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Molecular Characterisation of Microbial Diversity Associated with Oysters within a Commercial Oyster Farm

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Abstract

The diversity of microbial communities associated with oysters within a commercial mariculture system was examined using molecular analysis of 16S rRNA Illumina sequencing. The aim of the study is to identify microbial diversity using molecular-based techniques, and compare the results obtained using three sampling points. Results showed, the microbial diversity associated with farmed oysters was dominated by *Vibrio* spp., *Photobacterium* spp., and *Pseudoalteromons* spp., while *Psychrilyobacter* spp., *Prolixibacter* spp., *Cytophaga* spp., *Planococcus* spp., and *Exiguobacterium* spp. were minor in abundance. These findings provide valuable information on the microbial community, demonstrating the microbial diversity in oysters and its major abundant species.

Keywords*:* Oyster; microbial diversity; 16S rRNA gene; illumine; sequencing

Introduction

The microbiotas of oysters are shown to be dependent on bacterial colonization during feeding intake and also environmental conditions (Garland, Nash, Summer, & McMeekin, 1983), this is almost similar with other marine animals such as fish (Neuman *et al.*, 2016; Olafsen, 2001; Ringø & Birkbeck, 1999; Ringø, Sperstad, Myklebust, Refstie, & Krogdahl, 2006; Ringø, Strøm, & Tabachek, 1995; Zarkasi *et al.*, 2016). The need for a better understanding in microbial composition associated with oyster and influenced by the environment and feed intake has led to many studies and investigations on microbial communities and their diversity and dynamics (Deepanjali, Kumar, & Karunasagar, 2005). According to some researchers, the most dominant bacterial genera isolated from oysters are *Vibrio* spp., *Pseudomonas* spp., *Achromobacter* spp., *Flavobacterium* spp., and *Cytophaga* spp. (Colwell & Liston, 1960; Dupont, Jehl-Pietri, & Mnard, 1992; Pujalte, Ortigosa, Macián, & Garay, 1999). When molecular-based techniques were applied, the most common bacterial genera found were similar with traditional methods, but other microorganisms such as cyanobacteria were clearly identified (Green & Barnes, 2010).

Molecular-based methods like PCR and 16S rRNA sequencing for population analyses have been applied by many studies this day compared to the traditional methods such as conventional culture-based techniques, that use cultivation and selective/non-selective media, followed by isolation and phenotypic characterisation (Romero, Garcia-Varela, Laclette, & Espejo, 2002; Garnier, Labreuche, Garcia, Robert, & Nicolas, 2007). This molecular approach is highly accepted in the study of microbial ecology and has become more advanced (Lyons, Turnbull,

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Dawson, & Crumlish, 2016; Zarkasi *et al.*, 2016). The transition from traditional methods to advance molecular-based methods is due to several factors which limit the traditional methods such as being time consuming, very selective, not providing the entire microbial diversity and not able to detect certain microbiota such as unculturable microorganisms (Hovda, Lunestad, Fontanillas, & Rosnes, 2007; Zarkasi *et al.*, 2014). The aim of this study is to use 16S rRNA sequencing to describe the microbial diversity and community dynamic of farmed oysters. In addition, we wanted to identify the number of bacteria present using a culturable-based approach.

Materials and Methods

Sample Collection

Pacific oysters (*Crassostrea gigas*) were collected from an oyster farm in Clifton Beach, Sandford, Tasmania, Australia during low tides. Samples were collected according to their size and age; and only 36 matured oyster were collected randomly from 3 different cage rows identified as Row A, Row B and Row C. The oyster samples were then transported to the laboratory on ice and processed within three hours (Neuman *et al.*, 2016). Other farm data obtained include water temperature.

Microbial Enumeration and Cultivation

The oyster samples were cleaned using a brush under cold running tap water to remove any sand and mud on the oysters' shells. Then, the raw oysters were aseptically shucked using a sterile oyster knife with the intact bodies and liquor. They were placed into a sterile full filter blender bag and massaged through the bag by hand for 1 minute to separate the excess shelf from the liquor and intact bodies. Then, the samples were transferred to a new full filter blender bag to remove any remaining shells. This process is important to prevent leakage of the plastic bag during the homogenisation process. A liquor of 3% sea salt peptone water (around 450ml) was added and homogenised by the stomacher machine for two minutes (Green & Barnes, 2010; Lorca, 2000). Samples (5 mL) were taken and processed for microbial enumeration and DNA extraction respectively, and serial dilutions were performed and spread onto two types of agar media of Brain-Heart Infusion (BHI) Agar with 3% sea salt and Marine Agar (MA) with 3% sea salt. Plates were incubated according to aerobic and anaerobic atmosphere (using AnaeroGen kit by Oxoid) respectively at 20°C for 48-72 hours. The Thiosulfate-Citrate Bile salts-Sucrose (TCBS) agar by Oxoid was also used in this research for the detection for any growth of *Vibrio* spp. which is normally found to be associated with oysters and other marine organisms (Hara-Kudo *et al.*, 2001). After 48-72 hours of incubation, all plates were read and examined using the standard plate count method.

Direct Total DNA Extraction

Total microbial DNA was extracted directly from the 36 oyster samples using the QIAamp DNA Stool Mini Kit (QIAGEN Sciences, Germantown, MD, US) following the manufacturer's instructions. The direct DNA extraction was performed soon after sampling or on samples that were maintained frozen at temperature -80°C.

16S rRNA Gene Sequencing

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The sequencing of the 16S rRNA gene amplicon was applied to the 36 samples collected from the oyster farm, to examine the microbial communities and diversity present in each of the samples. Sequencing was carried out by Research and Testing Laboratories (Lubbock, Texas, USA) using the Illumina MiSeq platform. Pair-ended PCR amplification of the 16S rRNA gene V3 region was carried using 341F and 907R primers. FASTQ files generated were merged using PEAR (Zhang, Zhang, Liu, Han, & Zhou, 2012), these were then trimmed to remove the primer, barcode and adapter regions using an internally developed algorithm at Research and Testing Labs (Lubbock, Texas, USA). The seed sequence for each cluster was then sorted by length and clustered with a 4% divergence cut-off to create centroid clusters. Clusters containing only <2 sequences or <100 bp in length were then removed. Seed sequences were again clustered at a 4% divergence level using USearch to confirm whether any additional clusters appeared. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads was then analyzed to remove chimeras utilizing UCHIME in the *de novo* mode (Edgar, Haas, Clemente, Quince, & Knight, 2011). After chimera removal, each consensus sequence and its centroid cluster were denoised in UCHIME in which base position quality scores of >30 acted as the denoising criterion. Sequence de-replication and OTU demarcation was further performed in USEARCH and UPARSE to yield OTUs that were aligned using MUSCLE (Edgar, 2004) and FastTree (Price, Dehal, & Arkin, 2010) that infers approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang, Garrity, Tiedje, & Cole, 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.*, 2006).

Statistical Analysis

The relative abundance of taxa at the genus level was compared using unsupervised hierarchical clustering using Cluster 3.0 (de Hoon, Imoto, Nolan, & Miyano, 2004). Clustering was based on complete linkage comparisons utilizing uncentred correlations. The derived cluster matrix was then used to create heat maps using Java Tree View version 1.1.6r2 (Saldanha, 2004). PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK), respectively were used to conduct non-metric multidimensional scaling (MDS) and analysis of variance (ANOVA) (Anderson *et al.*, 2005). For this analysis sequence read data organised at the genus-level was normalised as percentages, square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. ANOVA was conducted using an unrestricted permutation of the data (n=9999), fixed terms summed to zero, and utilizing partial sum of squares. MDS were conducted using default settings, and ANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered marginally significant.

Results

Culturable Microbial Population Structure and Environmental Conditions

The water surface (5 m) temperature recorded during sampling ranged between 16-19.9°C in the summer season. The average total viable counts from the BHI, MA and TCBS plates for the three different row cages groups varied, ranging between 3.0 to 4.1 Log CFU/g for BHI, 2.9 to 3.8 Log CFU/g for MA, and 2.5 to 3.2 Log CFU/g for TCBS.

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Oyster Microbial Communities are Dominated by Members of the Family *Vibrionaceae* and *Pseudoalteromonadaceae*

The pyrotag read data indicated that oysters' (*Crassostrea gigas*) microbial diversity collected from the oyster farm was dominated by bacterial genera belonging mainly to the family *Vibrionaceae* (42.5-44.1% of total reads) and *Pseudoalteromonadaceae* (28.0-28.8% of total reads) (Table 1). The distribution of taxa in samples demonstrated that, the members of the family *Fusobacteriaceae*, *Bacteroidaceae* and *Prolixibacteraceae* were also abundant in the oyster samples (Table 1). The distribution and diversity of bacteria and community structure associated with oysters across the different row were very similar and there was no clear separation as visualised by the MDS plots (Fig. 1). The MDS plots of mean for microbial composition distribution according to the different rows indicated that the microbial community structure and diversity were similar between the three cage rows of sample collection from the oyster farm (Fig. 1).

The Dominant Bacterial Species from The Farmed Oysters

The main species of *Vibrionaceae* present includes *Vibrio gigantis*, *Vibrio Splendidus*, *Vibrio ichthyoenteri*, *Vibrio harveyi*, *Aliivibrio wodanis* and *Photobacterium phosphoreum* (Fig. 2), while for the main species of *Pseudoalteromonadaceae* are *Pseudoalteromonas undina*, *Pseudoalteromonas tetraodonis* and *Pseudoalteromonas marina* (Fig. 2). Those identified bacterial species can be considered typically isolated from fish and other marine animals (Neuman *et al.*, 2016; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). Other bacterial species (identified up to species level) found also includes *Prolixibacter bellariivorans*, *Cytophaga fermentans*, *Psychrilyobacter atlanticus*, *Planococcus maritimus*, *Exiguobacterium oxidotolerans* and *Paracoccus marcusi* (Fig. 2).

The Interesting Presence of Cyanobacterial Species from the Farmed Oysters

An interesting observation was the significant number of cyanobacterial reads (10% of reads on average, Table 1 and Fig. 2). These reads included what were mainly filamentous or coccoidal forms of cyanobacteria, mainly taxa related to the genera *Hydrocoleum*, *Cyanothece*, *Chroococcidiopsis*, *Chroococcus*, *Tolypothrix* and *Cylindrospermum*.

Discussion

The use of molecular approach for the identification of microbial communities in fish and marine animals has become more important this day since the advancement of identification/morphology technology and its capability to detect uncultivable bacteria and other bacteria requiring special growth conditions (Tarnecki, Burgos, Ray, & Arias, 2017). Moreover, traditional morphological and biochemical criteria had failed to differentiate between isolates as well as some of the bacterial species which are cultivable (Hovda *et al.*, 2007). Moreover, the API system is insufficient for the identification and morphological process.

The highest amount of bacterial genera strains identified in this study comprised of *Vibrio*, *Aliivibrio* and *Pseudoalteromonas* which made up a high proportion of strains, while *Psychrilyobacter*, *Exiguobacterium*,

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Paracoccus, *Planococcus* and *Photobacterium* were detected at lower levels. The high incidence of *Vibrio* spp., *Aliivibrio* spp., and *Pseudoalteromonas* spp., may indicate the influence of environmental factors in oyster farms since the farms are located at the open bay. Moreover, according to some researchers, these bacteria favour elevated temperatures (>18°C) (Lorca, 2002). The same bacterial species were also detected from the salmon fish and other fishes based on the molecular-based characterisation approached (Hovda *et al.*, 2007; Neuman *et al.*, 2016; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). *Vibrio* spp., and *Aliivibrio* spp., are important because some of their species can cause food-borne illnesses to oyster consumers and frequent incidents have been reported across the globe (Urbanczyk, Ast, Higgins, Carson, & Dunlap, 2007). Previous studies discussed the importance of *Vibrio* genera in aquaculture and its presence in fish farms and other aquaculture organisms (Austin, 2006; Austin & Zhang, 2006). The major problems were associated with *Vibrio parahaemolyticus* and *Vibrio splendidus* (Lorca, 2000). However, disease incidence depends on the number of bacteria present and whether it possesses specific virulence determinants (Cao, Xue, Liu, & Xue, 2009). Moreover, this study did not identify *Vibrio parahaemolyticus* and *Vibrio splendidus* which may indicate that the farm is free from harmful potential foodborne diseases.

The genus *Pseudoalteromonas* was the second major genera strain group found in this study. According to Romero, Gonzalez, & Espejo (2002), *Pseudoalteromonas* spp., is the most abundant bacteria found in spoiled oysters, thus it may answer the question of why some oyster products have a shorter lifespan, and it could be due to the presence of these bacteria inside the oysters (Romero, Garcia-Varela, Laclette, & Espejo, 2002; Romero, Gonzalez, & Espejo, 2002). Another major strain group found in this study is related to *Psychrilyobacter* spp., where these strains were previously isolated from low temperature marine environments (Zhao, Manno, & Hawari, 2009). The result from this study showed this bacterium is commonly found in oysters farmed in Tasmania, possibly due to the low Tasmanian sea temperature (Piquer, 2010). Other bacterial species such as *Exiguobacterium spp.*, *Planococcus* spp., *Photobacterium* spp., can be isolated from marine environments (Yoon, Weiss, Kang, Oh, & Park, 2003) and had been found by a previous study on fish bacteria (Zarkasi *et al.*, 2016). *Photobacterium* is known as a marine bacterium, found in salmon fish intestines (Holben, Williams, Saarinen, Särkilahti, & Apajalahti, 2002). The bacterium is also known for its spoilage potential of fish fillets and other aquaculture products (Reilly & Kaeferstein, 1998). The microbial flora from freshly-caught oysters could reflect the microbial flora in the surrounding environment (Hatje, Neuman, Stevenson, Bowman, & Katouli, 2014; Zarkasi *et al.*, 2014). The surrounding environment can influence the microbial diversity of oyster through many factors such as seawater temperature, seasonal period, pollution, and farming techniques (Zarkasi *et al.*, 2014). Identifying this bacterium as part of the oyster can be of interest to determine contamination routes and possible pollution from the surrounding environments.

The dynamism in the oysters' microbial community structure is more interesting due to the significant level of cyanobacteria present, despite the presence of high levels of *Vibrionaceae*. The number of the cyanobacteria is in the range of 10% (Table 1, Fig. 2). Even though their number is low, it is interesting to explore why they have a significant presence. Cyanobacterial predominance in samples is clear during the summer, showing substantial variation between individual oysters and potentially reflecting stochastic exposure patterns between individuals. We did not identify the source of cyanobacteria directly. One assumption is that they could be derived from the environment, such as those present in the water during high water temperatures (summer season). According to

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Fulton (2009), Tasmanian waters can experience frequent cyanobacterial blooms that worsen during the period of drought on land. The growth and development of cyanobacteria on the water surface especially during the summer could be concerning, since certain cyanobacteria species produce toxins that may impact fish health (Sellner, 1997). In fish farm cases, cyanobacteria appear to frequently occur in fish faeces. For example farmed carp faecal samples were found to have up to 60% of cyanobacterial reads (Wu *et al.*, 2012), presumably derived from their pond environment.

Conclusion

In this study, we described the predominant members of family *Vibrioneceae* and *Pseudoalteromonadaceae* in farmed oysters' (*Crassostrea gigas*) microbial communities.

The most common microbes abundant in oysters are *Vibrio* spp., *Photobacterium* spp., and *Pseudoalteromons* spp., while minor but common microbes are *Psychrilyobacter* spp., *Prolixibacter* spp., *Cytophaga* spp., *Planococcus* spp., and *Exiguobacterium* spp. Those abundant bacterial species found in this study can be considered as typically isolated from the fish and other marine animals (Neuman *et al.*, 2016; Zarkasi *et al.*, 2014; Zarkasi *et al.*, 2016). The sequence data obtained could be used to compare oyster aquaculture management strategies as well as mariculture practiced in different regions that may have similar or different climactic conditions. It is also useable for understanding environmental condition effects on oyster farms. Further studies of this nature could reveal important links between oyster farming, environmental factors, and husbandry strategies.

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Table 1. Relative abundances (in % of total reads) of the most abundant microorganisms at family level in farmed oysters.

Family	**Row A** (% of total reads)	**Row B** (% of total reads)	**Row C** (% of total reads)
Vibrionaceae	42.5	44.1	43.7
Pseudoalteromonadaceae	28.0	28.5	28.8
Fusobacteriaceae	2.3	1.1	1.6
Bacteroidaceae	4.4	2.5	2.4
Prolixibacteraceae	6.3	5.9	5.5
Cyanobacteria	9.0	11.0	11.0
Other microorganisms	7.5	6.9	7.0

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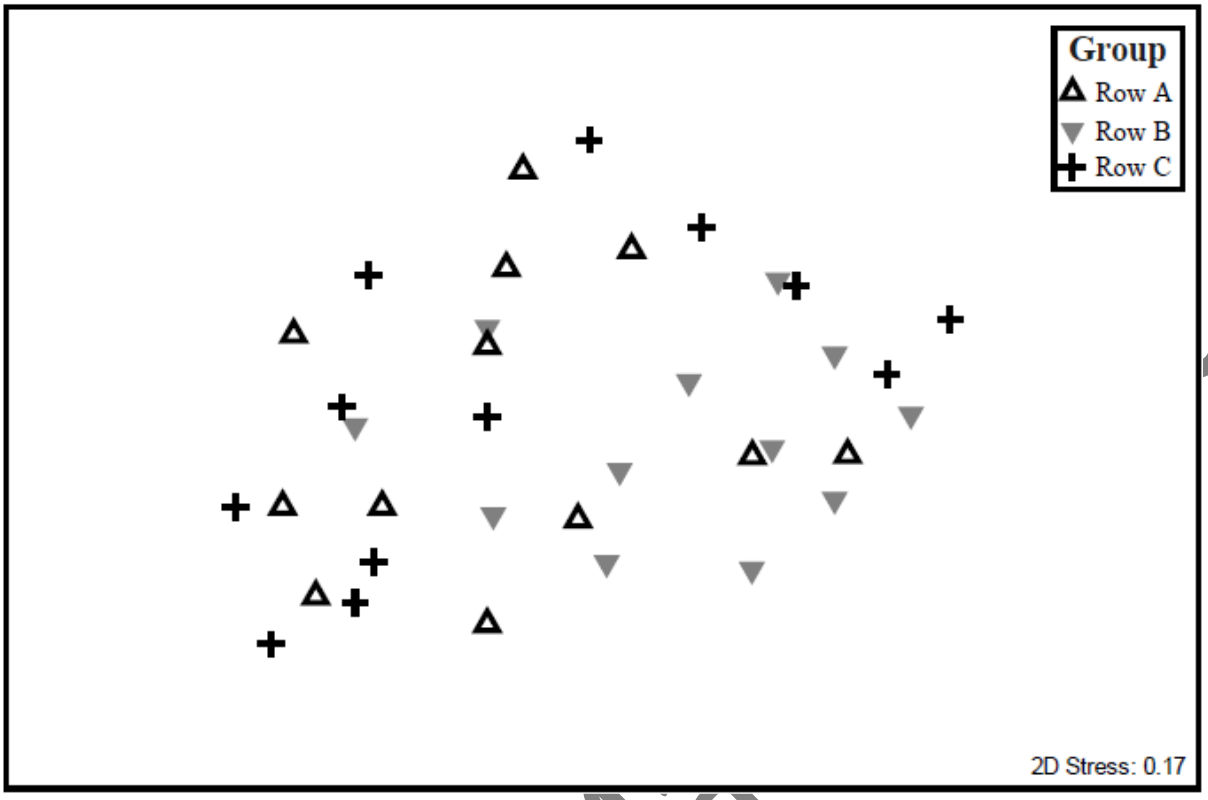

Figure 1. MDS plots showing microbial community similarity on the basis of cages row.

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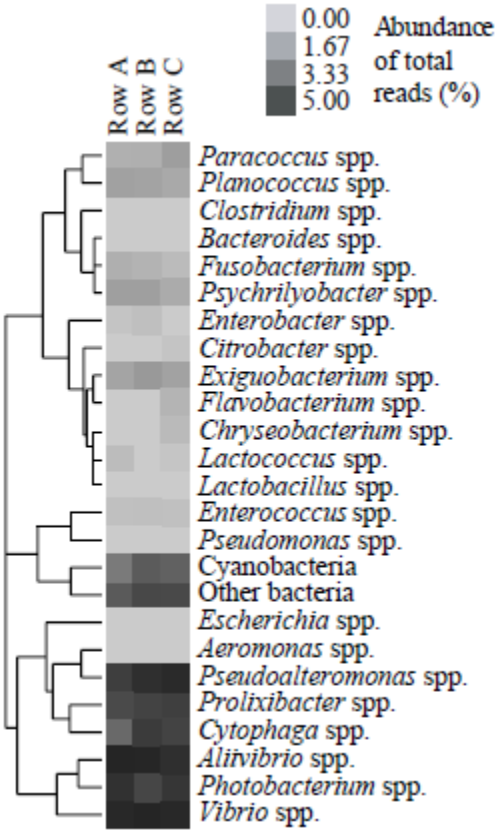

Figure 2. Heat map and hierarchical clustering plot of the 16S rRNA gene compositional distribution of oysters' microbial communities identified via pyrosequencing.