DETERMINATION OF TOTAL MICROORGANISMS COUNT AND *VIBRIO* SPECIES IN CRUSTACEANS

by

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LIST OF ABBREVIATIONS

ABBREVIATIONS	MEANING
CFU	Colony forming units
FDA	Food and Drug Administration
KKM	Kementerian Kesihatan Malaysia (Ministry of Health of Malaysia)
TCBS agar	Thiosulfate Citrate Bile Salts Sucrose agar
USA	United States of America
WHO	World Health Organization

PENENTUAN JUMLAH MICKROORGANISMA DAN PROFIL *VIBRIO*DALAM KRUSTASIA

ABSTRAK

Mikroorganisma *Vibrio* biasa ditemui pada bahagian pantai, maka ia selalunya ditemui dalam makanan laut. Mikroorganisma yang sentiasa ditemui pada makanan laut, spesifiknya krustasia ialah *V. parahaemolyticus*, *V. cholerae*, dan *V. vulnificus*. Namun begitu, di Malaysia masih tiada standard untuk had mikroorganisma *Vibrio* yang dibenarkan dalam krustasia. Oleh itu, matlamat kajian ini adalah untuk menentukan kehadiran mikroorganisma ini pada krustasia yang tersedia ada di Malaysia. Metodologi yang telah diguna untuk kajian ini diolah daripada kaedah analisis bakteria yang disediakan oleh pentadbiran makanan dan ubatan (FDA). Untuk kajian ini, 10 spesies krustasia telah dibeli daripada pasar sekitar Kota Bharu, Kelantan dan telah dianalisis untuk menentukan jumlah mikroorganisma dan profil *Vibrio.* Keputusan kajian ini telah menunjukkan bahawa *Metapeneopsis barbata,* atau udang pasir adalah spesises krustasia yang mempunyai kiraan mikroorganisma paling tinggi pada 1.33×10^{10} cfu/g apabila dibandingkan bersama krustasia lain. 30% sampel krustasia mempunyai kiraan mikroorganisma yang lebih tinggi daripada had standard yang telah ditetapkan oleh Kementerian Kesihatan Malaysia di mana had kiraan yang dibenarkan adalah kurang daripada 10^6 cfu/g. Hal ini bakal memberi kesan buruk kepada pengguna yang tidak terdedah akan risiko yang mungkin mereka hadapi. Dalam 60% krustasia telah membuktikan kewujudan *V. parahaemolyticus* dan *V. cholerae,* yang boleh menyebabkan masalah kesihatan jika tidak diurus dengan baik, terutamanya kepada pengguna yang mempunyai imuniti yang lemah. Kesimpulannya, dengan menyebarkan maklumat ini kepada populasi umum, keracunan makanan disebabkan oleh

mikroorganisma *Vibrio* akan berkurang dan pengurusan krustasia yang sesuai ketika penyediaan makanan akan sentiasa dipraktikkan.

DETERMINATION OF TOTAL MICROORGANISMS COUNT AND *VIBRIO* PROFILE IN CRUSTACEANS

ABSTRACT

Vibrio microorganisms are very common in coastal environments, thus they are usually present in seafood. The most prevalent *Vibrio* microorganisms discovered in seafood, particularly crustaceans are *V. parahaemolyticus, V. cholerae,* and *V. vulnificus*. However, currently in Malaysia, there is no set standard for the permissible limit of *Vibrio* microorganisms in crustaceans. Hence, the aim of this study was to determine their presence in crustaceans available in Malaysia. The methodology being utilized in this study was as according to Bacteriological Analytical Methods from the Food and Drug Administration (FDA). For this study, 10 different species of crustaceans were purchased from markets around Kota Bharu, Kelantan and were analyzed both for the total microorganisms count and *Vibrio* microorganism's profile. The results had shown that *Metapemeopsisbarbata,* or sand prawn was the type of crustaceans which had the highest microorganisms count at 1.33×10^{10}cfu/g as compared to the other tested crustaceans. 30% of the samples tested had a higher microorganisms count than the established standard, in which that the limit for total microorganisms count in fishery products stated by Ministry of Health Malaysia is at most at 10^6cfu/g. This may posed a threat to those who are unaware of the risk that may be present. Around 60% of the crustaceans harboured both *V. parahaemolyticus*and *V. cholerae,* which could lead to health complications if prepared inappropriately, particularly to those with compromised immune system. In conclusion, by making this information accessible to the general population, the incidence of food poisoning due to *Vibrio* microorganisms could be decreased and proper handling of crustaceans during preparation could be widely practiced.

CHAPTER 1: INTRODUCTION

1.1 BACKGROUND OF STUDY

Globally, more than 6.3 million tons of seafood are caught and consumed annually. Seafood in this context includes all fishery products, such as fish, shellfish and crustaceans. In the United States of America (USA) alone, more than 2 billion kg of seafood was consumed in 2002, which were then gradually increasing over the past several decades (Butt, Aldridge and Sanders, 2004). While in Malaysia, the fisheries sector had played a big role in economic development by providing employment, becoming a source of foreign exchange and protein supply for its' populations. In 2012, it was found that Malaysia is one of the top fish-consuming countries in the world with an average consumption of 52.0 kg per person per year. In the same year, the fisheries sector had contributed around RM 11,440 million to the nation's economy (Sekaran, 2014).

Outbreaks of foodborne diseases have been documented on every continent in the past decade, which shows the public health and social significance of these diseases. Foodborne diseases, including food poisoning and infections due to food-related diseases are diseases caused by the consumption of contaminated foods. The clinical characteristics of food poisoning generally include gastroenteritis, non-inflammatory or inflammatory diarrhea, neurological symptoms, and systemic diseases (KKM, 2006; World Health Organisation, 2014). In the USA alone, there were around 76 million foodborne diseases outbreaks annually, in which 10-19% of these outbreaks were due to seafood consumption that had caused about 9% deaths. 9% of these outbreaks were caused by bacterial agents. Parallel to the rise in consumption of seafood in the population's general diet, there was also a rise in seafood-related infectious disease outbreaks, which was virtually exclusive due to contamination of *Vibrio, Salmonella, Aeromonas, Plesiomonas, Listeria, and Clostridium botulinum* microorganisms (Butt, Aldridge and Sanders, 2004). There had been reports of outbreaks of intestinal infectious diseases due to ingestion of crustaceans and shellfish contaminated with salmonella, *Listeria monocytogenes*, and pathogenic *Vibrio spp.* (Sagoo, Little and Greenwood, 2007). The cause of foodborne illnesses would be due to the presence of either indigenous, non-indigenous, or bacteria being introduced to the seafood during processing (Feldhusen, 2000).

In a study conducted in the UK to assess the level of *Salmonella* spp., *Escherichia coli, Staphylococcus aureus,* and *Vibrio parahaemolyticus* in cooked shellfish and crustaceans, it had been illustrated that a larger proportion of cooked molluscan shellfish had unsatisfactory level of these microorganisms. This could be due to the ability of the shellfish to concentrate these microorganisms as they are a type of filter feeder in the ocean. Moreover, there was also found that shrimp and prawns, which are part of the crustaceans, had unsatisfactory levels of microorganisms. The researchers had also indicated that *Vibrio* microorganisms could be found in shallow coastal waters and were the common contaminants of seafood, and this might provide the reason how foodborne illnesses caused by *V. parahaemolyticus* were almost exclusively associated with consumption of raw, improperly cooked or cooked, re-contaminated shellfish (Sagoo, Little and Greenwood, 2007). In a review on the bacterial infections related to the ingestion of seafood, the researchers had found that infectious illnesses are common when the afflicted patients had ingested *Vibrio* microorganisms after consumption of seafood, in which most deaths were due to *V. vulnificus*. They had also proposed that the ingestion of *V. parahaemolyticus,* followed by *V. cholerae* with the genus non-O1 are the most commonly associated with human disease, while *V. cholerae* O1 epidemics were uncommon in the USA. There had been reports of foodborne illnesses outbreaks due to contamination with *V. parahaemolyticus*, particularly in the Pacific Northwest in 1997. Other than that, in 1998, Italy had also reported that shellfish was the incriminated seafood in 7.1% of the 84 notified foodborne illnesses outbreaks. In the USA, this microorganism had also been implicated in causing 14 outbreaks of food poisoning (Butt, Aldridge and Sanders, 2004; Normanno *et al.*, 2006).

1.2 PROBLEM STATEMENT

Researchers had found that multiple microorganisms in seafood would lead to foodborne diseases outbreaks, in which some of the most commonly found microorganisms were *Salmonella* spp., *E. coli, S. aureus,* and *V. parahaemoyticus.* The presence of these contaminants could be due to several factors, which would be related closely to environmental conditions and microbiological quality of water. The microbiological quality of water is depended upon the water temperature, salt content, distance between localities of catch and polluted areas, natural occurrence of bacteria in the water, ingestion of food by the seafood, methods of catch, and chilling conditions. The researchers had postulated that there were three groups of microorganisms that could cause foodborne diseases outbreaks; indigenous bacteria, non-indigenous bacteria, and bacterial contamination during processing. Indigenous bacteria includes *V. parahaemolyticus, V. cholerae,* and *V. vulnificus,* while non-indigenous bacteria are *E. coli* and *Salmonella* spp. Bacterial contamination due to practicing unsafe food handling could be due to the presence of *S. aureus* and *L. monocytogenes. Vibrio* microorganisms had been proven to be one of the most prevalent microorganisms found in fish and shellfish, however, there is no official standard for the microbiological level of *Vibrio* microorganisms in crustaceans and other food items due to the absence of ideal identifying kits to identify these microorganisms (Collins, Lyne and Grange, 1989; Feldhusen, 2000; Butt, Aldridge and Sanders, 2004; Sagoo, Little and Greenwood, 2007).

Hence, it is crucial for researchers to determine the best method in identifying *Vibrio* microorganisms in food items, which had shown an increasing trend of consumption to reduce the incidence of seafood-related food poisoning in the population, and also to determine the total number of microorganisms that are commonly found in crustaceans.

1.3 OBJECTIVES OF THE STUDY

1.3.1 General Objectives

To determine the total microorganisms count and V*ibrio* microorganisms profile in crustaceans.

1.3.2 Specific Objectives

1. To determine and compare the total microorganisms count in different species of crustaceans.
2. To compare the total microorganisms count to the permissible limit set by the microbiological standard of Malaysia.
3. To determine and compare the profile of *Vibrio* microorganisms in different species of crustaceans.

1.4 RESEARCH QUESTIONS

1. What is the total microorganisms count in crustaceans?
2. What is the difference of total microorganisms count between different species of crustaceans?
3. What is the difference of total microorganisms count in the crustaceans with the permissible limit set by Malaysian standard?
4. What is the profile of *Vibrio* microorganisms in different species of crustaceans?

1.5 HYPOTHESES

1) **Null hypothesis:** The median total plate counts of ten samples of crustaceans are not different.

   Alternative hypothesis: At least one pair of crustaceans have different median of total plate counts.

2) **Null hypothesis:** There is no significant difference in total plate count and the permissible limit set by the microbiological standard of Malaysia.

   Alternative hypothesis: There is significant difference in total plate count and the permissible limit set by the microbiological standard of Malaysia.

3) **Null hypothesis:** There is no statistically significant association between the profile of *Vibrio* microorganisms and different species of crustaceans.

   Alternative hypothesis: There is statistically significant association between the profile of *Vibrio* microorganisms and different species of crustaceans.

1.6 SIGNIFICANCE OF THE STUDY

Vibrio microorganisms are very common in coastal environments, and quite a number of these organisms are pathogenic to humans, thus they are among the commonly isolated microorganisms from seafood. Among the many species of *Vibrio,* the most universally recognized as pathogenic are *V. parahaemolyticus, V. cholerae,* and *V. vulnificus* (Feldhusen, 2000).

Thus, this study would be very significant to several crucial parties, namely researchers, government, food and beverage industries, and consumers. The researchers would be able to assess and determine the risks posed by unsanitary and unsafe food handling, thus establishing a safe limit for the presence of *Vibrio* microorganisms. Based on the research that had been conducted, the government could use the information generated to implement stringent policy in overseeing the adherence to safe food handling practices. For the food and beverage industries, the retailers would be able to practice safe food handling in order to keep selling their produce, thus avoiding the possibility of contamination. Lastly, the consumers would also be able to make informed choice in selecting crustaceans with a safe level of microorganisms and also would also pay more attention in practicing safe food handling.

CHAPTER 2: LITERATURE REVIEW

2.1 Statistics on Seafood Consumption and Its' Economic Contributions

Globally, it was found that more than 6.3 million tons of seafood are caught and consumed annually. Seafood in this context includes all fishery products, such as fish, shellfish and crustaceans. In the United States of America (USA) alone, more than 2 billion kg of seafood was consumed in 2002, which were then gradually increasing over the past several decades (Butt, Aldridge and Sanders, 2004). In the United Kingdom (UK), the value of their shellfish production in 2003 was estimated to be worth more than £167 million, in which 35% was attributed to the consumption of crabs and scallops. The main types of crustaceans that were imported into the UK were shrimps and prawns (Sagoo, Little and Greenwood, 2007). While in Malaysia, the fisheries sector has been playing a big role in economic development by providing employment, becoming a source of foreign exchange and protein supply for its' populations. In 2012, it was found that Malaysia is one of the top fish-consuming countries in the world with an average consumption of 52.0 kg per person per year. In the same year, the fisheries sector had contributed around RM 11,440 million to the nation's economy (Sekaran, 2014).

2.2 Statistics on Seafood-Related Food Poisoning

Outbreaks of foodborne diseases have been documented on every continent in the past decade, which shows the public health and social significance of these diseases. Foodborne diseases, including food poisoning and infections due to food-related diseases are diseases caused by the consumption of contaminated foods. Foodborne diseases will cause repercussions to people's health, wellbeing, and negative economic consequences for the individuals, families, communities, business, and the country itself (Sekaran, 2014).

Due to an increase in seafood consumption, consequently there is also an escalation of seafood-related infectious disease outbreaks. In the USA, there were around 76 million foodborne diseases outbreaks annually, in which 10-19% of these outbreaks were due to seafood consumption that had caused about 9% deaths. 9% of these outbreaks were caused by bacterial agents (Butt, Aldridge and Sanders, 2004). There had been reports of outbreaks of intestinal infectious diseases due to ingestion of crustaceans and shellfish contaminated with salmonella, *Listeria monocytogenes*, and pathogenic *Vibrios*. *Vibrio parahaemolyticus* and other *Vibrio* spp. are found in shallow coastal waters and they are the common contaminants of seafood (Sagoo, Little and Greenwood, 2007).

The rapid increment of seafood consumption could also be due to the recommendation proposed by the Food and Drug Administration (FDA) and Environmental Protection Agency (EPA) on the average consumption of seafood for adults to be increased to 8 to 12 ounces of a variety of seafood types, in which for children and pregnant mothers need to choose seafood with low mercury content. The FDA and EPA had put forward this recommendation as they had found that polyunsaturated omega-3 fatty acids, namely docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA) are abundant in a variety of seafood to exhibit extraordinary and unique health benefits. These unique health benefits include reducing inflammation and severity of heart and retinal diseases, lower blood triglycerols, reduced arrhythmias, and decreased the risk of sudden death due to heart diseases. It also had been shown that DHA and EPA to benefit children, in which when the mother had consumed low-mercury seafood during pregnancy, the children will experience better functioning brain and nervous systems *(EWG's consumer guide to seafood: Why eat seafood? And how much*?, 2014)

The microbial safety of seafood after being procured is closely related to environmental conditions and microbiological quality of water; water temperature, salt content, the distance between localities of catch and polluted areas, natural occurrence of the microorganisms in water, ingestion of food by the fish and crustaceans, methods of catch, and chilling conditions. The hazards that are typically associated with human pathogenic bacteria in crustaceans can be divided into two: bacteria naturally present in the aquatic environment (indigenous bacteria), and bacteria that are present due to contamination with human or animal feces or otherwise introduced into the aquatic environment (non-indigenous bacteria). Safety hazards may also occur via the introduction of bacteria during the post-harvest handling or processing. Usually, these indigenous pathogenic bacteria, when found to be present in freshly caught products, were at fairly low levels in which if these products are adequately cooked, food safety hazards might be insignificant (Feldhusen, 2000).

There were at least ten genera of pathogenic bacteria that had been associated with foodborne diseases; namely indigenous bacteria such as *V. parahaemolyticus, V. cholerae, V. vulnificus, L. monocytogenes* and *Clostridium botulinum*; non-indigenous bacteria such as *Salmonella* spp., pathogenic *E. coli, Shigella* spp., and *Campylobacter* spp.; bacterial contamination during processing such as *Bacillus cereus, S. aureus,* and *Clostridium perfringens.* Thus, there had been reports of outbreaks of intestinal infectious diseases due to ingestion of crustaceans and shellfish contaminated with salmonella, *Listeria monocytogenes*, and pathogenic *Vibrios* (Sagoo, Little and Greenwood, 2007).

As there is an increasing trend in the consumption of fishery products, it is crucial that the government bodies to focus upon the regulation of microorganisms levels in these products due to their ability to harbor pathogenic bacteria. The European Union had published a journal for the European Communities, which had put forward the limit of 10 cfu/g of *V. parahaemolyticus* among any 5 food samples that had been tested for its' presence using the Most Probable Number (MPN) method. This European Communities' Journal is to provide a guideline for the fisheries sector of the Europe in approving the import of fishery products into the European countries for safer food consumption (*Commission recommendation of 10 January 2003 concerning a coordinated program for the official control of foodstuffs for 2003*, 2003).For the Southeast Asian region, the only country that had provided the minimum level of *V. parahaemolyticus* in fishery products is the Philippines, in which frozen raw crustaceans' level of this microorganism is at 10^2cfu/g from any of the five samples taken (Food and Drug Administration Philippines, 2013).However, in Malaysia, there is no guideline on the level of *Vibrio* microorganisms on crustaceans, in which Malaysia had only focused on the levels of *E. coli* and coliform count (Malaysia, 1985). These microbiological standards had reflected whether the countries mentioned are stringent or not in the level of *Vibrio* microorganisms in crustaceans and also other fishery products. Hence, it is crucial for Malaysia to follow suit in establishing the standard of other microorganisms that would be crucial in identifying the causative agent in foodborne illnesses in the near future.

However, the full burden of foodborne diseases have never been successfully quantified globally due to the under-reporting nature of these diseases. Besides, the policy makers of the countries need science-based, reliable estimates on the burden of foodborne diseases to inform their decision to the general population and mobilize their resources, in which it would be a long and lengthy process. In Malaysia for example, to verify that there is a food outbreak occurrence, the healthcare providers need to define the cases of food poisoning based on the clinical, laboratory, and episodes of food outbreak. Then, they need to notify the cases to the district health offices and also provide the sample of the food that had been consumed to be tested for agents of contamination. The district health officer then needs to mobilize the Rapid Response Team (RRT) to investigate and control the occurrence of foodborne outbreak at the notified place by collecting the food sample, and conducting inspection on the food premise. Then, the officer-in-charge needs to prepare a report of the occurrence of the foodborne diseases to the district health offices, and to continuously observe the food premise for three days. This would be the reason of under-reporting of the incidence of food outbreaks as the process itself is very time-consuming. The clinical characteristics of foodborne diseases are different, in which we will be focusing more on food poisoning. The clinical characteristics of food poisoning generally include gastroenteritis, non-inflammatory or inflammatory diarrhea, neurological symptoms, and systemic diseases (KKM, 2006).

2.3 Crustacean

Crustaceans generally belonged in the phylum of Arthropods, together with insects and arachnids; in which they are characterized together due to possessing hard exoskeletons or shells, segmented bodies, and jointed limbs. Crustaceans are differentiated from other Arthropods by certain characteristics that are only unique to them, namely their biramous appendages, larvae, eyes, labrum, head, and baby teeth. In this study, the type of crustacean of interest is from the family of decapods, which is usually the type of crustaceans being commonly consumed at mealtimes, mainly crabs, lobsters, and shrimp. There are approximately 15,000 known species of decapods, both habituated the marine and freshwater ecosystem. Crustaceans are found to habituate lagoons and estuaries, and these places are considered as nurseries and refugees for them (Tew *et al.*, 2008). However, this study only focuses upon the decapods that are usually consumed in Malaysia, which would be easily available in the markets (Brandão, Hammock and Ferrari, nd). In this study, the focus were upon 10 species of crustaceans that are commonly consumed and available in Malaysia as to determine their total microorganisms count and also to identify the profile and count of *Vibrio* microorganisms.

A study conducted in the UK in 2003, aimed to determine the microbiological safety of cooked crustaceans and molluscan shellfish from the retail and production premises. The microorganisms that had been determined were *Salmonella* spp., *S. aureus,* and *E. coli* which were then compared criteria as outlined in Recommendation 2003/10/EC (EC 2003a), and *V. parahaemolyticus* which was then used as a guideline to the criterion proposed in the EC 2003a. They had found that shrimp and prawns had unsatisfactory microbiological status, while there were no significant differences found on the microbiological status of crustaceans being cooked on or off the premises. However, these crustaceans were then found to have unsatisfactory microbiological status if they were not being consumed in one hour after cooking. Crustaceans that were stored at or below 8°C and unfrozen were found to have unsatisfactory microbiological status. In both the production and retail establishments, a small portion of tested seafood samples had unsatisfactory levels of *E. coli* and *V. parahaemolyticus.* It was also found that in both establishments, molluscan shellfish had unsatisfactory levels of microorganisms compared to crustaceans. The researchers had stated that cooked crustaceans were not the significant source of Salmonella infection in Europe. They had also indicated that *S. aureus* and E. *coli* to be the process hygiene criteria which would define the acceptability of process for cooked crustaceans, in which these microorganisms would indicate either inadequate cooking process or post-process contamination, or both. The presence of *V. parahaemolyticus* demonstrated the need for good hygiene practices during food processing. The researchers had also emphasized the importance of microbiological status of cooked shellfish and crustaceans as these seafood products are typically able to support the growth of a wide variety of microorganisms (Sagoo, Little and Greenwood, 2007).

2.4 Vibrio Microorganisms

Vibrio microorganisms are gram negative, comma-shaped or straight rods bacteria. It is motile in liquid media, which could observe using the hanging drop method. By carrying out oxidase test for *Vibrio* microorganisms, it will exhibit a positive result. They are also a type of facultative anaerobic bacteria, which means that they could survive in both aerobic and anaerobic environment. Most *Vibrio* microorganisms are halophilic, which means that require additional NaCl for optimal growth. *Vibrio* microorganisms are very common in estuarine and coastal environments. They are particularly prevalent in tropical waters and temperate zones in summer months. However, the presences of these microorganisms are not indicative of fecal contamination, but it was found that they are more common in clean areas far from sewage outflows. *Vibrio* microorganisms are one the most frequent isolate from seafood, specifically shellfish. Among the many species of these microorganisms, *V.*

cholerae, V. parahaemolyticus, and *V. vulnificus* are the ones that are widely recognized as pathogenic to humans. *V. cholerae* is the etiological agent of cholera, which could cause severe dehydrating diarrhea and ultimately death in those who had consumed foods that are contaminated with this microorganism. *V. cholerae* strain that is found to cause cholera is known as cholera toxin-producing, the *V. cholerae* O1 strain. This organism may be transmitted from the environment to humans via several routes, including the consumption of undercooked seafood. In Southeast Asia, both *V. cholerae* O1 and non-O1 were isolated in brackish water cultured shrimp and also shrimp that are cultured in India. *V. parahaemolyticus* is the most commonly isolated "non-cholera" *Vibrio* microorganisms and it is prevalent in tropical and sub-tropical regions. *V. cholerae* could be differentiated from other *Vibrio* microorganisms as it is the only *Vibrio* that could thrive without salt addition in its' environment (Collins, Lyne and Grange, 1989; Feldhusen, 2000; Butt, Aldridge and Sanders, 2004).

In a review conducted in 2004, the researchers had focused on infectious illnesses related to the ingestion of seafood, which include finfish, shellfish and crustaceans. The infectious illnesses due to the ingestion of *Vibrio* microorganisms are common when the population had consumed shrimp, clams, oysters, mussels, cockles, crab, lobster, and scallops. They had proposed that *V. parahaemolyticus* is the most commonly associated with human disease, followed by *V. cholerae* non-O1. The ingestion of *Vibrio* microorganisms could cause gastroenteritis (80% of the cases), wound infections, followed by primary septicaemia. Most patients that had contracted septicaemia after ingestion of seafood containing *Vibrio* microorganisms had an underlying liver disease or immunosuppression, and accounted for most deaths. Foodborne illnesses outbreaks due to *V. parahaemolyticus* had been reported. In the Pacific Northwest in 1997, there were 209-culture confirmed cases of *V. parahaemolyticus. V. cholerae* O1 epidemics are uncommon in the USA as there were only a small percentage of reported seafood-related foodborne illnesses caused by this microorganism. Other important pathogenic *Vibrio* microorganism is *V. vulnificus,* which has the potential to cause more severe disease. This microorganism give rise to two distinct conditions, namely septicaemia with high mortality, and severe necrotizing wound infections due to exposure to water or handling fish with contamination of this *V. vulnificus.* The severe forms of diseases were usually contracted by people with pre-existing liver disease or were immune-compromised. *V. cholerae* O1 have the tendency to cause large epidemics, by causing the patients to contract cholera. Cholera would lead to profuse watery diarrhea with significant loss of fluids and electrolytes then may result in hypovolemia and shock in a short period of time. If it is leave untreated, it may lead to death (Butt, Aldridge and Sanders, 2004).

The distribution of *V. parahaemolyticus* in the marine environments were related to water temperatures, in which they thrived in water temperatures of 15°C or higher. This was proven by a study conducted in the Chesapeake Bay, in which Kaneko and Colwell (1973) had found that the microorganism survived in the sediments during the winter season and was then released into the water column when the water temperatures had risen during late spring or early summer. In another study conducted by Duan and Su (2005) had found a positive correlation between the occurrence of *V. parahaemolyticus* in an oyster-growing environment and the seawater temperatures (Su and Liu, 2007). Outbreaks due to contamination of *V. parahaemolyticus* are associated with cross-contamination or time-temperature abuse of cooked seafood. Food contamination due to the presence of *V.*

parahaemolyticus is characterized by diarrhea, headache, vomiting, nausea, low fever, and abdominal cramps, otherwise known as acute gastroenteritis (Su and Liu, 2007). In Japan, it was identified that *V. parahaemolyticus* was the cause of at least a quarter of total foodborne diseases due to the Japanese inclination to consume sushi with undercooked fish in their diet. In the Europe, 25% of shelled frozen prawns have been shown to be contaminated with this microorganism. In addition, *V. parahaemolyticus* was also recognized as one of the leading causes of human gastroenteritis in the USA after seafood consumption. However, for people with underlying medical condition such as liver disease or are immunocompromised, infection with this microorganism may lead to septicaemia, a type of blood infection disease that could be life-threatening to those afflicted (*Assurance of seafood quality*, no date; Collins, Lyne and Grange, 1989; Feldhusen, 2000; Butt, Aldridge and Sanders, 2004; Su and Liu, 2007). *V. parahaemolyticus* thrives in the temperatures ranged from 5 to 43°C, with an optimal growth pattern at 37°C (Food and Environmental Hygiene Department, 2005).

V. cholerae was the causative agent of Asiatic, otherwise known as epidemic cholera. In 2005, the WHO had had reported an astounding numbers of 132,000 cases of cholera outbreaks in 52 countries, with a death toll of over 2,000 deaths. Toxigenic strain of *V. cholerae* was seldom isolated from the environment, the non-toxigenic strains were the normal inhabitants of aquatic environment. In marine environments, this microorganism was found to attach to surfaces of plants, filamentous green algae, insects, and crustaceans (Blackwell and Oliver, 2008). *V. cholerae* thrives in temperatures of 10 to 43°C, with an optimal growth observed at 37°C (Food and Environmental Hygiene Department, 2005).

2.4.1 Risk Factors of Contamination of *Vibrio* microorganisms

The general risk factors of contamination with this microorganism are raw and undercooked shellfish, water, ice, rice, and food bought from street vendors in endemic countries (Butt, Aldridge and Sanders, 2004).

Among the many risk factors of contamination of food with *Vibrio* microorganisms specifically *V. cholerae* include street vended foods and water supplies that had been contaminated by *V. cholerae* bacterium. It is also occasionally spread by eating raw or undercooked shellfish and crustaceans, which had always been implicated in seafood-related foodborne diseases. People who are more likely to be exposed to cholera are healthcare personnel who are treating cholera patients, cholera response workers, and travelers in areas that had been known as active sites for cholera transmission. These people would be more at risk if they are not paying close attention to safe food and water precautions, and personal hygiene procedures (Centers for Disease Control and Prevention, 2016).

The WHO had postulated that certain populations would be more susceptible to *V. cholerae* infections and these population are those who are lacking access to improved sanitation. Based on their study, they had found that an estimate of 1.4 billion of the world's population are those at risk for cholera infection (Mohammad Ali *et al.*, 2011).

For the risk factors of contamination by *V. parahaemolyticus*, these factors include consumption of raw or undercooked shellfish and crustaceans in certain populations, and exposure to warm coastal waters. Shellfish that had always been implicated is oysters that are eaten raw. The populations that would be more at risk for adverse effects from *V. parahaemolyticus* infection are those with liver diseases, iron overload disease, diabetes, cancers, stomach disorders, or any illness that would compromised the immune system (*Vibrio infections*, nd). Other risk factors that had been identified are cross contamination, improper storage at temperatures lower than freezing temperatures, inadequate reheating at temperatures less than 43°C, and food that has been prepared too far in advance without proper warming temperatures (Food and Environmental Hygiene Department, 2005).

2.5 Vibrio Microorganisms and Crustaceans

In marine environment, it was found that *Vibrio* either adhered to multiple substrates, particularly organisms which were rich with chitin, such as crustaceans; or motile as a free-ranging microorganisms. When the *Vibrio* had adhered themselves to a zooplankton, they would be protected from the external environment and ultimately would also gained advantage of their improved nutritional conditions, as reported by Kaneko and Colwell (1975), Colwell and Huq (1994), and Hood and Winter (1997). It was also suggested that crustaceans, particularly small ones would be the most plausible aquatic reservoir of *V, cholerae* and other species of *Vibrios*, as by adhering to the chitin exoskeleton, the microorganisms would stand a chance to persist longer in the water as a special mode of survival. This adherence of *Vibrios* to crustaceans would also lead to its' concentration reaching an infectious dose. Free-ranging *Vibrio* was prevalent in the warmest months, particularly in spring and summer seasons (Covazzi Harriague *et al.*, 2008).

V. cholerae is a microorganism responsible for causing cholera which would lead to severe, dehydrating diarrhea, and death in those consuming foods that are contaminated with this microorganisms. It might be transmitted from the environment to humans via a number of routes, including consumption of undercooked or improperly cooked seafood. During the

US-Gulf coast-associated cholera cases, it was found that crabs had been the implicating animal which had harbored *V. cholerae* (Feldhusen, 2000).

The consumption of improperly prepared and inadequate cooking of crustaceans were found to increasing the prevalence of *V. parahaemolyticus* contamination, leading to acute gastroenteritis in particularly in India. It was also found that among the many species of crustaceans, *V. parahaemolyticus* was more commonly isolated from shrimp as compared to crabs. This highlighted the issue of public health safety as people in India commonly consumed crustaceans as a cheaper source of protein (Parthasarathy, Das and Kumar, 2016).

V. parahaemolyticus had been most commonly implicated "non-cholera" vibrios in the consumption of seafood, particularly molluscan shellfish and crustaceans. However, there is an absence of official standard of the permissible load of this microorganism in seafood, in which only the basic standard is being implemented in the Europe and Philippines by the Most Probable Number (MPN) method. To determine the load of this microorganism, only the MPN method is made available due to the lack of identification kits in identifying vibrios (Collins, Lyne and Grange, 1989; Food and Drug Administration Philippines, 2013). Other than that, *V. parahaemolyticus* when ingested could cause adverse effects to the health of the consumer by causing gastroenteritis, wound infections, and septicaemia. Septicaemia is the most common cause of death in foodborne illnesses due to ingestion of vibrios, in which it may affect those with underlying liver disease and immunosuppression the most (Butt, Aldridge and Sanders, 2004).

2.6 Measures that could be taken to minimize or prevent *Vibrio* microorganisms contamination

In order to minimize the risk of *Vibrio* microorganisms' contamination in crustaceans, it is advisable for retailers to practice good manufacturing and handling practices. Attention should be paid more to the hygienic quality of seawater used in storing live crustaceans and other seafood, and source of the seawater itself is paramount since *Vibrio* microorganisms had been found to thrive in coastal waters more than the open sea. Other recommendations for trading of seafood are divided into several aspects, namely handling of raw materials, manufacturing and storage, and equipment, utensils and personal hygiene (Food and Environmental Hygiene Department, 2005).

In the aspect of handling of raw materials, the retailers should purchase raw materials from trusted and reliable suppliers, and to buy shellfish and crustaceans with intact shell and are free from abnormal odour. The retailers should also store the seafood separately according to species to prevent cross contamination to other types of seafood, complete with proper filtration and disinfection systems that should always be regularly maintained. For the aspect of manufacturing and storage, the retailers should practice the "First In, First Out" principle, or the FIFO principle in storage of raw materials and to pay close attention to the storage temperatures. The retailers should also avoid holding chilled ingredients and finished products at above 4°C for more than 2 hours. A specific parts of the refrigerator for storage of seafood to be eaten raw should be reserved with proper wrapping. Foods should be cooked thoroughly before serving, and high risk foods such as oysters needs to be boiled for not less than five minutes, and for crustaceans flesh, it should turn opaque to determine that it had been thoroughly cooked. For buffet settings, the seafood should be displayed at 4°C or below, or 60°C or above (Food and Environmental Hygiene Department, 2005).

Lastly, for the aspect of equipment, utensils and personal hygiene, the retailers are advised to establish a clean-up and disinfection program to clean and sterilize equipment that are used in handling seafood. Other than that, the food handlers should observe good personal hygiene by washing their hands with potable water and soaps for thorough cleaning. Lastly, the retailers and food handlers should pay close attention in preventing cross contamination between fish tank water, raw seafood, and other foods (Food and Environmental Hygiene Department, 2005).

For the general population, the measures to be taken are also divided into three different aspects, namely purchase, preparation, and consumption. During purchasing, the consumers need to purchase these seafood from reputable and reliable suppliers and to avoid buying seafood from street hawkers as their supply may not be guaranteed to be safe. Consumers should also only buy seafood that are fresh with intact shell and free from abnormal odour (Food and Environmental Hygiene Department, 2005).

For preparation, the consumers should wash the seafood thoroughly before cooking process by scrubbing and rinsing them in clean water. Leftovers should be discarded or store them properly in the refrigerator at temperatures lower than 4°C, and to consume the leftovers, reheat them thoroughly. Consumers should also observe good personal hygiene and prevent cross contamination. For consumption, the consumers should consume the cooked seafood as soon as possible to prevent recontamination. Lastly, pregnant women, children, the elderly, and those with compromised immune system should be careful in selecting food of high risk, such as sashimi and oysters (Food and Environmental Hygiene Department, 2005).

CHAPTER 3: METHODOLOGY

3.1 RESEARCH DESIGN

Quantitative experimental design was used in this study. This research design was chosen as in the determination of microbiological counts. It is crucial to carry it out in a controlled and structured environment of the laboratory. This study focused on determining the presence of overall microorganisms and the profile of *Vibrio* microorganisms in crustaceans. A comparative test was also conducted, in which the microbiological count and *Vibrio* microorganisms profile were compared between different species of crustaceans.

In this study, 10 different species of crustaceans were purchased from wet markets around the area of Kota Bharu, Kelantan, specifically from the markets of Siti Khadijah, Pasir Puteh, and Rural Transformation Centre (RTC). The selected crustaceans samples were brought back to the Nutrition laboratory of Universiti Sains Malaysia, Kubang Kerian at about 25°C room temperature and would then be stored frozen in the freezer at -18°C. The procedures involved in determining the microorganisms count and *Vibrio* microorganisms profile were studied carefully based on previous literatures. The instrument, apparatus, reagents, and media that were used was available, both from Science Laboratory Management Unit (UPMS) and the Nutrition laboratory. This study was conducted and supervised by laboratory assistant to ensure the adherence to the safety guidelines when conducting this study in the laboratory. In accomplishing this study, each sample was analyzed in triplicate and underwent multiple microbiological analyses; Total Aerobic Plate Count to determine the total microbiological count and lastly, determination of *Vibrio* microorganisms' profiles utilizing methodologies extracted from the FDA. The Total Aerobic Count was conducted using the standard plate count while the Thiosulfate Citrate

Bile Salts Sucrose (TCBS) agar was used to isolate *Vibrio* microorganisms as TCBS agar is a type of selective media specifically used in the isolation of *Vibrio* microorganisms.

3.2 DATA COLLECTION

Data collection was performed as according to the schematic flow diagram as shown below:

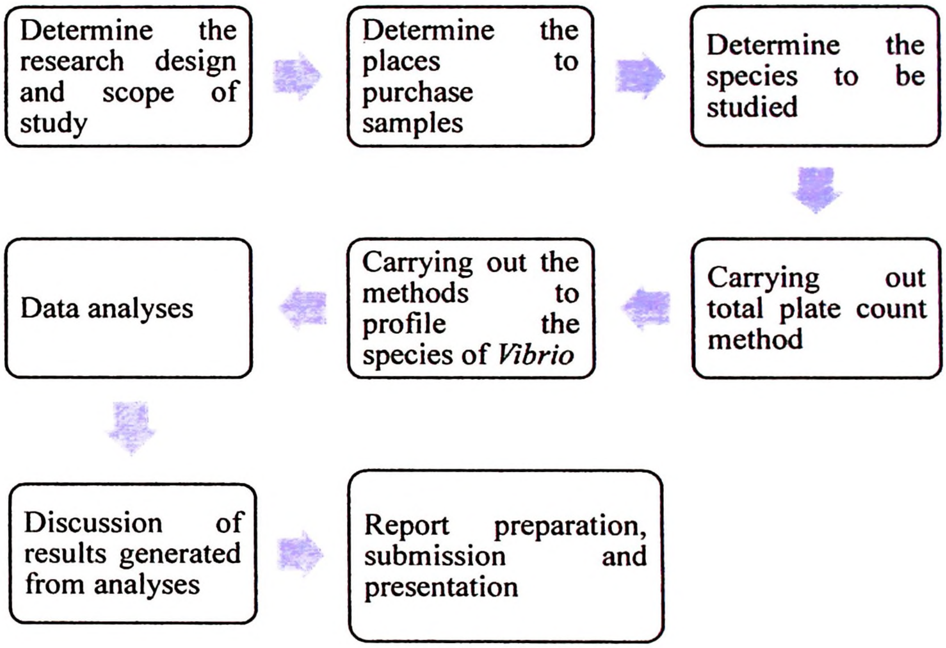

Figure 3.1: Flow chart for data collection