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Molecular Characterization of Microbial Community Diversity Associated with Blood Cockles (*Anadara granosa*) in Blood Cockle Farms

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Abstract

Microbial communities of blood cockles (*Anadara granosa*) collected from blood cockle farms at the coastal area of Northern Malaysia were examined using a standard cultivation method and 16S rRNA Illumina sequencing analysis. This study is aimed at identifying the major abundance of blood cockles' microbes and their potential relationship with different farm locations and environmental conditions. 16S rRNA Illumina sequencing and culturable microbial numbers were found to be slightly different among the samples in two different farms probably because of some environmental factors such as pollution, fresh water load, and the distance or closeness between the farm location and agricultural and industrial zones. The results indicated that most of the microbes found were typically present in blood cockles and in highly dynamic communities. The results revealed that there were slight similarities among the sampling times, and significant differences regarding the microbes' numbers between the different farm locations. Based on these results, the blood cockle microbial communities were highly dynamic and were greatly predominant by *Vibrio* spp., *Klebsiella* spp. and *Bacillus* spp. Other microbial genera found were *E. coli*, *Aliivibrio* spp., *Pseudomonas* spp., *Staphylococcus* spp., *Pseudoalteromonas* spp., and *Micrococcus* spp. existing in a minor abundance. These findings demonstrate the microbial diversity and the major abundant species in blood cockles. Hopefully, this study provides a good understanding of blood cockle microbial communities.

Keywords: Blood cockle, Environment, Coastal area, 16S rRNA genes, Sequencing.

1. Introduction

Microbial communities in marine and estuarine environments are diverse and dynamic. Some microbes can be easily found in marine and estuarine environments including *Vibrio* spp., *Pseudomonas* spp., *Klebsiella* spp., *Bacillus* spp., *Micrococcus* spp., *Photobacterium* spp., and *Aliivibrio* spp. (Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Mala *et al.*, 2014; Zarkasi *et al.*, 2017a; Zarkasi *et al.*, 2017b; Zarkasi and Nazari, 2018; Lau *et al.*, 2019). Allochthonous is the most common microbial species associated with blood cockles, since blood cockles consume the surrounding water and are directly exposed to the marine environments, where those microorganisms are present (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Ghaderpour *et al.*, 2014; Zarkasi *et al.*, 2017a). Therefore, the presence of pathogenic microorganisms in blood cockles has become a major concern because they may put the consumer's health at risk and jeopardize farmers and fishermen's sources of income. Such pathogenic microorganisms in blood cockles can also serve as an indicator for faecal pollutions for the respective locations.

Contamination with certain microbes such as *Vibrio vulnificus*, *E. coli* and *Vibrio parahaemolyticus* may cause blood cockle illnesses as reported by other studies (Sarkar *et al.*, 1987; Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Mala *et al.*, 2014). Human infections with *V. parahaemolyticus* are usually linked to improper food handling or raw seafood consumption (Johnson *et al.*, 1984; Robert-Pillot *et al.*, 2014). Moreover, *V. parahaemolyticus* is an important causative agent of gastroenteritis in humans. In addition, elements of weather and climate (Khan Chowdhury *et al.*, 2009), pollution (DePaola *et al.*, 1990), faecal pollution, storage, handling and management practices (Khan Chowdhury *et al.*, 2009) can also determine the variability of incidence and distribution of food poisoning/illnesses associated with the blood cockles in spite of the fact that most strains of seafood and environmental isolates are likely to be virulent (Khan Chowdhury *et al.*, 2009; Norhana *et al.*, 2016).

Nowadays, major blood cockle farms are located near agricultural lands, or close to residential and industrial areas. Previous studies have found out that blood cockle microbial communities are highly dynamic and sensitive to environmental factors and management practices (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011;

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Woodring *et al.*, 2012). Therefore, studying and understanding blood cockle microbiota and their influences can potentially lead to the protection of the environment, as well as the improvement of storage, transportation, and management practices of blood cockle products which may eventually contribute to blood cockle farming and help boost industrial sustainability. The aim of this study is to identify the microbial communities associated with blood cockles (*Anadara granosa*) and to study the potential factors that may influence these microbial communities. The primary questions which the current study attempts to answer are: what are the most abundant microbes associated with blood cockles (*Anadara granosa*), and whether the sampling location and the surrounding environments affect blood cockles' microbial communities or not?

2. Materials and Methods

2.1. Sample Collection

Blood cockle samples were collected from blood cockle farms located at coastal area in Juru and Kerian, Malaysia in August and October, 2017. Juru area is located near to an industrial zone, and is already known for its big pollution problem (Yap *et al.*, 2008), while Kerian area is located near an agriculture zone (paddy field) and a residential area. The samples collected from Juru are referred to as 'JU', while samples collected from Kerian are referred to as 'KR'. A total of twenty-four samples (twelve samples per sampling location) were randomly collected. They were, then, immediately transported in a chilling ice box to the laboratory and were processed within three hours.

2.2. Microbial Enumeration and Cultivation

Based on the sampling location, the blood cockle samples were divided into two different groups (JU and KR). The blood cockle samples were then examined thoroughly, and their colour, smell, and gross appearance were all recorded. The samples were then cleaned with a brush under running tap water to remove any sand, debris or mud on their shells. After that, the raw blood cockles were aseptically shucked, and the intact bodies and liquor were placed and pooled into a sterilized filter blender bag. The bag was massaged through by hand for one minute to separate the excess shell from the liquor and intact bodies. In a process to remove the remaining shells, the samples were then transferred into a new full filter blender bag, to which 450 ml of 3 % sea salt peptone water was added. The samples were allowed to homogenize for two minutes (Zarkasi and Nazari, 2018). Samples of 5 ml were taken and processed for microbial enumeration and DNA extraction respectively. Then, serial dilutions were performed and spread onto three different types of agar media, namely Marine Agar (MA), Brain-Heart Infusion (BHI) Agar with 3 % sea salt and thiosulfate-citrate-bile salts-sucrose (TCBS) agar (Zarkasi and Nazari, 2018). After that, the plates were incubated at 25ºC for 24-72 hours according to aerobic and anaerobic atmospheres (AnaeroGen kit by Oxoid). All plates were examined by the standard plate count method after 24-72 hours of incubation. One loop of the suspected growing colony was taken and streaked onto a Nutrient Agar (NA) media to get a pure colony for characterization and identification. In total, thirty-one colonies were chosen for identification.

2.3. DNA Extraction and 16S rRNA Gene Analysis for Pure Colonies

Using Eppendorf tubes containing sterile distilled water, a single colony from a pure culture was transferred into the tube and heated to 70°C for ten minutes, and was then centrifuged (4000 x g, 1 min). PCR was then performed using 2 µL of the heated extract with final concentrations of the PCR reaction mix including 1 µL(20 pmol) of each of primers 341F (5' CTA CGG GAG GCA GCA G) and 907R primer (5' AAA CTC AAA GGA ATT GAC) (GeneWorks, Australia), 1 µL of bovine serum albumin, 12.5 µL of ImmoMix (Bioline, UK), and 7.5 µL of sterile distilled water to a final volume of 25 µL. Thermocycling was performed using a C1000 Thermal Cycle (Bio-Rad, California, US) at 95°C for ten minutes, 94°C for one minute, 55°C for one minute, 72°C for one minute; repeated for twenty-three cycles,72°C for ten minutes, and soaked at 15°C (Zarkasi *et al.*, 2014; Zarkasi and Nazari, 2018). The purified amplicons were then sequenced using an ABI 3730 automated sequencer using the Big Dye direct cycle sequencing kit. A comparison of individual rRNA gene sequences to those published in the BLAST database (http://blast.ncbi.nlm.nih.gov/) was done to determine the microbial genera (Neuman *et al.*, 2016).

2.4. Direct Total DNA Extraction

The total microbial DNA was extracted directly from the twenty-four blood cockle samples using the QIAamp DNA 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.

2.5. 16S rRNA gene Sequencing Using Illumina MiSeq Platform

The sequencing of the 16S rRNA gene amplicon was applied to the twenty-four samples collected from blood cockle farms, to examine the microbial communities and diversity present in each of the samples. Sequencing was carried out using the Illumina MiSeq platform (Zarkasi *et al.*, 2018). Pair-ended PCR amplification of the 16S rRNA gene V3-V4 region was carried out using 341F and 907R primers. The FASTQ files generated were merged using PEAR (Zhang *et al.*, 2012), and these were then trimmed to remove the primer, barcode, and adapter regions. The seed sequence for each cluster was then sorted by length and clustered with a 4 % divergence cut-off to create centroid clusters. The clusters containing only <2 sequences or <100 bp in length were then removed. The 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 *et al.*, 2011). After chimera removal, each consensus sequence and its centroid cluster were denoised in UCHIME in which the 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 *et al.*, 2010) which infers approximate maximum likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang *et al.*, 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.*, 2006; Hussin *et al.*, 2018).

2.6. Statistical Analysis

PRIMER6 and PERMANOVA+ (Primer-E, Ivybridge, UK) respectively were used to conduct analysis of variance (ANOVA) and Multidimensional scaling (MDS) to assess the influence of different factors on community compositions. The ANOVA-derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered marginally significant (Zarkasi *et al.*, 2016).

3. Results

3.1. Culturable Microbial Population Structure

The water surface (5 m) temperature recorded during sampling ranged between 27 and 29°C. The results from culturable plates show that the average viable counts of JU and KR collected from MA, BHI and TCBS were varied. For JU, the average viable counts were 7.17 log cfu/g on MA, 7.13 log cfu/g on BHI and 7.56 log cfu/g on TCBS, while the KR samples average viable counts were 4.66 log cfu/g on MA, 4.76 log cfu/g on BHI and 4.53 log cfu/g on TCBS (Figure 1). However, the following month, the average viable counts were almost the same compared with the previous month. The average viable counts for JU were 7.20 log cfu/g on MA, 7.19 log cfu/g on BHI and 7.50 log cfu/g on TCBS, while for KR they were 4.69 log cfu/g on MA, 4.78 log cfu/g on BHI and 4.49 log cfu/g on TCBS (Figure 1).

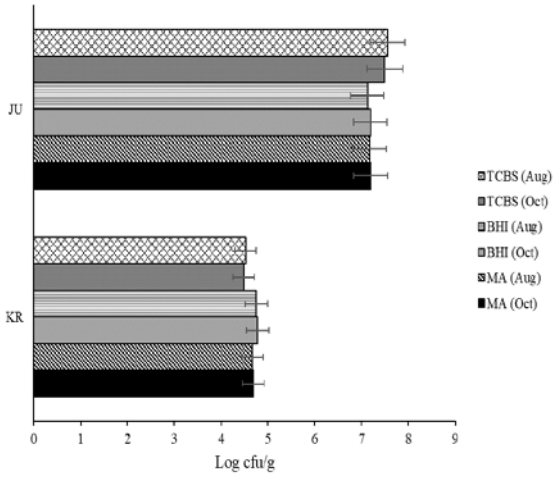

Figure 1. Total viable counts (TVC) populations derived from the colony numbers appearing on marine agar, BHI agar and TCBS agar, for bacterial cultured from blood cockle (*Anadara granosa*) according to the location of sampling. The bars equal to the Standard Error (SE).

3.2. Microbial Communities of Blood Cockles are Dominated by Members of the Family Vibrionaceae and Enterobacteriaceae

The major dominant microbes described from this study based on 16S rRNA Illumina gene analysis were bacterial groups belonging to the family of Vibrionaceae (*Vibrio, Aliivibrio* and *Photobacterium*) making up >36 % of the total numbers. These were followed by Enterobacteriaceae (*Escherichia, Klebsiella* and *Citrobacter*) making up >32 % of the total numbers, and also Bacillaceae (*Bacillus* and *Geobacillus*), which constituted >19 % of the total numbers (Figure 2).

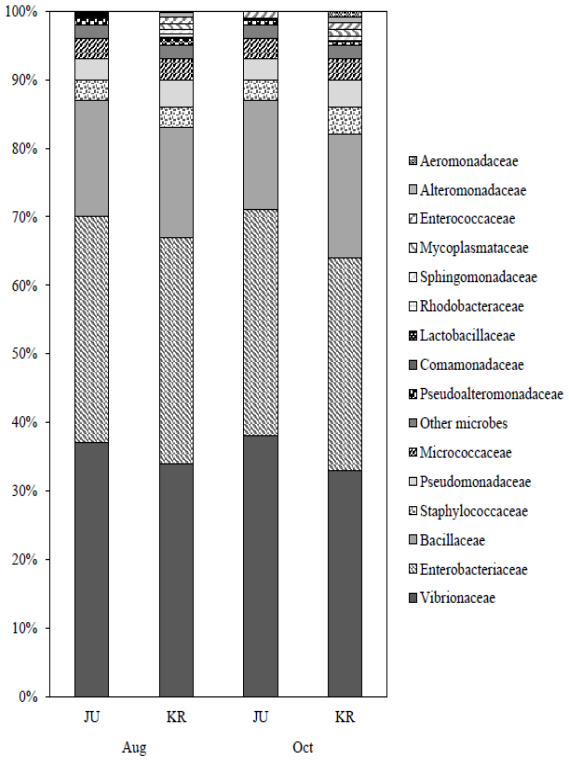

Figure 2. Relative abundances (in % of total numbers) of the most abundant microorganisms at family level associated with blood cockle.

The results show consistency among the JU and KR samples indicating its population dynamics. Other microbe families such as Staphylococcaceae, Pseudomonadaceae, Aeromonadaceae, Alteromonadaceae, Enterococcaceae, Mycoplasmataceae, Sphingomonadaceae, Rhodobacteraceae, Lactobacillaceae, Comamonadaceae, Pseudoalteromonadaceae, and Micrococcaceae were also identified making up >13 % of the total numbers (Figure 2). Down to the genera level, the most abundant microbial genera were *Vibrio* spp., *Klebsiella* spp., and *Bacillus* spp. making up ~28 %, ~20 % and ~16 % of the total number respectively (Figure 3). Besides, *E. coli* (~15 % of the total number), *Staphylococcus* spp. (~4 % of the total number), *Micrococcus* spp. (~3 % of the total number), *Pseudomonas* spp. (~3 % of the total number), and *Pseudoalteromonas* spp. (~2 % of the total number) were also significantly present as visualised by the heat map (Figure 3). Other microbial genera such as *Sphingomonas, Paracoccus, Enterococcus, Lactobacillus, Chryseobacterium, Flavobacterium, Citrobacter, Enterobacter* and *Streptococcus, Clostridium, Geobacillus,* and *Aeromonas* were also identified making up >7 % of the total numbers

(Figure 3). An interesting observation was the significant number of coliform bacterial reads (~15 % of reads on average (Figure 3). These reads were dominated by the *Escherichia coli*, the most popular coliform bacteria.

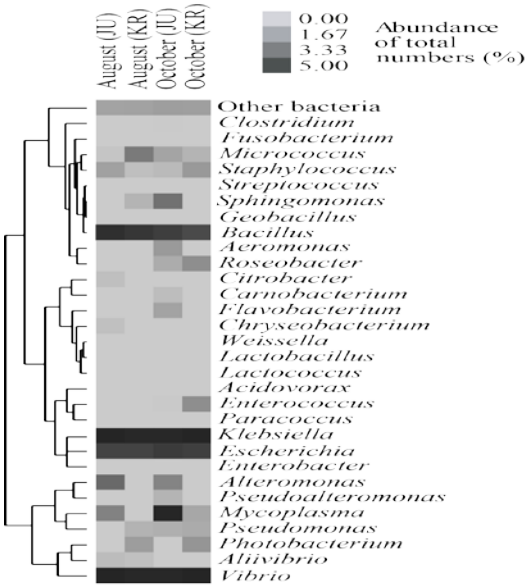

Figure 3. Heat map and hierarchical clustering plot of the blood cockle bacterial communities identified via 16S rRNA Illumina gene analysis.

3.3. Microbial Diversity

The distribution and diversity of microbial community structure associated with blood cockles in the different locations were slightly different, and there was a small clear separation as seen through the MDS plots based on 16S rRNA Illumina gene data (Figure 4). This is supported by the analysis of variance (ANOVA) results which indicated that the sampling locations were slightly different ($0.01 < P < 0.05$). However, as for the sampling time, there was no significant difference ($P > 0.05$). Further analysis using pairwise tests showed that the populations varied and were marginally significant ($0.01 < P < 0.05$). Marginal separation was observed between the JU and KR ($P = 0.04$) (Figure 4).

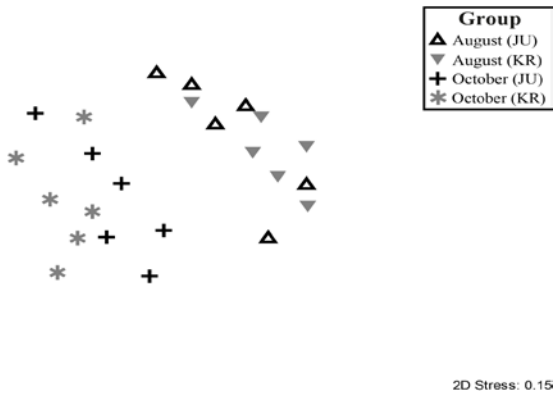

Figure 4. MDS plots showing blood cockle microbial communities.

4. Discussion

The traditional morphology, culture-dependent identification, and biochemical criteria had limitations in terms of comparisons between the isolates and non-culturable microbes because some of the microbial species are not cultivable (Hovda *et al*., 2007). Therefore, the use of a molecular approach such as the next generation sequencing especially for the microbial identification in fish and marine animals has become more important nowadays because the advancement of identification and morphology technology as well as its capability to detect uncultivable bacteria and other bacteria require special growth conditions (Tarnecki *et al.*, 2017).

The numbers of microbial genera groups present in the blood cockles (*Anadara granosa*) that were collected from blood cockle farms, located at coastal area in northern Malaysia, were investigated and analysed in this study. This study maintains that the microbial communities in the blood cockles are dynamic as explained earlier in response to some environmental factors, product handling, transportation, storage, and management practices (Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Zarkasi and Nazari, 2018). This study shows that blood cockle bacteria were allochthonous, in which the dynamic influence of microbial communities was due to external factors (Neuman *et al.*, 2016; Zarkasi *et al.*, 2016) such as temperature, season, human or animal activity, geographical location, and pollution. Both results from cultivation and 16S rRNA Illumina gene sequencing produce almost similar results. However, 16S rRNA Illumina gene sequencing data provide more details of microbial communities since 16S rRNA Illumina gene sequencing can identify non-cultivation microbes. This is the benefit of the application of 16S rRNA Illumina gene sequencing techniques in the study of microbial ecology to get a deeper understanding of microbial communities (Zarkasi *et al.*, 2018).

The environmental factors and management practices in blood cockle farms, transportation, handling and storage may influence the microbial communities and its population dynamics. This indication is supported by the high numbers of *Vibrio* spp., *Klebsiella* spp. and *Bacillus* spp. (Figure 3). The significant difference between microbial communities from different farms indicates that environmental factors do affect the microbial communities associated (Mohamad Suhaimi *et al.*, 2019) with blood cockles. The JU farm was located near an industrial estate, while the KR farm was located near an agricultural (paddy) and residential area. That explains why the microbial communities in the two farms were distinct, since the JU farm location was reported by previous studies to be polluted by the industrial activities (Yap *et al.*, 2008). Leavitt, (2009) explains how microbial contamination in the coastal area could have been caused by environmental factors such as pollution, or being located close to a residential, industrial, or farming area (Heath *et al.*, 1995). According to some researchers, these bacteria were easily found in blood cockles (Areerat, 2000).

The presence of *E. coli*, *Vibrio* spp., and *Staphylococcus* spp. may have been caused by environmental factors in blood cockle farms since the farms are located at coastal estuaries. Moreover, the coastal areas in northern Malaysia are known to be exposed to pollution coming from the industrial zone, residential areas, or agricultural farms (paddy field) nearby (Yap *et al.*, 2008; Khan Chowdhury *et al.*, 2009; Sutthirak and Boonprasop, 2011; Woodring *et al.*, 2012; Ghaderpour

et al., 2014; Shahunthala, 2015). Those bacterial genera detected by this study were also found in other marine organism (Hovda *et al.*, 2007; Zarkasi *et al.*, 2016) , and these bacteria are well-known to cause food spoilage and food-borne illnesses to the consumers. This study recommends that any research conducted on pollution must also discuss microbial communities present at the targeted locations in addition to analyzing the effect of chemical compounds. Frequent incidences were reported across the globe and raised concerns regarding blood cockle consumption (Urbanczyk *et al.*, 2007).

The bacterial genera of *Vibrio* spp., and *Escherichia coli* in aquaculture are important because they are considered as an indicator for faecal pollution and can cause food-borne illnesses to blood cockle consumers (Eng *et al.*, 1989; Austin, 2006; Austin and Zhang, 2006; Norhana *et al.*, 2016). However, disease incidence depends on the number of bacteria present and whether it possesses specific virulence determinants (Cao *et al.*, 2009). The interesting part in this study is the presence of *Pseudoalteromonas* spp. (Zarkasi and Nazari 2018) because this bacterium is known to be found in spoiled blood cockles. This provides an answer to the question why some blood cockle products have a shorter lifespan, which is attributed to the presence of these bacteria inside the blood cockles (Norhana *et al.*, 2016). The dynamism in the blood cockle microbial community structure is more interesting due to the significant level of coliform bacteria presence, despite the presence of high levels of Vibrionaceae. Coliform bacteria predominant in the samples as in the JU farm, show the substantial variation between individual blood cockles and potentially reflect stochastic exposure patterns between individuals, and environmental influences.

This study shows distinct results from different farms, and the morphological and 16S rRNA Illumina analysis results provide almost identical results, which gives a conclusive evidence of the microbial community dynamics in the blood cockles by this study. The dominant bacteria identified can be considered typical to marine organisms including blood cockles (Mala *et al.*, 2014; Zarkasi and Nazari, 2018). Based on the results (Figure 4), the microbial community from blood cockles reflects and is influenced by the surrounding environments and the location of the sampling site (Hatje *et al.*, 2014). The sampling location in the JU area was close to an industrial zone, while the KR farm was located close to an agricultural and residential area. Moreover, according to Llewellyn *et al.*, (2016) and Sullam *et al.*, (2012), the microbial communities in marine animals such as fish and blood cockles are influenced and shaped by the surrounding environments and geographical locations. Furthermore, the blood cockles collected from blood cockle farms at coastal/estuaries in northern Malaysia are possibly polluted by the surrounding environment (Ghaderpour *et al.*, 2014; Khodami *et al.*, 2017); according to this study, the microbial diversity in blood cockles is influenced by location and environmental factors. Similarly, previous also maintained that the surrounding environment can influence the microbial diversity of blood cockles, oyster and fish through many factors such as seawater temperature, geographical location, seasonal period, pollution, and farming techniques (Zarkasi *et al.*, 2014). Identifying this bacterium as part of the blood cockles can be of interest to determine contamination routes and study possible pollution from the surrounding environments.

5. Conclusion

This study concludes that *Vibrio* spp., *Klebsiella* spp., and *Bacillus* spp. are the most predominant bacterial genera associated with blood cockles (*Anadara granosa*), while *E. coli*, *Pseudomonas* spp., *Aliivibrio* spp., *Staphylococcus* spp., *Pseudoalteromonas* spp., and *Micrococcus* spp. are also commonly abundant. The findings of this study confirm that these bacteria are typically isolated from blood cockles and other marine animals with highly dynamic microbial communities (Yap *et al.*, 2008; Khan Chowdhury *et al.*, 2009; Woodring *et al.*, 2012; Ghaderpour *et al.*, 2014) and their presence raise real concerns. The results obtained could be used to improve choices regarding blood cockle farming locations, product storage and handling procedures by retailers and distributors for the sake of maintaining blood cockles' quality before reaching consumers and also improving seafood safety. This study could also be useful for understanding how environmental conditions can affect blood cockle farms. Further studies of this nature could reveal important links between blood cockle farming, environmental factors, and husbandry strategies.

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