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*Research Article*

# Effect of pH on the bacterial community present in larvae and spat of *Crassostrea gigas*

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**ABSTRACT.** Changes in marine environments, including pH changes, have been correlated to alterations in the physiology and disease susceptibility of cultured organisms at the early stages of development. In this study, high-throughput sequencing of the V3-V4 region of the 16S rRNA gene was performed to evaluate the bacterial biodiversity of *Crassostrea gigas* pediveliger larvae and spat under acidic stress compared to that of larvae at normal pH value. The evaluation was performed in an experimental system with continuous water flow and pH manipulation by $CO_2$ bubbling to simulate acidification (pH 7.38 ± 0.039), using the current ocean pH conditions (pH 8.116 ± 0.023) as a reference. The results indicated that the bacterial communities associated with both pediveliger larvae and spat were modified in response to acidic conditions. The families Rhodobacteraceae and Campylobacteraceae were the most affected by the change in pH, with increases in Vibrionaceae in pediveliger larvae and Planctomycetaceae and Phyllobacteriaceae in spat detected. The results of this study demonstrate that the bacterial communities associated with *C. gigas* pediveliger larvae and spat are responsive to changes in ocean acidification.

Ke**ywords:** *Crassostrea gigas*; pediveliger larvae; spat; ocean acidification; bacterial communities

## INTRODUCTION

Stress factors that are directly caused by climate change are of great importance to the marine system, particularly the associated decrease in pH (Kroeker *et al.*, 2010, 2013). Ocean acidification is a result of the increase in $CO_2$ levels and is an effect of global climate change (Caldeira & Wickett, 2005), with a decrease of 0.3-0.5 pH units estimated to occur by the end of the century (IPCC, 2013). Alterations to the oceans, including changes in temperature and increases/ decreases in $CO_2$ concentrations, disrupt the host-pathogen-environment balance, and have serious consequences for the survival of organisms (Burge *et al.*, 2014). Ocean acidification affects organisms in variety of ways, including causing changes in calcium deposition, acid-base balance, energy distribution and metabolic equilibrium, embryonic development, growth and reproductive success (Allen & Burnett, 2008; Parker *et al.*, 2009), with these changes having been observed in crustaceans (Travers *et al.*, 2009), corals (Haarvell *et al.*, 2002) and mollusks (Cooley & Doney, 2009).

Bivalve mollusks belong to a group of benthic and calcifying organisms that have been studied to understand the effect of acidification and climate change (Kurihara *et al.*, 2007; Barton *et al.*, 2012; Waldbusser *et al.*, 2013). The cultivation of these orga-

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nisms is a profitable economic activity throughout the world (Barton *et al.*, 2015). The larval stage is among the most susceptible stages of mollusk development and is greatly affected by the chemistry of carbonates in seawater since mollusk larvae structure their larval shell (prodissoconch I) with the most soluble calcium carbonate (aragonite) (Stenzel, 1964).

The combination of stressors, such as low pH and exposure to bacterial pathogens, can have detrimental effects on the normal physiological processes of mollusks, including energy allocation and survival (Dorfmeier, 2012). Krause *et al.* (2012) reported that small changes in pH have a direct effect on the composition of the bacterial community, and the composition of microbial communities can be strongly affected and regulated by the external environment (Fierer & Jackson, 2006).

Stress can alter microbial communities, causing an ecological succession and the appearance of pathogenic microorganisms that can cause disease (Zilber-Rosenberg & Rosenberg, 2008; Asmani *et al.*, 2016). Thus, it is necessary to study the bacterial communities associated with organisms that are under environmental stress. Therefore, the objective of this study was to analyze the microbiota of *Crassostrea gigas* larvae and juveniles grown at a low pH value (7.38) to test whether there is an effect on the structure of the resident communities of the host organism.

## MATERIALS AND METHODS

### Acidification system of seawater

The pH of seawater was manipulated following the $CO_2$ bubbling method described by Fangue *et al.* (2010). Clean air was mixed with $CO_2$ using Micro-Trak 101® to achieve a pH of 7.38 and Smart-Trak 100C® mass flow controllers (MFCs) to control the flow of $CO_2$ and air, respectively (Sierra Instruments, Monterey CA, USA). The flow of $CO_2$ and air were calibrated to be maintained at 5.00 and 2.63 L $min^{-1}$, respectively. For the modified pH treatment (pH 7.38), $CO_2$ and air mixture were incorporated into filtered seawater (1 µm membrane and sterilized with ultraviolet light) in a mixing vessel rather than by bubbling these gases directly into the culture vessels. For the treatment under environmental conditions (pH 8.1), the water was pumped directly from a 1,000 L reservoir of filtered seawater. The $CO_2$-air and filtered seawater were mixed in a 19 L high-density polyethylene bucket using a submersible aquarium pump to provide vigorous circulation, and a Venturi device was used to direct the filtered seawater through a small orifice, creating negative pressure that aspirated the gas mixture into a very fine stream of bubbles for efficient mixing.

### Experimental design

Pediveliger larvae of *Crassostrea gigas* Thunberg, 1793 (eyespots and active foot) with an average initial size of 335 ± 6 µm were subjected to two pH treatments (8.1 and 7.38). Larvae were placed in 2 inch PVC tubes with a 180 µm mesh Nitex$^{MR}$ bottom, without substrate, allowing the pediveliger larvae to attach to the mesh bottom and the walls of the tube. The larvae were seeded at an initial density of 5 larvae $mm^{-2}$, and the tubes were placed in 3 L plastic containers in triplicate, with a continuous downwelling-type flow of seawater (0.5 L $min^{-1}$) that had been filtered through a 1 µm membrane and sterilized by UV radiation, with a temperature of 25 ± 1°C and a salinity of 40. The larvae were incubated for 24 h, and the adhered organisms were directly quantified with the aid of magnifying glasses. The non-adhered organisms were placed in the tubes for an additional 24 h, and samples were withdrawn for bacterial microbiota fingerprinting (16S rRNA metabarcoding) at 0, 18 and 48 h after the start of the experiment.

A second experiment was performed by subjecting *C. gigas* spat (1,450 ± 220 µm) to two pH values (8.1 and 7.38) for seven days. Five hundred spat were placed in 3 L tanks in triplicate, and seed samples were withdrawn at 0, 48 and 168 h to evaluate the bacterial community. The larvae were fed a diet of the haptophyte *Isochrysis galbana* at cell densities of 30,000 cells $mL^{-1}$.

### Water quality

The pH was measured daily with a Beckman 32 pH m (accuracy ± 0.001) that was calibrated before each use. The total alkalinity (TA) was estimated by acid titration using the volumetric method with a phenolphthalein endpoint and methyl orange as an indicator, with sulfuric acid ($H_2SO_4$) used as the titrant (NOM-AA-36-1980). The salinity and temperature were determined with a YSI Model 85 multi-parameter measurement instrument. The chemical parameters of the carbonate system were calculated using the program $CO_2$SYS v.01.05 (Lewis & Wallace, 1998), applying the dissociation constant of Mehrbach *et al.* (1973) modified by Dickson & Milero (1987). Information on the specific values of the elements of the carbonate system, salinity and temperature in the experimental treatments are summarized in Table 1.

### DNA isolation

DNA was isolated from larvae and spat by filtering 10 mL of culture through a 300 µm sieve, which was co-

**Table 1.** Water chemistry parameters during the experiment. Salinity, temperature and pH were determined in samples of seawater from the experimental units every 48 h. Total alkalinity (µmol kgSW$^{-1}$), $pCO_2$ (µatm), $HCO_3$ (µmol kgSW$^{-1}$), $CO_3$ (µmol kgSW$^{-1}$), $\Omega_{cal}$ and $\Omega_{ar}$ were calculated using the $CO_2$SYS program. The data are presented as the means ± standard deviation.

| | T °C | Salinity | pH | TA (µmol kgSW$^{-1}$) | $pCO_2$ (µatm) | $HCO_3$ (µmol kgS W$^{-1}$) | $CO_3$ (µmol kgSW$^{-1}$) | Ωcal | Ωar |
|---|---|---|---|---|---|---|---|---|---|
| Control pH | 25 ± 1.0 | 40 ± 0.8 | 8.11 ± 02 | 2,030.42 | 263.9 ± 24.1 | 1432.45 ± 11.56 | 231.86 ± 22.15 | 5.343 ± 0.52 | 3.536 ± 0.34 |
| Low pH | | | 7.384 ± 04 | 2,008.49 | 1,864.46 ± 234.0 | 1,865.29 ± 12.87 | 55.96 ± 5.42 | 1.29 ± 0.13 | 0.853 ± 0.09 |

llected in 1.5 mL sterile tubes. For juveniles, 50 organisms were collected. All of the samples were washed with sterile seawater followed by the addition of 1 mL of 96% ethanol, after which the samples were stored at -20°C.

DNA was extracted using a combination of lysis buffer and a commercial kit DNA purification kit (Promega) following the manufacturer's instructions. The *C. gigas* larvae and spat were homogenized by mechanical disruption and incubated for 1 h at 37°C with a lysis buffer containing Tris-EDTA-SDS (100 mM NaCl, 50 mM Tris [pH 8], 100 mM EDTA [pH 8.0], sodium dodecyl sulfate 1% and 100 µL of lysozyme (50 mg mL$^{-1}$)). The homogenized sample was then incubated for 12 h at 65°C with 20 µL of proteinase K (20 mg mL$^{-1}$). Following lysis, 100 µL of 5 M NaCl was added, the mixture was stirred, and 80 µL of a CTAB/NaCl solution (10% CTAB in 0.7 M NaCl) was added and incubated at 65°C for 10 min. Followed by the extraction of DNA with the kit DNA purification Kit (Promega).

### Library preparation and Illumina MiniSeq sequencing

Twenty-five nanograms of DNA was used to generate amplicons spanning the V3 and V4 hypervariable regions of the bacterial 16S rRNA gene using the following primers: F-V3f 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG ACY CCT ACG GGR GGC AGC AG-3' and V4r 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3' (Klindworth *et al.*, 2013). Subsequently, the purified amplicon was used as template to amplify the V3 region of the 16S rRNA gene using the following primers: forward 5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG ACY CCT ACG GGR GGC AGC AG-3' and reverse 5'-GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTT ACC GCG GCT GCT GGC AC-3'. The total length of the final amplicon was ~300 bp (Huse *et al.*, 2008).

Indexed adapters were added to the ends of the 16S rRNA gene amplicons via limited-cycle PCR. The obtained DNA libraries were fluorometrically quantified using a Qubit dsDNA High Sensitivity Assay kit (Life Technologies). Subsequently, the DNA libraries were multiplexed and loaded onto an Illumina MiniSeq instrument (Illumina Biosystems, USA) following the manufacturer's instructions for 2×150 paired-end sequencing.

### Data analysis

The sequences were decompressed, cleaned, assembled and converted to FASTA format. First, the data sequences were filtered to remove the forward and reverse primers and the barcode sequences. To minimize the effects of random sequencing errors, low-quality (<Q20) fragments and sequences shorter than 150 bp were removed, as well as sequences that contained any undetermined nucleotides (N) and sequences >150 bp. Following denoising and chimera sequence removal, operational taxonomic units (OTUs) were classified using the EzBiocloud classifier (Yoon *et al.*, 2017). The α-diversity indices of the bacterial communities (Shannon, Simpson, evenness indices) were calculated using PAST 3.0 (Hammer *et al.*, 2001). The β-diversity of bacterial communities associated with *C. gigas* pediveliger larvae and spat were analyzed with non-metric multidimensional scaling (NMDS) plots using PAST 3.0 (Hammer *et al.*, 2001). The abundances of specific taxa in the bacterial communities were visualized used a shaded plot with a dendrogram representing Bray-Curtis similarities between bacterial taxa and the pH treatment.

## RESULTS

### Sequencing

The high-throughput sequencing analysis yielded 157,070 sequences for the 25 samples of oysters analyzed, which were assigned to 2,868 OTUs, including 1,578 OTUs for spat (Archaea, 0; Bacteria, 1,533; and Eukarya 45) and 1,355 OTUs for larvae (Archaea, 0; Bacteria, 1,290; and Eukarya, 65). Phylogenetic analysis of the sequences using the EzBiocloud classifier identified 14 phyla present in the

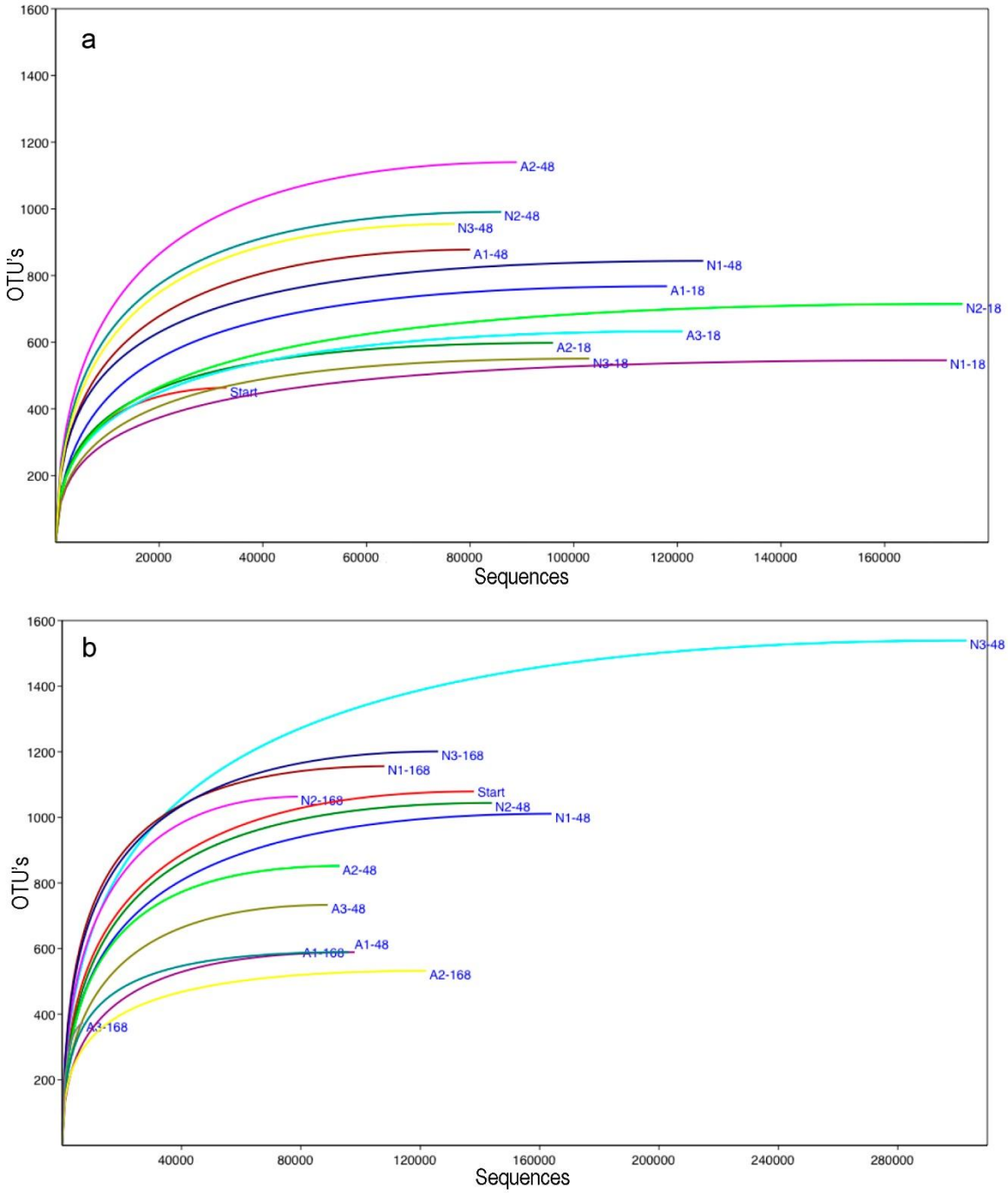


**Figure 1.** Rarefaction curves of the bacterial communities based on the 16S rRNA gene sequences generated using MiniSeq Illumina sequencing. a) Samples of pediveliger larvae, b) samples of a spat of *Crassostrea gigas.*

*Crassostrea gigas* larvae and spat samples (Acidobacteria, Actinobacteria, Bacteroidetes, Calditrichaeota, Chlamydiae, Chloroflexi, Chlorobi, Cyanobacteria, Firmicutes, Parcubacteria, Peregrinibacteria, Planctomycetes, Proteobacteria, and TM6). The results of a rarefaction analysis were of the OTUs suggested that the sequencing depth was adequate (Fig. 1).

## Bacterial communities in pediveliger larvae

The Proteobacteria phylum dominated in pediveliger larvae with 82.97% of the OTUs; Alphaproteobacteria and Gammaproteobacteria were the most dominant classes with 52.15 and 22.29%, respectively. The β-diversity analysis of all datasets showed that bacterial diversity changed after 18 h and varied depending on

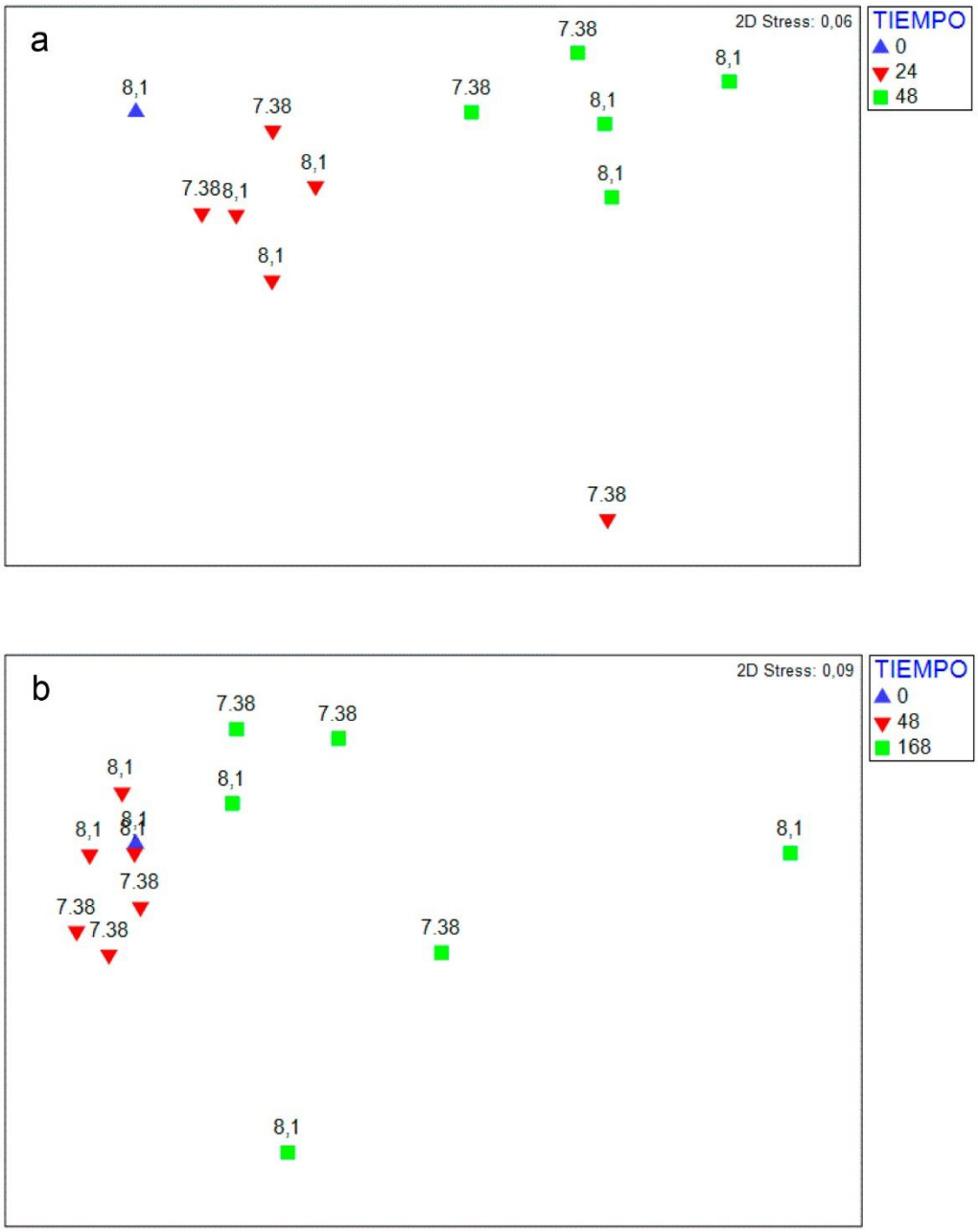


**Figure 2.** Nonmetric multidimensional scaling (NMDS) plots of the bacterial community structure of a) larvae and b) spat of *Crassostrea gigas* based on pairwise similarity estimate (Algorithm Bray-Curtis). Labels 8.1 and 7.38 indicate the pH treatment and the different culturing times (Start (▲), 18 h (♦), and 48 h (■), for larvae; and Start (▲), 48 h (▼) and 168 h (■) for spat).

the pH value of the water. The NMDS analysis indicated that changes occurred from 0-18 h and after 48 h, with a decrease observed in the number of families (Fig. 2a), as a reduction was observed in the percentages of the primary bacterial families present after 48 h of exposure to a low pH (7.38). Rhodobacteraceae dominated the bacterial community of the pediveliger larvae, exhibiting a relative abundance of 56.20%. Campylobacteraceae was the second most abundant family in all of the assayed samples and decreased in abundance between 18 and 48 h for pediveliger larvae. Vibrionaceae was the third most

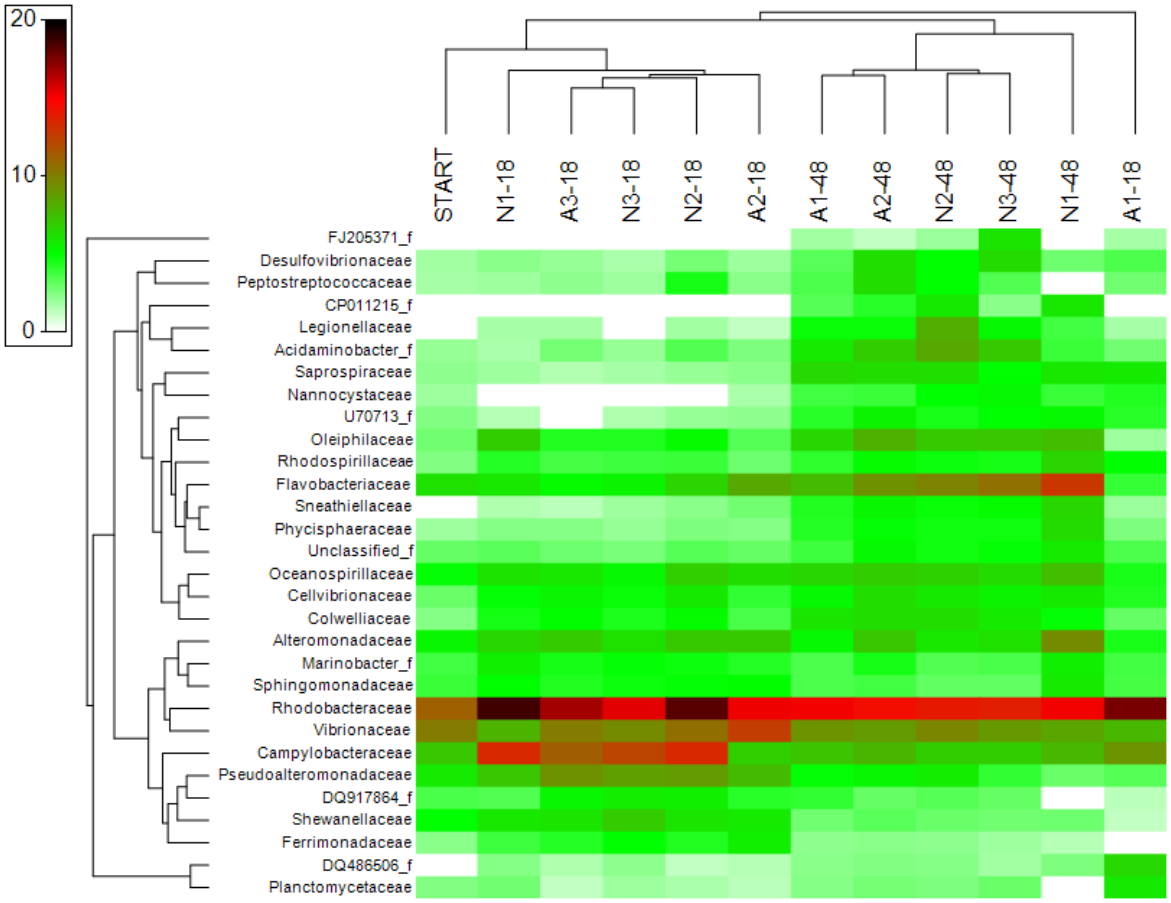


**Figure 3.** Shade plot of the 20 relevant families based on Bray-Curtis similarities in *Crassostrea gigas* pediveliger larvae cultured at different pH values (7.38 A1-A3: acidification, and 8.1 N1-N3: normal) at 0, 18 and 48 h. Color bar in the left indicate percentage abundance.

**Table 2.** Diversity indices of bacterial communities associated with *Crassostrea gigas* pediveliger larvae and spat. A: indicates the acidification treatment (pH 7.38), and C: indicates the normal treatment (pH 8.1) at different culturing times (pediveliger larvae: 0, 18 and 48 h; spat: 0, 48 and 168 h).

| | Pediveliger larvae | | | | |
|---|---|---|---|---|---|
| | | A | C | A | C |
| Diversity index | 0 h | 18 h | 18 h | 48 h | 48 h |
| Shannon H | 4.05 | 3.85 ± 0.22 | 3.48 ± 0.15 | 4.71 ± 0.39 | 4.61 ± 0.14 |
| Evenness e^H/S | 0.12 | 0.07 ± 0.02 | 0.05 ± 0.01 | 0.11 ± 0.02 | 0.11 ± 0.01 |
| Simpson 1-D | 0.96 | 0.93 ± 0.02 | 0.91 ± 0.01 | 0.97 ± 0.02 | 0.97 ± 0.01 |
| | Spat | | | | |
| | | A | C | A | C |
| | 0 h | 48 h | 48 h | 168 h | 168 h |
| Shannon H | 4.49 | 3.79 ± 0.41 | 4.38 ± 0.33 | 3.98 ± 0.65 | 4.93 ± 0.10 |
| Evenness e^H/S | 0.08 | 0.06 ± 0.01 | 0.07 ± 0.01 | 0.14 ± 0.14 | 0.13 ± 0.01 |
| Simpson 1-D | 0.97 | 0.90 ± 0.03 | 0.95 ± 0.02 | 0.93 ± 0.05 | 0.98 ± 0.00 |

abundant family and was detected at high levels the in pH 7.38 treatment at 48 h (7.67%) (Fig. 3). The values of the biodiversity indices (Table 2) indicated a high level of diversity for all of the samples, with marked differences observed between the treatments for the Shannon and Simpson 1-D indices, with the highest

values observed at 48 h in the pH 7.38 treatment ($H$ = 4.709, $S_{1\text{-}D}$ = 0.972).

**Bacterial communities in *Crassostrea gigas* spat**

The phylum Proteobacteria dominated spat, accounting for 83.3% of the OTUs, with α-proteobacteria (35.81%) and Gammaproteobacteria (34.88%) being the most dominant classes. The non-metric multidimensional scaling (NMDS) analysis was performed using the OTUs in each sample and was calculated to determine the similarity of the bacterial communities. The results revealed the presence of distinct microbial taxa associated with the pH 8.1 (normal) and pH 7.38 (acidification) samples after 168 h of exposure (Fig. 2b).

An increase in the relative abundances of specific taxa in the samples was observed at 168 h in compared to 0 and 48 h, although the differences were not substantial. Rhodobacteraceae dominated the sequencing libraries from spat (relative abundance of 68.20%), although the relative abundance was lower at 168 h (49.28%) than at 0 (84.7%) and 48 h (82.58 and 84.10% at the normal pH and pH 7.38, respectively).

The relative abundances of Planctomycetaceae and Phyllobacteriaceae increased at 168 h compared to that observed at 0 (2 and 0.71%) and 48 h (2.13 and 0.45%), with the highest levels observed at pH 7.38 (20.32 and 9.16%), respectively. The families Hyphomicrobiaceae, Sphingomonadaceae, and Rhodospirillaceae were more abundant after 168 h than at 0 h, representing more than 1% of the community (Fig. 4).

The values obtained for the biodiversity indices (Table 2) indicated high levels of diversity for all of the samples, with marked differences observed between the treatments for the Shannon and Simpson 1-D indices. In addition, the highest diversity values were observed in the samples from the normal pH ($H$ = 4.929, $S_{1\text{-}D}$ = 0.978) compared to those from the acidic treatment (H = 3.982, $S_{1\text{-}D}$ = 0.926) at 168 h.

## DISCUSSION

In the present work, the bacterial communities of *Crassostrea gigas* pediveliger larvae and spat under pH stress were analyzed, simulating the conditions present in estuaries where oysters are grown and of a hatchery located in northwestern Mexico, where the water has been reported to have a pH of 7.4 (P. Danigo, *pers. comm.*). Although these oceanic pH values are not expected to be reached until the year 2300 (Caldiera & Wickett, 2005; Doney *et al.*, 2009) the effects of ocean acidification are already being observed in these oyster growing areas and hatcheries in northwest Mexico (Páez-Osuna *et al.*, 2016; Rodríguez-Quiroz *et al.*, 2016) and in other parts of the world (Cai *et al.*, 2011; Cornwall *et al.*, 2013; Ginger *et al.*, 2013).

The results of this study showed that the bacterial communities in both pediveliger larvae and spat were modulated when subjected to acidic conditions. The larvae exhibited a remarkably higher bacterial diversity at the lower pH value, whereas the effect was opposite in spat since a reduction in diversity was observed. Counterproductive effects have been observed in oyster larvae that affect their survival, growth and calcification (Barton *et al.*, 2012, 2015; Kroeker *et al.*, 2013; Waldbusser *et al.*, 2015), and larvae with valves composed of aragonite are generally the most susceptible to dissolution under lower pH conditions (Ries *et al.*, 2009; Kroeker *et al.*, 2013). In this study, we observed an effect on the microbiota present in *C. gigas* larvae and spat that indicated a direct negative effect on some of the most dominant taxa. Oceanic acidification increases the colonization of pathogenic organisms in marine organisms because it increases the susceptibility of hosts by modifying the normal microbiota (Mouchka *et al.*, 2010).

Microorganisms adapt quickly to environmental changes due to resistance gene transfer, even in a homeostatic environment (Ochman *et al.*, 2000). Recently, *C. gigas* has faced new challenges to its survival, including the looming threat of ocean warming/acidification and the proliferation of pathogens. The decrease in pH was observed to have a negative effect on the bacterial community associated with both pediveliger larvae and spat, primarily affecting the relative abundance of Rhodobacteraceae, with that of Campylobacteraceae only affected in larvae. Rhodobacteraceae and Campylobacteraceae are considered to be susceptible to low pH values (Krause *et al.*, 2012), resulting in alterations in their abundances. Similar trends were also observed in coral reefs (Witt *et al.*, 2011). Rhodobacteraceae is considered to be an important bacterial lineage in marine environments, is naturally present in biofilms on flora and fauna (Elifantz *et al.*, 2013). Campylobacteraceae has been shown to grow over a wide pH range and can survive in contaminated food and support stomach transit (Murphy *et al.*, 2006; Reid *et al.*, 2008). Bivalve mollusks harbor an abundant bacterial microbiota that modulates in response to environmental factors. The influence of the microbiota on the health of host organisms has been documented as a crucial aspect in the development of diseases (Ruby *et al.*, 2004; Zhen *et al.*, 2017). Asmani *et al.* (2016) indicated that normal larval microbiota in *C. gigas* is primarily composed of Rhodobacteraceae (α-Proteobacteria) and a few members of the family Vibrionaceae.

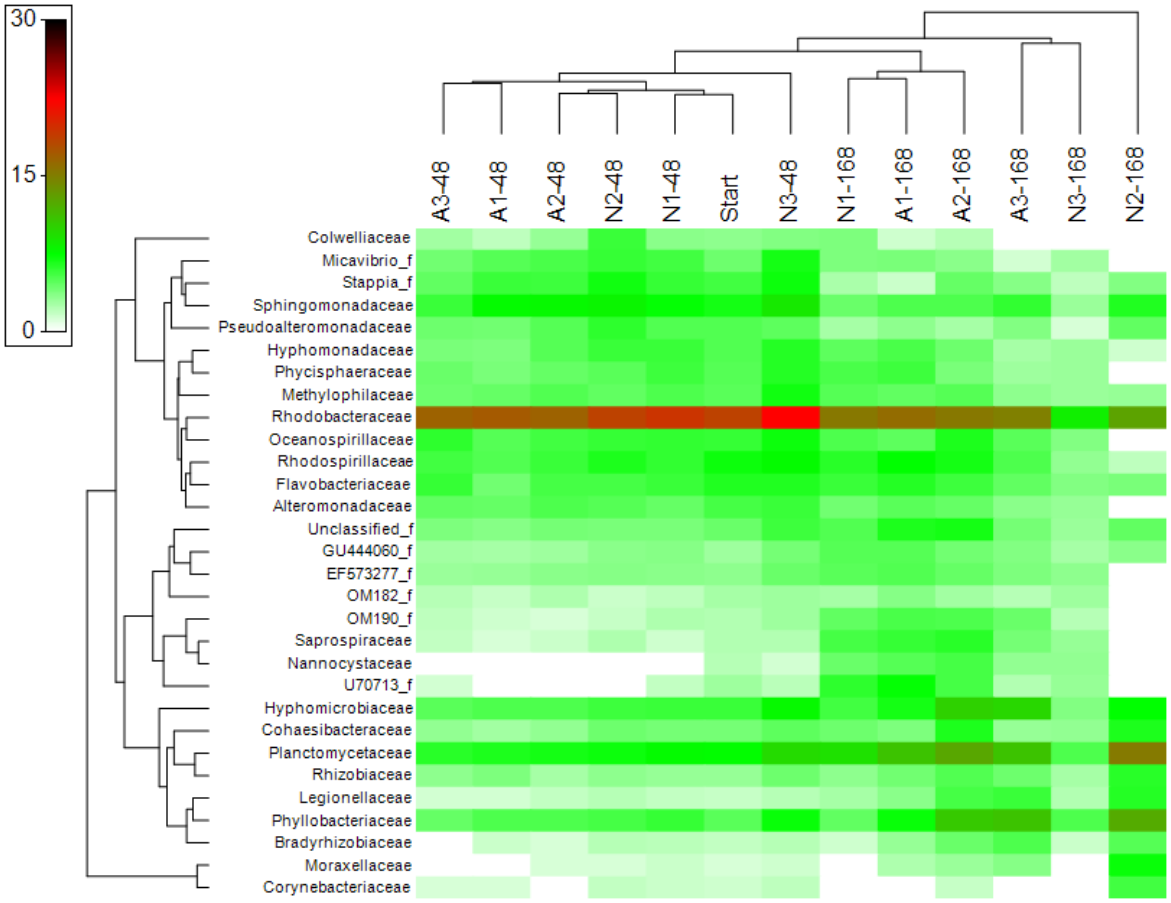


**Figure 4.** Shade plot of the 20 relevant families based on Bray-Curtis similarities in *Crassostrea gigas* spat cultured at different pH values (7.38 A1-A3: acidification; and 8.1 N1-N3: normal) at 0, 48 and 168 h. Color bar in the left indicate percentage abundance.

In this study, an increase in Vibrionaceae at a low pH value in pediveliger larvae was observed after 48 h of culturing compared to the normal pH value but not in the initial samples. This increase represents a negative effect on the development larvae, since a primary problem facing the culture of bivalve mollusks is the appearance of larval vibriosis, which can cause high larval mortality (Sainz-Hernández & Maeda-Martínez, 2005; Travers *et al.*, 2015; Asmani *et al.*, 2016). Furthermore, an increase of the virulence of *Vibrio tubiashii* toward the blue mussel has been observed at low pH (Asplund *et al.*, 2014) as well as an increase in the production of secondary metabolites and stress (Vega-Thurber *et al.*, 2009). Similarly, an increase in Vibrionales in the seaweed *Sargassum muticum* (Aires *et al.*, 2018) and corals (Meron *et al.*, 2012) has been observed. However, not all bacterial groups are susceptible to low pH, and only for those taxa that are key in the development of biofilms and are consider primary colonizers of marine environments, such as Rhodobacteraceae, has this effect been evidenced in bacterial communities present in seawater at lower pH (7.67) (Krause *et al.*, 2012). The decrease in members of this family may have implications for the stability of these communities, as it has been shown to be an initial colonizing family of marine bacterial communities (Elifantz *et al.*, 2013). Thus, this type of change in bacterial communities present in seawater can represent a risk for the development of the microbiota present in cultured organisms, since there is a direct relationship between the community present in the culture water and the microbiota of the organisms, as demonstrated by Trabal *et al.* (2014).

The families Planctomycetaceae and Phyllobacteriaceae exhibited the highest overall growth in a spat at pH 7.38 at 168 h. However, the family Rhodobacteraceae decreased at low pH over the same period and under the same treatment. Thus, the community structure, diversity, and dominance of families were directly influenced by culturing time and pH.

The results of this study demonstrate that bacterial communities change under low pH conditions. Specific

bacterial taxa exhibited significant shifts in abundance under acidic conditions, such as Vibrionaceae and Planctomycetaceae, and the levels Rhodobacteraceae and Campylobacteraceae. Meron *et al.* (2011) reported an increase in bacteria that possess antimicrobial activity in coral maintained at a low pH of 7.6. It is interesting to note that most of these antimicrobial active bacterial belonged to the family Vibrionaceae, suggesting that this may be one of the mechanisms that allow these species to increase their distribution within the microbial community.

In summary, the results of this study demonstrate that the microbiota associated with *C. gigas* pediveliger larvae and spat are responsive to changes in acidifycation. The abundances of families such as Vibrionaceae and Planctomycetaceae exhibited signifi-cant changes under conditions of acidification and adverse effects toward other groups, including Rhodobacteraceae and Campylobacteraceae.

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