Faculty of Resource Science and Technology

Distribution of Antibiotic Resistant Bacteria in Selected Aquaculture Farms in Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

There is a growing awareness on the importance of aquaculture for the social-economical livelihood for low- and mid-income community. Due to the domestic and global demand for fish meat as a preferred source of protein, this had generated a rampant growth of the aquaculture industry seen across the globe alongside a heavy use of antibiotics. These activities pose a risk of spreading antibiotic resistant bacteria (ARB) to the aquaculture environment, which tends to develop among environmental bacteria that is exposed to antibiotics. This study aimed to detect the distribution of ARB from five selected aquaculture farms in Sarawak. A total of 350 samples consisted of fish (108 samples), sediment (113 samples) and water (129 samples) were collected from five selected aquaculture farms within Sarawak. The samples were kept and transported immediately to the laboratory in an ice box. Upon arrival in the laboratory, the samples were plated on trypticase soy agar and incubated at 28 ℃ for 24 hours. After incubation, a few colonies were randomly picked, purified and stocked in glycerol. $(GTG)_5$-fingerprinting were employed to analyse the genetic differences of the bacterial isolates. A dendrogram was constructed based on the $(GTG)_5$-PCR patterns with similarity index ranged from 50% to 74% similarity. Based on the dendrogram, 50 isolates were chosen for species identification using 16S rRNA sequencing. These isolates were then tested with 25 antibiotics using a standard disk diffusion method. The degree of resistance of the isolates were categorised based on their area of inhibition zones whether they are resistant, intermediate and susceptible. About two hundred and four bacterial isolates were isolated and after species identification, 50 of these isolates constitute of 14 genera of bacteria including *Bacillus* (38%), *Exiguobacterium* (16%), *Enterobacter* (14%), *Aeromonas* (6%), *Acinetobacter* (4%), *Citrobacter* (4%), *Staphylococcus* (4%), *Achromobacter* (2%), *Chitinophaga* (2%), *Fictibacillus* (2%), *Plesiomonas* (2%),

Pseudomonas (2%) and *Pseudoxanthomonas* (2%) and *Stenotrophomonas* (2%). The antibiotic resistance analysis revealed that the highest percentage resistance recorded were against streptomycin (75.0%), followed by ampicillin (66.0%), ceftriaxone (50.0%), rifampin (43.3%), aztreonam (36.8%) and ceftazidime (31.6%). Resistance to more than two antibiotics was seen in 40.0% of the isolates with Multiple Antibiotic Resistant (MAR) index ranged from 0 to 0.79. Interestingly, all five farms have an MAR of more than 0.2, which suggested that the occurrence of MAR bacteria might originate from high-risk sources in all five aquaculture farms. Similar MAR patterns were observed in farm 4 and farm 5, suggesting that the distributions of ARB were dependent on the selective pressure of geographical location. A plethora of resistance patterns seen across these five farms warrant a careful examination on the dissemination of MAR in aquaculture farms. This should be of concerns for fish farmers, local communities and environmental authorities as the spreading of ARB would render the antibiotic helpless.

Keywords: Aquaculture, antibiotic resistance, $(GTG)_5$-fingerprinting, Multiple Antibiotic Resistant (MAR) index, standard disc diffusion

Taburan Bakteria Rentan Antibiotik di Ladang Akuakultur di Sarawak

ABSTRAK

Terdapat kesedaran yang semakin meningkat mengenai kepentingan akuakultur untuk kehidupan sosio-ekonomi untuk masyarakat berpendapatan rendah dan sederhana. Oleh kerana permintaan domestik dan global untuk daging putih sebagai sumber protein yang sihat, ini telah menghasilkan pertumbuhan industri akuakultur yang meluas di seluruh dunia bersama dengan penggunaan antibiotik yang berleluasa. Ini menyebabkan persekitaran akuakultur berisiko merebak bakteria tahan antibiotik yang cenderung berkembang di kalangan bakteria alam sekitar sebagai tindak balas terhadap pendedahannya kepada antibiotik. Tujuan kajian ini adalah untuk mengesan bakteria rentan antibiotik dari lima ladang akuakultur di Sarawak. Sejumlah 350 sampel terdiri daripada ikan (108 sampel), sedimen (113 sampel) dan air (129 sampel) dikumpulkan dari lima ladang akuakultur di Sarawak. Sampel dibawa ke makmal sebaik sahaja selepas pensampelan. Apabila tiba di makmal, sampel dibiakkan atas soya trypticase agar dan diinkubasi pada 28 ℃ selama 24 jam. Selepas pengeraman, beberapa koloni dipilih secara rawak, dibersihkan dan disimpan dalam gliserol. Cap jari $(GTG)_5$ digunakan untuk menganalisis perbezaan genetik dari isolat bakteria. Dendrogram dibina berdasarkan corak dari $(GTG)_5$-PCR dengan indeks keserupaan berkisar antara 50% hingga 74% kesamaan. Berdasarkan dendrogram, 50 isolat dipilih untuk pengenalan spesies menggunakan urutan 16S rRNA. Pengasingan ini kemudian diuji dengan 25 antibiotik menggunakan kaedah penyebaran cakera piawai. Penentangan isolat dikategorikan berdasarkan zon perencatan kawasan mereka sama ada mereka rentan antibiotik dan mudah terdedah. Sekitar dua ratus empat isolat bakteria telah diasingkan dan selepas pengenalpastian spesies, 50 isolat ini terdiri daripada 14 jenis bakteria termasuk Bacillus *(38%),* Exiguobacterium *(16%),* Enterobacter *(14%),* Aeromonas

(6%) Acinetobacter *(4%),* Citrobacter *(4%),* Staphylococcus *(4%),* Achromobacter *(2%),* Chitinophaga *(2%),* Fictibacillus *(2%),* Plesiomonas *(2%),* Pseudomonas *(2%) dan* Pseudoxanthomonas *(2%) dan* Stenotrophomonas *(2%). Analisis rintangan antibiotik mendedahkan bahawa rintangan peratusan yang tertinggi direkodkan berbanding streptomisin (75%), diikuti oleh ampisilin (66%), ceftriaxone (50%), rifampin (43.3%), aztreonam (36.8%) dan ceftazidime (31.6%). Rintangan kepada lebih daripada dua antibiotik dilihat dalam 40.0% daripada isolat dengan indeks kepelbagaian rentanan antibiotik dari 0 hingga 0.79. Menariknya, semua lima ladang mempunyai MAR lebih daripada 0.2, yang menunjukkan bahawa semua bakteria MAR mungkin berasal dari sumber risiko tinggi. Pola MAR yang serupa diperhatikan di ladang 4 dan ladang 5, menunjukkan bahawa taburan bakteria rentan antibiotik bergantung kepada tekanan dari lokasi geografi. Sebilangan besar pola rintangan yang dilihat di lima ladang ini memerlukan pemeriksaan yang teliti terhadap penyebaran MAR di ladang akuakultur. Ini harus menjadi kebimbangan bagi petani ikan, masyarakat setempat dan pihak berkuasa alam sekitar kerana penyebaran bakteria rentan antibiotik akan menyebabkan antibiotik tidak berdaya.*

Kata kunci: *Akuakultur, rintangan antibiotik, cap jari* $(GTG)_5$*, indeks kepelbagaian rentanan antibotik, penyebaran cakera piawai*

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LIST OF ABBREVIATIONS

%	Percentage
bp	Base pair
℃	Degree Celsius
AMR	Antimicrobial resistance
AR	Antibiotic Resistant
ARB	Antibiotic Resistant Bacteria
ARGs	Antibiotic Resistant Genes
AST	Antibiotic Susceptibility Testing
BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
CLSI	Clinical laboratory Standards Institute
DNA	Deoxyribonucleic acid
DOF	Department of Fisheries Malaysia
dNTP	Deoxyribonucleotide triphosphate
ETBR	Ethidium bromide
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FAO	Food and Agriculture Organization
GIS	Geographical Information System
GLASS	Global Antimicrobial Surveillance System
HGT	Horizontal gene transfer
JPG	Joint Photographic Experts Group
MALDI-TOF-MS	Matrix-Assisted Laser Desorption Ionization with Time-of-flight Mass Spectrometry

MAR	Multiple Antibiotic Resistance
MHA	Mueller-Hinton Agar
MRSA	Malaysia Remote Sensing Agency
NACA	Network of Aquaculture Centres in Asia-Pacific
NCBI	National Centre for Biotechnology Information
NEKMAT	National Fishermen's Association
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
Rep-PCR	Repetitive element palindromic – polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
sp.	Species
TBE	Tris-Borate-EDTA
TSA	Trypticase Soy Agar
TSB	Trypticase Soy Broth
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Introduction

The rapid growth of aquaculture is seen across nations, predominated in Asian countries which are driven by pre-existing aquaculture practices, growth of population and economic, relaxed regulatory framework and expanding export opportunities (Bostock et al., 2010). Thus, reliance on antibiotics to eradicate bacterial diseases in aquatic organisms are inevitable (Bostock et al., 2010; Cabello et al., 2013; FAO, 2016). Antibiotics used in the aquaculture industry are seen globally by means of treatment, control, prevention of disease (prophylactic) and as growth promoters (Philips et al., 2004; Bush et al., 2011; Romero et al., 2012). The spread of bacterial infection in aquaculture is expected as it is part of natural selection and subsequently there will be an abusive use of veterinary drugs in the aquaculture industry (Haya et al., 2000; Cabello, 2006; Paulson et al., 2016). This has become a global problem because the misuse of antibiotics in aquaculture has been identified to drive the emergence and widespread of ARB and led to an unwelcoming implication to the public health (Schmidt et al., 2000; Heuer et al., 2009; Deng et al., 2016; Patil et al., 2016; Paulson et al., 2016).

Antibiotic resistance is part of a natural phenomenon that develop in response to the exposure of bacteria to antibiotics in an environment. Several studies have shown that ARB may have originated from aquaculture and agriculture itself (Cabello et al., 2013; Shah et al., 2014; Done et al., 2015; Tomova et al., 2015). ARB carries antibiotic resistance genes (ARGs) by mobile genetic elements such as, integrons and plasmids that have shown to be shared between aquatic bacteria and terrestrial animals and human pathogen (Cabello et al.,

2013; Cantas et al., 2013). Still, the correlation between the misuse of antibiotics and resistance genes are not easy to follow, as antibiotic resistance is a complex phenomenon affecting both human and animal health (Galvin et al., 2013). Moreover, the aquaculture industry are associated with an increasing number of large farms, high density of fish and poor sanitary condition (Barton and Fløysand, 2010). These association could only lead to greater levels of resistance in the human commensal microbiota (Schmidt et al., 2000; Deng et al., 2016).

The question that underlies this study is: to what extend will ARB be able to distribute within the aquaculture environment? Thus, a surveillance study of ARB is of grave importance to monitor and identify the point and pattern of dispersion of bacterial population that is exposed to antibiotics. A spatial analysis of ARB based on Geographical information systems (GIS) platform is proven to be useful in epidemiological studies (Galvin et al., 2013; Samarasundera et al., 2012). Also, there is a need for surveillance on the use of antibiotics in aquaculture with a comprehensive regulatory framework for the registration of antibiotics drug, as current standards varied widely from one country to another (Cabello et al., 2013; Watts et al., 2017). An active enforcement by the Ministry of Health is of vast importance to ensure its safety and effectiveness. Concurrently, it is of considerable gravity that people working near the aquaculture industry to learn how to use antibiotics in such a way that maximise their efficacy while minimising the increased frequencies of resistant variants that are automatically a consequence of their use (Smith, 2008).

A clear view on the development and spread of antibiotic resistance in aquaculture in protecting the human, animal, and ecological health can be achieved by a better understanding of antibiotic resistance (AR) ecology through characterisation of ARB and antibiotic resistance prevalent in an aquaculture environment. This study discusses the

hypothesis that ARB sharing similar aquaculture environment may represent an important element in the many facets of antibiotic resistance dissemination possibly through mobile genetic elements. The objectives of this study are:

i. To isolate and detect antibiotic resistant bacteria from selected aquaculture environment in Kota Samarahan, Bau, and Kuching, Sarawak.

ii. To determine the resistance distribution (susceptible or resistant) of the identified bacteria.

iii. To develop a preliminary spatial data on the risk level of antibiotic contamination in the aquaculture farms in Kota Samarahan, Bau and Kuching, Sarawak.

CHAPTER 2

LITERATURE REVIEW

2.1 Aquaculture at a global scale

The aquaculture industry is still one of the fastest growing food production sectors in the world, dating back as early as 1100 B.C where China was considered the first country to implement their freshwater environment as a source of food (FAO, 2003). Aquaculture is an important source of nutrition and income for the community who depends on it for their social-economical livelihood (Béné et al., 2015). In 2016, the global aquaculture production of food fish amounted to 110 million tonnes, of which 64 million tonnes are produced from China alone, which account for 58.0% of the total aquaculture production. Asia countries altogether had contributed to about 92.14% of the shared total aquaculture output (FAO, 2018). The rapid growth of fish farming seen in China and other Asian region is driven by the growth of population and economic, relaxed regulatory framework and expanding export opportunities (Bostock et al., 2010). According to FAO data, Asia has been dominating the aquaculture industry with production of 92.14% due to the long history of aquaculture practices, followed by Americas (3.05%), and Europe (2.06%) as shown in Figure 2.1 which depicts the total number of aquaculture production output among continent in 2016.

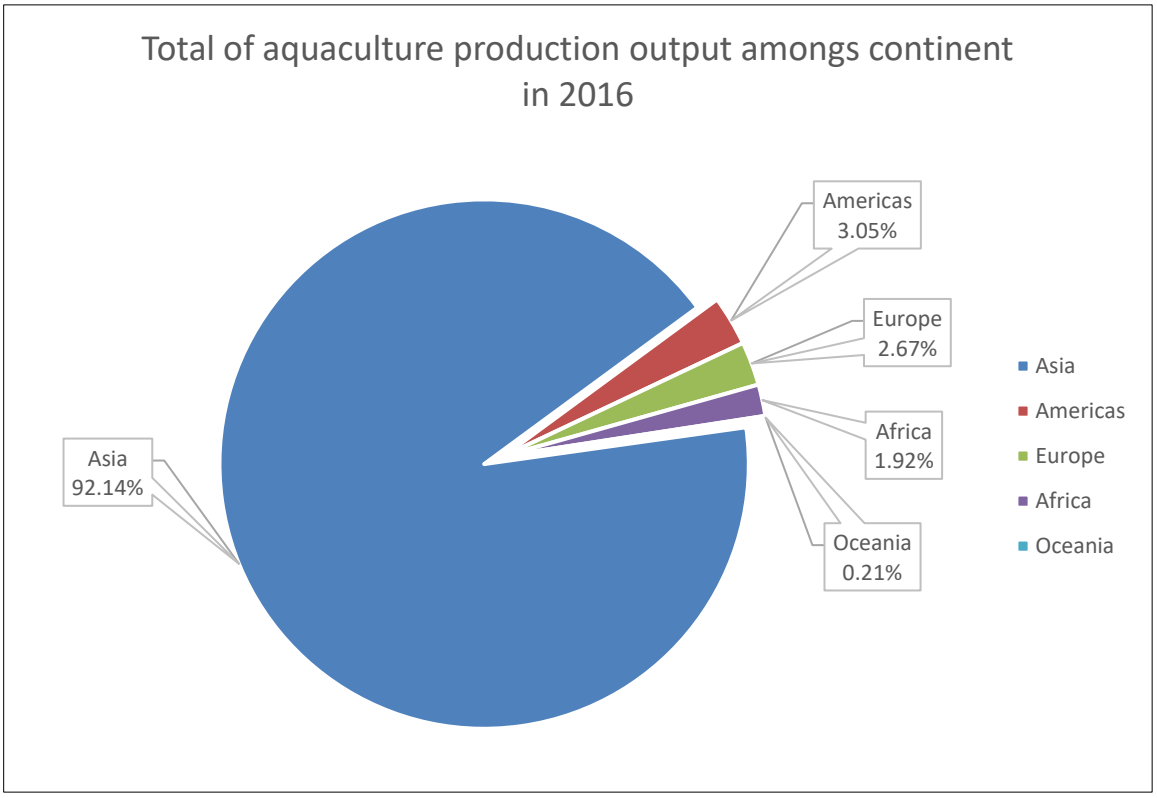

Figure 2.1: Total number of aquaculture production output among continent in 2016 (FAO, 2018)

Aquaculture covers a wide range of species and methods from simple traditional systems, in which fish or other aquatic animals are reared in a small pond for domestic consumption to the intensive industrial-scale production system (Heuer et al., 2009). Inland aquaculture in earthen ponds is the largest contributor from aquaculture to food security and nutrition in the developing nation (FAO, 2016). Aquaculture system can be subdivided into; extensive aquaculture, where predators are removed and competitor fish are controls; semi-intensive, where food is supplemented and enhanced; and intensive aquaculture, where all food needed are supplied (Watts et al., 2017).

2.2 Malaysian aquaculture

Malaysia is placed on the 17th ladder on the top 20 countries for aquaculture production which is 0.41%. Whereas neighbouring countries like Indonesia account for 16.6%, the Philippines at 2.2%, and Thailand at 0.96% as shown in Figure 2.2.

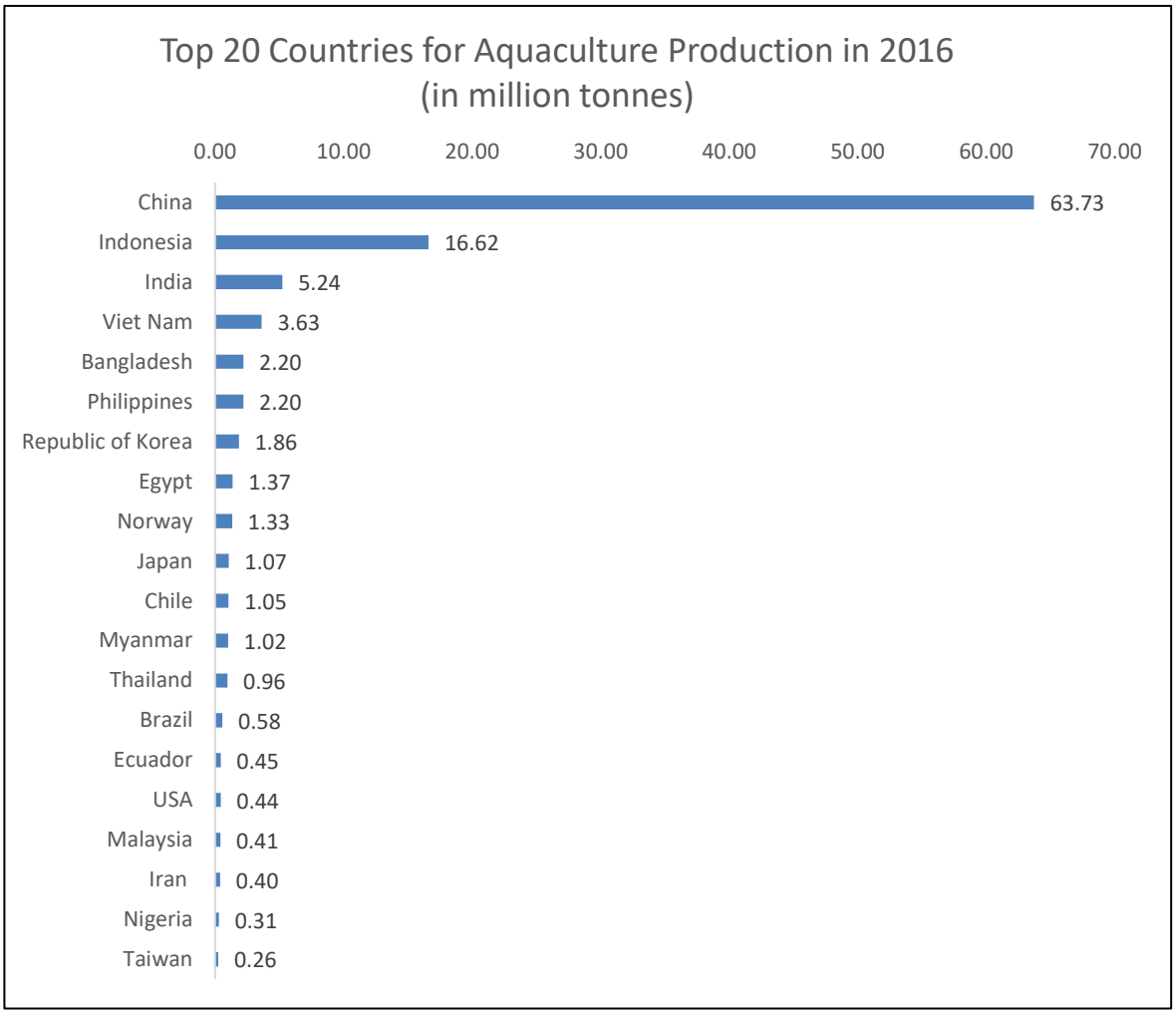

Figure 2.2: Top 20 countries for aquaculture production in 2016 (in million tonnes) (FAO, 2018)

Malaysian aquaculture practice three types of culture environment for farming of aquatic organisms which are the freshwater, brackish water and marine aquaculture (Hamdan et al., 2012). The development of aquaculture in Malaysia began in the 1920s with

the farming of freshwater fishes, such as the Chinese carp and common carp (*Cyprinus carpio*) predominate in Perak and Selangor, of Peninsular Malaysia (Tan and Khoo, 1980; Hamdan et al., 2012). Meanwhile, the growth of aquaculture in East Malaysia (Sabah and Sarawak) only started in the 1990s (Hamdan et al., 2012).

The domestic and global demand for fish meat has caused consumers preference for a healthy source of animal protein. This has generated a financial and livelihood driver for the growing population of Malaysia (Yusoff, 2015). With government encouragement and aid through the provision of loans scheme (Special Agricultural Credit Scheme and Fund for Food Scheme in Malaysia), often subsidised, channelled to fisheries association has enabled the small-scale farmer to embrace the aquaculture sector (Dey et al., 2008).

In 2016, Malaysia aquaculture production (freshwater, brackish water, marine environment) amounted to 407 thousand tonnes. This production is considerably low when compared to neighbouring countries like Indonesia and Vietnam with a staggering estimation of 16.6 million tonnes and 36.3 million tonnes respectively. Malaysia freshwater, brackish water, and marine production output contribute to 25%, 21%, and 54% respectively (Figure 2.3).

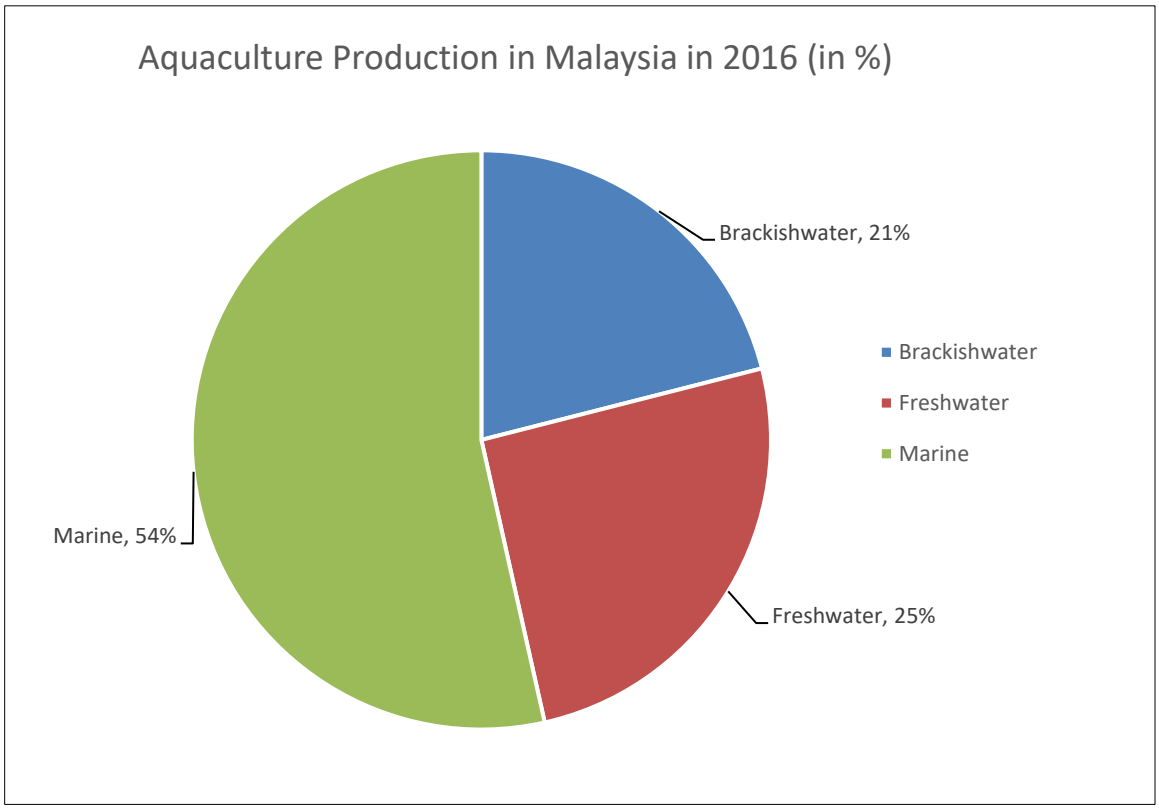

Figure 2.3: Aquaculture production in Malaysia in 2016 (in %) (FAO, 2018)

The top 20 freshwater species output is dominated by catfish species (Torpedo-shaped catfish, pangas catfish, Asian Redtail catfish) and tilapia species (Tilapias nei, Nile tilapia). The medial freshwater output is carp species (Bighead carp, Hoven's carp, and Common carp) as it is a species of a long tradition of Asia's aquaculture (Allison, 2011).

Data from Allison (2011) shows that Malaysia, a transitional economy of Asia have a noticeably higher fish-protein consumption as compared to poorer countries. It can be postulated that the increase of people with a substantial income had driven to the increasing demand for fish-protein (Béné et al., 2015).

2.2.1 Malaysian aquaculture regulation

Malaysia had adopted a legal framework on the regulatory use of antimicrobial drugs in the aquaculture and livestock industry that is stipulated in Malaysia's Food Act 1983 (2014 amendment) and Food Regulations 1985 (2012 amendment). Malaysian government have enforced a Maximum Residue Limits (MRLs) for aquaculture sector with the aim to provide guidelines on the maximum concentration of antibiotics for therapeutic purposes to be accepted by the community (Rodgers and Furones, 2009; Romero et al., 2012).

2.3 Remote Sensing and Geographical Information System

There have been a growing awareness surrounding the importance of aquaculture as a source of livelihood, food security and poverty alleviation as mentioned by international authorities such as the Food and Agriculture Association (FAO), World Fish, United Nation (UN), and Malaysia's governments; Department of Fisheries Malaysia (DOF); and Fisheries Development Authority of Malaysia (LKIM) (Allison, 2011; Malaysia Remote Sensing Agency (MRSA) Annual Report, 2012; Meaden and Aguilar-Manjarrez, 2013).

Ottinger et al. (2016) have recognised the potential of earth observation to support aquaculture management (aquaculture site selection, environmental monitoring, and aquaculture inventory). Nonetheless, Geographic Information system (GIS) is a tool well utilize in aquaculture since the mid-1980s used by means for aquaculture siting and management (Nayak et al., 2014; Puniwai et al., 2014), environmental impact assessment (Nuckols et al., 2004). Despite all these efforts, GIS application in inland fisheries (freshwater aquaculture) is remarkably limited as mentioned by Fisher (2007).

Doughty and McPhail (1995) had emphasised the need of closer collaboration between farmers and regulatory bodies on improving and sustaining fish farms since both

are a concern with the impact of environmental of aquaculture at a regional and global scales. This collaboration has been implemented by the Malaysia Remote Sensing Agency (MRSA) with DOF, LKIM and National Fishermen's Association (NEKMAT) that had benefited the regulators and stakeholders by implementing Fishing Site Identification (FSI) system to support small scale aquaculture and fisheries research, planning and management (Malaysia Remote Sensing Agency (MRSA) Annual Report, 2012).

2.3.1 Surveillance of antibiotic resistant bacteria in aquaculture

The accessibility of the internet is seen widespread across Asia, but the lack of information technology surveillance for ARB is environmental settings is still limited (Marti et al., 2014; Vong et al., 2017). According to Fisher (2007), the limited inland aquaculture data are due to the different challenges of the use of remote sensing and sensor network by fisheries experts, biologists, and ecologists of the geostatistical modelling of inland fisheries (rivers, streams, lakes and reservoirs) compared to the fish habitat in marine aquaculture system.

Network of Aquaculture Centres in Asia-Pacific (NACA) is an ongoing project that involves in promoting sustainable aquaculture, but currently not collection antimicrobial resistance (AMR) surveillance data (Ashley et al., 2017). This lack of data had been emphasised by the WHO report concerning AMR problem in Southeast Asia as often neglected (WHO, 2014). However, the sparse monitoring on AMR in Asia, especially in developing countries does not mean the problem is not as burgeoning as it is in developed countries (Hong, 2015).

A Global Antimicrobial Surveillance System (GLASS) developed by WHO was launched in 2015, aims to promote informed decisions to drive local, national and regional

action on AMR and sharing data among participating countries (WHO, 2017b). Vong et al. (2017) have suggested a strategic and practical plan on using GLASS to harmonise the AMR surveillance data for South East Asian countries. Continuous communication and coordinate mechanism between the intermediate and local level is crucial in solving issues concerning AMR data.

2.4 Antibiotics

The terms “antibiotics” and “antimicrobials” are often used synonymously in various journals. The term “antimicrobial” is referred to as a natural, semisynthetic or synthetic chemical that kills or nullifying the growth of bacteria, fungi, virus and protozoa. Whereas, the term “antibiotic” is used to describe the subset of antimicrobials that target bacteria (Rothrock et al., 2016). For this study, the term “antibiotic” (not antimicrobial) is used to describe the resistant group of microorganisms that are limited to bacteria and therefore to antibiotic resistance.

2.4.1 Antibiotic mechanisms

Antibiotics are naturally produced by microorganisms with a very low concentration in a natural environment (Gullberg et al., 2011). In an environment, bacteria compete against each other for resources by manufacturing antibiotic, a harmful compound that they direct against each other, killing the neighbouring bacteria (bactericidal) or simply nullifying the bacteria growth (bacteriostatic) (Walsh, 2000). Table 2.1 shows the targets, mode of action and mechanisms of resistance of the main classes of antibacterial drugs. However, bacteria have developed a series of mechanisms to resist the effects of antimicrobials through the bacterial and bacteriostatic mechanism. The bactericidal effect takes place when the antibiotics interfere with the bacterial cell wall or its bacterial protein synthesis. Whereas in

bacteriostatic effect, the antibiotics interfere with the multiplication of the bacteria by hindering its protein production, DNA replication or cellular metabolism. Such mechanism can be seen in tetracyclines, sulphonamides, chloramphenicol and macrolides (Romero et al., 2012).

Table 2.1: Targets, mode of action and mechanisms of resistance of the main classes of antibacterial drugs (Walsh, 2000)

Antibiotic	Target	Mode of Action	Resistance mechanism
Cell wall			
β-Lactams	Transpeptidases/transglycosylases (PBPs)	Blockade of cross-linking enzymes in the peptidoglycan layer of cell walls	β-Lactamases, PBP mutants
Vancomycin	D-Ala-D-Ala termini of peptidoglycan and lipid II	Sequestration of substrate required for crosslinking	Reprogramming of D-Ala-D-Ala to D-Ala-D-Lac or D-Ala-D-Ser
Protein synthesis			
Macrolides of the erythromycin	Peptidyl transferase, the centre of the ribosome	Blockage of protein synthesis	rRNA methylation, drug efflux
Tetracyclines	Peptidyl transferase	Blockage of protein synthesis	rRNA methylation, drug efflux
Aminoglycosides	Peptidyl transferase	Blockage of protein synthesis	Enzymic modification of drug
Oxazolidinones	Peptidyl transferase	Blockage of protein synthesis	Unknown
DNA replication/repair			
Fluoroquinolones	DNA gyrase	Blockade of DNA replication	Gyrase mutation to drug resistance

2.4.2 The use of antibiotic in aquaculture

Ever since the discovery of antibiotics for its prophylactic capabilities and as a growth promoter, it has transformed the humanity approached not only to eradicate infectious diseases among humankind but also for better-quality livestock (Phillips et al.,

2004; Bush et al., 2011; Romero et al., 2012). However, Cabello (2006) argues that antibiotics in aquaculture are not meant to be used as a growth promoter but to prevent further spreading of bacterial infections in fish and invertebrates.

Any aquaculture farming that relies on the regular use of antibiotics, in the long term are considered unsustainable (Smith, 2008). McArdell et al. (2003) demonstrated that there is a strong correlation between human activities such as farming and urbanisation with the amount of antibiotics release in different water bodies and water treatment plants. In aquaculture, antibiotics are administered to aquatic animals generally by medicated feed (oral), immersion (bath), and injection, with the immersion ramifying in the largest amount of released in the grow-out phase of production (Smith, 2008; Sekkin and Kum, 2011; Price and Morris, 2015).

A global survey done by Tuševljak et al. (2013) against 604 respondents with varying expertise in aquaculture identified that tetracycline and quinolone are widely used across the globe. It was reported that fish farms in Malaysia commonly used oxytetracycline and erythromycin antibiotics which are used to prevent further infection of *Streptococcus agalactiae*, which happened to be fatal towards tilapia *Oreochromis* species which are common in warm water and intensive aquaculture system (Musa et al., 2009; Sun et al., 2016). Following the aquaculture production, figures indicate that there is a 8.7% decline in the production of tilapia species in freshwater aquaculture between the year 2011 to 2015 (FAO, 2015).

The occurrence of antibiotics in aquatic environments is a major concern because of its potential spread of antibiotic resistance to the ecosystem (Levy, 1992; Corno et al., 2014). Such concern had led the European Union to ban all antibiotics associated with growth

promoter in animal feeds in 2006 (Cogliani et al., 2011). Also, many antibiotics used in aquaculture are used in human medicine which is categorised into three levels of importance; critical important, highly important, and essential in human medicine in accordance to Defoirdt et al. (2011), as shown in Table 2.2. These data were revised from "Critically Important Antimicrobials for Human Medicine" (WHO, 2017a).

Table 2.2: Properties of the major classes of antibacterial agents (Yan and Gilbert, 2004; Defoirdt et al., 2011)

Antimicrobial agent (drug class)	Route of administration in aquaculture	Importance of antimicrobial class in human medicine
Amoxicillin (aminopenicillins)	Oral	Critically important
Ampicillin (aminopenicillins)	Oral	Critically important
Chloramphenicol (amphenicols)	Oral/bath/injection	Highly important
Florfenicol (amphenicols)	Oral	Highly important
Erythromycin (macrolides)	Oral/bath/injection	Critically important
Streptomycin, Neomycin (aminoglycosides)	Bath	Critically important
Furazolidone (nitrofurans)	Oral/bath	Important
Nitrofurantoin (nitrofurans)	Oral	Important
Oxolinic acid (quinolones)	Oral	Critically important
Enrofloxacin (fluoroquinolones)	Oral, bath	Critically important
Flumequine (fluoroquinolone)	Oral	Critically important
Chlortetracycline, tetracycline (tetracyclines)	Oral/bath/injection	Highly important
Sulphonamides (sulphonamides)	Oral	Highly important

The magnitude of antibiotics and the genes rendering resistance in nature had been reported by Sengupta et al. (2013) and Corno et al. (2014), where the former highlighted that there is a clear impact of low doses of antibiotics in nature, whereas the latter reported that

a low dose of antibiotics in the environment could impact the bacterial communities, thus raising the concerns about the release of antibiotic into nature. Besides, an alternative to antibiotics such as vaccine is one of many ways to restrict the dissemination of ARB in the environment (Rodgers and Furones, 2009; Pridgeon, 2012). For example, Malaysia recently developed a feed-based adjuvant vaccine against *Streptococcus agalactiae* infection in tilapia in 2013. The principal of vaccine is that it would not need to be kept and the duration of protection exceeds those antibiotics. Vaccine is essentially discovered for the improvement of quality and sustainability of an aquaculture production (Firdaus-Nawi et al., 2013).

2.4.3 Mechanism of antibiotic resistance

Antibiotic resistance occurs when bacteria do not respond to the drugs that are meant to kill them (Centers for Disease Control and Prevention, 2017). In assessing the antibiotic resistance problem, Levy (1994) had coined it as The Drug Resistance Equation (Figure 2.4), whereby the antibiotic resistance problem will only emerge if both the antibiotics and resistance traits are present.

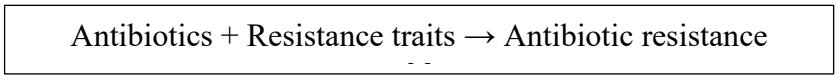

Figure 2.4: Drug Resistance Equation

Antibiotic resistance genes are transferable. The genes that carry the resistance traits can be transferred by mobile genetic elements such as bacteriophages, plasmids, naked DNA or transposon among bacteria of different ecological groups. Of well over 15 classes of antibiotics, the resistance mechanism will likewise varied (Levy and Marshall, 2004). The common mechanisms of antibiotic resistance essentially involve reducing antibiotic uptake

(permeability or efflux), enzyme-mediated inactivation, and target sites mutations (Walsh, 2000; Russell, 2002; Bhullar et al., 2012).

The widespread of ARB which typically arose within bacterial populations is due to ARGs getting collected on the plasmids (Davies, 1994). The plasmid can autonomously replicate within cells and can be transferred between different strains in the process of conjugation and transformation. Thus, this indicate that the plasmid is one of the modes for bacteria to acquire resistance (Walczak and Donderski, 2004).

The emergence of ARGs in human pathogens such as *qnr*, *tet*G, and *tet*C, *flo*R and some β-lactamase genes have been identified and have potentially originated from the aquatic environment (Tomova et al., 2015). These ARGs and mobile genetic elements which include integrons and plasmids have been shown to be shared between aquatic bacteria and terrestrial animals and human pathogen (Cabello et al., 2013; Cantas et al., 2013). However, the role of antibiotic usage in aquaculture in the development of resistance and dispersion of ARGs is still poorly understood (Walczak and Donderski, 2004; Done et al., 2015).

2.4.4 Multiple antibiotic resistant bacteria

Watanabe (1963) reported that resistance to multiple antibiotics was first detected among enteric bacteria, namely, *Escherichia coli*, *Shigella* and *Salmonella*. These are among bacterial species of a public health concern as they are food and the water-borne pathogens that infect both human and animal (Watanabe, 1963; Okere et al., 2014).

Multiple antibiotic resistant (MAR) bacteria are ubiquitous in aquatic environmental settings, where they were identified in the aquaculture waters (Okere et al., 2014; Huang et al., 2015), coastal waters (Dada et al., 2013; Alves et al., 2014), rivers (Nontongana et al., 2014; Delgado-Gardea et al., 2016;), estuaries (Ghaderpour et al., 2015), and even in the

Antarctic lakes (Tam et al., 2015). Regardless of the environmental setting, bacteria are resistant to antibiotics despite minimal or no exposure to antibiotics, due to intrinsic and selected antagonistic interactions among bacteria (Lo Giudice et al., 2007).

Furthermore, these MAR bacteria create a problem especially towards developing nations with small health care budgets that hindered the access to new, effective, and costly drugs causing a severe clinical problem and high mortality rate (Levy and Marshall, 2004). According to Cabello et al. (2013), ARB found at aquaculture sites for a prolonged period after antimicrobial use, further suggesting the relevance of this selection over time. Moreover, exposure to one antibiotic can give rise to bacterial resistance to other antibiotics that are not been exposed in the area. With the unrestricted use of antibiotics on and dissemination of ARGs, this will only lead to global resistance threats. As Chan (2015) explained,

> *The rise of antimicrobial resistance is a global health crisis. Medicine is losing more and more mainstay antimicrobials as pathogens develop resistance. Second-line treatments are less effective, costlier, more toxic, and sometimes extremely difficult to administer. With few replacement products in the pipeline, the world is heading towards a post-antibiotic era in which common infections will once again kill.*

2.5 Bacterial diversity in aquaculture

Aquaculture system harbour a diversified bacterial community which exists in combination with the current and past use of antibiotics. Moreover, a bacterial community in aquaculture is made up of bacteria originated from different sources such as from human,

livestock and environmental which favour horizontal gene transfer (HGT) and recombination (Di Cesare et al., 2013; Watts et al., 2017). HGT involves the exchange of resistance determinants between aquatic and terrestrial bacteria through conjugation and conjugative transposons (Cabello, 2006). With this in mind, a study was conducted by Tomova et al. (2015) reported the presence of similar quinolone resistant genes in both marine bacteria and in patients living in the aquacultural region and imposed a threat of contamination with ARB from livestock to human.

2.5.1 Surface water

A typical aquaculture pond will generally be of 1 meter in depth in which it is considered as an optimal level for fish growth of cultured species like tilapia, crabs and shrimps (Baluyut and Balnyme, 1989). Due to the shallow nature of the freshwater pond, there is usually no significant difference in bacterial diversity between the surface and bottom layer. Based on a study done on the bacterial community on aquaculture, results had shown that there is no significant difference in bacterial diversity between the surface and bottom layers which constitutes of four dominant phyla that are; *Proteobacteria*, *Cyanobacteria*, *Bacteroidetes,* and *Actinobacteria* (Qin et al., 2016).

2.5.2 Sediment

According to Wang et al. (2012), sediment is a special realm in an aquatic environment which includes of high microbial diversity. The microbial community in freshwater sediment had shown the most diverse in comparison with intertidal sediment and marine sediment with the latter two categorised as medium and the least respectively. Earlier studies have demonstrated that different temperatures and oxygen concentrations also

suggestively alter the microbial community composition in freshwater sediment (Bryant et al., 2012; Wu et al., 2013).

2.5.3 Fish gills

The fish gill is a multifunctional organ involved in gas exchange, osmoregulation, hormone production, acid-base balance, and immune defence (Rombough, 2007). Fish gills were believed to have a more diversified microbiota, qualitatively and quantitatively, due to their direct contact with the water as demonstrated in tilapia species (Rocha et al., 2014). Due to the thin respiratory epithelium of the gills, it is an obvious entry point for the fish pathogen that is readily spread in the water (Secombes and Wang, 2015).

According to Austin (2006), gills tissue has been found to harbour high bacterial populations with an estimated up to 106 bacteria. A study by Ghosh and Mandal (2010) in West Bengal, India reported that the viable counts of resistant bacteria from gills were higher than from those of intestinal content.

2.5.4 Fish intestinal tract

It is well established that an intestinal tract of fish posed as an excellent reservoir for bacteria microbiota. Del'Duca et al. (2015) have reported that the bacterial abundance in the fish intestine is significantly higher in comparison to the water and sediment. Also, the bacterial community of the intestinal tract of tilapia species has shown greater similarity to water microbiota than of pond sediment. Besides, the intestinal tract of freshwater fish is associated with a wide range of taxa and include *Aeromonas* (Apun et al., 1999; Hassan et al., 2017); *Escherichia, Enterobacter, Klebsiella* (McPhearson et al., 1991; Apun et al., 1999; Austin et al., 1999), *Citrobacter, Vibrio* (Akinbowale et al., 2006), *Bacillus, Listeria* (Ertafi, 2005) and *Staphylococcus* (Ali, 2014).

2.6 Discernment of unknown isolates using Polymerase Chain Reaction

Polymerase chain reaction (PCR) is a method to amplify specific sequences more simply and quickly by the direct enzymic process. This method would require a sample of DNA that includes a region to be amplified, primer pair in large molar excess, deoxynucleotide triphosphates (dNTPs), and a heat-stable DNA polymerase (normally *Taq* polymerase isolated from the thermophilic bacterium *Thermus aquaticus*). Generally, PCR is carried out using a PCR machine or a thermal cycler, and a typical set of reactions might have an initial melting carried out at 94 ℃, followed by 30 cycles each comprises melting at 94 ℃ (denaturation phase), renaturation or annealing phase at 60 ℃ and DNA synthesis at 72 ℃ (extension phase) and then after the 30 cycles a final extension round at 72 ℃ (Howe, 2007). Due to the high sensitivity rate of PCR, it will theoretically result in a 10^5 to 10^6 - fold amplification of the targeted DNA after 20 to 30 cycles (Brunk and Li, 2001).

PCR-based analysis of the well-known 16S rRNA genes is an essential tool for the studies of bacterial diversity, community structure, evolution and taxonomy. It enables us to detect and identify as-yet unculturable bacteria, thus increasing our knowledge of bacterial ecology and taxonomy (Hongoh et al., 2003). However, Hongoh et al. (2003) advised that we need to interpret the PCR method with great concern, as PCR naturally results in biases and errors, attributes from complex factors such as preferential annealing between primers and templates, self-annealing between PCR products, formation of artefact and primer-template mismatches (Hongoh et al., 2003; Mao et al., 2012).

2.6.1 $(GTG)_5$ - PCR fingerprinting

Repetitive element palindromic (rep-PCR) uses primers to target conserved repetitive elements found in the bacterial chromosome (Stern et al., 1984; Versalovic and

Lupski, 2002; Healy et al., 2005). Examples of these repetitive elements are the repetitive extragenic palindromic (REP) sequences, the enterobacterial repetitive intergenic consensus (ERIC) sequences, the BOX sequences, and the Penta-GTG oligonucleotide $(GTG)_5$ sequence (Healy et al., 2005).

Gevers et al. (2001) revealed that $(GTG)_5$-PCR was useful for screening a large number of *Lactobacillus* strains, and useful for intraspecies differentiation. $(GTG)_5$-PCR fingerprinting technique also offers identification possibilities based on DNA fragment size instead of DNA sequence, thus reduce the cost of requiring sophisticated laboratory materials (Braem et al., 2011). According to Braem et al. (2011), the typeability and the accuracy of this method were at 94.7% and 94.3% compared to identifications based on gene sequencing, but with these two methods combined, a higher increase in resolution can be achieved.

$(GTG)_5$-PCR fingerprinting has been successfully used in classifying and identification a number of bacterial species which includes *Lactobacilli* sp. (Gevers et al., 2001), *Virgibacillus* sp. (Heyrman et al., 2003), *Vibrio coralliilyticus* (Ben-Haim et al., 2003), *Enterococcus* sp. (Švec et al., 2005), *Salmonella* sp. (Rasschaert et al., 2005), *Campylobacter* (Matsheka et al., 2006), *Escherichia coli* (Mohapatra et al., 2007) and *Acetobacter* sp. (De Vuyst et al., 2008). $(GTG)_5$-PCR is considered as a valuable genotyping tools in epidemiological surveillance study (Mohapatra et al., 2007; Braem et al., 2011).

2.7 Antibiotic susceptibility testing

Antibiotic susceptibility testing (AST) is an essential test to guide clinicians in selecting the most effective antimicrobial therapy for bacterial infections. A variety of laboratory approach can be used to measure the in vitro susceptibility of bacteria to a range

of antibiotics. The common approach for AST would be the standard microdilution and agar disk diffusion methods (Jorgensen and Ferraro, 2009; Mahon et al., 2011).

In broth microdilution test, the bacteria are tested for their ability to produce visible growth on a series of broth (broth dilution) or of agar plates (agar dilution) that contain the dilution of antibiotics. In series of doubling dilution of antibiotic concentrations, the lowest concentration of antibiotics, which prevents the appearance of the visible growth of microorganism is known as minimum inhibitory concentrations (MICs) (Lalitha, 2004). This is generally performed in a 96-well format measured in mcg/mL, where test can be read manually or in an automated specialized machine (Mahon et al., 2011).

Disk diffusion test, also commonly known as the Kirby Bauer Test, is a standard qualitative test that allows the AST to be performed in a qualitative manner. Susceptibility is visualised by zone of inhibitions around an antibiotic diffused disc that is placed onto Mueller Hinton agar that has been swabbed with a standard concentration of a bacterium. In addition, disk diffusion method as it is relatively inexpensive and easy to set up (Jorgensen and Ferraro, 2009).

The susceptibility of the bacterium is appointed into three categories, namely susceptible, intermediate and resistance. Since different bacteria reacted differently with different antibiotics, the diameter of each antibiotic can be interpreted using the standard of Clinical and Laboratory Standards Institute (CLSI) or The European Committee on Antimicrobial Susceptibility Testing (EUCAST).

2.7.1 Clinical and laboratory standards institute

A standardized protocol to assess antimicrobial resistance or susceptibility is detailly describe by the Clinical and Laboratory Standards Institute (CLSI) method. It is a

standardized test to evaluate the antibiotic susceptibility of *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Acinetobacter* sp., *Burkholderia cepacian* complex, *Stenotrophomonas maltophilia*, other Non-*Enterobacteriaceae*, *Staphylococcus* sp., *Enterococcus* sp., *Haemophilus influenzae* and *Haemophilus parainfluenzae*, *Neisseria gonorrhoeae*, *Streptococcus pneumoniae* (β-Hemolytic group and Viridans group), *Neisseria meningitidis*, and Anaerobes (CLSI, 2017).

The European Committee on Antimicrobial Susceptibility Testing (EUCAST) also has a subcommittee on methods for detection of resistance mechanisms of clinical and epidemiological importance (Brown et al., 2015). Kassim et al. (2016) had done a comparison on AST using EUCAST and CLSI 2015 on *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*. They discovered a similar antibiotic susceptibility pattern using both procedures. EUCAST and CLSI may have the same objective that is to provide a breaking point guidelines used in antimicrobial susceptibility testing but may differ in a number of ways; 1) EUCAST provide clinical breakpoints and epidemiological cut-offs whereas, CLSI only provides clinical breakpoints and 2) EUCAST are not directly involved in decision making but play a more consultative role whereas CLSI play a more prominent role in decision-making process (Brown et al., 2015). Antibiotic resistant bacteria from high and low risk sources.

Multiple antibiotic resistance (MAR) index was create as a supplementary for the standard method for the detection of *Escherichia coli* in water, intendent to provide an additional information about the origin of contamination (Krumperman, 1983). For the differentiation of a high- and low-risk, a value of 0.2 is arbitrary, where the isolates might originate from high-risk sources such as human, poultry, piggeries, and cattle. They are considered high risk for they primarily carry disease causing microorganism such as

Escherichia coli, *Salmonella* sp. and *Shigella* sp. Whereas, a value of 0.19 and lower indicates the isolates might originated from low-risk sources such as domestic animals (Adenaike et al., 2016), where no known disease outbreak have been associated.

2.8 Scenario of antibiotic resistance in aquaculture

Proper mixing of antibiotics usage theoretically should yield better results (Bergstrom, 2004). However, insufficient knowledge on the proper use of antibiotics lead to a random mixing of antibiotics without proper toxicological and disease diagnosis. This in turn have caused incidents that coincide with antibiotic resistant. For example, in Indonesia, there was an outbreak of koi herpes virus in the year 2005 due to misdiagnosed of a bacterial pathogen which easily will turn to antibiotics (Sunarto and Cameron, 2005). Recently, a spread of Tilapia Lake Virus was detected in Israel, Ecuador, and Egypt (Bacharach et al. 2016; Fathi et al. 2017), where farmers were unaware of it being a viral disease which cause an upsurge used of antibiotics. Yang et al. (2013) had detected 58 resistance genes conferring resistance to 11 antibiotics collected from marine aquaculture sediment and describe that aquaculture could play a pivotal role in the exchange of antibiotic resistance globally.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study location

Samples of water, sediment, and fish were collected from farm 1 (1°26'57.80" N 110°24'21.30"E), farm 2 (1°32'53.43" N 110°32'53.99"E), farm 3 (1°24'02.40" N 110°19'50.60"E), farm 4 (1°26'59.5"N 110°10'12.0"E), and farm 5 (1°26'09.6"N 110°10'10.0"E) within Sarawak were generated and visualised using ArcMap 10.3 as shown in Figure 3.1.

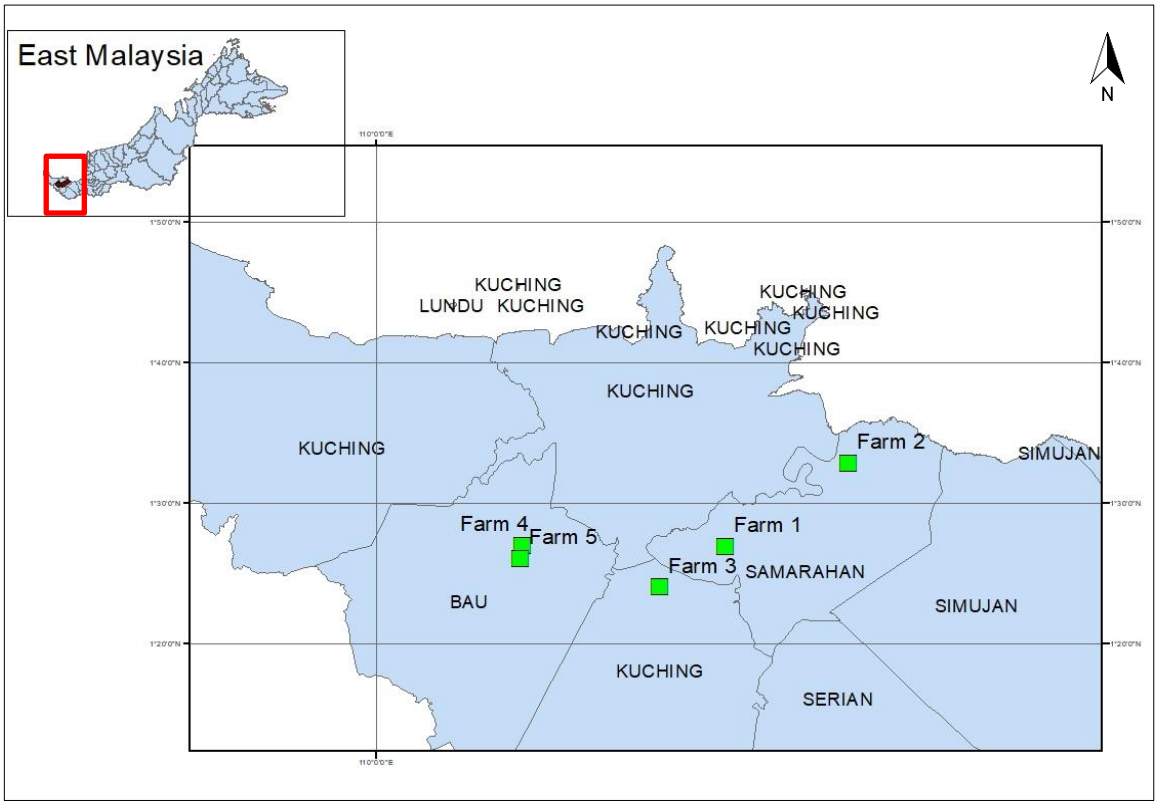

Figure 3.1: Sampling locations of five different farms in Sarawak

3.2 Sample collection and processing

In each sampling site, two sampling trips were conducted to collect pond water, sediment, and fish samples. The cultured organism (fish) was collected by hook and line or cast net. For certain fish, either intestinal content or scale swabs were collected. The fish scales were swabbed with the aid of swab sticks and collected in a sterile 15 ml Falcon tube containing 5 ml phosphate saline buffer (PBS). All samples were kept in a cooler box during transportation and processed within 24 hours. The fish intestinal contents were processed first after collection and aseptically extruded into a sterile 15 ml Falcon tube by the method recommended by Huys (2003). An approximately 0.5 g of intestine was suspended in 4.5 ml PBS solution and vortexed for 1 min. From the vortexed suspension, 3 ml of supernatant was collected and transferred into a sterile 15 ml tube. The process was repeated thrice to reach a final combine volume of 9 ml, and ten-fold serial dilution was performed. For each dilution, 0.1 ml of aliquots were inoculated (spread-plated) on Trypticase Soy Agar (TSA) and aerobically incubated at 28 ℃ for 18 to 24 hours (Hassan et al., 2017).

According to Huys (2003), 100 ml of water samples were taken from three random points at a pond using a sterile Schott bottle. Two ponds were selected from farm 1, 3, and 4, whereas three ponds were selected from farm 2 and 5 due to their relatively large farm size. Sediment was collected from the top of the sediment surface that was directly beneath the water sample by using a polyvinyl chloride pipe and immediately introduced into a zip lock plastic bag. Water samples were homogenised in a sterile Schott bottle by manual inversion (10 times) for each sampling station and then ten-fold serial dilutions was made from 10^1, 10^2 and 10^3 dilution in duplicates.

Sediment samples of 1 g were transferred into 9 ml PBS and homogenised. After vortexing, 3 ml of the supernatant were transferred into an empty tube. This was performed

for all three sediment samples per pond to obtain a final volume of 9 ml. This was continued with a ten-fold serial dilution. Using a micropipette, 100 µl of each dilution was spread plated on TSA agar and incubated in aerobic incubators at 31-33 ℃ for 18-24 hours.

3.2.1 Bacterial colony count

After incubation, bacterial colonies will be counted manually and expressed as colony forming unit per millilitre (CFU/ml) based on the formula shown in Figure 3.2. Representatives of five to ten colonies were re-streaked on fresh (1-7 days) TSA plates until a pure culture was attained. For long term storage, the pure cultures were aliquoted in Trypticase Soy Broth (TSB) supplemented with 20% (v/v) glycerol stock at kept at -80 ℃ for further bacteriological identification, testing, and analysis.

$$\frac{CFU}{ml} = \frac{no.of\ colonies\ \times dilution\ factor}{volume\ of\ culture\ bacteria}$$

Figure 3.2: Formula for calculating CFU

3.3 Differentiation and identification of bacteria

These bacterial isolates (n = 204) were differentiated using (GTG)5-PCR. DNA extraction were carried out prior to each PCR amplification process. Next, the DNA amplicons profiles were analysed visually, and dendrograms were constructed with the aid of GelJ version 2.0 software package (Heras et al., 2015). Based on the dendrogram, a representative of the unknown bacterial isolates from each cluster (group) were selected and identified using 16S-PCR sequencing. Sequence data were then compared using Basic Local Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCBI) website. Figure 3.3 illustrate the workflow of bacteria identification before it subjected to antibiotic susceptibility testing (AST).

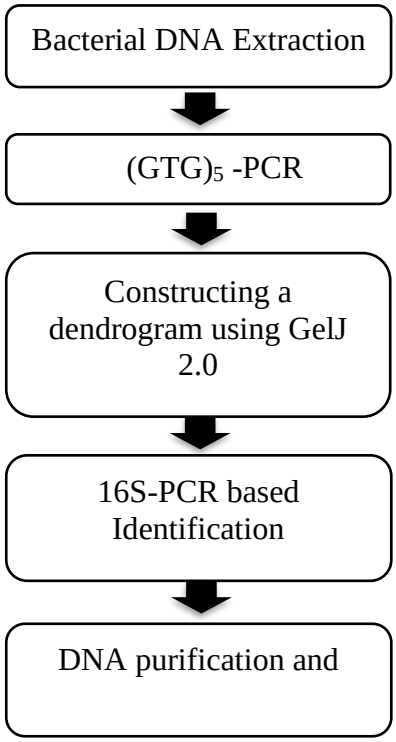

Figure 3.3: Workflow of Differentiation and Identification of bacteria

3.4 Bacteria DNA extraction

Chromosomal DNA was extracted from bacterial cultures grown in TSB using boiling-centrifugation method adapted from Soumet et al. (1994). Briefly, a broth culture was transferred into a sterile 2 ml centrifuge tube and was centrifuged at 10,000 rpm for 5 min. The supernatant was discarded, and the pellet was resuspended in 500 µl of sterile ddH_2O and vortexed. The suspension was then heated at 100 ℃ for 10 min using a heat block and then cooled on ice for 5 min. Next, the suspension was centrifuged at 10,000 rpm for 10 min. A 100 µl of supernatant was then transferred to a sterile tube and stored at -20 ℃ for further $(GTG)_5$ - PCR testing.

3.5 $(GTG)_5$ - PCR analysis using $(GTG)_5$ primer

$(GTG)_5$ - PCR analysis was performed according to Gomez-Gil *et al*. (2004) with slight modifications on the volume of *Taq* DNA polymerase, originally from 0.5 µl to 0.3 µl. The $(GTG)_5$ - PCR was carried out in a 25 µl volume containing 5 µl of 5 × PCR buffer solution, 3 µl of 25 mM $MgCl_2$, 0.5 µl of 25mM dNTPs, 0.5 µl of 25µM of 5'- GTG GTG GTG GTG GTG - 3' primer, 10.7 µl of sterile distilled water, 5 µl of DNA template and 0.3 µl of *Taq* DNA polymerase (Promega, USA) as shown in Table 3.1. The amplification was carried out using a thermocycler (SensoQuest, Göttingen, Germany) with the following PCR programme settings as shown in 3.2.

Table 3.1: $(GTG)_5$ - PCR mixture components

Components	Concentration	Volume per reaction (µl)
PCR Buffer Solution	5X	5
dNTPs	25 mM	0.5
$(GTG)_5$ primer	25 mM	0.5
$MgCl_2$	25 mM	3
DNA template	(20-30 ng)	5
Taq DNA polymerase	5U	0.3
double distilled water (ddH_20)	-	10.7
Final volume		25

Table 3.2: $(GTG)_5$ - PCR profile

Conditions	Time (min)	Temperature (℃)	Number of cycles
Pre-denaturation	2	95	1
Denaturation	1	95	30
Annealing	1	50	
Extension	1	72	
Final extension	5	72	1

3.6 Gel electrophoresis

The PCR amplification products were resolved in 1.2% agarose gel stained with 1 µl of ethidium bromide (ETBR). The agarose gel was electrophoresed for 75 minutes at 80 V, 400 mA, and visualised under an UV illuminator (Maestrogen, Hsinchu City, Taiwan). In every gel, sizes of amplicons were determined by comparison with a concurrently run DNA molecular 1 kb size DNA marker (Thermo Scientific, USA). A picture was taken and converted to a Joint Photographic Experts Group (JPG) format for further image analysis.

Afterwards, the image analysis of $(GTG)_5$ - PCR patterns were analysed using GelJ 2.0 Java software. A quantitative approach using similarity matrix based on dice coefficient with a tolerance value of 4 were used on the $(GTG)_5$ - PCR fingerprints of each isolate. The tolerance value served to decide whether the bands, dependent on their weight were matched. The threshold is computed using the maximum weight value and the minimum weight value of the GeneRuler 1kb DNA ladder (Thermo Fisher Scientific, USA). Thus, there were no

bootstrapping involved. Dendrograms were created using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) clustering algorithm (Heras et al., 2015).

3.7 16S rRNA gene sequencing

A PCR based approach using 16S rRNA gene sequencing were applied. The universal primer used for sequencing 16S RNA genes are; 27F (forward primer) and 519R (reverse primer) that were chosen for their reproducibility and ability to identify a wide range of bacterial family which includes *Bacteroidaceae, Enterobacteriaceae, Staphylococcacceae*, and *Pseudomonadaceae* (Hutter et al., 2003; Iñiguez-Palomares et al., 2007). These pair of primers are shown in Table 3.3.

Table 3.3: Primer used for 16S rRNA PCR

Primer	Sequences (5'−3')	Estimated target size (bp)	Reference
27F	CAG GCC TAA CAC ATG CAA GTC	492 bp	Iñiguez-Palomares *et al*., (2007)
519R	GWA TTA CCG CGG CKG CTG		Hutter *et al.*, (2003)

The 16S rRNA PCR mixture components are listed in Table 3.4. ExTEN mastermix was composed of *Taq* DNA polymerase, a proofreading enzyme, dNTPs, $MgCL_2$ and a reaction buffer. Table 3.5 illustrate the 16S rRNA PCR condition in accordance to Iñiguez-Palomares et al., (2007) with slight modification for the amplification of DNA template.

Table 3.4: 16S rRNA PCR mixture components

Components	Concentration	Volume per reaction (µl)
exTEN mastermix	5X	15
27 F	10 mM	1.2
519 R	10 mM	1.2
Sterile distilled water (dH_20)	-	3.6
DNA template	(20-30 ng)	9
Final Volume		30

Table 3.5: 16S rRNA PCR condition

Conditions	Temperature (°C)	Time (min)	Number of cycles
Initial denaturation	95	10	1
Denaturation	94	0.5	26
Annealing	55	1	
Extension	72	1.5	
Final Extension	72	10	1

Thirty microlitres of PCR amplification products were than resolved in 1% agarose gel stained with 1 µl of ethidium bromide (EtBr). The agarose gel was electrophoresed for 30 minutes at 90 V, 200 mA, and visualised under a UV illuminator (Maestrogen, Hsinchu

City, Taiwan). In every gel, sizes of amplicons were determined by comparison with a concurrently run DNA molecular 100 bp size DNA marker (Thermo Scientific, USA).

3.8 DNA purification and sequencing analysis

The PCR product was then recovered using Qiaquick Gel Purification Kit (Qiagen, Germany) according to the manufacturer's instructions. Firstly, the visible band on 1% agarose gel of 16S rRNA PCR was cut and put into a sterile 2.0 ml centrifuge tube and 300 µl QC buffer was added into the centrifuge tube. It was then placed into a water bath at 50 ℃ for 15 min until the gel was completely melted. Next, 100 µl of isopropanol was added, and the mixture was mixed by flicking the tube for 7 times. Then, the mixture was transferred into a new spin column and centrifuged at 10 000 rpm for 1 min, and the supernatant was discarded. Another 500 µl of QG buffer was added and centrifuged at 10 000 rpm for 1 min, and the supernatant was discarded. Next, 750 µl of PE buffer was added and centrifuged twice at 10 000 rpm for 1 min, and the supernatant was discarded. The spin column was transferred to a new sterile 2.0 ml microcentrifuge tube, and 50 µl of EB buffer was added and left for 1 min. Then, the microcentrifuge tube was centrifuged at 10 000 rpm for 1 min, and the spin column was removed, and the remaining supernatant was kept at -20 °C. The supernatant containing the purified DNA products were sent to Apical Sdn. Bhd. for sequencing. The DNA sequences data were compared with all known 16S rRNA sequences in the NCBI GenBank database using the BLASTN algorithm (Altschul et al., 1990).

3.9 Antibiotic susceptibility phenotypes

The identified bacteria were tested for antibiotic susceptibility using the disk diffusion method according to the recommendations of the Clinical Laboratory Standards Institute (CLSI, 2017) on all the 50 bacterial isolates. The bacteria were cultured in Muller-

Hinton Broth (HiMedia, India) and adjusted by dilution to an optical density of 0.10 (625 nm), with UVmini-1240 spectrophotometer (Shimadzu, Japan) which was equivalent to 0.5 MacFarland standard (1.5×10^8 CFU/ml). Briefly, a sterile cotton bud was dipped into the bacterial broth culture and spread evenly onto the surface of Mueller-Hinton agar (HiMedia, India) plates and let dry for 2-5 minutes before an antibiotic disk was placed on the agar surface using sterile forceps. These plates were then incubated at 35 °C for 18-20 hours aerobically.

The zone diameter was interpreted according to CLSI (2017) breakpoint tables and interpret as Resistant (R), Intermediate (1), or Susceptible (S). *E. coli* ATCC 25922 (Gram-negative) and *Staphylococcus aureus* ATCC 25923 (Gram-positive) were used as quality control strains that generally covers the two broad bacterial categories. Twenty-five antibiotics from eleven drug classes were tested in this study as which includes critically important (ampicillin and erythromycin) and highly important (chloramphenicol) antibiotics for human medicine (WHO, 2017a) as shown in Table 3.6. Note that, not all bacterial group were tested against all 25 antibiotics, as different bacterial groups were tested against 2 and up to 14 antibiotics which is shown in Appendix A (page 106). This is because different bacterial groups were intrinsically resistant to certain antibiotics. For example, *Stenotrophomonas* sp. were tested against 2 antibiotics because it is intrinsically resistant to 14 antibiotics as further described in CLSI (2017).

Table 3.6: Antibiotics used for Antibiotic Susceptibility Test

Antibiotics		Abbreviations	Concentration (µg)
(Aminoglycosides)	Amikacin	AK	30
	Gentamycin	CN	10
	Kanamycin	KA	30
	Streptomycin	S	10
(Tetracycline)	Doxycycline	DO	30
	Tetracycline	TE	30
(Penem)	Penicillin	P	10
	Piperacillin	PRL	100
	Ampicillin	AMP	10
(Phenicol)	Chloramphenicol	C	30
(Fluoroquinolone)	Levofloxacin	LEV	5
	Norfloxacin	NOR	10
	Ciprofloxacin	CIP	5
	Nalidixic Acid	NA	30
(Rifamycin)	Rifampin	RD	5
(Macrolide)	Erythromycin	E	15
(Sulfonamide)	Trimethoprim-sulfamethoxazole	SXT	1.25/ 23.75
(Cephalosporin)	Cefepime	FEP	30
	Ceftazidime	CAZ	30
	Ceftriaxone	CRO	30
	Cephalothin	KF	30
(Carbapenem)	Meropenem	MEM	10
	Ertapenem	E	10
	Imipenem	IPM	10
(Monobactam)	Aztreonam	ATM	30

3.10 MAR index

MAR index of the bacterial isolates were calculated as a/b where ‘a’ represents the number of antibiotics to which the particular isolate was resistant and ‘b’ the number of antibiotics to which the isolate was exposed to or using the formula a/b. MAR index value of more than 0.2 shows that the isolates are originated from high-risk sources; human, commercial poultry, piggeries, and dairy cattle (Krumperman, 1983). Whereas, a value of 0.19 and lower indicates the isolates are originated from low-risk sources; domestic animals and wild animals (Adenaike et al., 2016; Leong et al., 2019; Noorlis et al., 2011).

3.11 Analysis of zone diameter

After the given incubation time, the zone of inhibition of each isolate was measured to the nearest millimetre (mm). According to CLSI (2017), the different bacterial group were tested with a certain group of antibiotics according to the reference breakpoint values as listed in Appendix A (page 106). Since CLSI breakpoints latest version (2017) being freely available, it is used in this study to keep up to date with antibiotic susceptibility interpretation.

Whereas, the results of antibiotic susceptibility test on various bacterial species from water, sediment and fish samples are shown in Appendix B (page 109). The zone of inhibition for each bacterial isolate was recorded for calculating the percentage of antibiotic resistant and MAR index. The zone of inhibition that falls under “Intermediate” based on Table 4.6 was considered and calculated as “Resistant” in the MAR index. These MAR index values of isolates for each fish farms were then visualised on maps using ArcMap 10.3 to give a better picture of the point of dispersion of the resistant bacteria.

3.12 Fisher's exact test

Fisher's exact test were applied on *Bacillus* species using SPSS 24.0 statistical software (Chicago, USA) to determine if there were any significant difference in the proportion of *Bacillus* between the sample sources (sediment, water, and fish) with the risk level from antibiotic contaminated areas. *Bacillus* species were chosen as it accounts for the majority (38%, n = 19/50) of the isolates and significance was determined when $P < 0.05$.

3.13 Mapping using ArcGIS 10.3

The coordinate of sampling points for each farm were taken using a Global Positioning System, GPS (Garmin GPSmap 62S). Graphical representations of study locations and MAR index values for all sites were generated using ArcGIS 10.3 (ESRI, USA) as shown in Figure 3.1, Figure 4.7, Figure 4.8, Figure 4.9, Figure 4.10, Figure 4.11 and Figure 4.12 This study used Google maps as they were cloud free and freely available.

CHAPTER 4

RESULTS

4.1 Sample processing and isolation of bacterium

A total of 350 isolates consisted of fish (108 isolates), sediment (113 isolates) and water (129 isolates) were collected from the fish farms. The total number of pure isolates collected from each of the fives farm were: Farm 1 (40 isolates), Farm 2 (88 isolates), Farm 3 (66 isolates), Farm 4 (62 isolates), and Farm 5 (94 isolates). The sampling details are tabulated in Table 4.1. Whereas, the pH for studied farms ranged from pH 5.5 to 7.7, and the temperature of the sampling sites ranged from 28 to 32 ℃ (Table 4.2).

Table 4.1: Number of fish, sediment and water samples of farm sites

Sample Location	Fish	Sediment	Water	Total
Farm 1	14	13	13	40
Farm 2	18	28	42	88
Farm 3	22	22	22	66
Farm 4	20	20	22	62
Farm 5	34	30	30	94
Total	108	113	129	350

Table 4.2: Temperature and pH of farm sites

Sampling Location	Sampling Station	pH	Temperature (°C)
Farm 1	Pond A	5.8	28
	Pond B	6.3	29
	Pond C	6.9	30
Farm 2	Pond D	7.6	32
	Pond E	7.2	31
Farm 3	Pond F	6.3	30
	Pond G	6.6	30
Farm 4	Pond H	5.7	30
	Pond I	5.5	30
	Pond J	7.7	30
Farm 5	Pond K	7.3	30
	Pond L	7.3	30

Table 4.3: The mean colony counts of bacterial isolates isolated from water, sediment and fish samples from farm 1, farm 2, farm 3, farm 4 and farm 5 in Sarawak, Malaysia

Location	Sampling pond	Types of samples	Mean of bacterial colony counts (CFU mL-1)	Mean of bacterial colony counts (log CFU mL-1)
Farm 1	Pond A	water	1.09×10^4	4.04
		sediment	2.92×10^3	3.47
	Pond B	water	1.19×10^4	4.08
		sediment	5.17×10^4	4.71
		fish	2.33×10^5	5.37
Farm 2	Pond C	water	6.70×10^4	4.83
		sediment	6.65×10^4	4.82
	Pond D	water	2.85×10^4	4.45
		sediment	1.43×10^4	4.16
	Pond E	water	4.55×10^4	4.66

Table 4.3 continued

		sediment	3.02×10^4	4.48
		fish	4.50×10^3	3.65
Farm 3	Pond F	water	7.37×10^3	3.87
		sediment	1.32×10^4	4.12
	Pond G	water	1.18×10^4	4.07
		sediment	1.12×10^4	4.05
		fish	4.10×10^4	3.61
Farm 4	Pond H	water	2.55×10^3	3.41
		sediment	2.43×10^3	3.39
	Pond I	water	1.38×10^4	4.14
		sediment	3.42×10^3	3.53
		fish	2.05×10^4	4.31
Farm 5	Pond J	water	2.0×10^4	4.30
		sediment	1.25×10^4	4.10
	Pond K	water	1.33×10^4	4.12
		sediment	4.81×10^4	4.68
	Pond L	water	1.46×10^4	4.16
		sediment	3.15×10^4	4.50
		fingerlings	1.34×10^5	5.13

Based on Table 4.3, bacterial counts were taken from three main sources: water, sediment and fishes. Bacterial counts from pond water samples were the highest was seen in farm 2 in all three ponds; pond C, pond D, and pond E with bacterial counts of 4.83 log CFU mL^{-1}, 4.45 log CFU mL^{-1}, and 4.66 log CFU mL^{-1} respectively. For sediment samples, the highest bacterial counts were seen in one of the ponds in farm 2 with bacterial counts of 4.82 log CFU mL^{-1}, followed by farm 1 in pond B (4.71 log CFU mL^{-1}) and Farm 5 in pond K (4.68 log CFU mL^{-1}).

For fish samples, the highest bacterial counts were seen in Farm 1 with an average of 5.37 log CFU mL^{-1}. Whereas, the bacterial counts in Farm 2 is the lowest with an estimate of 3.65 log CFU mL^{-1}. The low bacterial counts in farm 2 could be attributed to the sample source, where swabbing technique from the fish scales was collected. Whereas, in other aquaculture farms, fish intestines were collected which have been proven to yield in more bacterial counts (Apun et al., 1999). The least bacterial counts were seen in farm 4 for both water and sediment samples with an estimated average of 3.41 log CFU mL^{-1} and 3.39 log CFU mL^{-1} both in pond H.

4.2 $(GTG)_5$ – PCR fingerprints

Two hundred and four of bacteria isolates from the aquaculture farms were then subjected to $(GTG)_5$-PCR analysis to assist in grouping the bacteria based on genetic differences. A range of three to eight clusters were generated according to the appropriateness size of the dendrogram through GelJ 2.0 software. PCR amplification products size ranged from 250 bp to 8,000 bp by comparison of 1 kb size DNA marker with DNA product ranged from 1 to 10 bands. The clusters from the dendrogram were created using a similarity index ranged from 50% to 74% similarity. The banding pattern and the dendrogram were visualised in Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4 and Figure 4.5.

4.2.1 $(GTG)_5$ – PCR fingerprint for isolates from farm 1

The banding pattern and the dendrogram of Farm 1 isolates are illustrated in Figure 4.1 Based on the dendrogram constructed, the bacterial isolates were grouped into four clusters. Cluster 3 with similarity index of 74% formed the largest clusters consist of 13 bacterial isolates: 6 isolates from water, 5 isolates from sediment and 2 isolates from fish. Cluster 1 with similarity index of 66% consisted of 6 bacterial isolates: 2 isolates from sediment, 2 isolates from fish, and 1 isolate from the water. Cluster 2 with similarity index of 66% consisted of 6 isolates from water and 1 isolate from sediment. Cluster 4 with similarity index of 72% consisted of 7 isolates from fish and only 1 isolate from water.

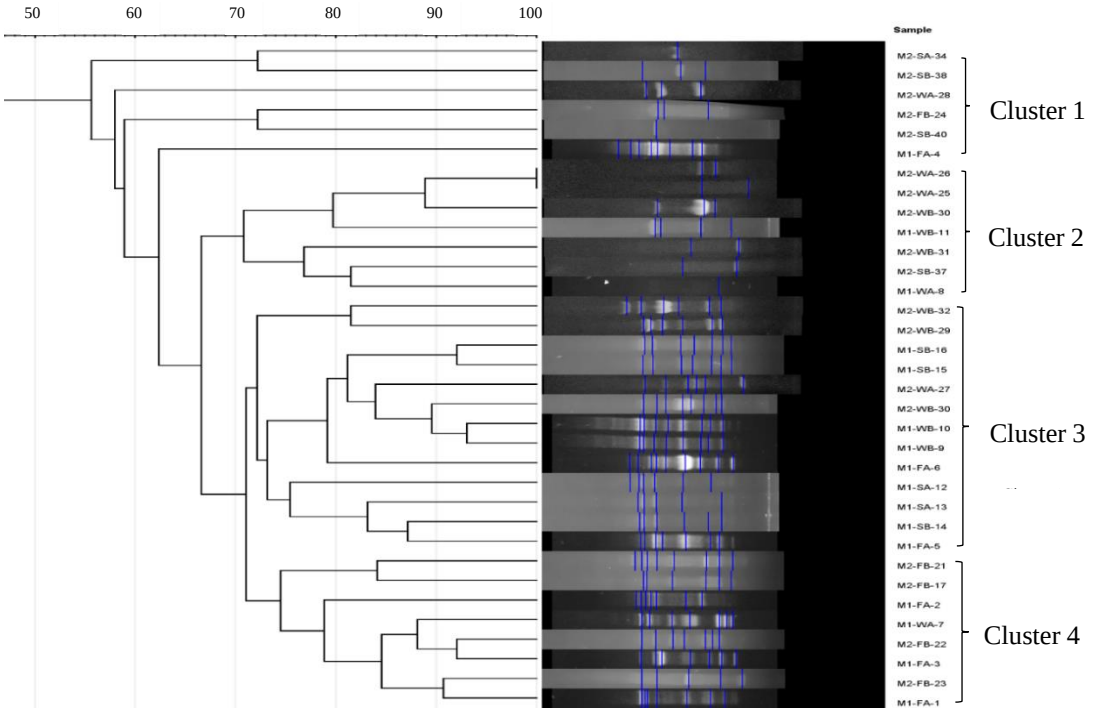

Figure 4.1: Dendrogram based on Dice similarity method with tolerance value of 4 using UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolated taken from Farm 1 aquaculture: Cluster 1: M2-SA-34, M2-SB-38, M2-WA-28, M2-FB-24, M2-SB-40, M1-FA-4; Cluster 2: M2-WA-26, M2-WA-25, M2-WB-30, M1-WB-11, M2-WB-31, M2-SB-37, M1-WA-8; Cluster 3: M2-WB-32, M2-WB-29, M1-SB-16, MI-SB-15, M2-WA-27, M2-WB-30, M1-WB-10, M1-WB-9, L1-FA-6, M1-SA-12, M1-SA-13, M1-SB-14, M1-FA-5; Cluster 4: M2-FB-21, M2-FB-17, M1-FA-2, M1-WA-7, M2-FB-22, M1-FA-3, M2-FB-23, M1-FA-1.

4.2.2 $(GTG)_5$ – PCR fingerprinting for isolates from farm 2

From Figure 4.2, seventy-seven bacterial isolates were isolated from farm 2 forming 4 major clusters. The largest cluster consisted of 36 bacterial isolates which are seen in cluster 3 with similarity index of 52%. Cluster 3 is made up of 23 isolates from water, 12 isolates from sediment, and 1 isolate from fish. Cluster 4 with similarity index of 60% consisted of 8 isolates from sediment, 9 isolates from water and 1 isolate from fish. Cluster 4 also consisted of very similar banding pattern with similarity index of 60%. Cluster 1 with similarity index of 50% was made up of 7 isolates consisted of 7 isolates from water, 4 isolates from fish and 1 isolate from sediment. The clusters formed ranged from 50% to 62%.

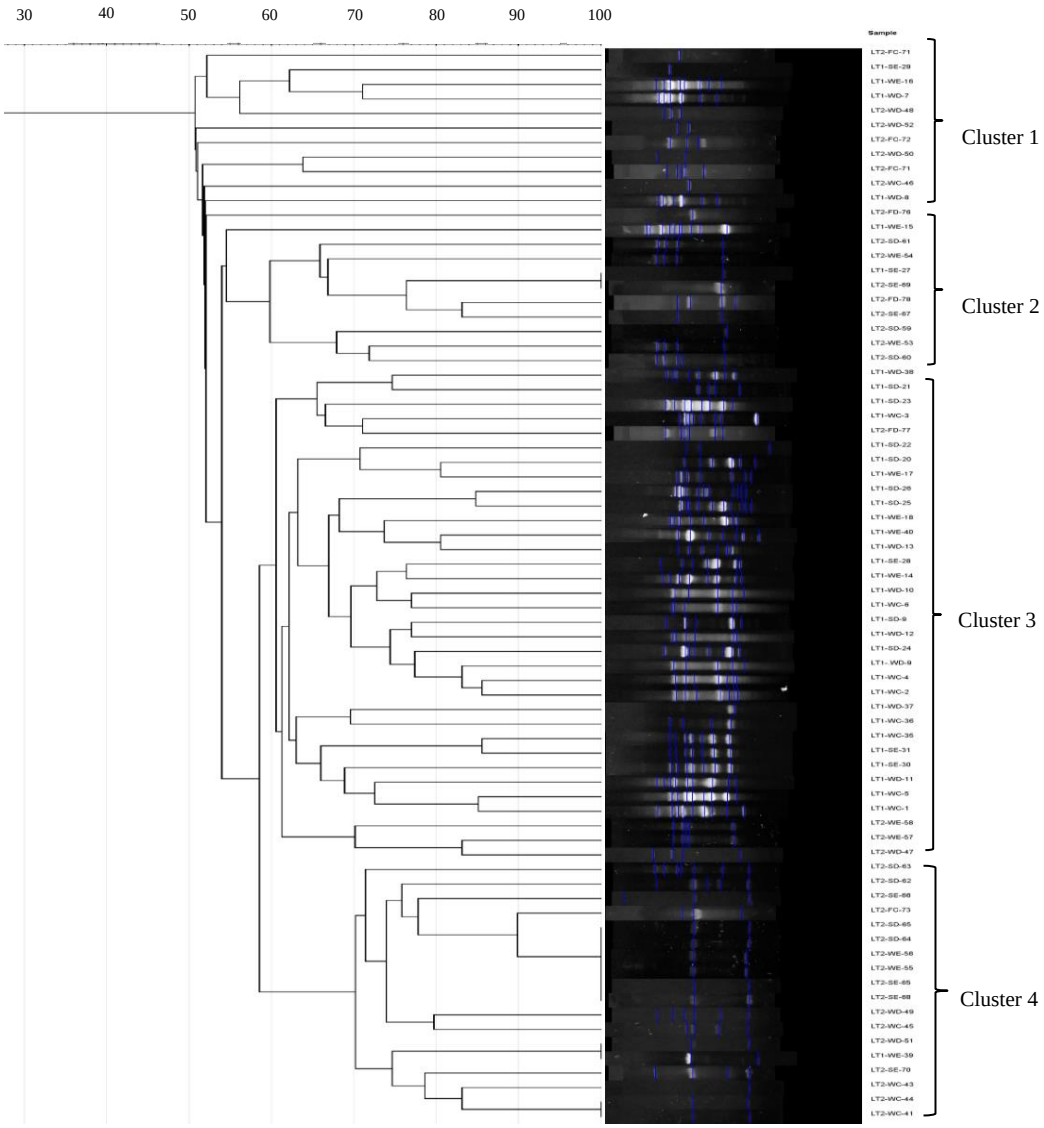

Figure 4.2: Dendrogram based on Dice similarity method with tolerance value of 4 using UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolated taken from Farm 2 aquaculture: Cluster 1: LT2-FC-71, LT1-SE-29, LT1-WE-16, LT1-WD-7, LT2-WD-48, LT2-WD-52, LT2-FC-72, LT2-WD-50, LT2-FC-71, LT2-WC-46, LT1-WD-8, LT2-FD-76; Cluster 2: LT1-WE-15, LT2-SD-61, LT2-WE-54, LT1-SE-27, LT2-SE-69, LT2-FD-78, LT2-SE-67, LT2-SD-59, LT2-WE-53, LT2-SD-60, LT1-WD-38; Cluster 3: LT1-SD-21, LT1-SD-23, LT1-WC-3, LT2-FD-77, LT1-SD-22, LT1-SD-20, LT1-WE-17, LT1-SD-26, LT1-SD-25, LT1-WE-18, LT1-WE-40, LT-WD-13, LT1-SE-28, LT1-WE-40, LT1-WD-13, LT1-SE-28, LT1-WE-14, LT1-WD-10, LT1-WC-6, LT-1-SD-9, LT1-WD-12, LT1-SD-24, LT1-WD-9, LT1-WC-4, LT1-WC-2, LT1-WD-37, LT1-WC-36, LT1-WC-35, LT1-SE-31, LT1-SE-30, LT1-WD-11, LT1-WC-5, LT1-WC-1, LT2-WE-58, LT2-WE-57, LT2-WD-47; Cluster 4: LT2-SD-63, LT2-SD-62, LT2-SE-66, LT2-FC-73, LT2-SD-65, L2-SD-64, LT2-WE-56, LT2-WE-55, LT2-SE-65, LT2-SE-68, LT2-WD-49, LT2-WC-45, LT2-WD-51, LT1-WE-39, LT2-SE-70, LT2-WC-43, LT2-WC-44, LT2-WC-41.

4.2.3 $(GTG)_5$ – PCR fingerprinting for isolates from farm 3

Using GelJ version 2.0 software, a combination of the dendrogram and $(GTG)_5$ banding pattern were constructed for Farm 3 consisting of 16 isolates where all 3 clusters have a similarity index of 65%. Three main clusters were formed as shown in Figure 4.3. Cluster 1 consisted of 3 isolates from sediments samples. Cluster 2 is made of 1 isolate from every water, sediment and fish. Cluster 3 is made up 6 isolates from water, 2 isolates from fish and 1 isolate from sediment.

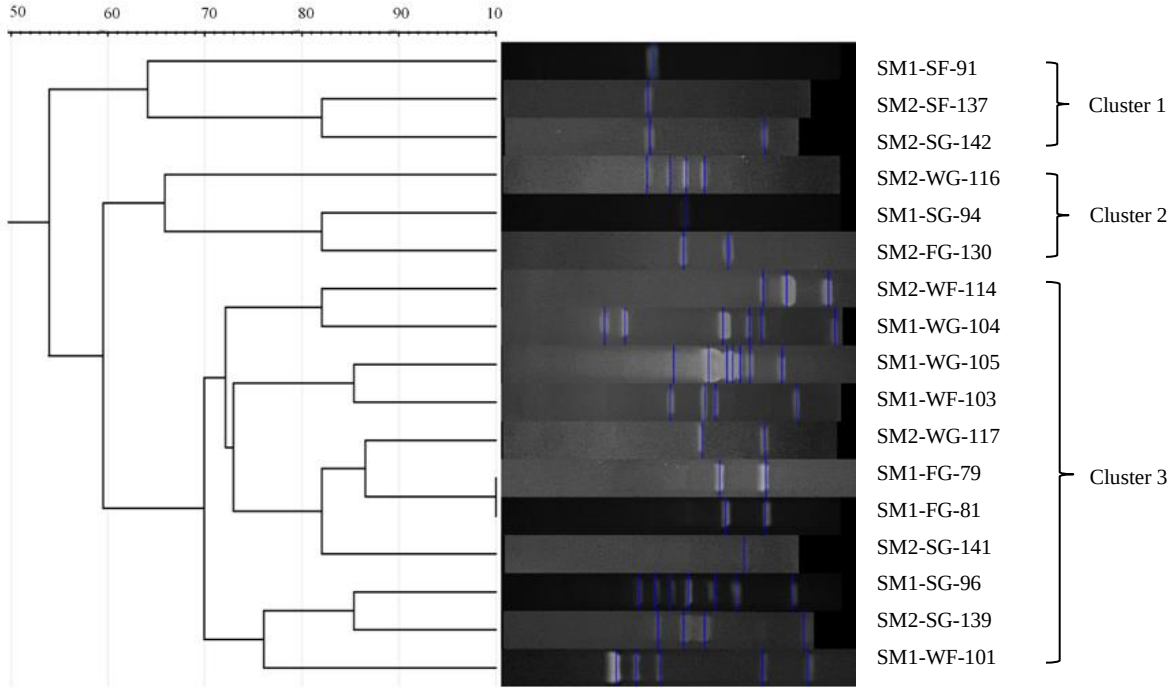

Figure 4.3: Dendrogram based on Dice similarity method with tolerance value of 4 using UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolated taken from Farm 3 aquaculture: Cluster 1: SM1-SF-91, SM2-SF-137, SM2-SG-142; Cluster 2: SM2-WG-116, SM1-SG-94, SM2-FG-130; Cluster 3: SM2-WF-114, SM1-WG-104, SM1-WG-105, SM1-WF-103, SM2-WG-117, SM1-FG-79, SM1-FG-81, SM2-SG-141, SM1-SG-96, SM2-SG-139, SM1-WF-101.

4.2.4 $(GTG)_5$ – PCR fingerprinting for isolates from farm 4

Twenty-eight bacterial isolates were subjected to $(GTG)_5$-PCR fingerprinting to formed 4 major clusters for Farm 4 with similarity index that ranged from 68% to 70% (Figure 4.4). Cluster 1 to 3 have a similarity index of 70%, whereas cluster 4 has a slightly lower similarity index of 68%. Cluster 1 consist of 6 isolates where 5 isolates originated from fish and 1 isolate from water. Cluster 2 consist of 3 isolates from water with very similar banding pattern and 1 isolate from fish. Cluster 3 consist of 4 isolates from fish and 3 isolates from water. Finally, cluster 4 is made up of 11 isolates where 5 isolates originated from sediment, 4 isolates from water, and 2 isolates from fish.

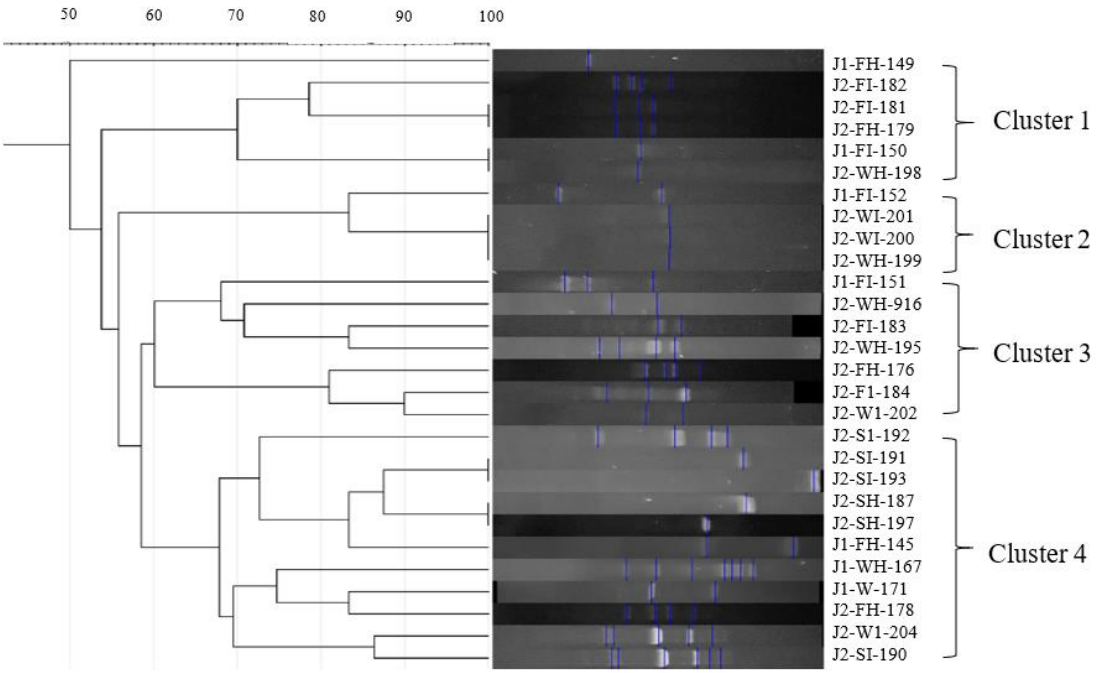

Figure 4.4: Dendrogram based on Dice similarity method with tolerance value of 4 using UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolated taken from Farm 4 aquaculture: Cluster 1: J1-FH-149, J2-FI-182, J2-FI-181, J2-FH-179, J1-FI-150, J2-WH-198; Cluster 2: J1-FI-152, J2-WI-201, J2-WI-200, J2-WH-199; Cluster 3: J1-FI-151, J2-WH-916, J2-FI-183, J2-WH-195, J2-FH-176, J2-F1-184, J2-W1-202; Cluster 4: J2-S1-192, J2-SI-191, J2-SI-193, J2-SH-187, J2-SH-197, J1-FH-145, J1-WH-167, J1-W-171, J2-FH-178, J2-W1-204, J2-SI-190.

Dendrogram for farm 5 consists of 8 Clusters with a similarity index with similarity index of 70% (Figure 4.5). Cluster 1 consisted of only 1 bacterial isolate originated from water. Cluster 2 consisted of 5 bacterial isolates: 2 isolates from sediment, 2 isolates from fish, and 1 isolate from water. Cluster 3 consisted of 11 bacterial isolates: 1 isolate from sediment, 5 isolates from fish, and another 5 isolates from water. Cluster 4 consisted of 5 bacterial isolates: 3 isolates from water, and 2 isolates from sediment. Cluster 5 consisted of 9 bacterial isolates. Whereby 6 of the isolates have very similar banding pattern originated from sediment, water, and fish samples. Cluster 7 consisted of 6 bacterial isolates where 2 isolates were from fish, 3 isolates were from water and 1 isolate from sediment. Cluster 8 consisted of 7 isolates from fish with very similar banding patterns and 2 isolates originated from sediment.

4.2.5 $(GTG)_5$ – PCR fingerprinting for isolates from farm 5

Dendrogram for farm 5 consists of 8 Clusters with a similarity index with similarity index of 70% (Figure 4.5). Cluster 1 consisted of only 1 bacterial isolate originated from water. Cluster 2 consisted of 5 bacterial isolates: 2 isolates from sediment, 2 isolates from fish, and 1 isolate from water. Cluster 3 consisted of 11 bacterial isolates: 1 isolate from sediment, 5 isolates from fish, and another 5 isolates from water. Cluster 4 consisted of 5 bacterial isolates: 3 isolates from water, and 2 isolates from sediment. Cluster 5 consisted of 9 bacterial isolates. Whereby 6 of the isolates have very similar banding pattern originated from sediment, water, and fish samples. Cluster 7 consisted of 6 bacterial isolates where 2 isolates were from fish, 3 isolates were from water and 1 isolate from sediment. Cluster 8 consisted of 7 isolates from fish with very similar banding patterns and 2 isolates originated from sediment.

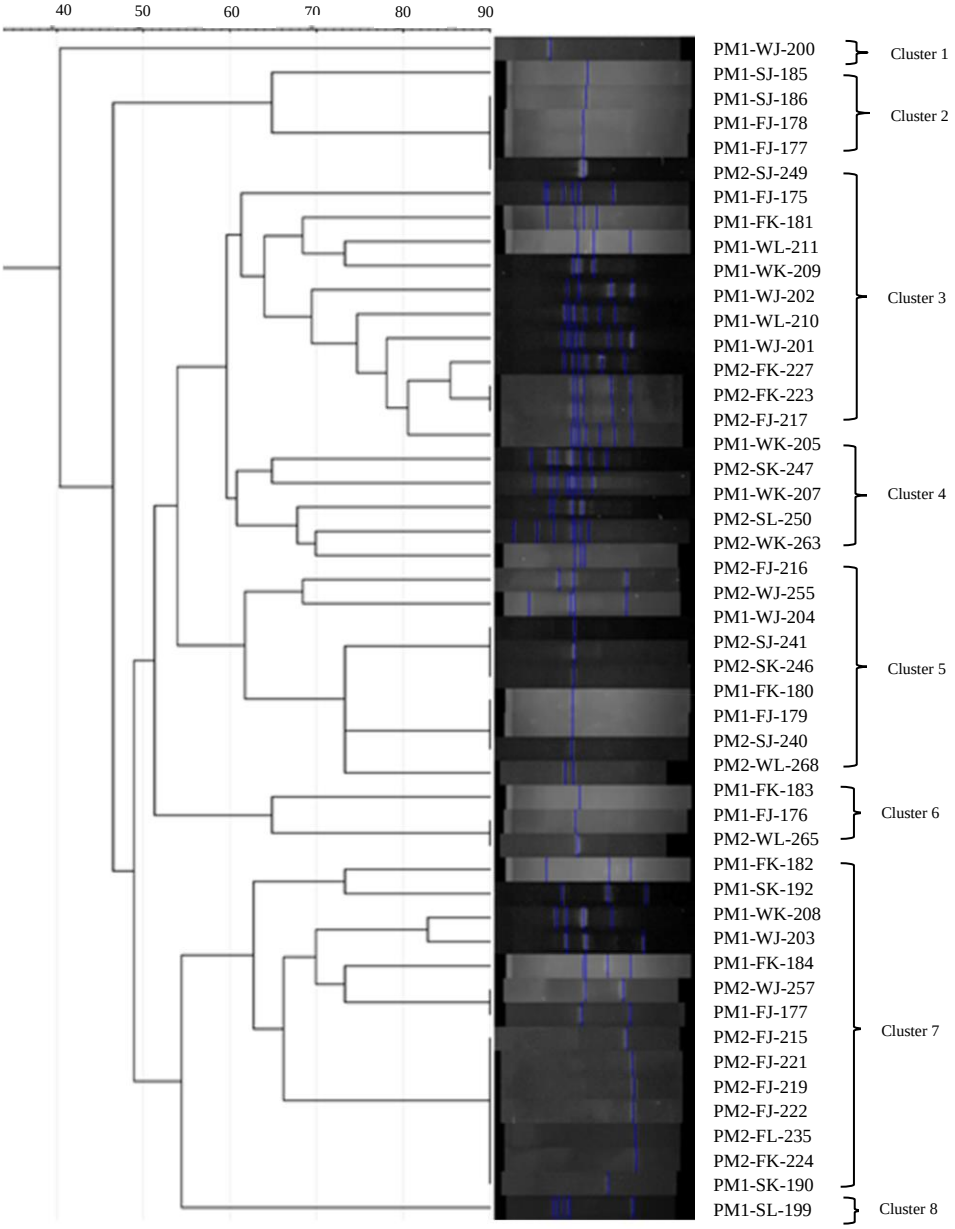

Figure 4.5: Dendrogram based on Dice similarity method with tolerance value of 4 using UPGMA linkage of $(GTG)_5$-PCR fingerprints obtained from bacterial isolated taken from Farm 5 aquaculture: Cluster 1: PM1-WJ-200; Cluster 2: PM1-SJ-185, PM1-SJ-186, PM1-FJ-178, PM1-FJ-177; Cluster 3: PM2-SJ-249, PM1-FJ-175, PM1-FK-181, PM1-WL-211, PM1-WK-209, PM1-WJ-202, PM1-WL-210, PM1-WJ-201, PM2-FK-227, PM2-FK-223, PM2-FJ-217; Cluster 4: PM1-WK-205, PM2-SK-247, PM1-WK-207, PM2-SL-250, PM2-WK-263; Cluster 5: PM2-FJ-216, PM2-WJ-255, PM1-WJ-204, PM2-SJ-241, PM2-SK-246, PM1-FK-180, PM1-FJ-179, PM2-SJ-240, PM2-WL-268, Cluster 6: PM1-FK-183, PM1-FJ-176, PM2-WL-265; Cluster 7: PM1-FK-182, PM1-SK-192, PM1-SK-192, PM1-WK-208, PM1-WJ-203, PM1-FK-184, PM2-WJ-257, PM1-FJ-177, PM2-FJ-215, PM2-FJ-221, PM2-FJ-219, PM2-FJ-222, PM2-FL-235, PM2-FK-224, PM1-SK-190; Cluster 8: PM1-SL-199.

4.3 Bacterial identification using 16S rRNA sequencing

A total of 50 bacterial samples were identified in all five farms using 16S rRNA gene sequencing following the $(GTG)_5$ PCR fingerprint analysis. There were 17, 19 and 14 bacteria identified that were originated from water, sediment and fish samples respectively. The sequence generated at approximately 500 to 550 bases were compared with those in the NCBI GenBank database. Analysis showed that the 16S rRNA gene sequences of the 50 bacterial isolates from the five farms had 84 to 100% similarities to their closest relatives as shown in Table 4.4.

Table 4.4: Sequence similarities results of bacterial isolates compared using NCBI GenBank database

Sample Source	Isolate code	Species	Location	Accession Number	Similarity (%)
Fish	M 1	*Bacillus altitudinis*	Farm 1	gi|1524754595| MK241863.1	99
Fish	M 4	*Bacillus* sp.	Farm 1	gi|1530829086| MG709201.1	100
Water	M 9	*Bacillus* sp.	Farm 1	gi|1532362179| MK280707.1	99
Water	M 11	*Acinetobacter junii*	Farm 1	gi|1247278441| KY049895.1	99
Sediment	M 14	*Enterobacter asburiae*	Farm 1	gi|1360449899| MH061358.1	99
Sediment	M 16	*Enterobacter asburiae*	Farm 1	gi|671722565| KJ937079.1	99
Fish	M 21	*Fictibacillus macauensis*	Farm 1	gi|1375377319| MH169269.1	99
Sediment	M 40	*Aeromonas veronii*	Farm 1	gi|1236049012| MF716714.1	100
Water	LT 16	*Stenotrophomonas* sp.	Farm 2	gi|1386719692| CP026004.1	99
Water	LT 17	*Bacillus pumilus*	Farm 2	gi|7862180|AF 260744.1	100
Water	LT 18	*Bacillus pumilus*	Farm 2	gi|7862181|AF 260745.1	99
Sediment	LT 21	*Bacillus aquimaris*	Farm 2	gi|1384038042| MH261098.1	99

Table 4.4 continued

Sediment	LT 23	*Enterobacter* sp.	Farm 2	gi|675822296|KM108474.1	92
Sediment	LT 26	*Bacillus cereus*	Farm 2	gi|820831982|KP284270.2	99
Sediment	LT 31	*Bacillus* sp.	Farm 2	gi|83630479|DQ299218.1	82
Water	LT 40	*Exiguobacterium profundum*	Farm 2	gi|1465463080|MG705821.1	99
Water	LT 43	*Acinetobacter* sp.	Farm 2	gi|393717569|JX047439.1	84
Water	LT 47	*Exiguobacterium* sp.	Farm 2	gi|1529654131|MH845741.1	99
Water	LT 52	*Exiguobacterium* sp.	Farm 2	gi|229467163|CP001615.1	99
Water	LT 54	*Exiguobacterium profundum*	Farm 2	gi|1465462998|MG705739.1	99
Sediment	LT 63	*Exiguobacterium profundum*	Farm 2	gi|838079647|KR137719.1	99
Sediment	LT 64	*Exiguobacterium aurantiacum*	Farm 2	gi|1041522627|KX458117.1	100
Fish	LT 78	*Enterobacter* sp.	Farm 2	gi|355343574|JN944751.1	95
Fish	SM 79	*Pseudoxanthomonas mexicana*	Farm 3	gi|1139272949|LT686970.1	99
Sediment	SM 91	*Bacillus cereus*	Farm 3	gi|939460273|LN890008.1	99
Sediment	SM 94	*Bacillus zhangzhouensis*	Farm 3	gi|1450319410|MH758787.1	99
Water	SM 101	*Chitinophaga* sp.	Farm 3	gi|952951644|KT154878.1	94
Water	SM 103	*Staphylococcus* sp.	Farm 3	gi|1480119682|MH935803.1	99
Fish	SM 130	*Bacillus pumilus*	Farm 3	gi|734915987|KM596792.1	99
Sediment	SM 139	*Staphylococcus haemolyticus*	Farm 3	gi|1409131228|MH532495.1	99
Sediment	SM 142	*Bacillus pumilus*	Farm 3	gi|7862180|AF260744.1	100
Fish	J 149	*Bacillus megaterium*	Farm 4	gi|1530829120|MG709235.1	99
Fish	J 151	*Enterobacter amnigenus*	Farm 4	gi|451170707|HF585318.1	99
Fish	J 152	*Achromobacter* sp.	Farm 4	gi|1384666853|MF319207.1	99
Sediment	J 162	*Bacillus* sp.	Farm 4	gi|1500055351|MK110364.1	99

Table 4.4 continued

Water	J 171	*Citrobacter* sp.	Farm 4	gi|1159332427| KY027163.1	99
Fish	J 176	*Citrobacter* sp.	Farm 4	gi|1369318811| MH109702.1	98
Fish	J 181	*Aeromonas jandaei*	Farm 4	gi|1352416542| MH010191.1	99
Fish	J 184	*Enterobacter cloacae*	Farm 4	gi|1401585411| MH470270.1	99
Sediment	J 187	*Bacillus* sp.	Farm 4	gi|1450319410| MH758787.1	99
Water	J 200	*Bacillus cereus*	Farm 4	gi|1446048862| MH734616.1	100
Fish	PM 183	*Aeromonas veronii*	Farm 5	gi|793967940| KP761691.1	99
Sediment	PM 185	*Bacillus indicus*	Farm 5	gi|1274590811| KY321480.1	99
Sediment	PM 199	*Pseudomonas* sp.	Farm 5	gi|850484150| KT203429.1	100
Water	PM 205	*Enterobacter* sp.	Farm 5	gi|1360449900| MH061359.1	96
Water	PM 207	*Bacillus* sp.	Farm 5	gi|1532362168| MK280696.1	100
Fish	PM 216	*Enterobacter* sp.	Farm 5	gi|733165552| LN624803.2	95
Sediment	PM 246	*Enterobacter* sp.	Farm 5	gi|528080873| KF307771.1	97
Sediment	PM 249	*Bacillus* sp.	Farm 5	gi|306451193| HM215458.1	100
Water	PM 257	*Plesiomonas shigelloides*	Farm 5	gi|1275509820| MG438515.1	100

4.4 Bacterial diversity in aquaculture farms

After the dendrogram was generated, 50 isolates were identified. Fourteen genera of bacterial species were identified in this study. As shown in Figure 4.6, the bacterial genera in this study consist of *Bacillus* (38%), *Exiguobacterium* (16%), *Enterobacter* (14%), *Aeromonas* (6%) *Acinetobacter* (4%), *Citrobacter* (4%), *Staphylococcus* (4%), *Achromobacter* (2%), *Chitinophaga* (2%), *Fictibacillus* (2%), *Plesiomonas* (2%), *Pseudomonas* (2%) and *Pseudoxanthomonas* (2%) and *Stenotrophomonas* (2%).

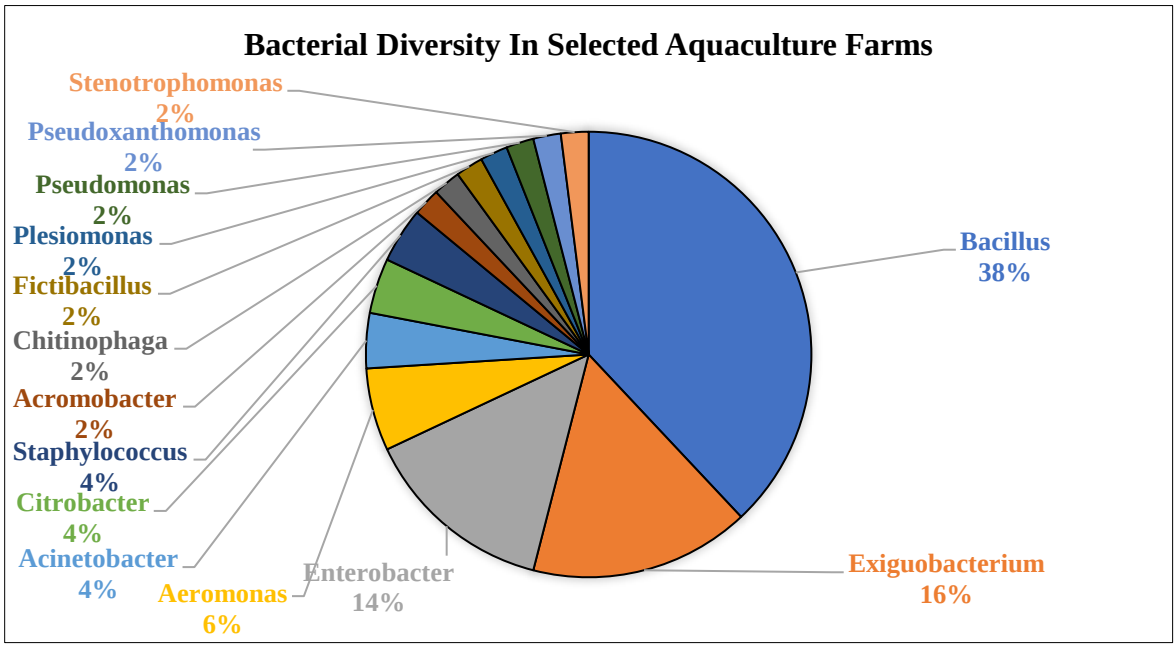

Figure 4.6: Percentage of bacterial diversity in selected Aquaculture Farms in Sarawak

Culture-based isolation and identification used in this study may have missed a larger number of bacterial species that were present in an aquaculture environment. The bacterial diversity present might represent a bias in data collection, but future studies may apply a wider range of culture media to give a clear picture of the bacterial diversity present.

4.5 Antibiotic susceptibility testing

The assessment of aquaculture bacteria susceptibility to twenty-five different antibiotics was presented in Table 4.5. Antibiotics selection were dependent on the bacterial genera, as a differential bacterial group may be intrinsically resistant to certain antibiotics which explained the unnecessary need to be tested to certain antibiotics. A total of 4 antibiotics (doxycycline, levofloxacin, trimethoprim-sulfamethoxazole and nalidixic acid) were completely susceptible toward aquaculture bacteria. A resistant of less than 10.0% were found in seven antibiotics; amikacin (8.7%), tetracycline (8.8%), gentamycin (8.2%), chloramphenicol (8.8%), norfloxacin (7.5%), ciprofloxacin (2.7%), meropenem (9.5%).

Table 4.5: Percentage of bacterial resistance based on antibiotics in all five farms

Antibiotics		Total of Resistant isolates (total isolates tested)	Percentage of resistance (%)
Streptomycin	S	9 (12)	75
Ampicillin	AMP	8 (12)	66.7
Ceftriaxone	CRO	2 (4)	50
Rifampin	RD	13 (30)	43.3
Aztreonam	ATM	7 (19)	36.8
Ceftazidime	CAZ	6 (19)	31.6
Erythromycin	E	8 (28)	28.6
Ertapenem	ETP	3 (12)	25
Cephalothin	KF	1 (4)	25
Penicillin	P	6 (28)	21.4
Cefepime	FEP	1 (5)	20
Piperacillin	PRL	3 (17)	17.6
Kanamycin	KA	2 (12)	16.7
Imipenem	IMP	1 (7)	14.3
Meropenem	MEM	2 (21)	9.5
Tetracycline	TE	3 (34)	8.8
Chloramphenicol	C	3 (38)	8.8
Amikacin	AK	4 (46)	8.7
Gentamycin	CN	4 (49)	8.2
Norfloxacin	NOR	3 (40)	7.5
Ciprofloxacin	CIP	1 (37)	2.7
Doxycycline	DO	0 (30)	0
Levofloxacin	LEV	0 (42)	0
Trimethoprim-sulfamethoxazole	SXT	0 (3)	0
Nalidixic Acid	NA	0 (12)	0

The highest percentage resistance recorded were of streptomycin (75.0%), followed by ampicillin (66.0%), ceftriaxone (50.0%), rifampin (43.3%), aztreonam (36.8%) and ceftazidime (31.6%). A resistance of between 30.0% to 10.0% were found in eight antibiotics; chloramphenicol (21.4%), erythromycin (28.6%), piperacillin (17.6%), cefepime (20.0%), kanamycin (16.7%), ertapenem (25.0%), imipenem (14.3%) and cephalothin (25.0%).

From Table 4.6, isolates from water, sediment and fish were resistant to seventeen, eleven and nineteen antibiotics respectively. The percentage of resistance among samples are highly variable; 0% to 100.0% (water), 0% to 50.0% (sediment) and 0% to 100% (fish). However, no relationship can be drawn out between the sample sources as not all the isolates were equally tested to the number of antibiotics due to the diversity of bacteria.

Table 4.6: Percentage of resistant strains from different sources (% of resistant; number of isolates)

Antibiotics	Water	Sediment	Fish
Amikacin	50.0 (2)	0	50.0 (2)
Tetracycline	100.0 (3)	0	0
Doxycycline	0	0	0
Gentamycin	50.0 (2)	25.0 (1)	25.0 (1)
Penicillin	50.0 (3)	33.3 (2)	16.7 (1)
Chloramphenicol	33.3 (1)	0	66.7 (2)
Levofloxacin	0	0	0
Rifampin	30.8 (4)	46.2 (6)	23.1 (3)
Norfloxacin	33.3 (1)	33.3 (1)	33.3 (1)
Erythromycin	25.0 (2)	50.0 (4)	25.0 (2)
Ciprofloxacin	0	0	100.0 (1)
Piperacillin	33.3 (1)	33.3 (1)	33.3 (1)

Table 4.6 continued

Trimethoprim sulfamethoxazole	0	0	0
Cefepime	0	0	100.0 (1)
Meropenem	50.0 (1)	0	50.0 (1)
Ceftazidime	16.67 (1)	50.0 (3)	33.33 (2)
Kanamycin	50.0 (1)	0	50.0 (1)
Nalidixic Acid	0	0	0
Streptomycin	33.3 (3)	22.2 (2)	44.4 (4)
Ampicillin	25.0 (2)	12.5 (1)	62.5 (5)
Aztreonam	14.1 (1)	28.6 (1)	57.1 (4)
Ertapenem	66.7 (2)	0	33.3 (1)
Imipenem	0	0	100.0 (1)
Cephalothin	100.0 (1)	0	0
Ceftriaxone	0	50.0 (1)	50.0 (1)

4.6 MAR index assessment

In the current study, a high percentage of isolates (74.0%, n = 37/50) have MAR index less than 0.2. Similar results were obtained from a study conducted by Kathleen et al. (2016), whereby most isolates (63.1%, n = 94) were found to be from a lower antibiotic contaminate sources. The overall results indicated that MAR index range from 0 to 0.79. Figure 4.7 illustrate the MAR frequency value of bacteria from different farms represented in pie charts of more than 0.2 (high risk sources) and less than 0.2 (low risk sources).

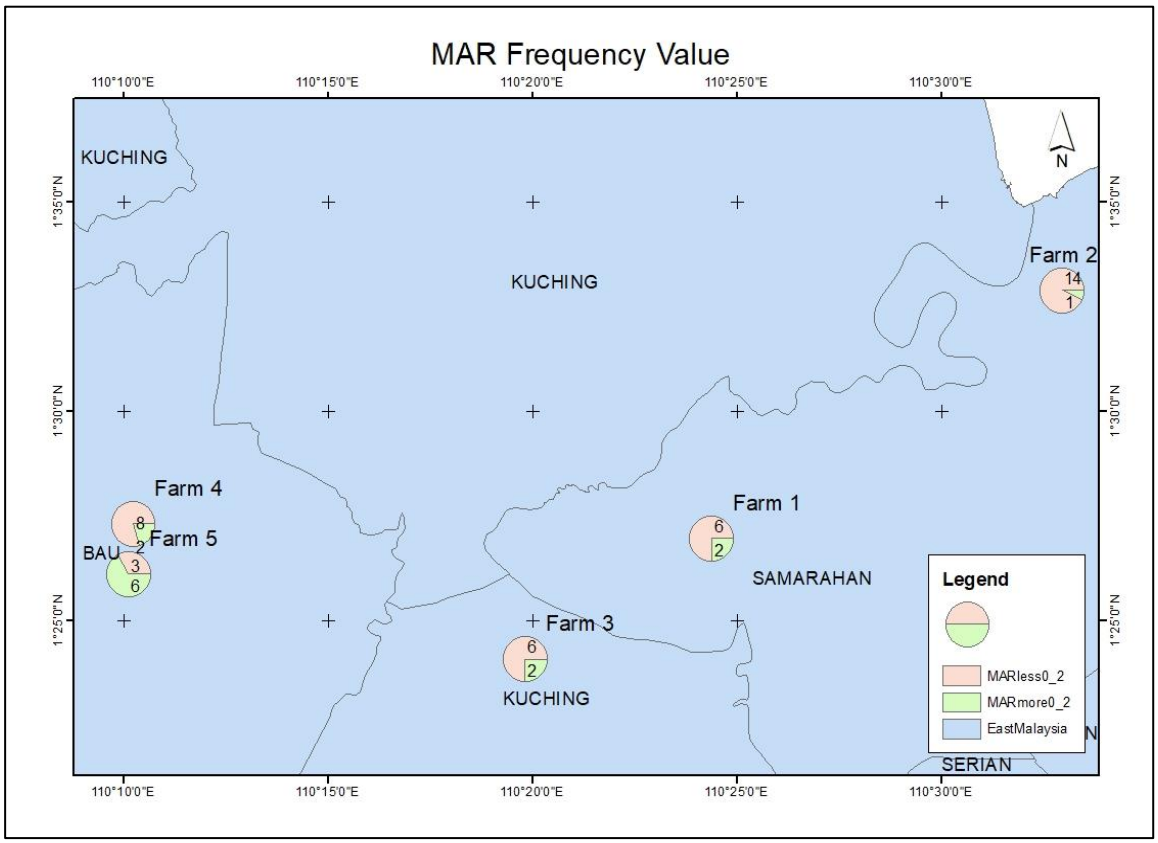

Figure 4.7: MAR index frequency value between different farms in Sarawak

Only a small percentage of 26.0% (*n* = 14/50) of isolates have a MAR index of more than 0.2. Bacterial isolates with MAR index more than 0.2 were attributed from *Aeromonas veronii*, *Aeromonas jandaei*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus* sp., *Chitinophaga* sp., *Enterobacter cloacae*, *Enterobacter amnigenus*, *Enterobacter* sp., *Exiguobacterium aurantiacum*, *Exiguobacterium aurantiacum*, *Exiguobacterium aurantiacum*, *Fictibacillus macauensis*, *Plesiomonas shigelloides* and *Pseudoxanthomonas mexicana* across all farms. This indicates that antibiotic resistance is not restricted to pathogenic bacteria but widespread among free living opportunistic aquatic bacteria identified in these farms.

Most of the bacterial isolates from Farm 1 had resistance indices of less than 0.2 (75.0%, *n* = 6/8), indicating the most of these bacteria originated from low risk sources that might originated domestic animals or wild animals. Only 25.0% (*n* = 2/8) of bacteria with resistance indices were more or equals to 0.2 which attribute from a high-risk source that might originate from the farmer's own poultry. Poultry has been regarded as a high-risk source because of it the primary reservoir of *Salmonella* sp. (Krumperman, 1983). These two bacteria identified as M40 and M21 were resistance to 3 and 6 antibiotics identified in *Aeromonas veronii* and *Fictibacillus macauensis* respectively. Presence of MAR bacteria in Farm 1 which is located within a residential area as shown in Figure 4.8, posed a high risk for people living within the vicinity of the area to acquire such resistance traits, and especially to the farm owner who is directly involved in the management of the aquaculture.

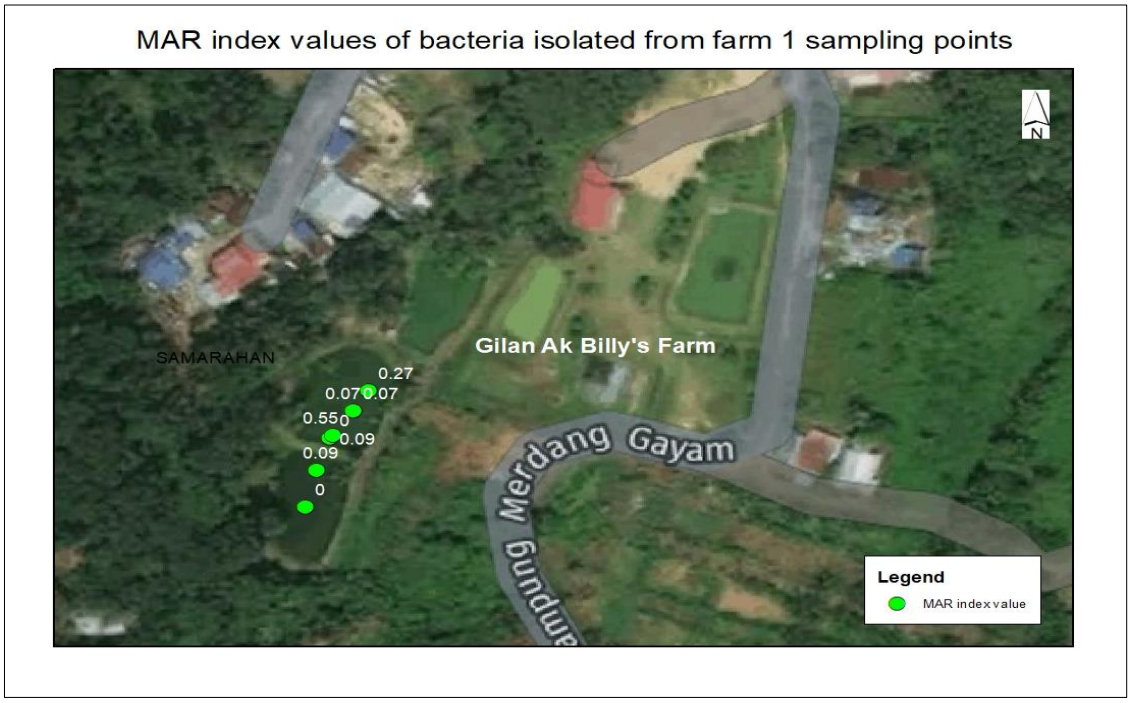

Figure 4.8: MAR index values of bacteria isolated from farm 1 sampling points

Figure 4.9 illustrate the distribution of MAR index values of bacteria isolated from two fishponds, and one from a cement tank. Only 1 bacterium out of 15 isolates tested in

Farm 2 have a MAR index of more than 0.2 identified as *Exiguobacterium aurantiacum* (LT 78). The isolate has an MAR index value of 0.21 which was resistant towards 3 antibiotics (ampicillin, piperacillin, and chloramphenicol) out of 14 antibiotics tested, as shown in Table 4.7. This suggests that most isolates originate from a lower antibiotic contaminated sources (Krumperman, 1983; Tanil et al., 2005), suggesting a low or no history of usage of antibiotics in the aquaculture environment (Maurice et al., 2018). Whereas another 14 isolates have an MAR index less than 0.2 which ranged from 0 to 0.18. A MAR index of 0 was identified in 8 isolates, where they are susceptible to all the antibiotics tested on them. Another 6 isolates were resistance from 1 to 2 antibiotics with a variety of resistance patterns; LT 17 and LT 26 were resistant to rifampin, LT 63 was resistant to erythromycin, LT 23 was resistant to ceftazidime and ampicillin, LT 52 was resistant to penicillin and erythromycin, and LT 64 was resistant to norfloxacin and erythromycin as previously depicted in Table 4.7. These resistance to a variety of antibiotics among different isolates might be to the highly efficient environmental bacteria to mobilize their resistance genes that circulate the aquatic system though transformation, conjugation, and transposition (Wright, 2007).

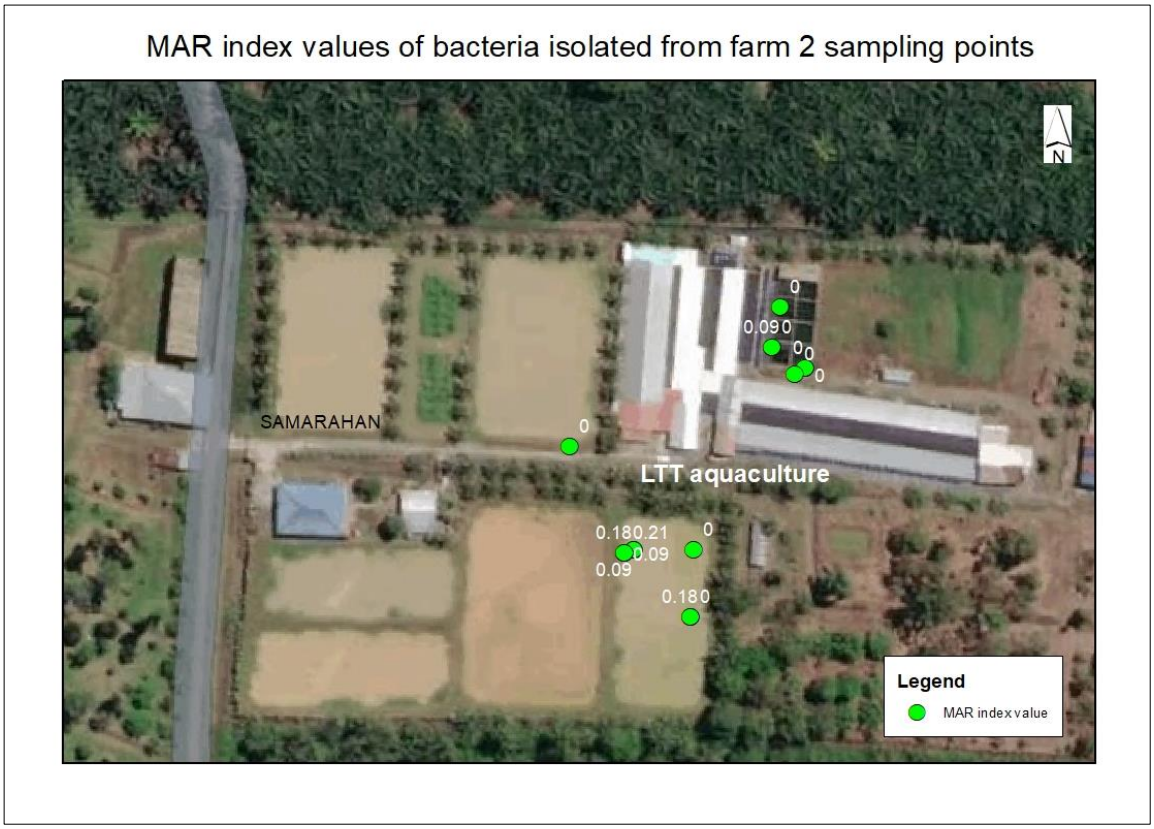

Figure 4.9: MAR index values of bacteria isolated at farm 2 sampling points

Approximately two isolates from farm 3 were above MAR 0.2 index as shown in Figure 4.10. *Chitinophaga* sp. recorded was resistance to 11 antibiotics out of 14 antibiotics, which have the highest MAR index value of 0.79. The second bacteria that were identified as *Pseudoxanthomonas Mexicana* have an MAR index of 0.78, which was resistance to 7 antibiotics out of 9 antibiotics tested. These considerably high MAR index value suggested that these two gram-negative environmental bacteria might compete for limited nutrients available and probably produce antibiotics to strive in the aquatic habitat (Bell et al., 2013). Whereas, another 6 isolates have a MAR index that range from 0 to 0.9, where 3 isolates were susceptible to all the antibiotics tested, another 3 isolates; SM 91, SM 103, and SM 142 were resistant to penicillin, tetracycline, and rifampin respectively as shown in Table 4.7. Interestingly, in a study by Grossman (2016) discovered that one of the mechanisms that

could possibly cause resistance to penicillin, tetracycline, and rifampin was induced by an efflux pump system, which might be responsible to some resistance of these bacteria as a form of an intrinsic mechanism

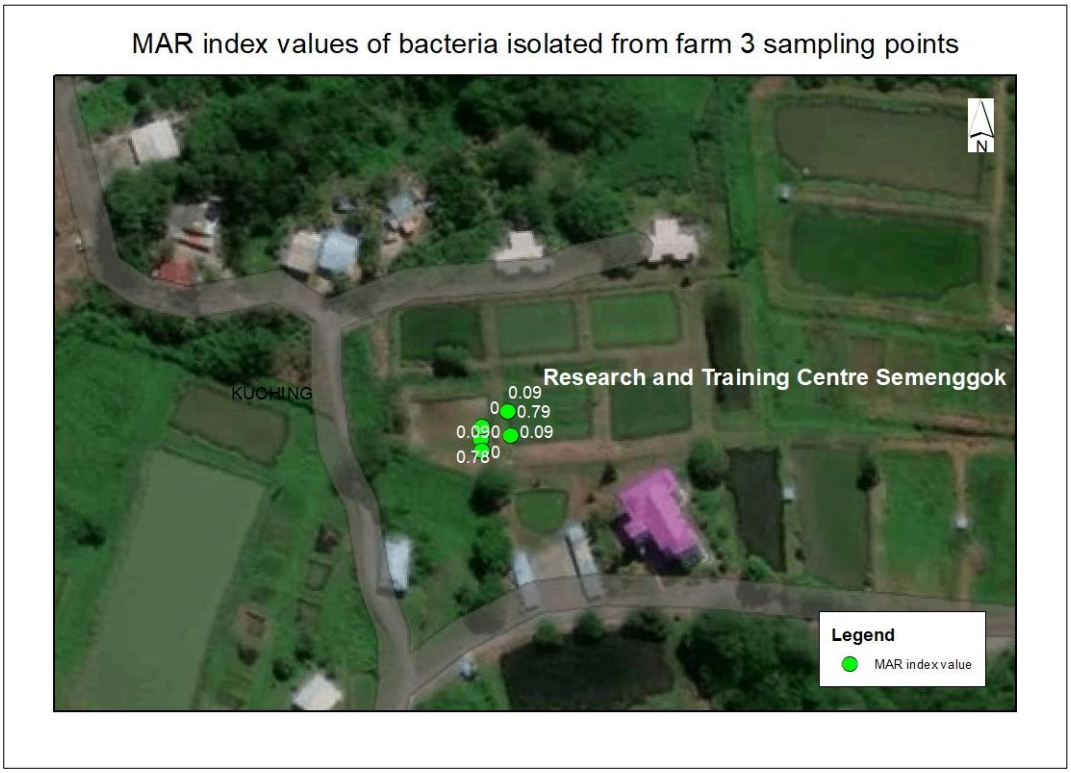

Figure 4.10: MAR index values of bacteria isolated from farm 3 sampling points

Meanwhile in farm 4 (Figure 4.11), only 2 isolates have a resistance index of more than 0.2, and another 8 isolates were less than 0.2. Isolates in farm 4 were resistant to at least 1 antibiotic and up to 4 antibiotics with a range of resistance patterns. The bacteria identified in farm 4 were consist of *Bacillus* sp., *Enterobacter* sp., *Citrobacter* sp., *Aeromonas* sp., and *Achromobacter* sp. These free living environmental bacteria often have complex regulatory network that allow them to degrade and resist toxicity of antibiotics (Martinez, 2009). Besides, farm 4 was found relatively close to farm 5 as shown previously in Figure 3.1. This could potentially pose a risk of acquiring resistance strains between these two fish farms

through human-to-human interaction, favouring the emergence of ARB (Ashbolt et al., 2013).

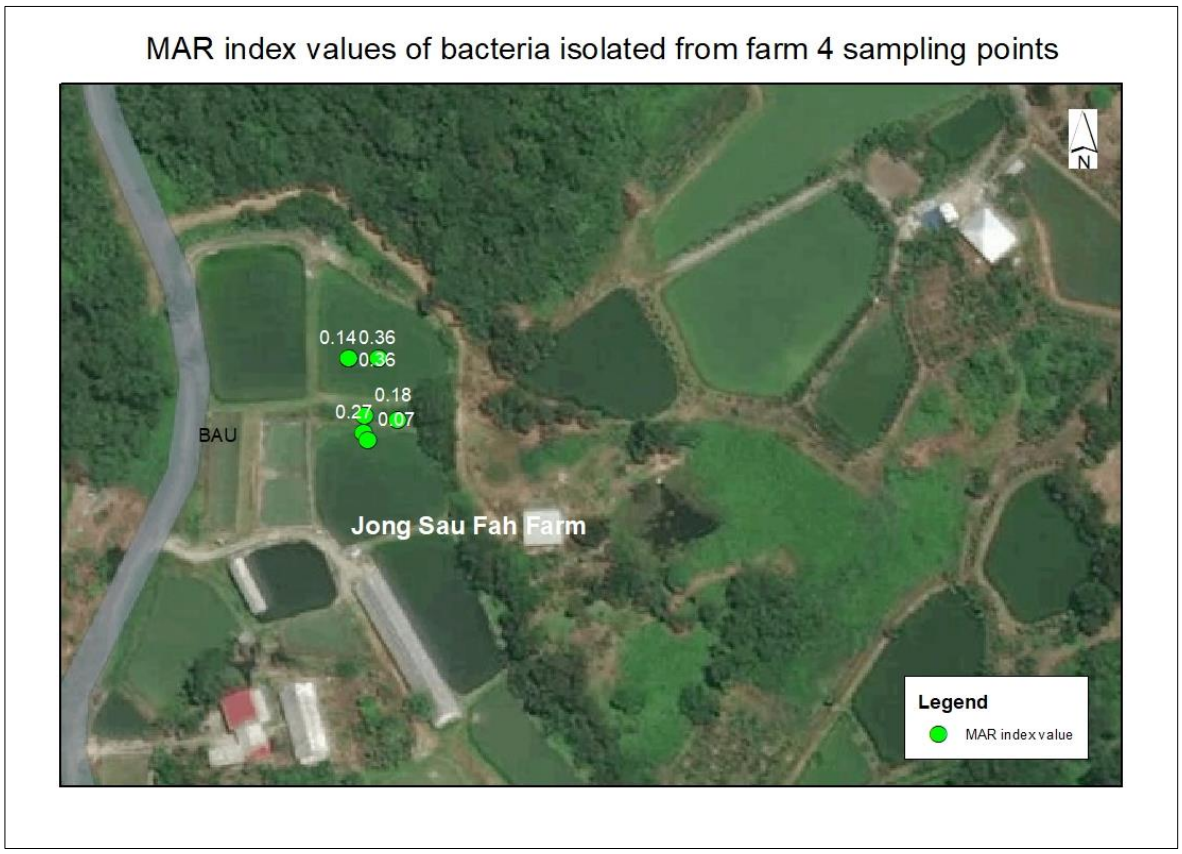

Figure 4.11: MAR index values of bacteria isolated from farm 4 sampling points

As shown in Figure 4.12, 6 out of 9 isolates have an MAR index value of more than 0.2, which ranged between 0.21 to 0.36, where each isolate was resistant to 3 to 4 antibiotics. Whereas, another 3 isolates have an MAR index between 0 to 0.09, where PM 199 isolate was susceptible to all 9 antibiotics, and PM 183 and PM 185 were resistant to aztreonam, and rifampin respectively. Even though, the number of bacteria with a MAR index value of over 0.2 out weight the number of bacteria with less 0.2, this might suggest that there were strong competitiveness among the environmental bacteria in farm 5, which in reflected on the a variety of resistance patterns (Bell et al., 2013).

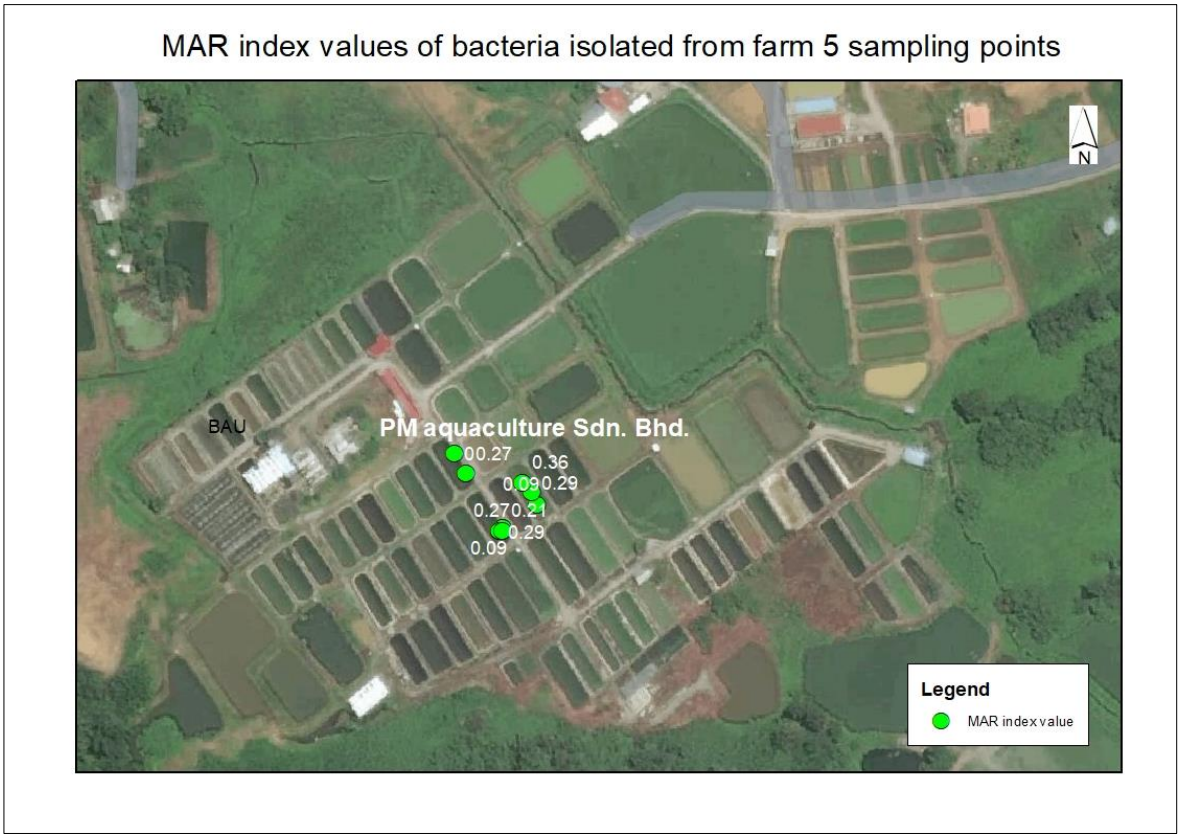

Figure 4.12: MAR index values of bacteria isolated from farm 5 sampling points

4.7 MAR patterns

In Table 4.7 shows the antibiotic resistant for all isolates, together with the percentage of isolates within the same pattern. There were twenty-four different resistant patterns found in this study which was highly variable. Resistance to at least one antibiotic was seen in 32.0% (16/50) of isolates in all the farms. Whereas, 40.0% (20/50) of isolates were found to be of multiple resistance. There were five resistant patterns shared by at least two or more bacteria. The variability of antibiotic resistant patterns exhibit by different bacterial species may suggest the dependent on the selective pressure exhibit in different geographical location which is in agreement with studied by Lesley et al. (2011) and Kathleen et al. (2016).

Table 4.7: Resistant patterns and MAR index of aquaculture bacteria

MAR Index	Resistant Pattern	Isolates code	Percentage of isolate (%)
0.79	NOR-AK-CN-C-PRL-CAZ-KA-S-AMP-ATM-ETP	SM 101	2.0
0.78	PRL-CN-FEP-MEM-CAZ-ATM-IMP	SM 79	2.0
0.55	C-E-CIP-AK-RD-NOR	M 21	2.0
0.36	CN-TE-P-RD AK-TE-CN- KF AK-CAZ-ATM-KF	J 200 PM 257 J 181	6.0
0.29	MEM-S-ETP-AMP KA-S-ETP-AMP	PM 205 PM 216	4.0
0.27	P-RD-E CRO- CAZ-ATM	PM 249, PM 207, J 149 M40	8.0
0.21	PRL-CAZ-ATM C-PRL-AMP	PM 246 LT 78	4.0
0.18	RD-E NOR-E P-E	J 187 LT 64 LT 52	6.0
0.14	S-AMP CAZ-AMP	J 184, J 151 LT 23	6.0
0.11	ATM	J 152	2.0
0.09	RD TE E P ATM	LT 26, LT17, PM 185, J 162, M 9, M 1, SM 142 SM 103 LT 63 SM 91 PM 183	22.0
0.07	S AMP	M 14, M 16, J 171 J 176	8.0
0	-	LT 18, LT 21, LT 40, SM 94, SM 130, SM 139, M 4, LT 54, LT 31, LT 47, M 11, LT 43, PM 199, LT 16	28.0

Nineteen *Bacillus* sp. were identified in the sediment, water and fish from the farms. Group sizes of *Bacillus* from the three-sample sources were unequal, where 10 *Bacillus* sp. were identified from the sediment, 5 isolates from water and 4 isolates from fish. At the conclusion of sediment source, nine isolates (90.0%, n = 9/10) had shown to be originate from low risk area, compared to three isolates (60.0%, n = 3/5) in water and three (75.0%, n = 3/4) from fish. Based from Fisher's exact test, there was no significant difference in proportions of *Bacillus* sp. that comes from low- or high-risk level from antibiotic contaminated areas in these three sources, P = 0.373.

CHAPTER 5

DISCUSSION

5.1 Microbial population in aquaculture farms

The bacterial counts in overall environmental samples from the fish farms ranged from 3.47 to 5.37 log CFU mL^{-1}. Pond water yielded mean bacterial counts in the ranged of 3.41 log CFU mL^{-1} to 4.83 log CFU mL^{-1}, sediment in the ranged of 3.47 to 4.82 log CFU mL^{-1} and fish samples in the ranged of 3.65 to 5.37 log CFU mL^{-1}. The results from pond samples were similar to the value recorded by Al-Harbi and Uddin (2003) in a tilapia pond culture (3.75 to 4.38 log CFU mL^{-1}). However, values recorded of bacterial counts from sediment samples and fish samples were lower from reports by Al-Harbi, and Uddin (2003), which recorded a ranged from 6.96 to 8.17 log CFU mL^{-1} and 6.53 to 7.76 log CFU mL^{-1}. The difference in bacterial counts could be due to differences in externally environment factors.

External environmental factors that could influence the bacterial counts might be from the sampling season and maintenance of the fishponds that could influence the variation in microbial growth in the study. Throughout the sampling sessions, all the samples were taken from September 2017 until January 2018, a relatively uniformly humid and wet season. Water temperature is an important positive regulator that influence the variation in bacterial growth (White et al., 1991). The range of temperatures recorded in this study was the normal ambient temperatures for a tropical climate (28.0 to 32.0 ℃). Cotner et al. (2013) stated that a bacterial growth rate decreases with increasing temperatures. Their study also stated that a bacterial growth rate in the tropical freshwater is lower when compared to bacterial growth

in the temperate one. This is due to the high temperature in the tropical countries, decreased the nutrient levels which explains a lower growth efficiency among bacteria.

Regarding the maintenance of the fishponds, farm 2 and 5 have the most well-managed aquaculture practices. It was located away from urban communities in a gated area. The aquaculture farm is located at higher ground levels near the hills, where water sources and rainwater from the hills were used for the pond maintenance. According to the aquaculture manager in farm 5, the water quality parameters such as the level of dissolved oxygen, ammonia, nitrite and hydrogen sulfide of the ponds are monitored accordingly to ensure healthy growth of cultured species. It is known that water quality parameters influence each other in a dynamic environment of the aquaculture ponds. Environmental factors, such as pH and temperature at unbalanced levels can affect ammonia and hydrogen sulphide toxicity to the cultured organisms (Wurts and Durborow, 1992).

Based on observation, Farm 1 is the only small-scale fish farms found near a residential area and wet markets. The other farms were located further away from the residential area. A permitted distance between the housing area and aquaculture ponds should be considered and implemented when operating an aquaculture farm. A study by Gilsdorf *et al*. (2008) reported that there is a positive relation to the distance travelled of a zoonotic bacteria, *Coxiella burnetiid* from an animal farm to the people living at closer proximity. Thus, the possibility of antibiotic resistant bacteria to travel to the nearby residential area via mobile genetic elements that could potentially cause a serious problem, if a disease outbreak were to take place.

5.2 Identification of aquaculture bacterial and bacterial diversity

To differentiate the bacterial isolate, $(GTG)_5$-PCR was applied to give an estimate of bacterial identification through the different banding pattern. Alongside, similar or close banding pattern were group together in a number of clusters (Figure 4.1, Figure 4.2, Figure 4.3, Figure 4.4, and Figure 4.5). This is to reduce the cost of sequencing of unknown isolates which share similar banding pattern. Only 50 isolates were selected for sequencing based on the dendrogram generated from the initial 204 unknown isolates subjected to $(GTG)_5$-PCR.

The banding pattern generated ranged from 1 to 10 of visualised PCR products. This was lower than expected compared to a study conducted by Gevers et al. (2001) which reported that the banding pattern using $(GTG)_5$-PCR ranged between 10 to 20 of visualised PCR product. It is worth noting that Gevers et al. (2001) study focused on *Lactobacillus* sp. which explains the difference in the number of banding patterns as this study focus on a broader range of unknown bacterial species. According to Braem et al. (2011), due to the differences in the number of $(GTG)_5$ elements in the genome of different bacterial species, this might influence the number of bands in the $(GTG)_5$-PCR fingerprints.

5.3 Microbial diversity in aquaculture farms in relation to antibiotic resistance

From $(GTG)_5$ fingerprinting, some bacteria from different sample type were classified under the same cluster. This might suggest that bacteria may have derived from the same sources, either from the water or sediment of the aquaculture system. Following the identification of the 50 isolates through 16S-PCR sequencing, 14 genera of bacteria were identified in this study. Microbial diversity in fish farms and in any other cultured species were diverse regardless of the size of the farm area. A large part of bacterial diversity was made up of *Bacillus* sp. (38%). Using 16S sequencing, 18 *Bacillus* species were identified

in all the farms in all of the water, sediment and fish samples which includes *Bacillus altitudinis, Bacillus aquimaris, Bacillus cereus, Bacillus pumilus, Bacillus zhangzhouensis, Bacillus megaterium, Bacillus indicus* and *Bacillus* sp. This was expected as *Bacillus* have the ability to grow rapidly, sporulate, and can tolerate a wide range of physiological condition (Hong et al., 2005). An abundance of the genus *Bacillus* was also found in other aquaculture systems (Del'Duca et al., 2015; Tam et al., 2015). In addition, *Bacillus* contains naturally occurring plasmid which encodes conjugative and mobile elements, allowing a potential risk of transferring antibiotic resistant genes to other environment and pathogenic bacteria (Rokana et al., 2017).

The genus *Exiguobacterium* is made up of 16.0% of the bacterial population that was found in water, sediment and fish samples. From this study, *Exiguobacterium* were phenotypically resistant to chloramphenicol, piperacillin, ampicillin, norfloxacin, erythromycin and penicillin. According to Yang et al. (2013), *Exiguobacterium* sp. is related phylogenetically to the *Bacillus* sp. Wiedenbeck and Cohan (2011) stated that horizontal gene transfer occurs more frequently between closely related species, which may potentially cause plasmid-encoding resistant genes to be transferred between *Exiguobacterium* and *Bacillus*.

Gram-negative *Enterobacter* sp. also accounts for 16% of the bacterial population which includes *Enterobacter cloacae*, *Enterobacter amnigenus* and *Enterobacter asburiae*. *Enterobacter* sp. exhibits phenotypically resistant to streptomycin, ampicillin, meropenem, ertapenem, kanamycin, piperacillin, ceftazidime and aztreonam. *Enterobacter* is one of the ESKAPE (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacter*), which was considered as one of the major pathogens describes to contribute to human health problems

(Rice, 2010). Resistant to meropenem and ertapenem were observed in this study, which posed as a great concern as both of these antibiotics belongs to carbapenem family which often used to treat infections that are resistant to most other antibiotics (Qiao et al., 2018).

A small percentage of bacterial species in the study area were made up of *Acinetobacter* (4%, *n* = 2), *Citrobacter* (4%, *n* = 2), and *Staphylococcus* (4%, *n* = 2). Bacteria from these three genera are ubiquitous in nature and are found in soil, water and aquatic environments (Cao et al., 2018; Saito et al., 2010; Sun et al., 2018). *Acinetobacter*, *Citrobacter* and *Staphylococcus* are the genera of bacteria that are notably responsible to cause bacterial disease to fish and humans. For example, an endotoxin that is produced from *Staphylococcus aureus* has been identified as a causative agent of Staphylococcal food poisoning (Saito et al., 2010). In this study, *Staphylococcus* were resistant to tetracycline. A similar study has reported that *Staphylococcus* found in shrimps were resistant to tetracyclines, as well as to aminoglycosides, β-lactams (Boss et al., 2016).

Whereas, there have been reported of an infection caused by *Citrobacter* species; for example, tilapia, rainbow trout and angelfish infected with *Citrobacter freundii*, leading to high mortality (Gallani et al., 2016; Jeremic et al., 2003; Thanigaivel et al., 2015). Two species of *Citrobacter* sp. identified in this study were resistant toward ampicillin and streptomycin respectively with low risk of contamination (< 0.2 MAR index). *Citrobacter* is an opportunistic pathogen and if there is an increase in the resistance prevalence were to happen, this could lead to serious economic losses for fish farmers.

Acinetobacter isolated in this study were completely susceptible to all the tested antibiotics. However, this does not mean *Acinetobacter* cannot harbour resistance genes from other bacteria or developed its resistance over time. A study by Petersen et al. (2002)

on antibiotic resistant *Acinetobacter* in an integrated farm, discovered that there was a significant increase in antibiotic resistant *Acinetobacter* towards oxytetracycline and sulfamethoxazole increasing from between 1% and 5% to 100% within the period of just mere 2 months. Thus, there is a possibility of *Acinetobacter* to develop resistance to antibiotics in the future. In addition, the genus *Acinetobacter* is considered an opportunistic pathogen and often associated with the nosocomial outbreak in clinical settings (Cao et al., 2018), and if *Acinetobacter* were to be resistant, this could evidently cause human health problems.

The bacterial diversity of aquaculture farms in this study accounts to only a small percentage of cultivable bacteria as to the actual bacterial community of an aquatic ecosystem. Nevertheless, MAR bacteria were seen in most of the bacterial genera across the farms with presumably no history of antibiotics usage. Thus, it can be speculated that the antibiotic resistance genes can be shared among aquatic bacteria in freshwater flocs, as a reservoir of antibiotic resistance (Drudge et al., 2012).

5.4 Antibiotic resistant development in relation to antibiotics tested

As previously shown in Table 4.5, a high percentage of resistance to streptomycin (75%; 9/12) and ampicillin (66.7%; 8/12) were observed among the environmental bacteria. This observation is consistent with Chelossi et al. (2003) study, which also recorded a high resistance towards streptomycin and ampicillin on benthic bacterial community in marine aquaculture. Whereas, the prevalence of streptomycin resistance was found in numerous fish and shrimp farms (Dung et al., 2008; Kian et al., 2012; Shah et al., 2014). High incident of ampicillin resistance were also found in *Vibrio* sp. in the tropical water (You et al., 2016), and in *Enterococci* in the recreational water in Malaysia (Dada et al., 2013). In another study conducted by Letchumanan et al. (2015), 82% of *Vibrio parahaemolyticus* in retail shrimps

in Malaysia displayed a high resistant to ampicillin. This suggest that high incident of streptomycin and ampicillin resistance were not restricted to a particular water body, but they are indeed widely distributed in an environment.

A high incident of susceptibility was recorded towards doxycycline, levofloxacin, trimethoprim-sulfamethoxazole and nalidixic acid. From this findings, a high susceptibility towards doxycycline was observed might suggest that doxycycline was not commonly used among fish farmers in a South East Asian country (Pham et al., 2015). Shaban et al. (2014) further reported that doxycycline was only commonly used on domestic animals. Similarly, high susceptibility of levofloxacin was observed in *Vibrio parahaemolyticus* isolated from retail shrimps by Letchumanan et al. (2015) and Saifedden et al. (2016). High susceptibility towards trimethoprim-sulfamethoxazole was also seen in *Aeromonas hydrophila* in catfish aquaculture (Paola et al., 1995) and bacteria isolated from sea bass (Bourouni et al., 2000). *E. coli* isolated from tilapia species in Brazil (Rocha et al., 2014) and *Vibrio* species isolated from aquaculture water in Sabah (Ransangan et al., 2013) also revealed a high sensitivity towards nalidixic acid. However, in a short period of time, these originally susceptible bacteria may become resistant through acquiring resistant genes via horizontal gene transfer or gene mutations (Allen et al., 2010).

Based on the results, MAR bacteria are resistance to a variety of antibiotic, which might be an offshoot of diverse genes that protects them against the therapeutic dose of antibiotics. These genes which is also known as the resistome have the potential to transferred to pathogens and there have been evidence that some clinically relevant resistance genes originated from environmental bacteria (Cattoir et al., 2008). Therefore, bacteria with antibiotic resistance genes get selective advantage over their antibiotic-sensitive bacteria in presence of antibiotics, and evidently creates a plethora of resistance

patterns exhibited by the aquatic bacteria. The fact that ARB were seen in all five fish farms where no known antibiotics were used further confirms that resistance genes exist naturally in the environment (Allen et al., 2010).

5.5 MAR patterns assessment

Only 25%, 14 out of 50 isolates identified might originate from high-risk sources, with MAR index values of more than 0.2. Interestingly, all five farms suggestively carry ARB from this higher than normal risk, where no known antibiotics were used, which require careful sifting. The resistant patterns of the identified isolates had been previously depicted in Table 4.7.

In farm 1, two isolates, M21 and M40 which have resistant patterns of "C-E-CIP-AK-RD-NOR" and "CRO-CAZ-ATM" respectively. These resistant patterns combined formed under 7 antibiotic classes, which includes carbapenem, cephalosporins, monobactam, aminoglycosides, phenicol, fluoroquinolone and rifampin. The first 3 antibiotic classes mentioned shared similar mechanism of affecting the bacterial cell wall synthesis by blocking the cross-linking enzymes in the peptidoglycan layer of the cell walls (Sultan et al., 2018). Whereas, aminoglycosides and phenicol antibiotics were known to bind to the bacterial ribosome and interfere with the protein synthesis. Given the diverse mechanism of bacterial resistance to aminoglycosides, the common mechanism of antibiotic resistance is through the inactivation of aminoglycosides by aminoglycoside-modifying enzymes (Garneau-Tsodikova and Labby, 2016). As for phenicol, the presence of chloramphenicol acetyltransferases (CAT) enzymes inactivates the phenicol antibiotics (Roberts and Schwarz, 2016). As for fluoroquinolone and rifampin antibiotics, it interferes with the bacterial DNA replication and inhibits the bacterial RNA polymerase synthesis respectively. Bacteria developed resistance to these interferences and inhibitions through the

mutation at the specific domain of their topoisomerase enzymes and DNA gyrase enzymes (Hooper and Jacoby, 2015). These enzymes which are coded and embedded in the bacterial DNA are highly transferable and their genes can be transfer to various bacteria via genetic mobile elements (Garneau-Tsodikova and Labby, 2016). This might explain the considerably high number of resistance patterns that is up to 7 antibiotic classes out of 11 classes tested.

In farm 2, only 1 isolate (LT 78) has an MAR index of 0.21 with resistant pattern of "C-PRL-AMP". This resistance formed under two antibiotic classes; phenicol and penem. Phenicol relies on the ability to inhibits protein synthesis. Whereas, penem inhibits the bacterial cell wall synthesis causing cell lysis (Munita and Arias, 2016). These bacteria in turn might developed resistance through overproduction of HSV and production of CAT enzymes as a mechanism for piperacillin and penem resistance respectively (Roberts, 2016; Han et al., 2019). Whereas, Farm 3 consist of two isolates (SM 101 and SM 79) with the highest MAR index among the isolated bacteria with a MAR index value of 0.79 and 0.78. These two isolates which were resistant to 11 and 7 antibiotics, that shared 4 similar resistant patterns which were "CN-PRL-CAZ-ATM". These four antibiotics were consisted of 4 different antibiotic classes; aminoglycosides (inhibit bacterial protein synthesis), cephalosporin, monobactam, and penem. The last 3 antibiotics mentioned have a similar ability to interfere with the bacterial cell wall synthesis (Mayers, 2009).

Two isolates, J 181 and J 200 found in farm 4 shared similar index value of 0.36, with very different resistant patterns which were "AK-CAZ-ATM-KF" and "CN-TE-P-RD". Together, these two patterns formed under 6 antibiotic classes; aminoglycoside, penem, rifampin, cephalosporins, monobactam and tetracycline. Bacteria shield themselves from these antibiotics through various mechanism. An example is through plasmids that carry

genes for resistance to many other antibiotics. Some genes code for enzymes that modify the agents, others alter the antibiotic targets in the cell or provide alternate biosynthetic pathways (Mayers, 2009).

Finally farm 5, consisted of 6 isolates with MAR index value of more than 0.2, whereby 1 isolate with a value of 0.21, 2 isolates with a value of 0.27, 2 isolates with a value of 0.29 and 1 isolate with a value of 0.36. Note that farm 4 and farm 5 were located at a close proximity as compared to other farms as illustrated in Figure 4.7. Interestingly, the resistant patterns found in isolates of farm 4 were also found scattered in 5 isolates in farm 5, namely, PM 257, PM 249, PM 207, J 149 and PM 246. Whereby, PM 257 resistant pattern of "AK-TE-CN-KF" shared similar resistant pattern to isolate J 181 "-TE-CN" and J 200 "-AK-KF". For isolates PM 249 and PM 207 with resistant pattern of "P-RD-E' shared similarity to isolate J 200 resistant pattern of "-P-RD". Also, isolate PM 246 with resistant pattern "PRL-CAZ-ATM' has shown similarity pattern with J 181 "-CAZ-ATM". This similar resistance patterns found in farm 4 and farm 5 suggested that MAR bacteria might had travelled between these farms via genetic mobile elements through physical factors such as wind and watershed that could accelerate the movement of ARB (Allen et al., 2010).

Overall, antibiotic resistance can be transferred between pathogenic and environmental bacteria and has been seen in numerous aquaculture settings (Ghosh and Mandal, 2010; Shah et al., 2012; Boonyasiri et al., 2014; Corno et al., 2014; Lim and Yong, 2014; Neela et al., 2015; Huang et al., 2017). The dissemination of antibiotic resistance seen in all 5 farms is cause for concern for fish farmers and public health. Thus, a careful examination on the prudent use antibiotics through consultation from qualified veterinarians and better management of hygiene and sanitation could lead to a better-quality fish production (Muhammad et al., 2010).

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Based on the summated data, two hundred and four isolates were subjected to $(GTG)_5$-PCR to differentiate the banding patterns amongst the unknown isolates. Fifty isolates were then selected to be identified using 16S sequencing, consisting of bacteria of 14 genera. A high percentage of environmental bacteria (74.0%, *n* = 37) have MAR index less than 0.2, suggesting most isolates originate from low antibiotic contaminated areas. The overall results indicated that MAR index ranged from 0 to 0.79. Among the five farms, farm 5 recorded the highest percentage (66.7%, *n* = 6/9) of bacteria isolated from high antibiotic contaminated sources, (MAR > 0.2).

A high percentage of resistance were seen towards streptomycin (75%; 9/12) and ampicillin (66.7%; 8/12) based on the AST data. Whereas, a high incident of susceptibility was seen towards doxycycline, levofloxacin, trimethoprim-sulfamethoxazole and nalidixic acid. In this study, 40.0% (20/50) of isolates were found to be of multiple resistance, and 32.0% (16/50) of isolates were resistant to at least one antibiotic. A multiple resistant seen in 40.0% of bacteria should still be of a concern, as no known antibiotics were used in these farms. Besides antibiotic resistance was widespread among environmental bacteria which often have large genomes that resides resistance determinant that could be transfer to other pathogenic bacteria. However, the answer to whether the antibiotic resistance patterns are acquired through gene transfer, physical force or occur intrinsically is still not clear. In conclusion, there is a need for public authorities to continuously ensure a systematic management of antibiotics in an aquaculture system to deter the effect of antibiotic

resistance. Implementation of routine screening of the presence of ARB in aquaculture could contribute to a better understanding of the role of aquaculture environment and seafood in the transmission of MAR among human pathogens.

6.2 Recommendations

It is worth noting that there are rooms for improvement in the workflow of this study if it were to be continued. Such as using an alternative method in the identification of a larger number of bacteria. Secondly, identification of bacterial isolates based on the spectral patterns using Matrix-Assisted Laser Desorption Ionization with Time-of-flight Mass Spectrometry (MALDI-TOF-MS) provide a rapid alternative method to the standard 16S sequencing. According to a recent study, MALDI-TOF-MS can also combine its functionality with AST through software based quantitative evaluation with reliable algorithm (Idelevich et al., 2017). Ultimately, this approach would provide a rapid differentiation between resistant and susceptible isolates.

Sampling period can be taken before and after the feeding time of the fish, as fishmeal could have unknown antibiotics alongside the nutrient needed by the fish. A recent study by Han et al. (2017) discovered that fishmeal contains traces of antibiotics and carried resistant genes and in consequence antibiotics in a very low level find their way both to the soil and aqueous environment. This could help explain the samples from different sources (water, sediment, fish) could harbour ARB even though no antibiotics were uploaded to be administered in the aquaculture field. Also, it would help gives a clearer picture of risk asessment overview of the prevelance of ARB in aquaculture.

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APPENDICES

Appendix A: Breakpoint values (Susceptible, Intermediate and Resistant) for different bacterial group tested

Bacterial group tested	References	Antibiotic agent	Zone diameter to the nearest whole (mm)		
			Susceptible	Intermediate	Resistant
Citrobacter sp. *Chitinophaga* sp. *Enterobacter* sp.	*Enterobacteriaceae* (CLSI, 2017)	Ceftazidime (CAZ)	≥ 21	18-20	≤ 17
		Kanamycin (KA)	≥ 18	14 -17	≤ 13
		Chloramphenicol (C)	≥ 18	13 - 17	≤ 12
		Levofloxacin (LEV)	≥ 21	17 - 20	≤ 16
		Norfloxacin (NOR)	≥ 17	13 - 16	≤ 12
		Amikacin (AK)	≥ 17	13 - 16	≤ 12
		Nalidixic Acid (NA)	≥ 19	14 - 18	≤ 13
		Piperacillin (PRL)	≥ 21	16 - 20	≤ 17
		Streptomycin (S)	≥ 15	11 -14	≤ 11
		Ampicillin (AMP)	≥ 17	14 -16	≤ 13
		Gentamicin (CN)	≥ 15	13 -14	≤ 13
		Meropenem (MEM)	≥ 23	20 - 22	≤ 19
		Aztreonam (ATM)	≥ 21	18 - 20	≤ 17
		Ertapenem (ETP)	≥ 22	19 - 21	≤ 18
Staphylococcus sp. *Exiguobacterium* sp. *Bacillus* sp.	*Staphylococcus* sp. *(CLSI, 2017)*	Amikacin (AK)	≥ 17	15 - 16	≤ 14
		Tetracycline (TE)	≥ 19	15 - 18	≤ 14
		Ciprofloxacin (CIP)	≥ 21	16 - 20	≤ 15
		Doxycycline (DO)	≥ 16	13 - 15	≤ 12
		Gentamicin (CN)	≥ 15	13 - 14	≤ 12
		Erythromycin (E)	≥ 23	14 - 22	≤ 13
		Penicillin (P)	≥ 29	-	≤ 28
		Chloramphenicol (C)	≥ 18	13 - 17	≤ 12
		Levofloxacin (LEV)	≥ 19	16 - 18	≤ 15
		Rifampin (RD)	≥ 20	17 - 19	≤ 16

Breakpoint values (Susceptible, Intermediate and Resistant) for different bacterial group tested

		Norfloxacin (NOR)	≥ 17	13 - 16	≤ 12
Pseudomonas sp.	*Pseudomonas aeruginosa (CLSI, 2017)*	Aztreonam (ATM)	≥ 22	16 - 21	≤ 15
Pseudoxanthomonas sp.		Ciprofloxacin (CIP)	≥ 25	19 - 24	≤ 18
Achromobacter sp.		Ceftazidime (CEF)	≥ 18	15 - 17	≤ 14
		Cefepime (FEP)	≥ 18	15 - 17	≤ 14
		Gentamicin (CN)	≥ 15	13 -14	≤ 12
		Levofloxacin (LEV)	≥ 22	15 - 21	≤ 14
		Meropenem (MEM)	≥ 19	16 - 18	≤ 15
		Piperacillin (PRL)	≥ 21	15 - 20	≤ 14
		Imipenem (IMP)	≥ 19	16 - 18	≤ 15
Stenotrophomonas sp.	*Stenotrophomonas (CLSI, 2017)*	Levofloxacin (LEV)	≥ 17	14 - 16	≤ 13
		Trimethoprim-sulfamethoxazole (SXT)	≥ 16	11-15	≤ 10
Acinetobacter sp.	*Acinetobacter sp. (CLSI, 2017)*	Doxycycline (DO)	≥ 13	10 - 12	≤ 9
		Gentamicin (CN)	≥ 15	13 - 14	≤ 12
		Piperacillin (PRL)	≥ 21	18 - 20	≤ 17
		Ciprofloxacin (CIP)	≥ 21	16 - 20	≤ 15
		Tetracycline (TE)	≥ 15	12 -14	≤ 11
		Trimethoprim-Sulfamethoxazole	≥ 16	11-15	≤ 10
		Amikacin (AK)	≥ 17	15 - 16	≤ 14
		Cefepime (FEP)	≥ 18	15 - 17	≤ 14
		Meropenem (MEM)	≥ 18	15 - 17	≤ 14
		Levofloxacin (LEV)	≥ 17	14 - 16	≤ 13
Aeromonas sp.	*Aeromonas (CLSI, 2017)*	Meropenem (MEM)	≥ 16	14 - 15	≤ 13
Plesiomonas sp.		Tetracycline (TE)	≥ 19	15 - 18	≤ 14
		Chloramphenicol (C)	≥ 18	13 - 17	≤ 12

Breakpoint values (Susceptible, Intermediate and Resistant) for different bacterial group tested

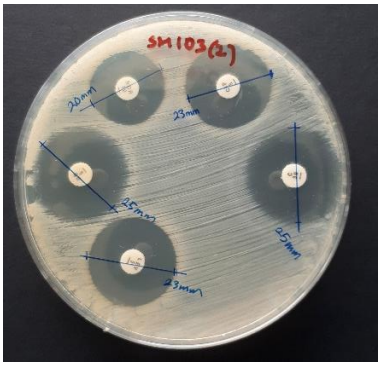
	Cephalothin (KF)	≥ 18	15 - 17	≤ 14
	Amikacin (AK)	≥ 17	15 - 16	≤ 14
	Gentamicin (CN)	≥ 15	13 - 14	≤ 12
	Aztreonam (ATM)	≥ 22	16 - 21	≤ 15
	Ciprofloxacin (CIP)	≥ 21	16 - 20	≤ 15
	Ceftazidime (CAZ)	≥ 18	15 - 17	≤ 14
	Ceftriaxone (CRO)	≥ 21	14 - 20	≤ 13
	Imipenem (IPM)	≥ 16	14 - 15	≤ 13

Appendix B: Zone of Inhibition of Antibiotic Susceptibility Test

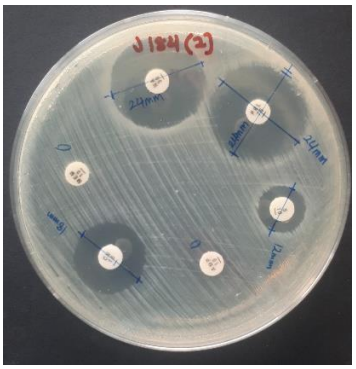
a)

b)

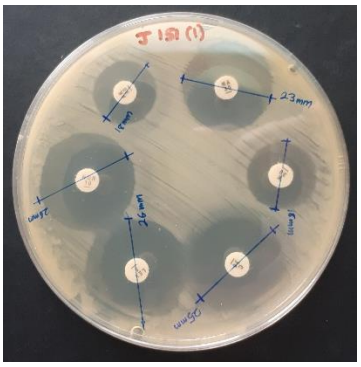
c)



d)



e)



f)

The antibiotic susceptibility test on aquatic bacteria in various farms isolated from aquaculture farms. a) *Bacillus pumilus*; b) *Enterobacter sp*.; c) *Bacillus pumilus*; d) *Bacillus cereus*.; e) *Enterobacter cloacae*; f) *Enterobacter amnigenu*

Appendix C: Antibiotic Susceptibility Profile of bacterial Isolates from aquaculture fish farms

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Acinetobacter* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibility Profile										Reference
		DO	CN	PRL	CIP	TE	SXT	AK	FEP	MEM	LEV	
M 10	*Acinetobacter junii*	29	25	29	35	27	29	23	28	32	31	*Acinetobacter* sp. (CLSI 2017)
		S	S	S	S	S	S	S	S	S	S	
LT 43	*Acinetobacter* sp.	32	25	22	28	27	29	25	31	32	30	
		S	S	S	S	S	S	S	S	S	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Pseudomonas Aeruginosa* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibility Profile									References
		ATM	CIP	CAZ	FEP	CN	LEV	MEM	PRL	IMP	
SM 79	*Pseudoxanthomonas mexicana*	8	28	9	10	7	35	7	18	7	*Pseudomonas Aeruginosa* (CLSI, 2017)
		R	S	R	R	R	S	R	I	R	
J 152	*Achromobacter* sp.	0	30	30	28	20	27	33	40	27	
		R	S	S	S	S	S	S	S	S	
PM 199	*Pseudomonas* sp.	30	44	25	35	24	40	41	36	40	
		S	S	S	S	S	S	S	S	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Staphylococcus* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibility Profile											Reference
		AK	TE	DO	CN	P	C	LEV	RD	NOR	E	CIP	
PM 249	*Bacillus* sp.	19	24	20	15	0	24	24	6	29	10	31	*Staphylococcus* sp. (CLSI, 2017)
		S	S	S	S	R	S	S	R	S	R	S	
LT 26	*Bacillus cereus*	27	38	36	23	36	34	34	19	26	28	28	
		S	S	S	S	S	S	S	I	S	S	S	
LT 17	*Bacillus pumilus*	20	32	32	16	33	20	20	17	30	30	33	
		S	S	S	S	S	S	S	I	S	S	S	
J 200	*Bacillus cereus*	18	8	26	14	7	22	22	12	22	24	24	
		S	R	S	I	R	S	S	R	S	S	S	
J 187	*Bacillus* sp.	24	33	34	19	29	19	19	18	29	22	33	
		S	S	S	S	S	S	S	I	S	I	S	
LT 18	*Bacillus pumilus*	20	31	34	17	33	21	21	20	31	26	33	
		S	S	S	S	S	S	S	S	S	S	S	
PM 207	*Bacillus* sp.	20	28	30	19	21	28	28	19	23	22	26	
		S	S	S	S	R	S	S	I	S	I	S	
LT 21	*Bacillus aquimaris*	24	31	38	24	35	34	38	22	28	23	33	
		S	S	S	S	S	S	S	S	S	S	S	
LT 40	*Exiguobacterum profundum*	22	32	33	22	36	28	29	28	27	26	27	
		S	S	S	S	S	S	S	S	S	S	S	
PM 185	*Bacillus indicus*	28	38	40	27	35	21	33	19	28	23	33	
		S	S	S	S	S	S	S	I	S	S	S	
SM 103	*Staphylococcus* sp.	21	18	23	22	30	25	23	25	20	24	23	
		S	I	S	S	S	S	S	S	S	S	S	
SM 94	*Bacillus zhangzhouensis*	25	34	36	25	32	23	32	22	27	30	30	
		S	S	S	S	S	S	S	S	S	S	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Staphylococcus* sp. CLSI (continue)

LT 63	*Exiguobacterium profundum*	24	29	28	20	40	25	28	37	24	11	28	*Staphylococcus* sp. (CLSI, 2017)
		S	S	S	S	S	S	S	S	S	R	S	
SM 91	*Bacillus cereus*	19	21	24	18	9	20	27	13	24	23	27	
		S	S	S	S	R	S	S	S	S	S	S	
SM 130	*Bacillus pumilus*	24	34	34	16	34	20	30	20	25	27	29	
		S	S	S	S	S	S	S	S	S	S	S	
SM 139	*Staphylococcus haemolyticus*	32	33	39	29	29	33	36	41	33	32	35	
		S	S	S	S	S	S	S	S	S	S	S	
J 162	*Bacillus* sp.	26	34	34	25	30	30	28	18	23	28	28	
		S	S	S	S	S	S	S	I	S	S	S	
J 149	*Bacillus megaterium*	17	27	23	16	0	26	23	9	23	9	26	
		S	S	S	S	R	S	S	R	S	R	S	
M 4	*Bacillus* sp.	23	31	31	20	33	22	32	20	24	28	28	
		S	S	S	S	S	S	S	S	S	S	S	
M 9	*Bacillus* sp.	23	32	35	18	34	21	34	18	29	24	32	
		S	S	S	S	S	S	S	I	S	S	S	
M 1	*Bacillus altitudinis*	28	33	32	25	34	22	34	18	30	24	34	
		S	S	S	S	S	S	S	I	S	S	S	
SM 142	*Bacillus pumilus*	26	33	32	25	32	23	27	18	21	25	25	
		S	S	S	S	S	S	S	I	S	S	S	
LT 54	*Exiguobacterium profundum*	21	30	31	20	36	25	26	30	24	25	26	
		S	S	S	S	S	S	S	S	S	S	S	
M 21	*Fictibacillus macauensis*	7	19	28	17	33	16	26	8	9	17	20	
		R	S	S	S	S	I	S	R	R	I	I	
LT 52	*Exiguobacterium* sp.	22	27	31	23	15	25	29	30	26	10	26	
		S	S	S	S	R	S	S	S	S	R	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Staphylococcus* sp. CLSI (continue)

LT 31	*Bacillus* sp.	26	26	35	25	31	31	35	20	31	30	35	*Staphylococcus* sp. (CLSI, 2017)
		S	S	S	S	S	S	S	S	S	S	S	
LT 47	*Exiguobacterium* sp.	31	39	41	30	44	33	33	21	30	30	35	
		S	S	S	S	S	S	S	S	S	S	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Enterobacteriaceae* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibility Profile														Reference
		CAZ	KA	C	LEV	NOR	AK	NA	PRL	S	AMP	CN	MEM	ATM	ETP	
M 14	*Enterobacter asburiae*	28	20	25	28	27	19	25	29	14	20	17	31	32	31	*Enterobacteriaceae* (CLSI, 2017)
		S	S	S	S	S	S	S	S	I	S	S	S	S	S	
M 16	*Enterobacter asburiae*	28	18	24	27	27	18	23	27	13	18	16	29	30	30	
		S	S	S	S	S	S	S	S	I	S	S	S	S	S	
J 171	*Citrobacter* sp.	36	21	28	34	34	21	29	26	0	18	20	36	40	36	
		S	S	S	S	S	S	S	S	R	S	S	S	S	S	
J 184	*Enterobacter cloacae*	28	18	24	28	27	18	24	25	12	0	18	32	38	35	
		S	S	S	S	S	S	S	S	I	R	S	S	S	S	
PM 205	*Enterobacter* sp.	30	19	29	25	24	19	20	25	13	0	17	20	23	21	
		S	S	S	S	S	S	S	S	I	R	S	I	S	I	
LT 23	*Enterobacter* sp.	20	36	38	38	32	33	34	28	25	11	33	29	28	30	
		I	S	S	S	S	S	S	S	S	R	S	S	S	S	
J 176	*Citrobacter* sp.	38	20	28	34	32	21	30	24	16	10	18	36	40	34	
		S	S	S	S	S	S	S	S	S	R	S	S	S	S	
SM 101	*Chitinophaga* sp.	0	0	10	25	16	0	36	11	0	0	0	42	0	15	
		R	R	R	S	I	R	S	R	R	R	R	S	R	R	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Enterobacteriaceae* sp. CLSI (continue)

J 151	*Enterobacter amnigenus*	27	18	25	26	26	18	23	27	14	9	17	36	32	29	*Enterobacteriaceae* (CLSI, 2017)
		S	S	S	S	S	S	S	S	I	R	S	S	S	S	
PM 246	*Enterobacter* sp.	14	30	34	38	32	30	36	19	19	19	27	31	0	23	
		R	S	S	S	S	S	S	I	S	S	S	S	R	S	
PM 216	*Enterobacter* sp.	24	17	22	22	23	18	19	24	13	0	17	23	23	19	
		S	I	S	S	S	S	S	S	I	R	S	S	S	I	
LT 78	*Enterobacter* sp.	23	20	6	26	29	19	23	25	11	0	16	32	31	30	
		S	S	R	S	S	S	S	S	R	R	S	S	S	S	

Antibiotic Susceptibilities Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Stenotrophomonas* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibilities Profile		Reference
		LEV	SXT	
LT 16	Stenotrophomonas sp.	25	18	*Stenotrophomonas maltophilia* (CLSI, 2017)
		S	S	

Antibiotic Susceptibility Profile of aquaculture bacteria (measured to the nearest millimetre) based on *Aeromonas* sp. CLSI

Bacterial strains	Bacterial species	Antibiotic Susceptibility Profile											Reference
		MEM	TE	C	KF	AK	CN	ATM	CIP	CAZ	CRO	IPM	
M40	*Aeromonas veronii*	34	39	34	19	31	26	0	37	14	14	38	*Aeromonas* (CLSI, 2017)
		S	S	S	S	S	S	R	S	R	I	S	
PM 257	*Plesiomonas shigelloides*	33	18	40	14	15	13	40	32	34	34	31	
		S	I	S	R	I	I	S	S	S	S	S	
PM 183	*Aeromonas veronii*	40	26	29	31	29	27	9	35	31	23	47	
		S	S	S	S	S	S	R	S	S	S	S	
J 181	*Aeromonas jandaei*	20	34	42	27	0	26	0	35	0	9	23	
		S	S	S	S	R	S	R	S	R	R	S	

Note: Symbol “S”: Susceptible, “I”: Intermediately Resistant, “R”: Resistant.