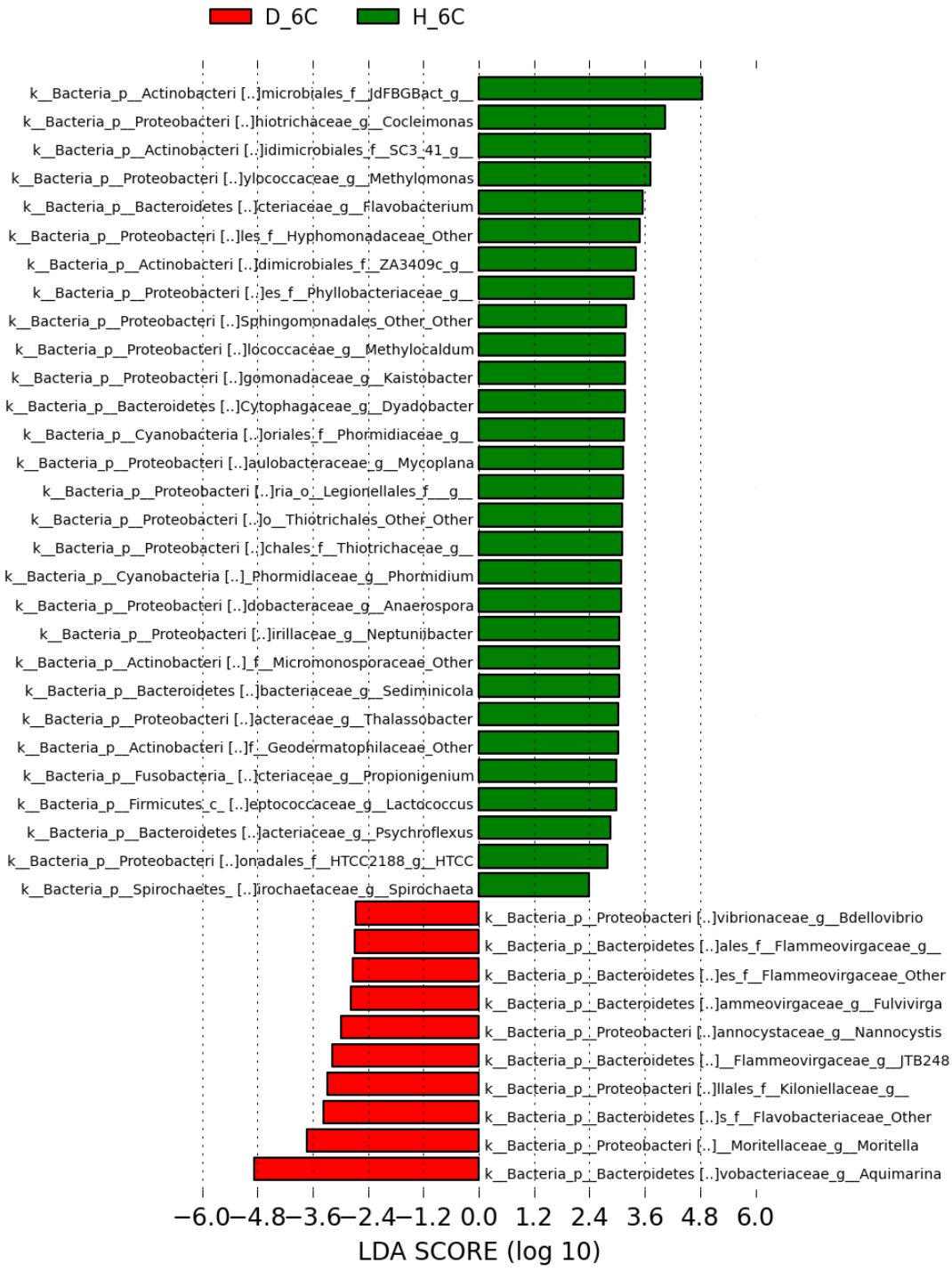
Appendix 7. LEfSe comparison of lesion samples of *Homarus americanus* kept in 6ºC and 18ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 6ºC environment in comparison lesion samples from the 6ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 18ºC environment compared to lesion samples from the 6ºC environment.



Appendix 8. LEfSe comparison of lesion samples of *Homarus americanus* kept in 12ºC and 18ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 18ºC environment in comparison lesion samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 12ºC environment compared to lesion samples from the 18ºC environment.

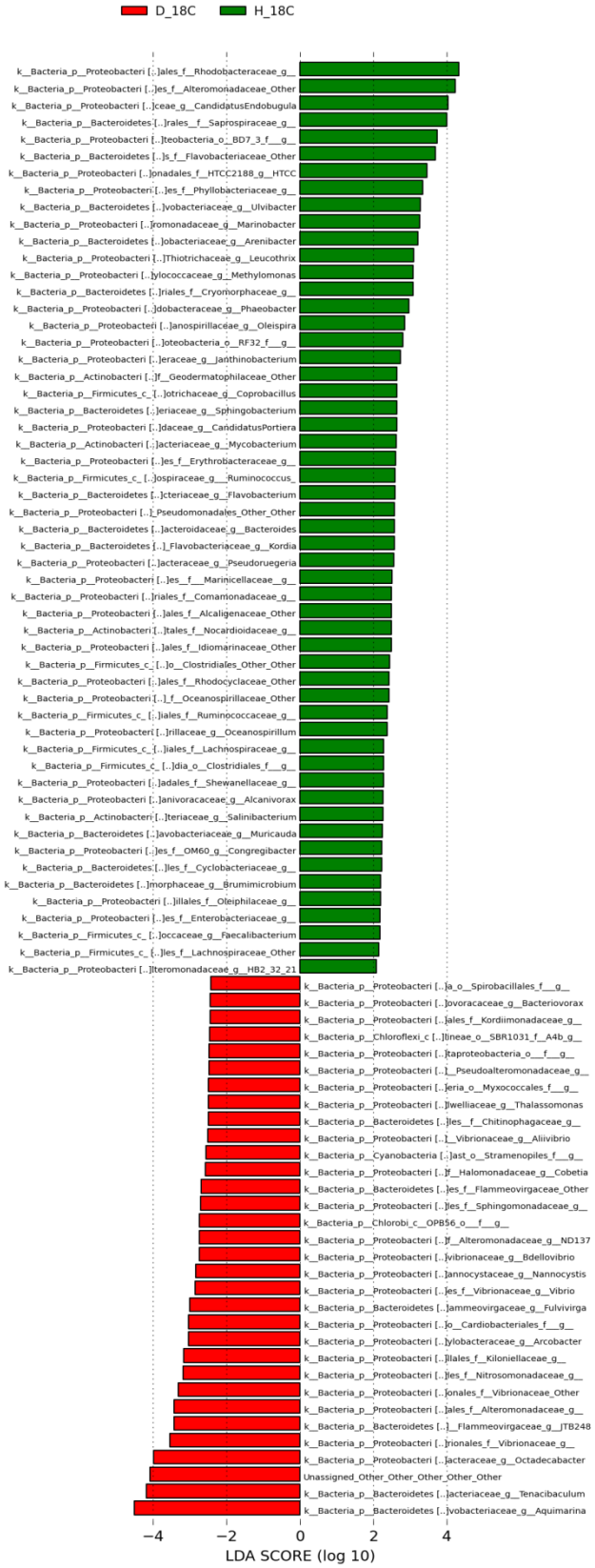


Appendix 9. LEfSe comparison of lesion samples of *Homarus americanus* kept in 12ºC and 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 6ºC environment in comparison lesion samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 12ºC environment compared to lesion samples from the 6ºC environment.



Appendix 10. LEfSe comparison of healthy and lesion samples of *Homarus americanus* kept in 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly

different bacteria in the healthy samples in comparison to lesion samples. The red bar represents the LDA scores of significantly different bacteria in the lesion samples compared to healthy samples.


D_18C
H_18C
k__Bacteria_p__Proteobacteri [..]ales_f__Rhodobacteraceae_g__
k__Bacteria_p__Proteobacteri [..]es_f__Alteromonadaceae_Other
k__Bacteria_p__Proteobacteri [..]ceae_g__CandidatusEndobugula
k__Bacteria_p__Bacteroidetes [..]rales__f__Saprospiraceae_g__
k__Bacteria_p__Proteobacteri [..]teobacteria_o__BD7_3_f__g__
k__Bacteria_p__Bacteroidetes [..]s_f__Flavobacteriaceae_Other
k__Bacteria_p__Proteobacteri [..]onadales_f__HTCC2188_g__HTCC
k__Bacteria_p__Proteobacteri [..]es_f__Phyllobacteriaceae_g__
k__Bacteria_p__Bacteroidetes [..]vobacteriaceae_g__Ulvibacter
k__Bacteria_p__Proteobacteri [..]romonadaceae_g__Marinobacter
k__Bacteria_p__Bacteroidetes [..]obacteriaceae_g__Arenibacter
k__Bacteria_p__Proteobacteri [..]Thiotrichaceae_g__Leucothrix
k__Bacteria_p__Proteobacteri [..]ylococcaceae_g__Methylomonas
k__Bacteria_p__Bacteroidetes [..]riales_f__Cryomorphaceae_g__
k__Bacteria_p__Proteobacteri [..]dobacteraceae_g__Phaeobacter
k__Bacteria_p__Proteobacteri [..]anospirillaceae_g__Oleispira
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k__Bacteria_p__Proteobacteri [..]eraceae_g__Janthinobacterium
k__Bacteria_p__Actinobacteri [..]f__Geodermatophilaceae_Other
k__Bacteria_p__Firmicutes_c_ [..]otrichaceae_g__Coprobacillus
k__Bacteria_p__Bacteroidetes [..]eriaceae_g__Sphingobacterium
k__Bacteria_p__Proteobacteri [..]daceae_g__CandidatusPortiera
k__Bacteria_p__Actinobacteri [..]acteriaceae_g__Mycobacterium
k__Bacteria_p__Proteobacteri [..]es_f__Erythrobacteraceae_g__
k__Bacteria_p__Firmicutes_c_ [..]ospiraceae_g__Ruminococcus_
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k__Bacteria_p__Proteobacteri [..]_Pseudomonadales_Other_Other
k__Bacteria_p__Bacteroidetes [..]acteroidaceae_g__Bacteroides
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k__Bacteria_p__Proteobacteri [..]acteraceae_g__Pseudoruegeria
k__Bacteria_p__Proteobacteri [..]es_f__Marinicellaceae_g__
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k__Bacteria_p__Proteobacteri [..]ales_f__Alcaligenaceae_Other
k__Bacteria_p__Actinobacteri [..]tales_f__Nocardioidaceae_g__
k__Bacteria_p__Proteobacteri [..]ales_f__Idiomarinaceae_Other
k__Bacteria_p__Firmicutes_c_ [..]o__Clostridiales_Other_Other
k__Bacteria_p__Proteobacteri [..]ales_f__Rhodocyclaceae_Other
k__Bacteria_p__Proteobacteri [..]_f__Oceanospirillaceae_Other
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k__Bacteria_p__Proteobacteri [..]rillaceae_g__Oceanospirillum
k__Bacteria_p__Firmicutes_c_ [..]iales_f__Lachnospiraceae_g__
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k__Bacteria_p__Proteobacteri [..]illales_f__Oleiphilaceae_g__
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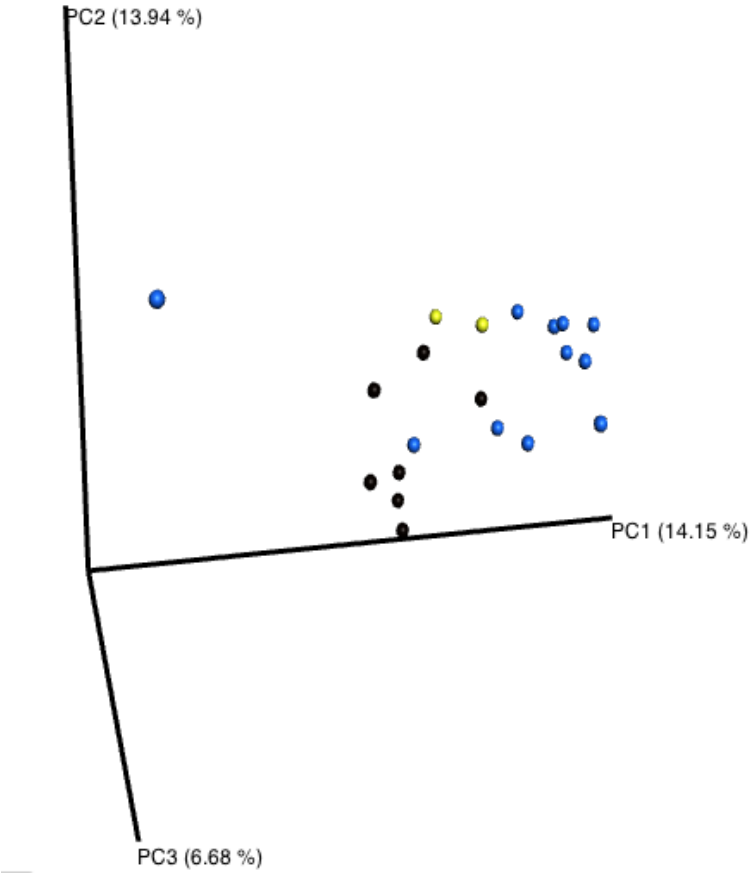
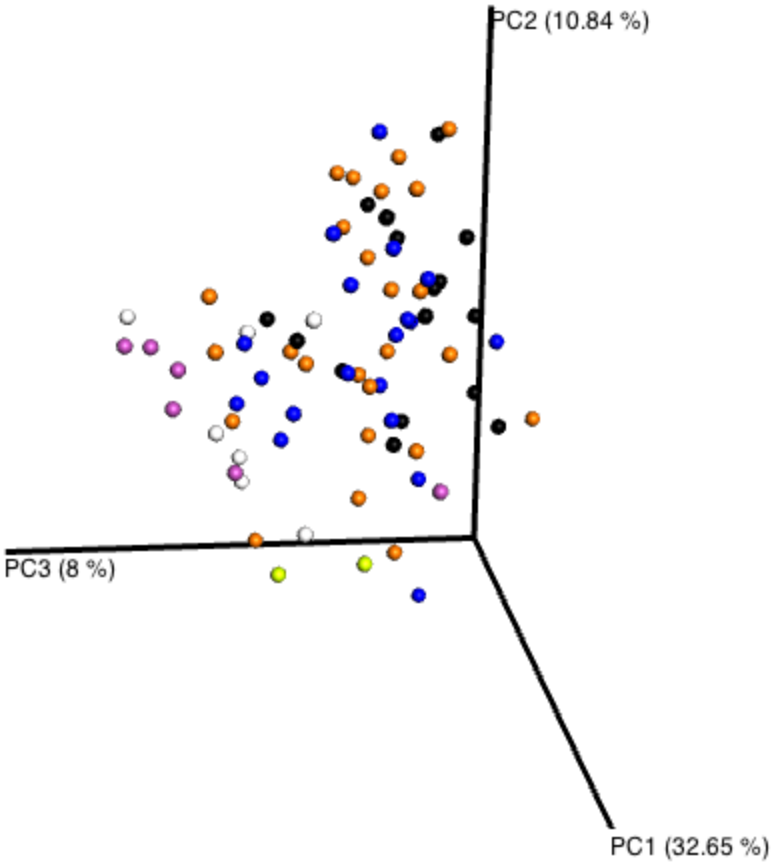
k__Bacteria_p__Proteobacteri [..]acteraceae_g__Octadecabacter
Unassigned_Other_Other_Other_Other
k__Bacteria_p__Bacteroidetes [..]acteriaceae_g__Tenacibaculum
k__Bacteria_p__Bacteroidetes [..]vobacteriaceae_g__Aquimarina
−4
−2
0
2
4
LDA SCORE (log 10)

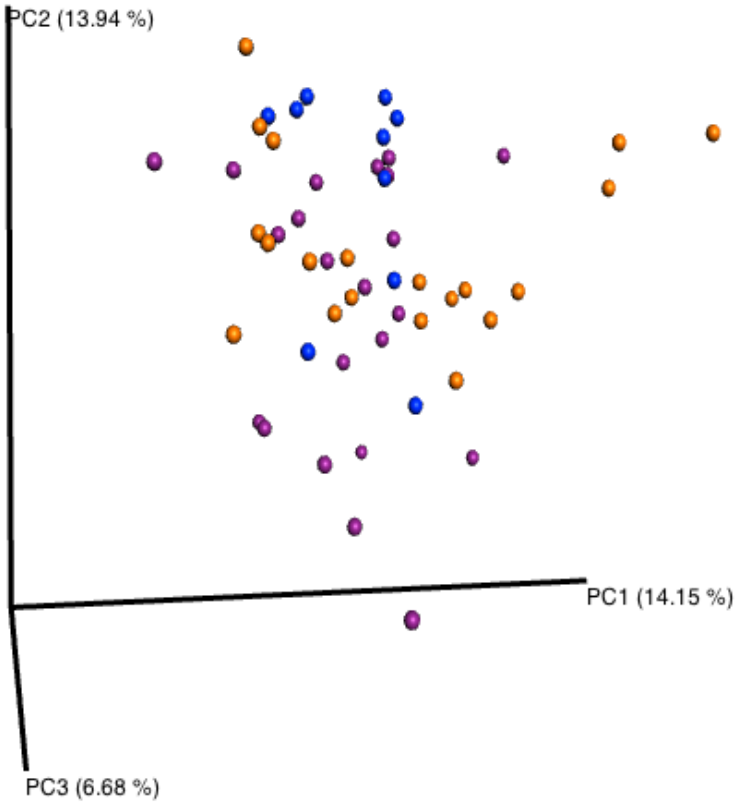
Appendix 11. LEfSe comparison of healthy and lesion samples of *Homarus americanus* kept in 18ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in the healthy samples in comparison to lesion samples. The red bar represents the LDA scores of significantly different bacteria in the lesion samples compared to healthy samples.



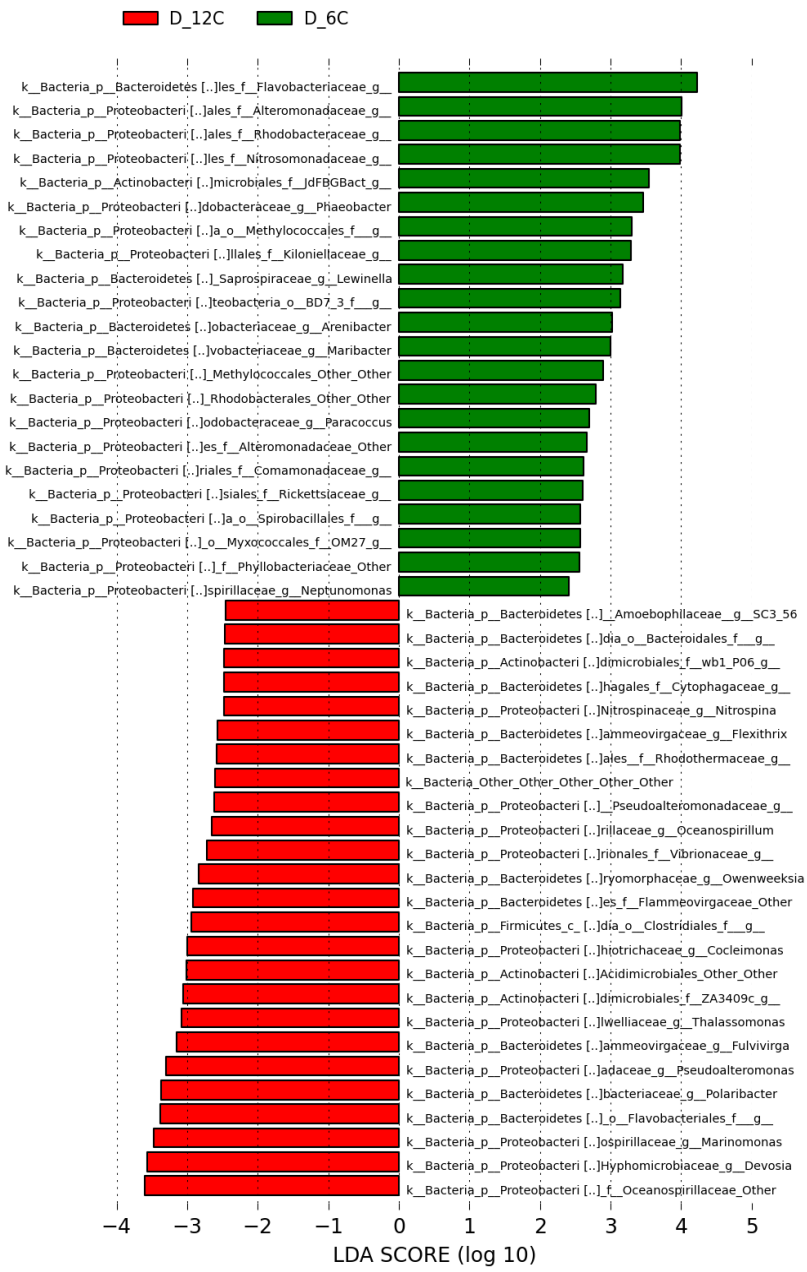
Appendix 12. PCoA of healthy shell bacteria at 6ºC, 12ºC, and 18ºC from the second sampling point. Healthy 12ºC samples = Blue, healthy 18ºC samples = Yellow, healthy 6ºC samples = Black. Principle Component 1 (PC1) explains 14.15% of the variance, Principle Component 2 (PC2) explains 13.94% of the variance, and Principle Component 3 (PC3) explains 6.68% of the variance. In sum, the Principle Components explains 34.77% of the variance of the dataset.



Appendix 13. PCoA of lesion and healthy samples at 6ºC, 12ºC, and 18ºC at the first sampling point. 12ºC lesion samples = Black, 18ºC lesion samples = Blue, 6ºC lesion samples = Orange, 12ºC healthy samples = White, 18ºC healthy samples = Purple, 6ºC = Yellow. Principle Component 1 (PC1) explains 32.65% of the variance, Principle Component 2 (PC2) explains 10.84% of the variance, and Principle Component 3 (PC3) explains 8% of the variance. In sum, the Principle Components explains 51.40% of the variance of the dataset.

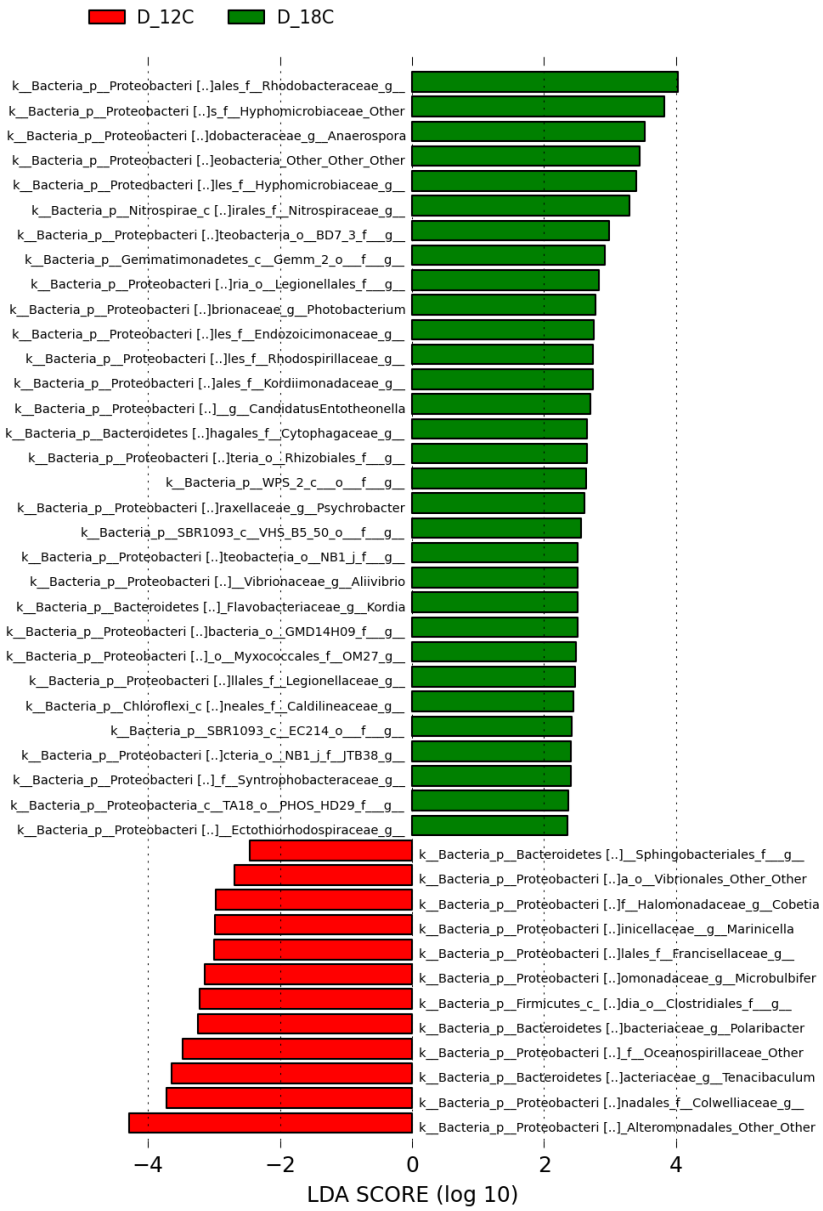


Appendix 14. PCoA of lesion samples at 6ºC, 12ºC, and 18ºC at the second sampling point. 12ºC lesion samples = Blue, 18ºC lesion samples = Orange, 6ºC lesion samples = Purple. Principle Component 1 (PC1) explains 14.15% of the variance, Principle Component 2 (PC2) explains 13.94% of the variance, and Principle Component 3 (PC3) explains 6.68% of the variance. In sum, the Principle Components explains 34.77% of the variance of the dataset.

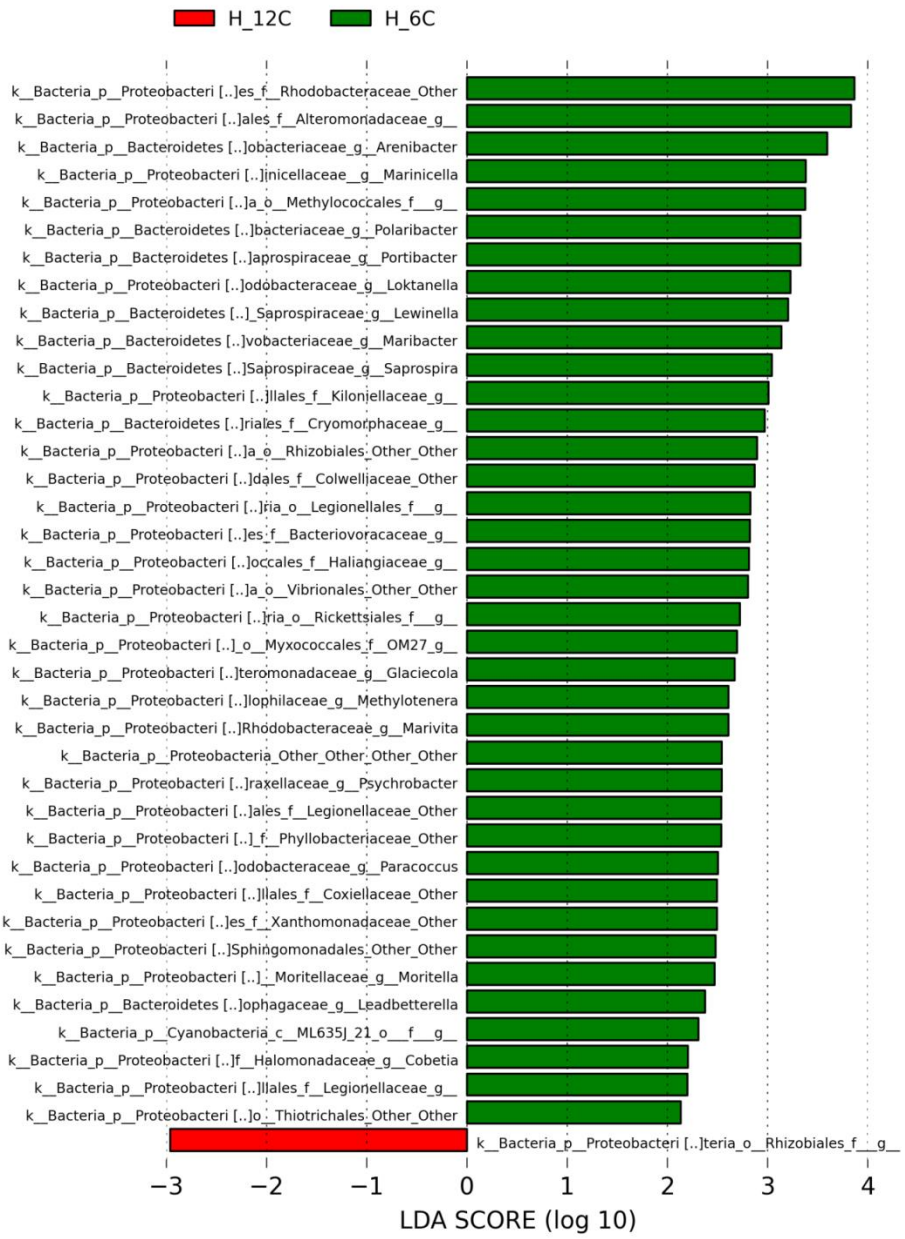


Appendix 15. LEfSe comparison of lesion samples of *Homarus americanus* kept in 12ºC and 6ºC water at the second sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 6ºC environment in comparison lesion samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 12ºC environment compared to lesion samples from the 6ºC environment.

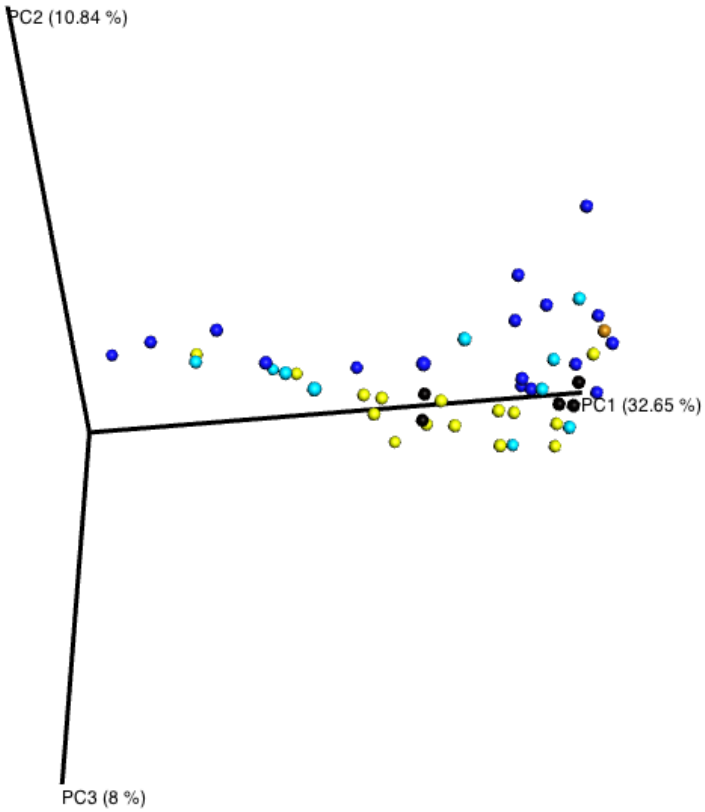
Appendix 16. LEfSe comparison of lesion samples of *Homarus americanus* kept in 18ºC and 6ºC water at the second sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 6ºC environment in comparison lesion samples from the 18ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 18ºC environment compared to lesion samples from the 6ºC environment.



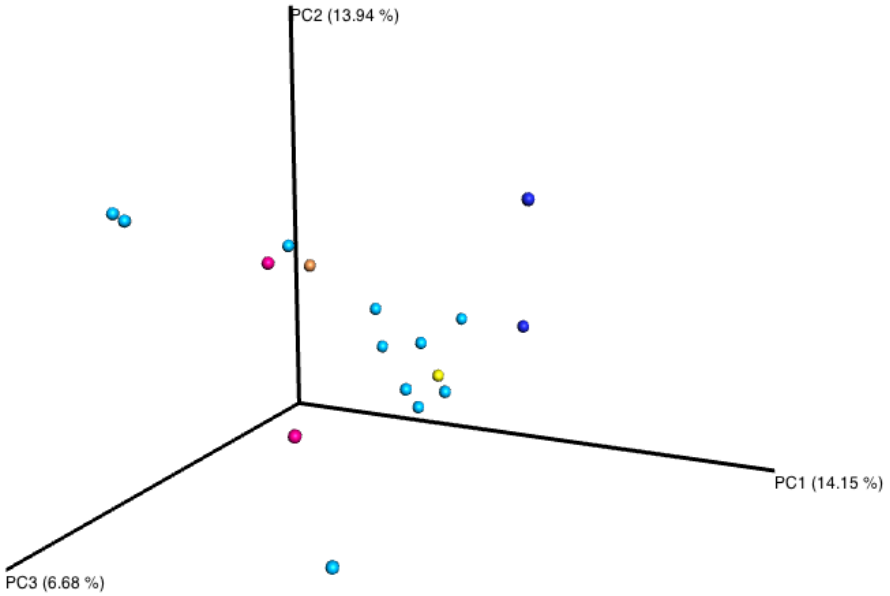
Appendix 17. LEfSe comparison of lesion samples of *Homarus americanus* kept in 18ºC and 12ºC water at the second sampling point. Green bars represent the LDA scores of significantly different bacteria in lesion samples from the 18ºC environment in comparison lesion samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in lesion samples from the 12ºC environment compared to lesion samples from the 18ºC environment.



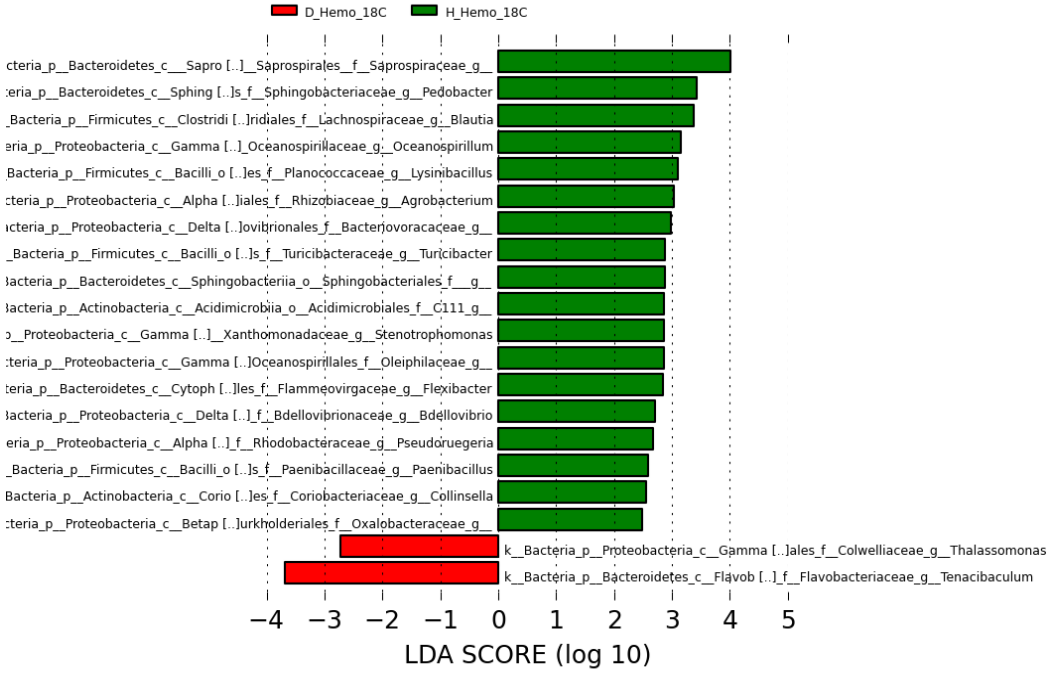
Appendix 18. LEfSe comparison of healthy samples of *Homarus americanus* kept in 12ºC and 6ºC water at the second sampling point. Green bars represent the LDA scores of significantly different bacteria in healthy samples from the 6ºC environment in comparison healthy samples from the 12ºC environment. The red bar represents the LDA scores of significantly different bacteria in healthy samples from the 12ºC environment compared to healthy samples from the 6ºC environment.



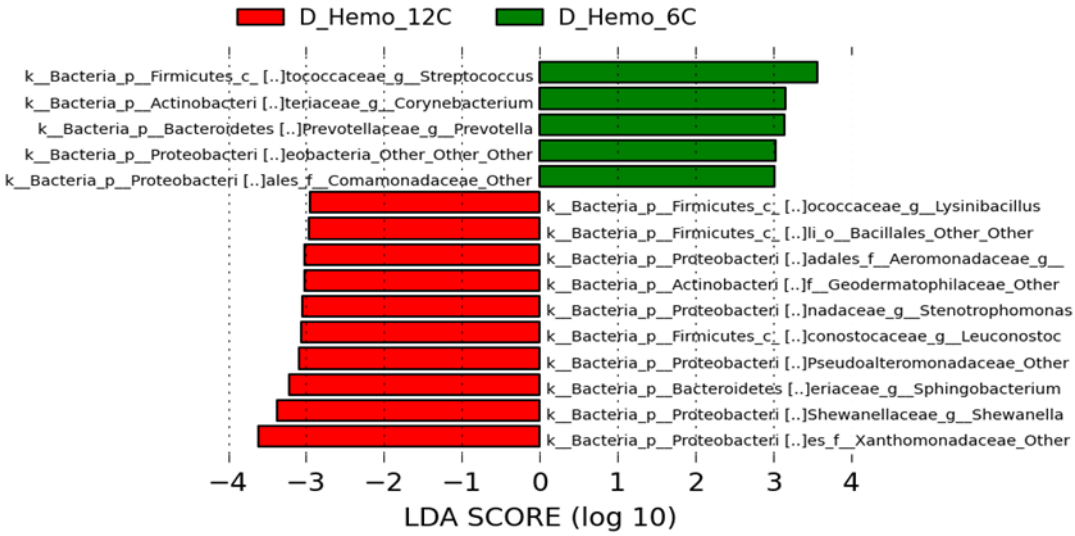
Appendix 19. PCoA of healthy and diseased hemolymph samples in different temperature environments from the first sampling period. Bacteria from diseased lobster hemolymph in 12ºC water = Light Blue, diseased samples from lobster hemolymph in 18ºC water = Yellow, from diseased lobster hemolymph in 6ºC water = Dark Blue, healthy hemolymph from lobsters in 12ºC water = Orange, healthy hemolymph from lobsters in 18ºC water = Black. Principle Component 1 (PC1) explains 32.65% of the variance, Principle Component 2 (PC2) explains 10.84% of the variance, and Principle Component 3 (PC3) explains 8% of the variance. In sum, the Principle Components explains 51.40% of the variance of the dataset.



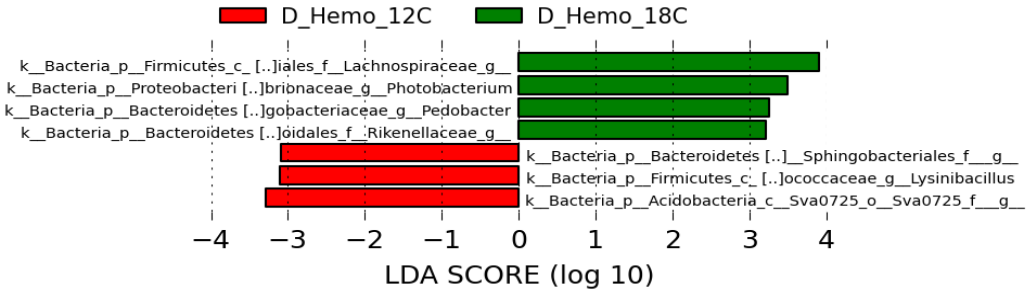
Appendix 20. PCoA of healthy and diseased hemolymph samples in different temperature environments from the second sampling period. Bacteria from diseased lobster hemolymph in 12ºC water = Dark Blue, diseased samples from lobster hemolymph in 18ºC water = Pink, from diseased lobster hemolymph in 6ºC water = Light Blue, healthy hemolymph from lobsters in 12ºC water = Orange, healthy hemolymph from lobsters in 6ºC water = yellow. Principle Component 1 (PC1) explains 14.15% of the variance, Principle Component 2 (PC2) explains 13.94% of the variance, and Principle Component 3 (PC3) explains 6.68% of the variance. In sum, the Principle Components explains 34.77% of the variance of the dataset.



Appendix 21. LEfSe comparison of hemolymph samples of *Homarus americanus* kept in 18ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in healthy hemolymph in comparison to diseased hemolymph. The red bar represents the LDA scores of significantly different bacteria in diseased hemolymph compared to healthy hemolymph.

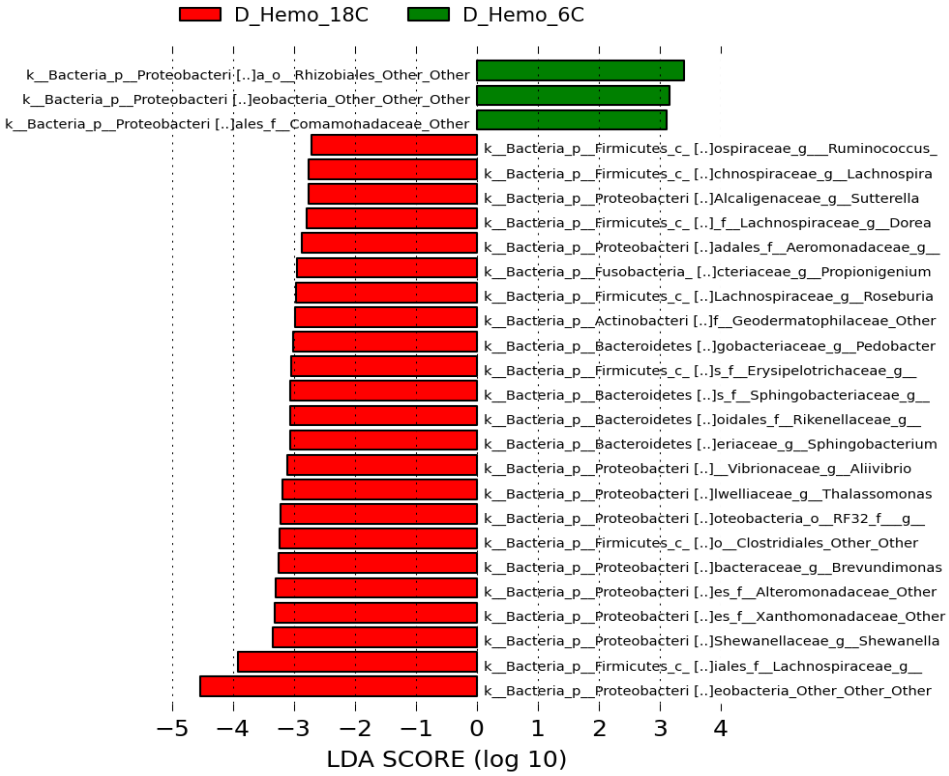


Appendix 22. LEfSe comparison of hemolymph samples of diseased *Homarus americanus* kept in 12ºC and 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in diseased hemolymph of lobsters kept in 6ºC water compared to those kept in 12ºC water. The red bar represents the LDA scores of significantly different bacteria in diseased hemolymph lobsters kept in 12ºC water compared to those kept in 6ºC water.



Appendix 23. LEfSe comparison of hemolymph samples of diseased *Homarus americanus* kept in 18ºC and 12ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in diseased hemolymph of lobsters kept in 18ºC water compared to those kept in 12ºC water. The red bar represents the LDA

scores of significantly different bacteria in diseased hemolymph lobsters kept in 12ºC water compared to those kept in 18ºC water.



Appendix 24. LEfSe comparison of hemolymph samples of diseased *Homarus americanus* kept in 18ºC and 6ºC water at the first sampling point. Green bars represent the LDA scores of significantly different bacteria in diseased hemolymph of lobsters kept in 6ºC water compared to those kept in 18ºC water. The red bar represents the LDA scores of significantly different bacteria in diseased hemolymph lobsters kept in 18ºC water compared to those kept in 6ºC water.

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