ANTIBACTERIAL ACTIVITY OF PRODIGIOSIN EXTRACT AGAINST OPPORTUNISTIC SHRIMP GUT BACTERIA ISOLATED FROM *Litopenaeus vannamei*

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by

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LIST OF ABBREVIATIONS

AHPND	acute hepatopancreatic necrosis disease
AMPs	antimicrobial peptides
BLAST	Basic Local Alignment Search Tool
CFU	colony forming unit
CLSI	Clinical and Laboratory Standards Institute
CpG ODN	cytosine guanine oligodeoxynucleotides
DCM	dichloromethane
DNA	deoxyribonucleic acid
EA	ethyl acetate
EHP	enterocytozoon hepatopenaei
EM	electron microscope
EMS	early mortality disease
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
FDA	Food and Drug Administration
GC-MS	gas chromatography mass spectrometry
h	hour
ha	hectare
HCL	hydrochloric acid
HMDS	hexamethyldisilazane
HPLC	high-performance liquid chromatography
HST	head soft tissue
Hx	hexane
IBRL	Industrial Biotechnology Research Laboratory
IHHVN	infectious hypodermal and hematopoietic necrosis virus
INT	p-iodonitrotetrazolium violet salt
LPS	lipopolysaccharide
MA	marine agar
MBC	minimum bactericidal concentration
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth

MIC	minimum inhibition concentration
mL	mililiter
MRL	maximum residue level
mt	million tonnes
MTT	dimethylthiazolydiphenyl-tetrazolium bromide
OIE	World Organization for Animal Health
OTU	operational taxonomic unit
PCR	polymerase chain reaction
RDS	runt-deformity syndrome
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
rpm	revolutions per minute
SEM	scanning electron microscope
SID_{50}	shrimp infectious dose with 50% endpoint
SOD	superoxide dismutase
ssRNA	single stranded ribonucleic acid
TCBS	thiosulfate-citrate-bile-salts-sucrose
TEM	transmission electron microscope
UV	ultraviolet
WSSV	white spot syndrome virus
XTT	methoxynitrosulfophenyl-phenylaminocarbonyl-tetrazolium hydroxide
YHV	yellow head virus

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AKTIVITI ANTIBAKTERIA OLEH EKSTRAK PRODIGIOSIN TERHADAP BAKTERIA USUS UDANG YANG BERSIFAT OPORTUNISTIK YANG DIPENCILKAN DARI *Litopenaeus vannamei*

ABSTRAK

Penyakit udang peliharaan sekarang ini dilihat sebagai masalah yang kritikal kepada pengeluaran akuakultur dunia. Penyalahgunaan antibiotik secara berlebihan untuk merawat jangkitan bakteria walau bagaimanapun telah menyebabkan berlakunya rintangan terhadap antibiotik. Isu ini telah menimbulkan pelbagai masalah bukan hanya kepada industri penternakan udang malah juga kepada persekitaran berhampiran. Tujuan utama kajian ini adalah untuk menaksir aktiviti antibakteria oleh ekstrak prodigiosin dari bakteria laut *Serratia marcescens* IBRL USM84 terhadap bakteria oportunis yang diasingkan dari usus udang *L. vannamei* yang berpenyakit. Ekstrak mentah ekstrasel telah dipilih apabila ia menunjukkan kesan perencatan yang lebih baik dengan kadar pembunuhan yang lebih pantas pada bakteria yang diuji berbanding ekstrak mentah intrasel. Aktiviti antibakteria oleh prodigiosin dinilai ke atas sembilan bakteria oportunis Gram-positif menggunakan kaedah kerentanan antimikrob. Purata diameter zon perencatan berada dalam linkungan 16 ± 0.51 mm to 28.3 ± 0.29 mm sementara nilai kepekatan perencatan minimum (MIC) dan kepekatan bakterisid minimum (MBC) yang dicatatkan oleh ekstrak mentah ekstrasel adalah 125 µg/ml. Kajian ini menunjukkan semua bakteria Gram-positif yang diuji adalah sensitif kepada ekstrak mentah ekstrasel tersebut. Keberkesanan ekstrak mentah ekstrasel telah dikaji dengan lebih mendalam dengan menentukan kadar pembunuhan ke atas *Exiguobacterium profundum* MA6. Apabila terdedah kepada nilai 2MIC, ekstrak menunjukkan kebergantungan pada kepekatan dan masa dengan mengurangkan

jumlah bilangan bakteria sehingga lebih daripada 3 log 10 CFU/ml dalam tempoh 4 jam pengeraman. Pada kepekatan yang lebih tinggi, ekstrak akan membasmi pertumbuhan sel-sel bakteria. Kesan bakterisid yang diperhatikan di bawah mikroskop elektron penskanan (SEM) menunjukkan sel-sel bakteria berada dalam keadaan stres selepas terdedah kepada ekstrak, yang menyebabkan berlakunya perubahan serius pada morfologi sel. Pendedahan yang lebih lama kepada ekstrak juga telah menunjukkan kerosakan yang teruk pada sel bakteria apabila berlakunya pembentukan rongga dan pengumpulan serpihan sel yang akhirnya merobohkan struktur sel. Kesan sinergi pada ekstrak mentah ekstrasel menghasilkan kesan rencatan yang lebih bagus terhadap bakteria usus udang yang bersifat oportunistik. Prodigiosin mempunyai sifat antibakteria yang menjanjikan dan berpotensi sebagai agen antibakteria untuk merawat jangkitan bakteria di akuakultur.

ANTIBACTERIAL ACTIVITY OF PRODIGIOSIN EXTRACT AGAINST OPORTUNISTIC SHRIMP GUT BACTERIA ISOLATED FROM *Litopenaeus vannamei*

ABSTRACT

Disease in cultivated shrimp nowadays has seen as the critical problem to the aquaculture world production. The excessive and abusive used of antibiotic to treat bacterial infection, however, have driven the development of antimicrobial resistant. These issues have raised many problems to the shrimp aquaculture industry and nearby environment. Therefore, searching of new treatment method is important especially from natural substances against opportunistic and pathogenic bacteria. The objective of this research study was to assess the antibacterial activity of prodigiosin extract of marine bacterium *Serratia marcescens* IBRL USM84 against opportunistic bacteria isolated from the infected shrimp gut of *L. vannamei*. Extracellular crude extract was selected as it showed a better inhibitory effect with faster killing rate on tested bacteria compared to intracellular crude extract. The antibacterial activity of prodigiosin was evaluated on the nine Gram-positive opportunistic bacteria using antimicrobial susceptibility methods. The mean diameter of inhibition zone was ranging from 16 ± 0.51 mm to 28.3 ± 0.29 mm meanwhile both the minimum inhibition concentration (MIC) value and minimum bactericidal concentration (MBC) value recorded by the extracellular crude extract was 125 µg/ml. The results from this study showed that all tested Gram-positive bacteria were susceptible to the extracellular crude extract. The effectiveness of the extracellular crude extract was further studied by determining the killing rate on *Exiguobacterium profundum* MA6. The extract demonstrated concentration-dependent by reducing significantly the number of bacteria within 4

hours of incubation greater than 3 log 10 CFU/ml when exposed to 2MIC level. At higher concentration, the extract would eradicate the growth of tested bacterial cells. The bactericidal effect exhibited by extracellular crude extract observed under scanning electron microscope (SEM) revealed that bacterial cells become stress after being exposure to the extract, leading to alteration in cell morphology. Prolonged exposure to the extract had also demonstrated the severe damage of bacterial cells with the formation of cavities and accumulation of cell debris which eventually collapsed the structure of the cells. Synergism effect in extracellular crude extract produced better inhibitory effect against opportunistic shrimp gut bacteria. Prodigiosin has promising antibacterial properties and could be used as potential antibacterial agent to treat bacterial infection in aquaculture.

CHAPTER 1

INTRODUCTION

1.1 General introduction

In the world of aquaculture sector, Pacific white shrimp which also known as *Litopenaeus vannamei* is recognized as the most dominant crustacean species accounting almost 70% of total shrimp production. United Nations estimates that by the year 2050, there will be an excess 9.7 billion people due to exponential growth in the global human population (Millard *et al.,* 2020). The growing demand of shrimp globally have made various types of cultured system being introduced starting from extensive to semi-intensive and intensive systems. As the largest shrimp producer that exporting this valuable species to many other developed and developing countries, Southeast Asia has expanding the aquaculture sites and increase the number of shrimps in single pond to intensify production.

However, high stocking density has been coupled with the emergence of devastating diseases. Shrimp growths are hindered by a range of numerous bacterial, viral, fungal and parasites diseases and until recently the latest bacterial disease become more aggressive causing 100% mortality leading to significant economic losses (Shinn *et al.,* 2018). Acute hepatopancreatic necrosis disease (AHPND) is a persistent and new emerging disease in farmed shrimp populations, initially detected caused by *Vibrio parahaemolyticus.* The latest update regarding AHPND reported rearing shrimp can be infected with multiple pathogens simultaneously which accelerate the rate of mortality and severity of co-infection. AHPND alone is categorized as a lethal disease, therefore, when pre-infected shrimp exposed to the secondary pathogenic bacteria, a more destructive effect is observed on vital organs and affected tissues (Han *et al.,* 2019; Han *et al.*, 2020).

Due to high disease burden, antibiotics have been applied together with shrimp feed or given directly to the water in the hope that this bacterial disease can be controlled and reduced the mortality rate among the rearing shrimp species. Unfortunately, antibiotic treatment does not resolve the disease problem. Instead, the problem getting more complicated after certain of period when antibiotics become less effective. In addition, the development of antimicrobial resistance also posed a threat to animal and human health (Cabello *et al.*, 2013). Several bacteria derived from the penaeid culture environment such as *Vibrio* spp., *Aeromonas hydrophilla*, *Klebsiella* sp., *Acinetobacter* spp., and *Bacillus* spp. have been reported to carry multi-resistance genes to the clinical antibiotics including sulfonamide, tetracycline and ß-lactams (Gao *et al*., 2012; Costa *et al.*, 2015; Pham *et al.*,2018).

Therefore, there is a need to replace antibiotics with other treatment option which is biodegradable and more eco-friendly that can help maintain the shrimp microbiome and improved overall shrimp health. In a meantime, the new antimicrobial agent may prevent the spreading of antimicrobial resistance among opportunistic and pathogenic bacteria. This research focused on the microbial metabolites called prodigiosin, that is a natural red pigment produced by a marine bacterium *Serratia marcescens* IBRL USM84, isolated from a marine sponge, *Xestospongia testunidaria*.

1.2 Rationale of study

Marine environment host vast variety of life forms as 95% of Earth's biosphere is covered by saline in nature (Bhatnagar & Kim, 2010). Interestingly, it provides unlimited biological resource from diverse groups of marine microorganisms that could be found living in different marine habitats (Stincone & Brandelli, 2020). Marine bacteria during the last decades have been acknowledged as a major source of untapped bioactive compounds. Their unique metabolic, functional and structural properties become attractive to many researchers for discovery of natural secondary metabolites. Marine bacteria possess complex characteristic features of adaptation to constantly survive in continuous competitive and hostile condition such as extreme temperature, hypersalinity, pH and various ecological pressures which leading to the development of biosynthesis of complex secondary metabolite by producing pigment.

Besides, microbial metabolite compounds found in ocean are more diverse and abundance compared to the terrestrial environment (Soliev *et al.*, 2011). Ocean has wide range of habitats for marine microorganism. Some of them are capable of living in unique ecosystem such as deep-sea hydrothermal vent (Pettit *et al.*, 2011) and Artic marine sediment (Poli *et al*., 2017) that are characterized by different physical and chemical factors. Furthermore, some of the bioactive compounds produced by marine bacteria are differ and may not found in terrestrial bacteria (Biswas *et al*., 2016). Bioactive compound from marine environment usually are larger in size, more hydrophobic and have more rotatable bonds and chains in their structures for easier adaptation in high pressure (Shang *et al.*, 2018). Hence, the exploration of marine bacterial pigment can be a great potential and promising sustainable source of new natural bioactive compounds.

Prodigiosin is recognized among fascinated marine bacterial pigment that possessed wide variety of pharmacological properties with red natural colour of pigment. The pharmacological activities include antimicrobial, antioxidant, anti-tumor and immunosuppressive (Suryawanshi *et al.,* 2014; Lapenda *et al*., 2015; Arivizhivendhan *et al*., 2018). It is considered secondary metabolites which synthesized by several Gram-negative bacteria with *Serratia marcescen* as a major producer. Prodigiosin can be produced through complex mechanism of quorum sensing whereby individual bacterial cells communicate with each other for secretion of pigment or also can be induced by several stress condition in external environment. Scientific literature represents that natural red pigment of prodigiosin play a role as a defense mechanism. Although the role of prodigiosin has been increasingly discovered, studies on the effect of prodigiosin on varying species of bacteria isolated from different sources are still limited. Moreover, the ability of prodigiosin as therapeutic drugs in aquaculture is not yet explored.

1.3 Research objectives

This research study was carried out based on several objectives:

a) To isolate and identify the bacteria causing shrimp disease specifically on AHPND infected cultured *Litopenaeus vannamei* using phenotypic and molecular identifications.
b) To perform extraction and fractionation of prodigiosin pigment produced by *Serratia marcescens* IBRL USM84 based on bio-assay guided fraction.
c) To determine the antibacterial activity of prodigiosin pigment against shrimp opportunistic and pathogenic bacteria.
d) To examine the effects of prodigiosin on a chosen bacterium, *Exiguobacterium profundum* MA6.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture industry

Aquaculture is an active cultivating method of various type of fish, shellfish, algae and other aquatic organism in a controlled environment. It involved breeding and rearing process in marine, brackish water or freshwater environments at certain period of time and harvesting to produce food for human consumption. Nowadays, aquaculture happens all around the world from Latin America to Southeast and Eastern of Asia including Malaysia. In the past of thousand years, aquaculture was known as ancient farming activity to China's population. Malaysia has the largest area approximately 17, 357 ha of brackish pond water abundant with bivalve molluscs, blood cockles, shrimp and some marine fishes; and 4, 769 ha is provided for freshwater aquaculture activity (FAO, 2008).

2.1.1 Shrimp production system

Basically, there are several types of mode of rearing and cultivating aquatic animals which can be divided into extensive, semi-intensive and intensive farming (Thornber *et al.*, 2020). Extensive polyculture system was first employed back then in the 1920s, that involved diversity of many crops, fish and crustacean species as water exchange largely depends on incoming tides (Ashton, 2008). It is an old traditional practice. Usually, extensive shrimp farming has approximately 100 ha size of pond, located on a coast or can be found in mangrove areas. This is the easiest way to culture aquatic animal species because it does not require any advanced technical skills and many labours, low production cost besides low stocking density per time ideal for daily consumption (Washim *et al*., 2020)

Rapid expansion and increasing demand had forced farmers to improve to semi-intensive and intensive farming systems for large scale commercial production business. They installed water pump, controlled temperature, acidity, oxygen level, salinity, adding artificial and supplementary feed in form of formulated pellets and fertilized the pond regularly. Semi-intensive farm typically yields 500 to 500 kg/ha annually suitable for medium stocking density meanwhile stocking density ranging from 25 to 50 per square meter of intensive farm produce 5 000-20 000 kg/ha. Shrimp is ready to harvest between 3 to 6 months using lift nets (Hashim *et al.*, 2005).

Shrimp world industry is quite competitive. Recently, super-intensive farming system was developed. The emergence of new development farming system primarily designed to improve water quality and provide efficient biosecurity from the existing commercial farm (Thornber *et al.*, 2020). Vietnam becomes a pioneer introduced better sustainable shrimp pond management by investing indoor environment with the pond being located in a greenhouse. Some other countries allow the aquatic farms to be operated in closed building (Xu *et al.*, 2021). These farms strictly operated on monoculture practice with target species *Litopenaeus vannamei*, prohibited usage of antibiotics and chemicals but encourage the utilization of probiotics and high-quality commercial fed pellet (Ferreira *et al.*, 2015). As water and sediment contamination were commonly arisen issues in shrimp pond areas, therefore opted to closed - tank pond system is a good choice to reduce contact with external environment. Super-intensive shrimp farming technology also is environmental friendly (Khoa *et al.*, 2020). Water discharged is treated properly in the discharged treatment zone.

2.1.2 Status of shrimp production

According to statistics reported by Tacon (2020) in 2017, a total of 112 million tonnes being produced globally represent aquaculture production from aquatic animals and plants species compared to production during the year 2000. Total production of 30 different species crustacean products including prawn, crab and lobster had produced 8.44 million tonnes (mt) worth USD 61.06 billion with marine whiteleg shrimp dominate crustacean aquaculture production, 4.46 mt (Tacon, 2020). Crustaceans have high commercial value and one of the most profitable market products. In Malaysia, annual aquaculture production during the year 2019 was estimated at 411 782 tonnes (DOF, 2019).

2.1.3 *Litopenaeus vannamei*

There are two genera under the family *Penaidaea* which are *Penaeus* and *Metapenaeus*, however only several species such as *Penaeus vannamei* (white leg shrimp) that contribute the most to the total revenue production local and international market and one of the most profitable species (Dugassa & Gaetan, 2018). Another species also includes *Penaeus monodon* (black tiger shrimp), *Penaeus japonicus, Penaeus merguiensis*, *Penaeus indicus* and *Penaeus orientalis* (Thitamadee *et al.*, 2016). *P. vannamei* is indigenous to the Eastern Pacific coast of America (Roshith *et al*., 2018). This euryhaline species can survive at temperature above 25℃ to 32℃ (Zheng *et al.*, 2016; Dugassa & Gaetan, 2018) and tolerate a broad range of environmental salinities (Roshith *et al*., 2018). It has a total of 20 segments of head that fused with thorax called cephalothorax, walking legs (pereopods), swimming legs (pleopods), compound stalked eyes and tail. Five pair of walking legs enables *P.vannamei* to hunt the food at the sandy and muddy bottom area during at night. Shrimp calcified-hard cuticle is important to protect the vital organs like gill, stomach, brain and heart from any damage (Dugassa & Gaetan, 2018).

2.1.4 Life cycle and feeding habit

Like other penaeid species, *P. vannamei* undergo metamorphosis and development during their life which constitutes into four stages mainly larvae, post larvae, juvenile and adult (Figure 2.1) (Wei *et al.*, 2014). They begin a new journey by periodically shedding their old skeleton and grow into their maximum size. The outer shell is primarily made up of protein, calcium and chitin (Gao *et al*., 2017), so when shrimp moulting, it re-absorbs all necessary nutrients from the old skeleton. In a meantime new skeleton emerge and hardening, shrimp expand their body and increase water intake leaving only a small space between the body and new shell. Shrimp are short lived animal hence hatching the egg occurs within 16 hours. After hatching, larvae remain in offshore water, unable to swim and depend mostly on plankton feeding microalgae. The zoeae larvae will then be metamorphosis into a mysis stage to post larvae in almost 10 days before they migrated from offshore to a low salinity area near estuaries or mangroves. They spent their juvenile and adults there up to five months (Rosle & Ibrahim, 2017). At this stage, shrimps eat various types of food. They are well known as carnivorous animal. Penaeid shrimp capture mollusc, crustaceans and detritus.

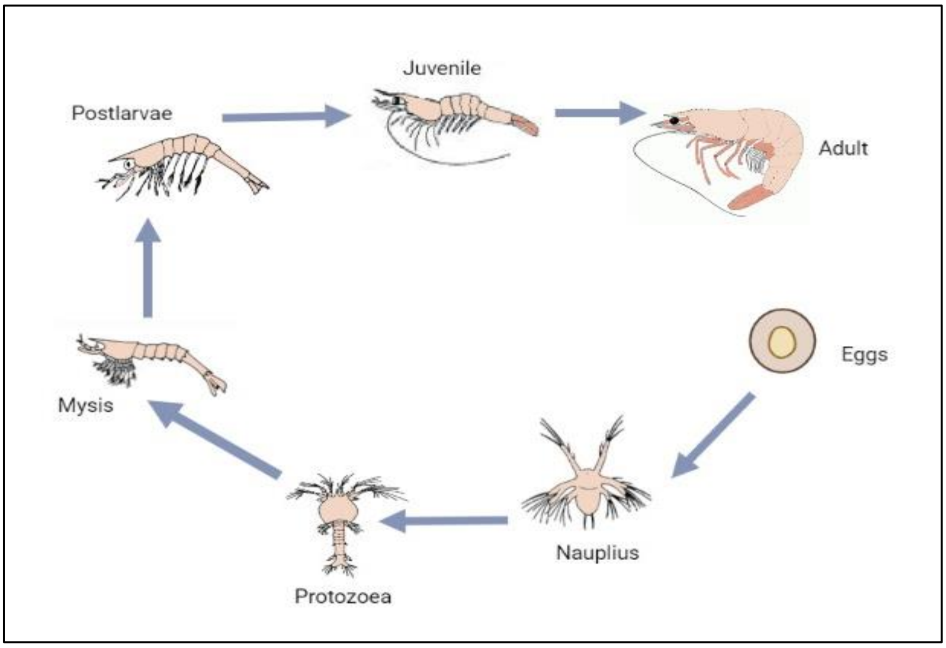

Figure 2.1: Life cycle of penaeid shrimp

2.2 Shrimp gut microbiome

The shrimp gastrointestinal tract which also known as gut is located between the mouth and anus has three main parts (Figure 2.2). The first part is the foregut that comprises of stomach and oesophagus, while the second part is called the midgut and it is the longest section where hepatopancreas and intestine are located. The third part is the hindgut with chitinous cuticle. Shrimp gut is a vital organ which carry out various life functions by assisting in digestion activity (Tzuc *et al.*, 2014), promote nutrient absorption (Fan *et al.*, 2019), maintain immune homeostasis (Jarchum and Pamer, 2011), stimulate immune response (Zhou *et al.*, 2019) and food storage.

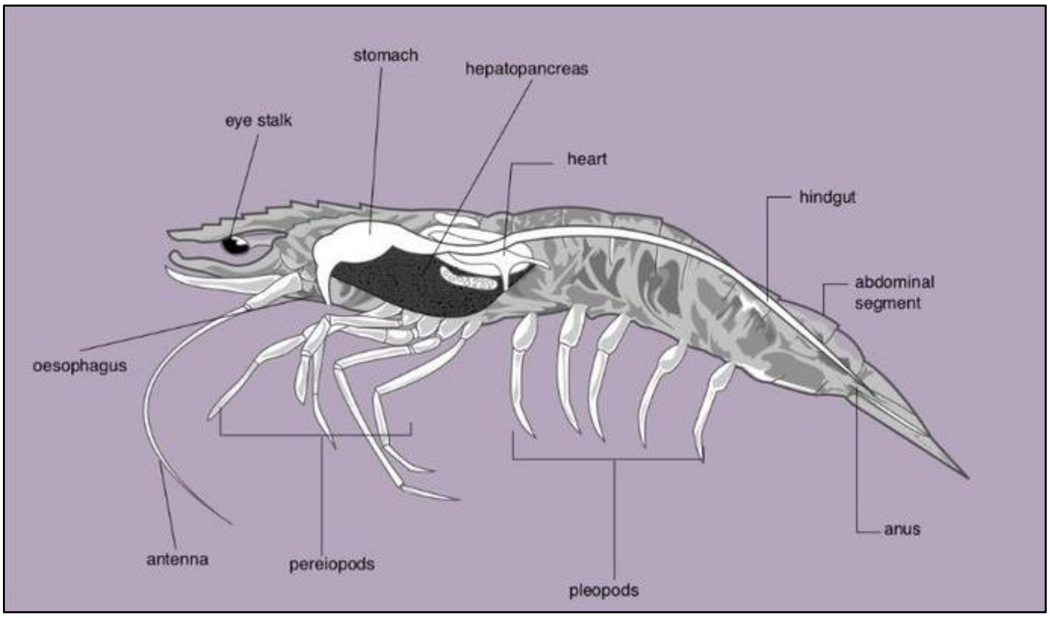

Figure 2.2: Internal and external anatomy of penaeid shrimp (Bondad-Reantaso *et al.,* 2001)

In the past decade, disease prevalence in marine and inland aquacultures has been greatly affected the performance and productivity of penaeid shrimp. Therefore, many researchers were trying to find out in their studies remedy to reduce loss of bulk shrimp either from nutritional aspect to environmental factors to neither diet components nor bacterial composition in shrimp gut itself. Ramirez *et al.* (2013) and Ringo *et al.* (2016) suggested that manipulation of microbial composition in the gut could produce a positive effect on the shrimp health as those bacteria that present in the aquatic environment can influence the composition of the gut microbiota. As for the rearing pond environment, bodies of water and sediments are two habitats where bacteria can be found living abundantly. Development of the gastrointestinal tract of shrimp is a gradual process yet its digestive system only fully developed once reach post-larvae stage. This also indicates the diversity of bacterial population in every shrimp growth stage is a dynamic process. The exogenous bacteria start to colonize inside the shrimp gastrointestinal tract when anal pore begin anal drinking movement during the nauplius stage (Tzuc *et al*., 2014).

In a healthy gastrointestinal tract of *L. vannamei*, there are undoubted various kind of different genera of microorganisms reside and inhabit the gastrointestinal tract including both opportunistic pathogenic and beneficial bacteria (Zheng *et al.*, 2017). Some of them are recognized as transient microbiota because their presence only lasts for a certain period of time where they come along with the ingested food and might cause the intervention in a shift of bacteria colonization. In contrast, resident bacteria are indigenous and permanent bacteria that adhere to the gut wall (Shade & Handelsman, 2012; Yukgehnaish *et al.*, 2020). It is considered as a normal microbiota if the composition of community among the opportunistic, pathogenic and beneficial bacteria is balanced with symbiotic and commensal relationships. To analyze and understand the microbiome within the shrimp gut (Tzuc *et al.*, 2014; Cornejo-Granados *et al.*, 2017; Holt *et al.*, 2020), it is compulsory to note that different geographical distribution, climate, environment (Yukgehnaish *et al.*, 2020), intrinsic, extrinsic (Li *et al.*, 2018; Kumar *et al.*, 2020) factors and shrimp phylogeny (Yu *et al.*, 2018) might contribute to the divergence of microbiome communities regardless of the same species.

2.2.1 Core bacterial population

Core bacteria can be defined as key bacteria colonize inside the shrimp gut. Based on several studies on penaeid shrimp species (Rungrassamee *et al.*, 2014), it is suggested that the core bacterial population of wild type caught and cultured showed quite similar bacterial populations at the phyla level with differences in bacterial richness and abundances classified using operational taxonomic unit (OTU). This perhaps could be due to the selective pressure from the environment (Jarchum & Pamer, 2011). In general, interaction in a wild habitat is unlimited compared to rearing pond area which explained the diverse of bacterial population found in a wild caught intestine of penaeid shrimp. There are five common bacterial phyla found in shrimp gut comprising of Proteobacteria, Bacteroidetes, Firmicutes, Fusobacteria and Actinobacteria (Rungrassamee *et al*., 2014; Oetama *et al*., 2016; Li *et al.*, 2018; Fan *et al.*, 2019). Among them, Proteobacteria is the most prevalent and abundant phylum reported in the intestine of *L. vannamei* and *P. monodon* that accounted for more than 40% (Rungrassamee *et al.*, 2014; Fan *et al.*, 2019). Li *et al.* (2018) proposed that the Proteobacteria as the most stable bacteria. This phylum also can be commonly seen in other crustaceans since Proteobacteria is the highly diverse and ubiquitous phylum that can be found anywhere including aquatic environment.

2.2.2 Shift in gut microbiome influence shrimp health

Imbalanced of bacterial community and decreased diversity affect major shrimp immunity and health. This abnormal condition that alters the bacterial richness and composition in a shrimp intestine is referred as dysbiosis. Diseased shrimp of *L. vannamei* revealed significantly in the increasing number of opportunistic and pathogenic bacteria from the members of the order Vibrionales (Yao *et al.,* 2018; Yu *et al.,* 2018) which dominated their intestines and hepatopancreas (Cornejo-Granados *et al*., 2017). This finding is also supported by the study carried out by Chen *et al*. (2017) to figure out the possible shift population in the healthy and AHPND-infected shrimp stomach. It seems that a healthy intestine has more complex bacterial interspecies interaction than the infected shrimp. In healthy gut, commensal microbiotas compete to each other for habitat and growth-limited resources. This antagonistic interaction has formed colonization resistance and is associated with stable and diverse bacterial community. Colonization resistance can offer shrimp fitness and protect the shrimp by enhancing its immune system from the overgrowth of pathogens (Xiong *et al.*, 2017). Nonetheless, about 33.7% of *Vibrio* and 11.6% of *Photobacterium* under the Vibrionaceae family had been detected present in the healthy shrimp intestinal gut reared in Malaysia and Vietnam pond areas which believed to be endogenous (Zoqratt *et al.*,2018). In other studies, *Vibrio* and *Photobacterium* were consistently detected in the disease-free shrimp dominated surrounding gastrointestinal tract, yet no symptom of infection was found (Rungrassamee *et al*., 2014; Zheng *et al.,* 2017). Bacteria from both genera originally are natural part of normal flora and prevalent in shrimp digestive tract. During the moulting stage, shrimp repeatedly shed off old exoskeleton, replaced and renewed with new lining. *Vibrio* spp. were apparently responsible in the production of chitinolytic enzyme to degrade chitin (Soonthornchai *et al*., 2015) and utilize chitin for its nutrient source. However, under certain circumstances such as malnutrition and stress (Zhu *et al.*,2016), vibrio could disrupt and shift bacterial population.

Apart from that, some bacterial groups in the order Actinomycetes and Sphingobacteriales could be considered as 'diseased indicator' (Xiong *et al*., 2015). They showed greater relative abundance in the intestine at the order level. At the family level, disease severity also correlated with the enriched of Rickettsiaceae (Zhou *et al.*, 2019). The abundance of Rickettsiaceae deteriorates the hepatopancreas and digestive tract causing shrimp loss of appetite. As a result, shrimp body size becomes seriously affected and there is an exclusively reduction in body weight leading to yield loss and unmarketable shrimps. Besides, very low abundance of Rhodobacteraceae and Flavobacteriaceae indicate shrimp health status since both usually are present in all growth stages as the dominant members under phyla Proteobacteria and Bacteroidetes respectively (Yao *et al.,* 2018). *Candidatus Bacilloplasma* (Tenericutes), *Phascolarctobacterium* (Firmicutes), *Pseudoalteromonas* and *Alteromonas* (Hou *et al.*, 2018; Alfiansah *et al.*, 2020) detected by 16S rRNA sequencing in the infected intestine and asymptomatic shrimp showing remarkable multiplied but reduce the overall diversity of the bacterial community.

As a conclusion, it could be said that the exact number and relative abundance of opportunistic and pathogen bacteria for major alteration of microbiota is still pretty much unclear, same applied to specific bacterial groups in certain phyla. Therefore, it is not easy to predict shrimp health status.

2.3 Diseases in shrimp aquaculture

Raising penaeid shrimp species has become a spotlight among agriculture farmers after the introduction of *L. vannamei* species to Malaysia in 2002 (Kua *et al*., 2018). However, intensification of stocking density has invited a high level of bacterial infection in intensive farming pond which later on deteriorates the growth and shrimp productivity (Walker & Winton, 2010). Subsequently, several measures have been taken to prevent and control the disease but still the outbreak is remaining concern. Shrimp can be infected via direct and indirect transmissions. Direct transmission is when a shrimp has a direct contact with infected live feeds and acquire the disease through ingestion while indirect transmission happens in the contaminated water environment (Raja *et al.*, 2015). The most common diseases infected the farmed shrimps are from viral and bacterial infections. The occurrence of massive mortality has led to economic loss especially to the low and middle classes countries in Asia (Kalaimani *et al.*, 2013; Shinn *et al.*, 2018).

2.3.1 Bacterial diseases

Back in 2009 mass mortalities have been detected occurring in farmed shrimp ponds in the southern part of China, and the disease was named acute hepatopancreatic necrosis disease (AHPND). Originally, it was known as early mortality disease (EMS). Shrimp is the fastest growing food sector where most developed and developing countries depend on its production. Thus, when EMS outbreak emerged, extensive research was carried out. Tran *et al.* (2003) in their studies reported that EMS was caused by a strain of halophilic Gram-negative marine bacterium. It colonized inside *P. vannamei* digestive tract which later identified as *Vibrio parahaemolyticus* using 16S rRNA sequencing. This opportunistic pathogen bacterium can be found abundant inside hepatopancreas, a vital organ that has a similar function as the liver and pancreas. The affected shrimp showed necrosis condition in hepatopancreas tissue, enlarged nuclei, sloughing and degradation of hepatopancreas tubules (Manan *et al*., 2015). Recent study revealed the etiological agent of AHPND by other *Vibrio* spp. such as *V. harveyi* (Kondo *et al.*, 2015), *V. campbelli* (Han *et al*., 2017), *V. owensii* (Xiao *et al.*, 2017) and *V. punensis* (Restrepo *et al.*, 2018). Besides, a study by Kua *et al.* (2018) showed that infection become exacerbate especially when *P. vannamei* under stress condition.

During routine polymerase chain reaction (PCR) screening, it has been found that *Vibrio parahaemolyticus* can drastically has impaired health status, weakened the immune system of shrimp and induce cell death. It harbors binary toxin genes, PirA and PirB containing 69 kb of virulent pVA1 plasmid (Kumar *et al.*, 2020). However, without the presence of extrachromosomal plasmid, the ability to cause disease is abolished (Lee *et al.*, 2015). Deletion and alteration in nucleotide sequence also are not able to generate AHPND-caused effect. This is due to the disruptions of PirA gene expression that prevent this strain from producing a required amount of binary toxin to cause AHPND pathology (Sirikharin *et al.*, 2015; Caro *et al.*, 2020). Although their disease-causing mechanisms remain unclear, in general, *V. parahaemolyticus* started to colonize in shrimp stomach after successfully enter via feeding route through ingestion (Prachumwat *et al.*, 2018). Once toxins are released and seeping into the tubule of hepatopancreas, it immediately activates virulent plasmid with pndA postsegregational killing system ensuring plasmid is inherited (Lee *et al.*, 2015). Horizontal gene spread of plasmid then conjugatively transferred between one vibrio cell to another cell receiving AHPND toxin gene via pilus, reproduce and replicate (Dong *et al*., 2019) before slowly induce sloughing of the tubule of epithelial cells in hepatopancreas. However, the amount of PirAB released is useful to indicate the level of AHPND virulence as some of the plasmid in non-AHPND strain do not contain virulence genes (Tinwongger *et al.*, 2016).

2.3.2 Viral diseases

2.3.2(a) White spot syndrome virus (WSSV)

Meanwhile, white spot syndrome virus (WSSV) is a viral infection. It is the most widely threat infection among marine invertebrate animal causing serious mortality of crustacean species. *P. monodon*, *P. vannamei*, *P. japonicus*, *P. chinensis*, *P. stylirostris* and *Macrobrachium rosenbergii* (giant river prawn) are seems to be highly susceptible to WSSV compared to marine crab, lobster and other crustacean species (Wang *et al.*, 2002). Severity of WSSV does not consider adverse if the WSSV alone without involving other stressors. A study by (Tsai *et al*., 1999) had discovered terminal infection of single WSSV can be detected after 13 months reared in pond at low intensity under low-stress culture condition. Many studies have been conducted since decades ago to determine the correlation between abiotic factors such as temperature, salinity, dissolved oxygen, weather, pH and disease outbreaks (Gao *et.al.,* 2011; Zhang *et al.*, 2016), as these external factors exerted extra high pressure to shrimp that would increase WSSV viral replication and massive mortality.

Early sign of WSSV can be detected during feed intake where shrimps only consume in small portions. Then circular white spots would appear around the exoskeleton, and the carapace started to detach from its body, and soon followed by the formation of dark reddish colour on their body surfaces. According to Lightner (1996), Wang *et al.,* (1999) and Gao *et al.,* (2011), the cumulative mortality of WSSV post-infection shrimp could reach about 90-100% within 2 to 10 days. Brood stock quality is the first indicator to prevent WSSV from spreading because it can be transmitted vertically direct from parents to offspring and has high possibility to rapidly spread this virulent virus to other shrimp in the same population via horizontal transmission (Iqbal *et al*., 2011).

2.3.2(b) Infectious hypodermal and hematopoietic necrosis virus (IHHNV)

From the 20 different shrimp viral diseases, IHHVN is among the five major viral pathogens listed by OIE infected penaeid shrimp which seriously impacted the sustainability of shrimp aquaculture (Walker & Mohan, 2009). IHHVN is classified under the family *Parvoviridae*. Recently, besides penaeid shrimp, this single-stranded DNA virus successfully penetrates freshwater prawn, *Macrobrachium rosenbergii* population in Malaysia (Khalid, 2013). Both species have been reported to induce the development and suffer from runt-deformity syndrome (RDS) with abnormalities around the rostrum. They also experienced in size variation, deformed sixth abdominal segment, cuticular roughness and wrinkled antennae (Jagadeesan *et al*., 2019) especially in *P. vannamei* during chronic infection. Chayaburakul *et al.* (2005) found that the infection of IHHVN in cell tissues of *P. vannamei* is generally more severe than *P. monodon.* Although a high prevalence of IHHVN usually was detected in blue shrimp *L. stylirostris,* the physical abnormalities and stunted growth rarely appear in this adult shrimp species, instead, heavy mortalities and acute epidemics at juvenile and sub-adults of *L. stylistrosis* (Seibert *et al.,* 2012).

2.3.2(c) Yellow head virus (YHV)

A study conducted by Wongteerasupaya *et al.* (1995) had identified the virus that caused YHV outbreak in *P. monodon* rearing pond. It belonged to order

Nidovirales, family *Ronoviridae*, *Okavirus* genus and positive-sense single-stranded RNA (+ssRNA). When observe under transmission electron microscopy (TEM), yellow head virus has rod-shaped structure virion with an inner helical nucleocapsid (Chantanachookin *et al.*, 1993). This virus begins its replication in the cell cytoplasm, spread and infected easily nine parts of tissues and organs of juvenile and subadult of penaeid. There are gills, lymphoid organ (Oka), nerve cord, heart, midgut, hepatopancreas, head soft tissues (HST), abdominal muscle and eyestalk (Munro & Owens, 2007; Navarro-Nava *et al.*, 2011). After the onset of the disease clinical signs, it only took between 3 to 5 days to wiped out the entire population as the cumulative mortality could reach 90%-100% resulted from necrotic cell death (Seibert & Pinto, 2012). This highly lethal virus epizootic and endemic in Thailand that cultivated *P. monodon* before widely spread to other Asian countries including Malaysia through international trade of frozen shrimp (Seibert & Pinto, 2012). The virus was named after the appearance of pale yellowish colour accumulated surrounding the cephalothorax area due to discoloration of gill and hepatopancreas (Munro & Owens, 2007).

2.3.3 Fungi and parasite diseases

Most of the reported shrimp infectious disease caused by the fungal parasite come from the phylum Microsporidia; unicellular intracellular parasite that possess unique survival capability of highly resistant spores. Generally, organisms within this phylum are able to reproduce only in the presence of animal host inside gut cells by directly transfer its sporoplasm through a polar tube of spore into the host cell cytoplasm. The main target tissue is epithelial cells in the hepatopancreas (Aranguren *et al.*, 2017). Enterocytozooan hepatopenaei (EHP) is a new parasitic pathogen that causing growth retardation of cultured black tiger shrimp (*P. monodon*) in Thailand and Malaysia (Anderson *et al.*, 1989; Chayaburakul *et al.*, 2004), kuruma shrimp in

Australia (*P. japonicus*) (Hudson *et al.,* 2001), white leg shrimp (*P.vannamei*) in Vietnam, Indonesia, India and Venezuela (Rajendran *et al.,* 2016; Tang *et al.,* 2017; Han *et al.,* 2020) without showing any single clinical symptoms. EHP could lead to unprofitable commercial shrimp farming because of the impact of the disease appearing late. However, the symptoms are easily determined with the accompaniment of other opportunistic infection known as co-infection. Aranguren *et al.* (2017) in their studies found that EHP and *Vibrio* sp. both attack and deteriorate hepatopancreas in different ways but EHP pre-infected *P. vannamei* would increase the favour of the establishment of vibrio associated with AHPND. Observation after 12 hours of post-infection had found that there was massive sloughing of hepatopancreas tubule cells which increase the damage to hepatopancreas. Unlike many other shrimp infections, EHP does not cause mortality and is difficult to eradicate. Table 2.1 summarizes the disease which commonly occurred in Asian shrimp pond.

Table 2.1: Summary list of shrimp disease

Type	Disease	Abbreviation	Etiological agent
Bacteria	Acute hepatopancreatic necrosis disease	AHPND	*V. parahaemolyticus*
			V. harveyi
			V. campbelli
			V. owensii
			V. punensis
Virus	White spot syndrome virus	WSSV	dsDNA virus from genus *Whispovirus*
	Infectious hypodermal and hematopoietic necrosis virus	IHHNV	ssDNA virus from family *Parvoviridae*
	Yellow head virus	YHV	ssRNA virus from genus *Okavirus*
Fungi/parasite	Enterocytozooan hepatopenaei	EHP	fungal parasite from phylum Microsporidia

2.4 Multiple infections

Great loss of shrimp becomes more severe when multiple infections in the aquaculture environment emerged. Compared to fish, knowledge and information regarding shrimp concurrent infection are still constrictive and limited even though co-infection occurs regularly in shrimp ponds. It is plausible as shrimp’s natural environment harbor varieties of living microorganisms. During co-infection with heterologous pathogens, a dose of 30 SID_{50} of WSSV was injected into juvenile *P. vannamei* shrimp followed by different doses of *Vibrio campbelli* with 10^3, 10^4, 10^5, 10^6 CFU shrimp^{-1} (Phuoc *et al.*, 2009). Based on these experimental challenges, shrimp died much faster in dual infection at least with 10^4 CFU shrimp-^{1} concentration compared to a single infection of WSSV or *V. campbelli*. The result obtained showed synergistic interaction between these two pathogens (Phuoc *et al.*, 2009). Rubio-Castro *et al.* (2016) also mentioned that simultaneous infection between WSSV and *V. parahaemolyticus* at the same time recorded higher cumulative mortality in addition to the short time taken for shrimp to die after concurrent post-infection. In this field study, shrimp died between 12 h and 72 h post-infection. Moreover, it was also found that the severity of dual infection is associated with the immune response which weakens the resistance of shrimp against pathogenic bacteria.

2.5 Relation between shrimp health and immune system

Unlike vertebrates, shrimp are considered unique because they only possess innate immunity as their defense mechanism (Rusaini *et al.*, 2010). Farmers in the aquaculture sector always encounter shrimp mass mortalities because of the disease incidence. To overcome this problem, we need first to understand and have knowledge about the shrimp immune system.

If a vaccine can be injected into the infected fish as one way of treatment, shrimp on the other hand does not capable to absorb the vaccine and give out the expected response. This is because shrimp lack of adaptive immune system (Vazguez *et al.*, 2009). This is supported by the finding on vaccination studies of juvenile *L. vannamei* after haemolymph was injected by formalin-inactivated *B. subtilis*. Vaccine is believed to boost the growth and welfare of shrimp however, this study shows there was no increase in the number of phagocytic cells and absence of antibacterial activity after exposure to *B. subtilis* (Pope *et al.*, 2011). In another word, shrimp does not record history on specific memory against invading foreign substances and pathogens after the first infection. Thus, it would not be able to recognize and remember the same pathogen. Instead, it relies completely on the innate immune system or passive immunity. Infection does not occur immediately, however, spreading virulence gene demands pathogen enough times to break shrimp first-line defence and breach into shrimp tissue. The first-line defence of shrimp is the hard-calcified exoskeleton made up of chitin that protects the entire body of shrimp (Aguirre-Guzman *et al*., 2009).

Cellular and humoral innate immune responses are vital after the pathogen managed to get past the physical barrier. Both involved different mechanisms. For instance, phagocytosis and encapsulation and nodulation in the cellular immune response trap, degrade and destroy foreign substances whereas humoral responses working with various immune molecules and proteins. These are including antimicrobial peptides (AMPs), serine proteinases, inhibitors, pattern recognition protein and oxidative enzymes (Bachere *et al*., 2004; Tassanakajon *et al.*, 2013).

Haemocyte is a type of cellular effector circulating in haemolymph (Aguirre-Guzman *et al*., 2009). Haemocytes play important roles as a defence bridge between immune response and pathogen. However, the number of haemocytes released by hematopoietic tissues depleted drastically during infection (Johansson *et al.*, 2000). Due to abnormal levels of haemocynin, fluctuation of haemocytes restricts the flow of oxygen in haemolymph (Noga, 2000). As a result, several organ malfunctions due to stressful conditions and favour the development of pathogen invasion (Boonyaratpalin *et al*., 2001). Usually, those organs are very important to the physiology, nutritional and immune status of shrimp. They are hepatopancreas, lymphoid organ (Van de Braack *et al.,* 2002), gills (Taylor & Taylor, 1992), intestine consists of foregut to hindgut (Jayabalan *et al.,* 1982) and stomach. Infected haemolymph would appear red colour whilst blue colour in the healthy haemolymph (Song *et al.,* 2003). Velazquez-Lizarraga *et al*. (2019) in their findings suggested that stress conditions also might be a precursor for excessive production of reactive oxygen species (ROS). Failure of antioxidant defense to remove the overwhelming ROS production will cause cellular and oxidative damages (Akbulut *et al*., 2014; Duan *et al*., 2015).

Hepatopancreas plays an important role during digestion and absorption of nutrients. Presumptive test and study on microscopic tissue of AHPND to further determine the stage of infectious disease have revealed shrimp hepatopancreas could be an initiator for deterioration of its health (Tran *et al*., 2013; Devadas *et al*., 2019; Santos *et al*., 2020). Another organ affected is the lymphoid organ (Oka). It is known as a phagocytic organ to trap and accumulate foreign material from entering the circulatory system. Interestingly, other crustacea do not possess lymphoid organ like shrimp do (Rusaini, 2020). This advantage remarkably aided in identifying viral infection as the lymphoid organ becomes spheroids after being invaded by the pathogen (Rusaini, 2020).

To enhance the shrimp non-specific immunity and control the occurrence of disease in aquaculture, different approaches have been identified. The most popular and promising method is immunostimulant (Apines-Amar *et al.,* 2015). It is widely commercialized in aquaculture with no direct effect on pathogens but enhanced the innate defense system. Like a vaccine, immunostimulant is used as a prophylactic agent (Apines-Amar *et al.,* 2015). Bacterial derivatives (probiotics, LPS), complex carbohydrates (prebiotics, glucans), nutritional dietary supplement (vitamin, carotenoid, trace element), animal and plant extracts (seaweed, herbs, chitin), hormones (growth hormone), cytokine (lactoferrin), synthetic sources (levamisole, CpG ODN) have been confirmed to perform in modulating the immune system of shrimp by directly interact with cells of the immune system and activate them (Wang *et al.,* 2005; Citarasu *et al.,* 2006; Flores-Miranda *et al.,* 2011; Qiao *et al.,*2011; Meena *et al.,* 2012; Apines-Amar *et al*., 2015; Mastan, 2015; Mohan *et al.,* 2019). Furthermore, both humoral and cellular immunities will cooperate in recognizing and eliminating pathogens. Immunostimulants can be administered through injection, immersion or oral administration. So far, immersion and oral methods have been shown to be the most effective method to stimulate/activate the innate immune system against pathogens. (Jadhav *et al.*,2006).

2.6 Antibiotics

Antibiotics era began in the 19th century following the discovery of effective drugs to treat many incurable and serious diseases by two British renowned researchers, Paul Ehrlick and Alexander Fleming. Paul Ehrlick embarked on the journey by thinking of practical chemotherapy that selectively targets only the disease-causing microbes without harming the other host cells until he discovered his ‘magic bullet’ (Aminov, 2010). Alexander Fleming, on the other hand, accidentally discovered antibiotic named penicillin on his uncovered grows culture plate in his laboratory after leaving it unattended for holiday. He was astonished upon contamination of a fungus *Penicillin notatum* on staphylococci that created free zone area and inhibited the growth of this bacteria. This initial discovery had led to much research for the development of new antibiotic drugs. Antibiotics have two primary properties namely bacteriostatic and bactericidal. Bacteriostatic is an action of stop and hinder the growth and multiplication of target bacteria whereas bactericidal has powerful capability in killing microorganisms.

2.6.1 Antibiotic application in aquaculture

The emergence of disease threatening shrimp productivity is a major problem faced by farmers nowadays. In many developed and developing countries that practicing intensive shrimp farming systems, the usage of antibiotics is common and