Bacterial community diversity associated with blood cockle (*Anadara granosa*) in Penang, Malaysia

Kamarul Zaman Zarkasi[1], Koh Fu Sheng[1], Teh Faridah Nazari[1], Nurfitri Amir Muhammad[1] and Amirul Al-Ashraf Abdullah[1]*

[1]School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Penang, Malaysia

**corresponding author email: kamarul.zarkasi@gmail.com*

Abstract

Bacterial communities of blood cockles (*Anadara granosa*) collected from wet market across Penang, Malaysia, were examined using a cultivation method. This study aimed to describe the major abundance of blood cockle bacteria and its relationship with different sampling locations. 16S rRNA gene analysis and culturable bacterial numbers were found to be slightly different between samples in two different locations potentially due to management, handling, transport and storage practices by the farmers, distributors and retailers. Results from this study indicated that most of bacteria found were typically present in blood cockles. The results revealed that there were slight similarities between sampling times; and slight differences on bacterial numbers between two different sampling locations. Based on the results, the blood cockle microbial communities comprised of members of the genera *Klebsiella* and *Bacillus*, which are greatly predominant, with highly dynamic of bacterial communities. Other bacterial genera found were *E.coli, Vibrio, Pseudomonas, Staphylococcus* and *Micrococcus*. The overall data demonstrated dynamic bacterial communities in blood cockles (*Anadara granosa*) and its diversity.

Index Terms: blood cockle; characterisation; bacterial community; diversity

1. Introduction

Marine and estuarine environments contain diverse microbial communities, such as *Vibrio* spp., *Pseudomonas* spp., *Klebsiella* spp., *Bacillus* spp., and *Aliivibrio* spp.[1-3] Most of the microbial species in blood cockles are allochtonous since blood cockles consume the surrounding water and are exposed to marine environments where those microorganisms are present.[2,3] The presence of certain pathogenic microorganisms are of concern; since it may be a health risk to consumers, and could be an indication of faecal pollutions. Blood cockle illnesses were previously reported due to contamination of *Vibrio vulnificus, E.coli* and *Vibrio parahaemolyticus*.[2-4] Human infections with *V. parahaemoiyticus* are usually linked to raw or mishandled seafood consumption[5] and is an important agent of human gastroenteritis. Despite that, there is high incidence and distribution variability in different regions, depending on the seasons,[1,2] pollution,[6] faecal pollution,[1,2] storage[1,2] and handling,[1,2] and management practices.[1,2] Hence, most strains of environmental and seafood isolates are likely to be virulent.[2] Understanding blood cockle microbiota and its influences can potentially lead to the improvements of sampling, storage, management practices and blood cockle farming, thus aiding in industrial sustainability. It was found that blood cockle microbial communities are highly dynamic[1-3] and sensitive to environmental and management factors[2].

The aim of this study is to investigate the microbial communities associated with blood cockles (*Anadara granosa*) and potential factors that influence the communities, such as environment, storage, handling, management practices, sampling time and location. The

primary question being asked, what is the main dominant of microbial communities associated with blood cockle (*Anadara granosa*) and the pathogenic microbes found from the blood cockles.

2. Experimental approach

2.1 Samples collection

Blood cockle samples were collected in October and December 2015 from wet markets in Bayan Baru and Relau, Penang, and only blood cockles from Penang's coastal/marine farms source were chosen. In this study, the samples are referred to as 'BB' (samples collected from Bayan Baru) and 'RL' (samples collected from Relau). Around 12 samples per sampling location (in total 24 samples) were randomly collected, and transported in a chilling ice box immediately to the laboratory. Blood cockle samples were examined thoroughly, their colour, smell and gross appearance recorded.

2.2 Microbial enumeration

The blood cockle samples were grouped into two different groups according to their sampling location (BB and RL). The samples were then cleaned with a brush under running tap water to remove any sand, debris and mud on the blood cockle's shell. Then the raw blood cockle were aseptically shucked using a sterile knife with intact bodies and liquor 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. Then, the samples were transferred into a new full filter blender bag to remove remaining shells. A liquor of 3% sea salt peptone water (around 450ml) was added and homogenised for two minutes.[7,8] Samples (5 mL) were taken and processed for microbial enumeration and DNA extraction respectively. Serial dilutions were performed, and spread onto three types of agar media; Brain-Heart Infusion (BHI) Agar with 3% Sea salt (for detecting pathogenic bacteria of fungi), Marine Agar with 3% sea salt (for detecting marine microbes) and thiosulfate-citrate-bile salts-sucrose (TCBS) agar. Plates were incubated at 20°C for 24-72 hours. The Thiosulfate-Citrate Bile salts-Sucrose (TCBS) agar by Oxoid was also used in this research for detecting and checking any of *Vibrio* spp. growth which are normally associated with marine organisms.[9] After 24-72 hours of incubation, all plates were read and examined by standard plate count method. One loop of suspected growing colony was then streaked onto the agar and various media (using same type of agar) and incubated to get a pure colony for characterisation and identification (Gram-staining, microscopic observations of cellular morphology, colonial characteristics, biochemical tests and 16S rRNA gene analysis). In total, 60 colonies were chosen for identification.

2.3 Microbial identification

Representative colonies were transferred onto new plates and later identified using a commercial identification kit of API 20E 25 Strips (bioMerieux USA, St. Louis, MO, US) by following the manufacturer's instructions and standard protocols. All isolated colonies were reconfirmed using Gram-staining examination for bacterial cell morphology and series of biochemical tests (tested for Indole, MRVP, nitrate reduction, citrate test and lactose, sucrose and dextrose test). 16S rRNA gene analysis were also applied.

2.4 DNA extraction and 16S rRNA gene analysis from pure cultures

A single colony from a pure culture were transferred into Eppendorf tubes containing sterile distilled water and heated to 70°C for 10 minutes and centrifuged (4000 x g, 1 min). PCR was then performed using 2 µl of the heat 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),[10] 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, United States) at 95°C for 10 minutes, 94°C for 1 minute, 55°C for 1 minutes, 72°C for 1 minutes,

repeated for 23 cycles; 72°C for 10 minutes, and soaking at 15°C.[10] The purified amplicons were then sequenced using an ABI 3730 automated sequencer using the Big Dye direct cycle sequencing kit. Comparison of individual rRNA gene sequences to those published in the BLAST database (http://blast.ncbi.nlm.nih.gov/) was done to determine the bacterial genera.

2.5 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.[10,11]

3. Results and Discussion

3.1 Culturable bacterial population structure

This study investigated and analysed the number of bacteria and bacterial genera group present in blood cockles (*Anandara granosa*) collected from wet markets in Penang, where the sources came from Penang coastal/estuaries area and blood cockle farms in Penang. We assumed that microbial communities in blood cockles would show dynamic presence as previously indicated in response to environmental factors, handling, storage and management practices.[1-3]

Average viable counts from MA, BHI and TCBS plates for the two different sampling location of BB and RL, and the different collecting months were varied. For samples collected on October; BB samples the average viable counts were 4.66 Log CFU/g on MA, 4.76 Log CFU/g on BHI and 3.53 Log CFU/g on TCBS, while RL samples the viable counts were 7.17 Log CFU/g on MA, 7.13 Log CFU/g on BHI and 3.56 Log CFU/g on TCBS (see ***Figure 1***). During the following months of December, the population of BB and RL were almost the same with previous months, the average viable counts for BB were 4.69 Log CFU/g on MA, 4.78 Log CFU/g on BHI and 3.49 Log CFU/g on TCBS, while for RL were 7.20 Log CFU/g on MA, 7.19 Log CFU/g on BHI and 3.50 Log CFU/g on TCBS (see ***Figure 1***).

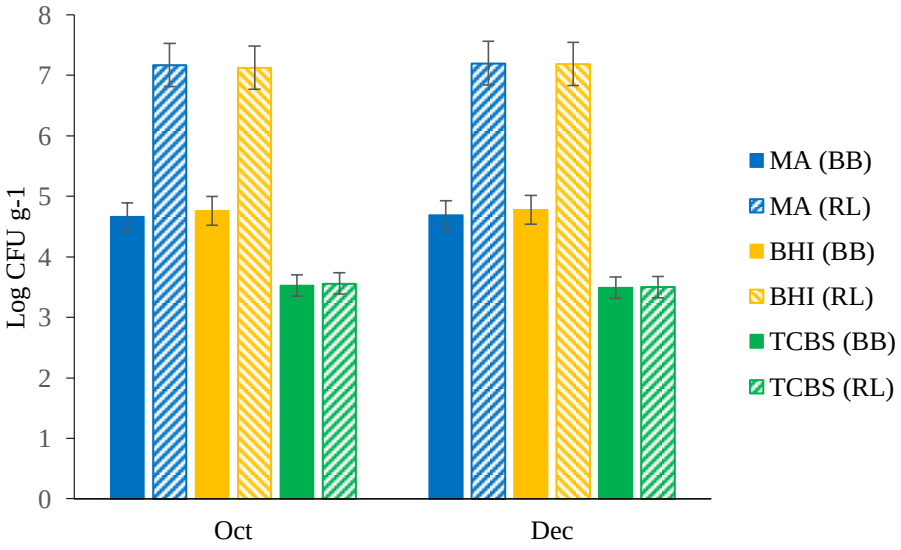

Figure 1. Total viable counts (TVC) populations for bacterial cultured from blood cockle (*Anadara granosa*) (n=12 for each location group) according to the time of sampling. TVC are derived from the colony numbers appearing on marine agar, BHI agar and TCBS agar.

3.2 Identification of blood cockle microbial communities

The results showed that the blood cockle bacteria were allocthonous, in which the dynamic influence was due to external and environmental factors.[11] The total 60 strains identified were dominated by bacterial groups belonging to the family *Enterobacteriaceae* (*Escherichia* and *Klebsiella*) making up >52% of total numbers), followed by the family of *Bacillaceae* (*Bacillus*), which made up >23% of total numbers (see ***Table 1***). The results are consistent among BB and RL samples, collected both in October and December (see ***Table 1***). The bacterial group belonging to the family of *Vibrionaceae* (*Vibrio* and *Aliivibrio*) were also identified, making up >7% of total numbers (see ***Table 1***).

Table 1. Relative abundances (in % of total numbers) of the most abundant microorganisms at family level associated with blood cockle.

Family	October		December	
	BB	**RL**	**BB**	**RL**
Enterobacteriaceae	51.7	53.3	50.0	51.7
Bacillaceae	23.3	25.0	21.7	23.3
Vibrionaceae	6.7	6.7	8.3	10.0
Staphylococcaceae	5.0	3.3	3.3	3.3
Pseudomonadaceae	5.0	3.3	3.3	1.7
Micrococcaceae	3.3	3.3	1.7	3.3
Other microorganisms	5.0	5.1	11.7	6.7

3.3 Microbial composition and diversity

Results indicated that the blood cockles (*Anadara granosa*) samples from BB and RL were dominated by the bacterial genera *Klebsiella* spp. and *Bacillus* spp., making up ~33% and ~23% of total number, respectively by morphological studies (see ***Table 2***) and 16S rRNA gene analysis (see ***Figure 2***). Other bacterial genera also found were *E.coli* (~18 % of total number), *Vibrio* spp. (~5 % of total number), *Staphylococcus* spp. (~3 % of total number), *Micrococcus* spp. (~3 % of total number) and *Pseudomonas* spp. (~3 % of total number) (see ***Table 2*** and ***Figure 2***), as visualised by the heat map (see ***Figure 2***). The results from October and December collection were similar and not significantly different. Results from the morphological and 16S rRNA gene sequences provide almost identical results, thus give conclusive evidence for bacterial identifications**.** The majority of identified bacterial genera from this study can be considered typically present in blood cockles and other marine animals.[10]

The high numbers of *Klebsiella* spp. and *Bacillus* spp., may indicate the influence of environmental factors and management practices in blood cockle farms, transportation and storage as well as conditions of the wet market. Farm management practices who are not control their workers movement, do not use treated water and clean their farms regularly might causes pathogenic contamination into their farm.[12,13] Beside, improper transportations and storage such as cockles are stored at warm temperature (not cold condition) could also cause contamination and spoilage.[13] Moreover, according to some researchers, these bacteria were easily found from blood cockles.[1,2] The presence of some bacterial species, such as *E.coli*, *Vibrio* spp., and *Staphylococcus* spp., could cause by the surrounding environments since the blood cockle farms and estuaries in Penang are known to be exposed to pollution from the industrial, residential and agriculture farms nearby.[1-3, 14,15]

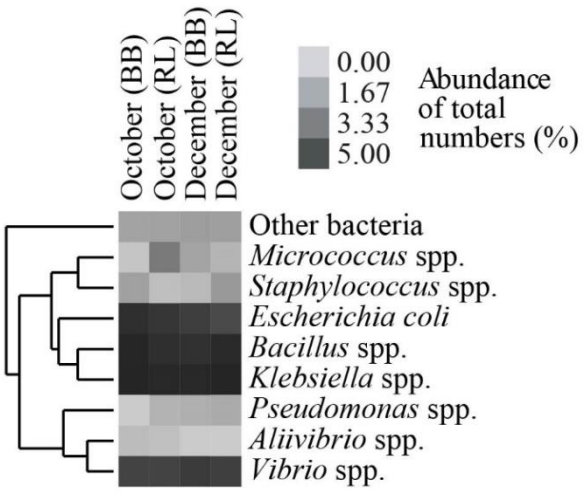

Figure 2. Heat map and hierarchical clustering plot of the blood cockle bacterial communities identified via 16S rRNA gene analysis.

The same bacterial genera were also detected from other marine organisms.[10,11,16] *E.coli*, *Staphylococcus* spp., and *Vibrio* spp., which are important because those bacteria can cause food spoilage and food-borne illnesses to blood cockle consumers. Frequent incidents were reported across the globe and raised concerned to the blood cockles consumptions.[17] Previous studies discussed the importance of the *Vibrio* and *Escherichia coli* in aquaculture and it presence in the aquaculture industry.[1-3,18-20] Both bacterial genera can be considered as an indicator for faecal pollution and may not be good to consume.

3.4 Analysis of variance (ANOVA) study of microbial diversity

The microbial community was influenced to a degree by location, management practices and environments according to the ANOVA analysis (see ***Figure 2***). Furthermore, the blood cockles from Penang coastal/estuaries are where knowingly affected by pollutants coming down from the industrial and residential estates as well as agriculture farms.[21] The different sampling locations produced an insignificantly different of analysis of variance (ANOVA) result ($P > 0.05$), moreover the interaction between sampling time was not significant too ($P = 0.11$), indicating bacterial diversity in this study was not influenced by the location and sampling time. Further analysis using pairwise tests showed that populations varied were not significant ($P > 0.05$). No separation was observed between the BB and RL ($P = 0.83$).

4. Conclusion

In this study, we described the predominant bacterial genera associated with blood cockles (*Anadara granosa*) are *Klebsiella* spp., and *Bacillus* spp., while others which commonly abundant were *E.coli*, *Vibrio* spp., *Pseudomonas* spp., *Staphylococcus* spp., and *Micrococcus* spp. Those abundant bacterial genera found in this study can be considered as typically isolated from the blood cockles and other marine animals[1-3,10] even though some of them could be of concern. The results obtained could be used to improve management strategies by the blood cockle farmers, distributors and retailers. Further studies on this topic is important to understand more about the bacterial communities associated with blood cockles and environmental factors that may shape bacterial diversity, especially in the tropic regions.

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Table 2. Identification of bacteria through biochemical characteristics.

Bacterial code	Indole	MR	VP	CU	NR	Lactose	Sucrose	Dextrose	Identified bacteria
BB1	+	+	-	-	+	+	+	+	*E.coli*
BB2	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB3	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB4	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB5	+	-	+		+	+	+	+	*Vibrio sp.*
BB6	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB7	-	+	-	-	+	+	+	+	*Staphylococcous* sp.
BB8	+	+	-	-	+	+	+	+	*E.coli*
BB9	+	+	-	-	+	+	+	+	*E.coli*
BB10	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB11	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB13	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB14	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB15	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB16	-	-	-	+	+	-	-	-	*Pseudomonas* sp.
BB17	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB18	+	+	-	-	+	+	+	+	*E.coli*
BB19	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB20	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB21	+	+	-	-	+	+	+	+	*E.coli*
BB22	-	-	-	-	-	-	-	-	*Micrococcus* sp.
BB23	+	+	-	-	+	+	+	+	*E.coli*
BB25	-	-	-	-	+	-	-	+	*Bacillus* sp.
BB27	-	+	-	+	+	+	+	+	*Klebsiella* sp.
BB28	+	-	+		+	+	+	+	*Vibrio sp.*
BB29	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL1	+	+	-	-	+	+	+	+	*E.coli*
RL2	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL3	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL4	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL5	-	-	-	+	+	-	-	-	*Pseudomonas* sp.
RL6	+	+	-	-	+	+	+	+	*E.coli*
RL7	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL8	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL9	+	-	+		+	+	+	+	*Vibrio sp.*
RL10	-	-	-	-	-	-	-	-	*Micrococcus* sp.
RL11	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL12	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL14	+	+	-	-	+	+	+	+	*E.coli*
RL15	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL17	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL19	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL21	-	+	-	-	+	+	+	+	*Staphylococcous* sp.
RL22	+	+	-	-	+	+	+	+	*E.coli*
RL24	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL25	+	-	+		+	+	+	+	*Vibrio sp.*
RL26	-	-	-	-	+	-	-	+	*Bacillus* sp.

RL27	-	+	-	+	+	+	+	+	*Klebsiella* sp.
RL28	-	-	-	-	+	-	-	+	*Bacillus* sp.
RL29	+	+	-	-	+	+	+	+	*E.coli*
RL30	-	+	-	+	+	+	+	+	*Klebsiella* sp.

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