UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF COMMON BACTERIA IN ASIAN SEABASS Lates calcarifer (BLOCH, 1790) CULTURED IN FLOATING NET-CAGES AT PULAU KETAM, SELANGOR, MALAYSIA.

ATIQAH BINTI ZULHISAM

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ATIQAH BINTI ZULHISAM

A project paper submitted to the

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CERTIFICATION

It is hereby certified that we have read this project paper entitled "Determination of Common Bacteria in Asian Seabass *Lates calcarifer* (Bloch, 1790) Cultured in Floating Net-Cages at Pulau Ketam, Selangor, Malaysia.", by Atiqah Binti Zulhisam and in our opinion it is satisfactory in terms of scope, quality, and presentation as partial fulfilment of the requirement for the course VPD4999 – Final Year Project.

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DEDICATIONS

This project paper is dedicated to

Allah S.W.T.

My Beloved Family

My Teachers and Lecturers

My Friends

All the Animals on Earth

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Besides that, I would also like to thank my two co-supervisors, Dr. Norhariani Binti Mohd Nor and Dr. Nur Indah Binti Ahmad for your ideas, enlightenment, knowledge and support during this whole project. The feedback that I gathered from both of you had really pushed me to move forward and to improve my way of tackling issues that I faced during this research project.

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LIST OF ABBREVIATIONS

AUP	Animal Utilisation Protocol
IACUC	Institutional Animal Care and Use Committee
TSA	Trypticase Soy Agar
TCBS	Thiosulfate-citrate-bile salts-sucrose
API	Analytical Profile Index
TBL	Total Body Length
SBL	Standard Body Length
WHO	World Health Organisation
CFU	Colony Forming Unit
APC	Aerobic Plate Count
TPC	Total Plate Count
SPC	Standard Plate Count
TFTC	Too Few To Count
TNTC	Too Numerous To Count
n.d.	No date

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4999-Projek

PENENTUAN BAKTERIA LAZIM DALAM IKAN SIAKAP *Lates calcarifer* (BLOCH, 1790) YANG DITERNAK DI DALAM SANGKAR JARING TERAPUNG DI PULAU KETAM, SELANGOR, MALAYSIA.

Oleh

ATIQAH BINTI ZULHISAM

2021

Penyelia Utama: Prof. Madya Dr. Hassan Hj. Mohd Daud

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Dr. Nur Indah Ahmad

Penternakan akuakultur secara intensif yang kini semakin giat dijalankan merupakan satu faktor tercetusnya kenaikan kadar penyakit yang menyumbang kepada penurunan hasil akuakultur dan peningkatan kos pengeluaran. Kajian ini dijalankan bagi mengenal pasti bakteria lazim di dalam ikan siakap yang diternak dalam sangkar terapung di Pulau Ketam, Selangor, Malaysia. Sampel daripada hepar, ginjal, usus dan sebarang lesi luka diambil daripada 10 ekor ikan siakap. Inokulum daripada semua sampel dicoretkan atas Thiosulfate-citrate-bile salt- sucrose agar (TCBS) dan Trypticase Soy Agar marin (TSA yang ditambah dengan 1% natrium klorida) dan dieramkan pada suhu 25-30°C selama 24 jam secara aerobik. Parameter kualiti air diambil di bahagian depan dan belakang sangkar,

mengikut aliran air. Koloni tulen yang diperoleh daripada agar-agar diwarnakan dengan pewarnaan Grams dan morfologi sel dihuraikan berdasarkan pemeriksaan mikroskopik. Identifikasi bakteria dijalankan menggunakan beberapa ujian biokimia seperti oksidase, katalase, triple sugar iron, ujian pergerakan dan API20E untuk mengenal pasti genus dan spesis bakteria. Seterusnya, kuantifikasi bebanan bakteria dilakukan dengan pengiraan jumlah plat melalui kaedah penyebaran atas plat di mana beberapa siri pencairan dicoretkan ke atas TSA marin. Keputusan menunjukkan *Vibrio alginolyticus* mempunyai peratusan prevalen yang tertinggi iaitu 41.38%, sementara *Stenotrophomonas maltophilia* adalah 34.48%, disusuli oleh *Photobacterium damselae* (20.69%) dan *Shewanella putrefaciens* (6.9%). Jumlah pembentukan unit koloni (CFU) yang dikira daripada ginjal ikan siakap mempunyai julat daripada 2.9 x 103/ml hingga 1.9 x 104/ml pada siri pencairan 2-5, hepar 7.4 x 103/ml pada siri pencairan 2-4 dan usus 4.7 x 104/ml pada siri pencairan 2-5. Berdasarkan keputusan yang diperolehi, pelbagai cadangan perihal cara untuk menguruskan penyakit berjangkit yang disebabkan oleh bakteria patogenik dan oportunistik diutarakan.

Kata kunci: Sangkar jaring, Lates calcarifer, Pulau Ketam, bakteria marin, kuantifikasi

ABSTRACT

An abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4999 – Project.

DETERMINATION OF COMMON BACTERIA IN ASIAN SEABASS *Lates calcarifer* (BLOCH, 1790) CULTURED IN FLOATING NET-CAGES AT PULAU KETAM, SELANGOR, MALAYSIA.

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Intensive aquaculture farming was one of the reasons for the rising number of disease outbreaks which could contribute to the decline in aquaculture production and increases the production cost. This research was carried out to determine the common species of the marine aquatic bacteria in Asian seabass reared in floating net-cages located at Pulau Ketam, Selangor, Malaysia. Samples of liver, kidney, intestines and from abnormal lesions were taken from ten Asian seabass. The inoculum from each sample was then streaked on the thiosulfate-citrate-bile salts-sucrose agar (TCBS) and marine trypticase soy agar (TSA added with 1% sodium chloride) and incubated at 25-30°C for 24 hours, aerobically. Water quality parameters were taken in the cages at the forward and the astern side of the farm, according to the water flow. Pure colonies obtained were stained with Gram's stain and based on the microscopic examination, the cell morphologies were described. Identification of bacteria was conducted by using several biochemical tests such as oxidase, catalase, triple sugar iron, motility test, and API 20E test kit was used to confirm the bacteria genus and species. Then, the quantification of the bacterial loads was done with total plate count by using the spread plate method where a series of dilutions were

plated on marine TSA. The results showed that *Vibrio alginolyticus* has the highest prevalence of 41.38%, while *Stenotrophomonas maltophilia* was 34.48%, followed by 20.69% of *Photobacterium damselae* and 6.9% of *Shewanella putrefaciens*. The total colony-forming unit (CFU) calculated from the Asian seabass kidney ranged from 2.9 x 10^3/ml to 1.9 x 10^4/ml in 2^{-5} dilution, in liver 7.4 x 10^3/ml in 2^{-4} dilution and in intestines 4.7 x 10^4/ml in 2^{-5} dilution. Based on the result findings, various recommendations on how to manage infectious disease outbreaks by pathogenic and opportunistic bacteria were suggested.

Keywords: Net-cage, *Lates calcarifer*, Pulau Ketam, marine bacteria, quantification

1.0 INTRODUCTION

Lates calcarifer (Bloch, 1790) is a euryhaline and catadromous fish species from the Centropomidae family that populates such as freshwater, brackish and marine. The common name for this species varies from sea bass, barramundi to giant sea perch according to the region. Asian seabass were first reared in floating net-cages in Malaysia back in the 1980's and was commercially reared in the middle of 1990's (Ali et al. 1996). Over the year, Asian seabass had become one of the most sought-after cultured marine species due to its fine-flavoured flesh leading to high domestic demand for the species which contributes to the economical rise in the aquaculture industry. Most farmers prefer to culture this fish species in floating net cages compared to ponds due to the dynamic structure of the cage and better performance and production.

Both male and female seabass have similar physical appearance and colours. In addition, the anatomical features of the juvenile and adult Asian seabass comprises of fusiform lateral body shape with packed cross section of the muscle arrangement. The dorsal head profile is concavely shaped with a superior mouth structure. The body is largely covered with ctenoid scales and the caudal fin is rounded (Department of Fisheries Sabah, n.d.). The salinity and depth range are between 30–32 ppt and 10–15m, respectively. Sexually mature fish which can be found in the river mouths, lakes or lagoons (Mathew, G. 2009). The marketed weight of this species can range from 350 g to 3 kg within six to two years while the length is between 250 mm to 600 mm.

Although Malaysia aquaculture industry had thrived for the past few decades but the total aquaculture production had decreased more than 150,000 metric tonnes (MT) from 373,350 MT in 2010 to 217,894 in 2018. Moreover, the marine aquaculture production also faced the

same reduction in numbers as in 2010, the production is 117, 723 MT compared to 55, 506 MT in 2018 (FAO, n.d.). Diseases outbreak, climate changes, poor management, increase in production cost, decrease in fish stock and market demand are some of the major reasons that lead to decline in aquaculture production in Malaysia (Fathi et al. 2018). The problem statement for the current research is that there is a lack of study on the bacterial diseases in marine food fishes which strike concern as it leads to negative impact on aquaculture production and fish quality. There is also no study on the bacterial load quantification (CFU/g) or (CFU/mL) in Asian seabass.

The objectives of this research are as stated below:

1. To determine the common types of marine bacteria species in Asian seabass *Lates calcarifer* (Bloch, 1790) cultured in floating net-cages at Pulau Ketam, Selangor, Malaysia through isolation and characterization the bacteria isolated from the kidney, liver, intestine and any abnormal lesions presence.
2. To quantify the bacterial load (CFU/g or CFU/mL) in the samples of kidney, liver and intestines taken from healthy and moribund Asian seabass.

Hypothesis:

H_o 1: There are no distinctive types of common marine bacteria that can be isolated from kidney, liver, intestine and lesion samples in healthy and moribund Asian seabass.

H_a 1: : There is a distinctive type of common marine bacteria that can be isolated from the kidney, liver, intestine and lesion samples in healthy and moribund Asian seabass.

H_o 2: There is a low bacterial load (CFU/g or CFU/mL) in the samples of kidney, liver and intestines taken from Asian seabass.

H_a 2: There is a high bacterial load (CFU/g or CFU/mL) in the samples of kidney, liver and intestines taken from Asian seabass.

2.0 LITERATURE REVIEW

2.1 Asian Seabass

Lates calcarifer (Bloch 1790) is known as Asian seabass or Giant seaperch in Asia and Barramundi in Australia. The species belongs to the Perciformes order and under the Centropomidae family. The species usually occupies the freshwater, the estuarine and the coastal area and they are extensively distributed from the Arabian Gulf to China, Taiwan, Northern Australia, Papua New Guinea and throughout the Indo-West Pacific region. In the 1970's, the aquaculture production of *Lates calcarifer* began in Thailand before swiftly expanded to all over Southeast Asia (Vijayan et al. 2015).

According to the FAO (n.d.), the biological features of the Asian seabass is that it has pointed head with curved dorsal profile that becomes arched cranial to the dorsal fin. The mouth is a bit diagonal and enormous with the upper jaw reaching the region caudally to the eye. Other distinctive characteristics such as absence of canines and the teeth are villi form. The body is compressed and elongated, with a profound caudal peduncle and covered with large ctenoid scales. There is no presence of bars or spots on the fins or the body. The pre-operculum consists of a strong spine at the lower edge while the operculum consists of a small spine with serrated flap above the beginning of the lateral line. The dorsal fin consists of seven to nine spines and 10 to 11 soft rays with a profound notch, nearly separating the spiny part from the soft part of the fin. The pectoral fin is short and rounded and has a few short and tough serrations at the pre-basal area. The anal and the caudal fins are rounded and anal fin consists of three spines and seven to eight short rays. Scaly sheath can be observed on both dorsal and anal fin.

Figure 1: Mature Asian seabass, *Lates calcarifer* sampled from floating net-cage in Pulau Ketam, Selangor, Malaysia.

Furthermore, Asian seabass has excellent hardy quality which means it can survive in high stocking rate, high growth and fecundity, amendable to formulate and trash fish diets as well as high adaptability towards salinity changes (Matthew 2009). Currently, the market price of Asian seabass or 'Siakap' in Malay fluctuates between RM 17.00 to RM 19.00 per kg from January until September of 2020 (based on the weekly price reported by the Fisheries Development Authority of Malaysia , 2020). Despite the price, Malaysian still prefer to consume this fish species especially during festive season or huge celebration which makes it in highly demand marine food fish.

2.2 Marine cage cultured system

Some of the small fish farmers in Malaysia still prefer to use ponds to rear Asian seabass, however, the mass production of Asian seabass is largely derived from the floating net-cages culture. This is due to factors such as cages are more flexible and easy to build because the farmers can change the farm structure, size of cages

and location easily to fit farm purposes. Hence, low investment is required to build a versatile net-cages and the farm management can be handled smoothly throughout the whole rearing cycle (Buendia, 1997). The structure of the floating net-cages requires a few wooden platforms, fishnet and barrels, where the cages size is about (3 x 2 x 1) m. The fishnet is attached to plastic barrels to help the fishnet to stay afloat and the cages come in a variety of shapes and sizes that are easy for the farmers to construct the whole cages. According to Mojjada (2012), open sea floating-cages were more beneficial than inshore floating net-cages because of less stress, better nutrition and good photoperiod for the fish. Thus, fishes had better growth performance and higher survivability from lack of disease outbreak.

Unfortunately, this rearing technique has its own set back where high stocking density and inadequate fish farm health management leads to unsuccessful fish production. Immense stress and immunosuppressive conditions can also happen due to the failure of the farmer to provide an adequate nutritional diet and satisfactory water quality which eventually increase the risk of disease infection for the fishes (Gopakumar , 2009). Poor water quality and high-stocking density of fishes cultured in net-cages leads to hazardous and stressful conditions that eventually contribute to the cause of the outbreak (Kousar et al. 2019).

2.3 Common marine bacteria dan diseases

Aquatic marine bacteria can be divided into two groups which are pathogenic and non-pathogenic bacteria where the relationship between the microorganism and the aquatic fishes can either be mutualistic or morbific. Pathogenic bacteria is a type of bacteria that can cause disruption in the normal physiological function of the fishes which may lead to numerous health complications and diseases. On the contrary, non-pathogenic bacteria like the normal flora or environmental bacteria

can live together with other organisms symbiotically without causing any detrimental health issues. Therefore, infection by the pathogenic bacteria are more of concern as compared to non-pathogenic bacteria.

Both groups of bacteria are normally divided into other distinctive groups based on their Gram's staining reactions. Gram positive and Gram negative bacteria are differentiated by the presence of peptidoglycan where the outer membrane of Gram positive bacteria consists of thicker layer peptidoglycan compared to Gram negative bacteria (Amils, 2011).

Toranzo et al. (2005) stated that *Listonella anguillarum, Photobacterium damselae, Tenacibaculum maritimum, Streptococcus iniae* and *Mycobacterium marinum* are among the economic significant aetiological agents that are affecting aquatic marine fish like Asian seabass by causing infections and diseases. According to Falaise et al. (2016), the bacteria species such as *Vibrio sp.*, *Pseudomonas sp.*, and *Aeromonas sp*. were few of the infectious fish diseases that had an impact on the economy of marine fish culture.

In Malaysia, several notable bacterial fish diseases in Asian seabass were caused by *Vibrio sp., Streptococcus sp., Aeromonas sp.* and other bacteria species as mentioned by Chiew et al. (2019). While in Sabah, Malaysia, a report of a disease outbreak caused by *Vibrio harveyi* was made by Ransangan and Mustafa (2009). Generally, bacteria diseases in marine fish were associated with clinical signs of acute septicaemia, dermal ulceration, haemorrhages, organomegaly, corneal opacity or chronic focal lesions in fish (Ina-Salwany et al. 2020). Meanwhile, Austin (2019) stated that inappetence, emaciation, behavioural changes, scale loss or dermatitis, abscesses, erosion, ulceration, haemorrhages, necrosis, exophthalmia, abdominal distension and paralysis were the examples of gross lesions caused by bacteria diseases.

2.4 Bacterial diseases in Asian seabass

Vibriosis caused by *Vibrio* sp. in Asian seabass is among the bacterial diseases that force the farmers to sustain their profit loss due to drop in fish production when major disease outbreak occur. The clinical signs for vibriosis are necrosis and haemorrhagic ulcerations and exopthalmia which lead to high mortality and reduce in the fish quality (Alshaharani et al., 2015). Buller (2004) stated that one of the notable bacteria species that caused bacterial diseases in Asian seabass was *Photobacterium damselae* ssp. *damselae* which caused fish pasteurellosis. This bacterial disease caused severe systemic signs where haemorrhagic septicaemia occurred with lack of abnormal gross lesions observed on the fish physically. *Streptococcus iniae* can also cause bacterial disease in Asian seabass with clinical signs of subcutaneous abscess, torticollis, ulcers, granulomas, and organomegaly as well as systemic signs which would not be cost-effective to treat in major outbreaks. Bacterial diseases can be determined by isolating the bacteria through bacteria culture and identifying the bacteria species by using biochemical or molecular tests. By understanding the clinical signs manifested by distinctive bacterial diseases, it can help the clinician to come out with the differential diagnosis, plans to rule out the disease and the most appropriate treatment plan as well as recommendation for the farmers to improve their fish production.

2.5 Bacterial load quantification in fish organs

Bacterial load quantification is a quantitative analysis used to determine the quatity of bacteria present in organ, and calculated as colony-forming unit (CFU) form on inoculated agar. The procedure is beneficial as to determine the acceptable levels of microbiological quality of food samples that is safe for human and animal consumption (Fasnz, 2001). The bacterial population of a sample that grows aerobically at mesophilic temperatures were indicated by using Aerobic plate count (APC) which also known as the Total plate count (TPC), Standard plate count (SPC), Aerobic colony count (ACC) and Mesophilic count. The APC is a good indicator for raw fish meat quality depending on situations but it does not indicate the entire bacterial population in the organ and does not differentiate between bacteria species.

Allen et al. (2002) reported that APC can be conducted by using either pour plate or spread plate methods. According to the Malaysia Food Regulations (1985), the standard bacterial load (cfu/g) for TPC at 37°C for 48 hours for fish and fish products that are appropriate for consumption, except for fish and fish products in airtight containers which shall not exceed 10^6 cfu per gram. If the TPC of the fish or fish products exceed 10^6 cfu per gram, the fish are deemed unsafe to be consumed and should be disposed properly to ensure sanitary and hygienic surrounding environment.

2.6 Fish immunity against bacterial infection

Fish immunity is quite similar to the immunity of the terrestrial animals whereby the immune response is divided into specific and nonspecific immunity. The nonspecific immunity is the fundamental defence system in aquatic animals as they rely mostly on the innate immunity for survival and to protect them from various pathogenic microorganisms (Rombout et al., 2005). It is also vital because

of their poikilothermic nature and the limitations of the adaptive immunity in combating pathogens such as short antibodies repertoire, slow lymphocytes proliferation and maturation and poor immune memory (Whyte, 2007).

When an opportunistic pathogen such as the environmental bacteria attached themselves on the host epidermis, the defense mechanisms of the physical barrier i.e. the scales, skin mucous and gills will inhibit the entry of pathogens by producing antibacterial peptides, lysozymes, complement proteins and immunoglobulin M (IgM) (Boshra et al., 2006; Saurabh and Sahoo, 2008). Moreover, the epidermis maintains its integrity and osmotic balance while the cellular components like macrophages, granulocytes and agranulocytes continue to protect the host. Then, if the pathogenic bacteria somehow manage to penetrates through the defence and innate immune system, the adaptive immunity will take over by developing specific immune responses against distinct bacterial antigens through a series of mechanisms involving a complex network of specialized cells, proteins, genes and biochemical messages that provide the infected host with high specificity and affinity antibodies and effector cells to fight off the infective agents (Uribe et al., 2011).

2.7 Zoonotic potential and public health concern of aquatic marine bacteria

Environmental bacteria as well as the normal flora in the marine habitat can manifest a budding possibility to give rise to zoonotic diseases in humans.

In fact, several clinical cases had been recorded involving zoonotic wound infections due to post exposure to contaminated seawater, seafood and fish where some of the patients required immediate hospitalization (Hundenborn et al. 2013). He also mentioned that 9.4% of the hospitalized patients caused by the zoonotic wound infections had a terminal consequence. Acha and Szyfres (2003) also

reported that the transmission of the infectious agents that cause bacterial diseases can be carried out through direct contact with the infected or contaminated fish, seafood or any water bodies or through oral ingestion of the infected fish or associated contaminants.

Gram-negative bacteria species such as *Vibrio parahaemolyticus* and *V. vulnificus* were the most frequently isolated species duo in Europe (Baker-Austin et al. 2010) while in the United States, *Vibrio sp.* like *V. parahaemolyticus, V. vulnificus, V, alginolyticus* and *V. cholerae* as well as other Gram-negative species like the *Aeromonas hydrophila* can cause wound contamination and infection (Hlady et al. 1996; Oliver, 2005). Major aquatic bacteria such as *Aeromonas hydrophila, Escherichia coli, Salmonella typhi, S. paratyphi, V. cholerae O1, V. parahaemolyticus and V. vulnificus,* had become the common pathogens to cause foodborne diseases that lead to public health concern according to the WHO (2007).

3.0 MATERIALS AND METHOD

3.1 Bacteria Media Preparation

Marine trypticase soy agar (TSA added with 1% sodium chloride) and Thiosulfate-citrate-bile salts-sucrose agar (TCBS) were used to culture the marine bacteria. The TSA and TCBS powders were mixed with reverse osmosis water and were sterilised in an autoclave at 121°C for 15 minutes. Then, the molten sterilised agar was poured into sterile disposable plates until half full with approximate of 2.5 ml per plate inside a laminar flow cabinet. Then, the plates were left inside the laminar flow cabinet at room temperature until fully solidified. All the plates were later packed and sealed inside a sterile plastic bag and were kept at 4°C for storage purpose.

3.2 Sampling location

The sampling location was at a commercial floating net-cage farm at Pulau Ketam, Selangor, coded as SL-S-87 farm, located at latitude of 3°00’57°N and longitude of 101°14’54”E (Figure 3.2). The floating net-cages were made out of wooden platforms, floated on plastic containers and steel drums. There were 60 cages culturing marine food fishes such as snapper, hybrid grouper, Asian seabass mangrove jack, sweetlips and giant trevally.

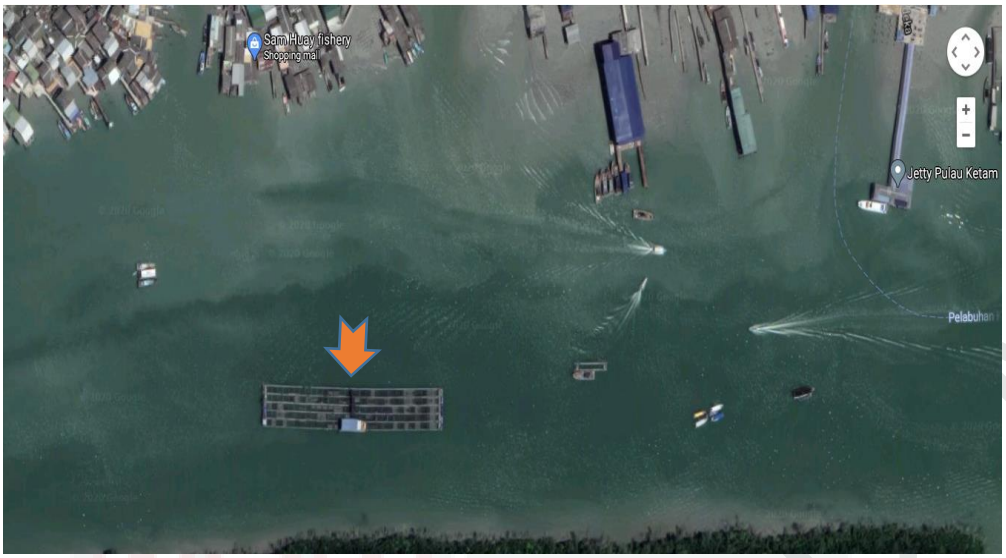

Figure 2: The SL-S-87 marine fish farm from a satellite image (Google satellite maps; 10/09/2020)

The samples Asian seabass were morphometrically measured (standard and total body length; body weight) on the farm. The fish were wrapped with a towel to prevent it from slipping from the cutting board and a thorough physical examination was performed on each fish to observe for any abnormalities presence on the fishes before it was euthanized. Euthanasia was performed by severing the cervical vertebrae column with sterilized blade or scissors. Then, the fishes were kept inside a sterile plastic bag prior to putting them inside a polystyrene box (37 x 33 x 30 cm) packed with ice cubes. The fish samples were brought back to the Aquatic Animals Health Unit, Universiti Putra Malaysia for bacterial isolation where the journey took about one and a half hours.

3.3 Bacterial Isolation

The plates were first labelled according to the type of agar, fish ID, organ samples, date, and person-in-charge. Next, a line was drawn on the agar plate base to divide the area on the agar for inoculation of different source of samples from

the same fish. The organ samples that were taken were from liver, intestine, kidney as well as any abnormal lesions. Abnormal lesions such as ulcerations on the body were first assessed and taken for bacteria isolation. The fish were dissected by using a scalpel blade and a pair of scissors at the ventral midline from the operculum until the anus. Precaution steps were taken not to accidentally contaminate the internal organ with faecal material. The visceral organs surface were observed *in situ* for the presence of any atypical lesions. The first organ that was inoculated onto the agar for bacteria isolation was the liver, followed by the intestine and the kidney.

A small cut was made to expose the inner surface of the organ by using a sterile blade. Then, a metal inoculating loop was sterilized under the flame before obtaining the inoculum from the organ samples. The inocula were then streaked in a zig-zag manner on the TSA (with 1% NaCl) and TCBS agars. All the plates were sealed with parafilm and incubated under a control temperature of 30°C for 24 hours where a mixed growth will be observed on the agar overnight.

3.4 Gram's and May-Grünwald +Giemsa (MGG) stainings

Squashed smears of the organ were made by cutting a small piece of tissue using a sterile scissor and putting it on a clean glass slide. Another glass slide was then overlaid onto the glass slide that had the tissue sample and the sample was squashed between the two slides until a thin layer was formed. Both glass slides were fixed in 100% methanol for 1 minute before each slide were stained according to the two staining protocols i.e. Gram's and MGG (as stated in the appendix)

3.5 Colony morphology

The individual colony morphologies of the bacteria that successfully grown on both marine TSA and TCBS agar were recorded. The bacteria colonies characteristics such as the size, shape, colour, elevation, surface, margin and texture were described. On the marine TSA, the colonies are usually white, cream or translucent in colour while on the TCBS agar, the colonies can appear either in yellow or green colour. All the colonies seen were then stained with Gram's stain as described in the appendix (American Society of Clinical Pathology, 2004).

3.6 Species Identification Using Biochemical Tests

The primary biochemical tests that were used included oxidase, catalase and triple sugar iron (TSI) tests while the secondary biochemical test used was API20E test kit that aids in identifying the bacterial species. Oxidase test was performed to detect organisms that has cytochrome oxidase or also known as indophenol oxidase (Shields and Cathcarts 2016). A bacteria colony was obtained from the culture agar by using sterilized inoculating loops and spread evenly on the membrane filter paper before adding the reagent onto the bacterial colony. Positive result will show purple colour development. While Catalase test is a biochemical test to detect the presence of catalase, an enzyme that breaks down hydrogen peroxide into water and oxygen (Reiner, 2010). A drop of hydrogen peroxide was put on a glass slide which was earlier smeared with a bacteria colony. Presence of bubbles indicated positive reaction while absence of bubbles indicated negative reaction. Motility tests were done by using a hanging drop method as adapted from Paydar (2013) where one drop of normal saline was put on the glass slide and a bacteria colony from the pure culture was added aseptically into the water droplet. A cover slip that was put a bit of petroleum jelly on all four edges before it was carefully placed on the droplet to allow it to hang in between the cover slip and the glass slide. It was viewed under

the microscope at 40x magnification. Positive reaction of motility test will shows the movement of alive bacteria indicating presence of flagella and locomotor appendages.

The TSI test is then used to test the ability of microorganisms to ferment carbohydrates like glucose, sucrose and lactose as well as do sulphur reduction in order to produce hydrogen sulphide. A bacteria colony was obtained from the pure culture by using a sterile platinum rod. The rod was stabbed into the TSI agar and was left inside the incubator for 24 hours at 30°C. Then, the colour of the slant and the butt were recorded. According to the adapted manual (Difco, 1984), a red slant and yellow butt showed an alkaline and acidic reaction, respectively, which indicates fermentation of dextrose while a yellow slant and yellow butt shows an acid and acid reaction which indicates the fermentation of dextrose, lactose and sucrose. Red slant and red butt shows an alkaline and alkaline reaction from the absence of carbohydrate fermentation. Presence of hydrogen sulphide is indicated as the presence of the blackening of the medium while presence of bubbles or cracks in the agar suggested the production of gas.

API20E is one of the Analytical Profile Index that is used specifically for Gram-negative rods bacteria and other non-fastidious bacteria from the family Enterobacteriaceae. The API20E test strip contains 20 distinctive microtubes that are filled with dehydrated chemical substrates. The bacterial suspensions in sterile saline are inoculated into biochemical substrates for 24 hours. Colour changes can be seen in positive reaction, either by adding exogenous reagents or through spontaneous reaction.

The prevalence of the bacteria species in the fish identified was calculated using the prevalence formula below where the number of infected fish at the given point

of time divided by the number of fish samples at the same point of time (Noordzii et al., 2010).

$$\text{Prevalence Formula} = \frac{\text{Number of infected fish at a given point in time}}{\text{Number of fish samples at the same point in time}}$$

3.7 Quantification of bacterial load

Total plate count by using spread plate method with two-folds of serial dilution was utilised to count the quantity of bacterial load in organ. The tissue samples from kidney, liver and intestine from all ten Asian seabass were taken aseptically by using a sterilized iris scissor. A piece of tissue weighing approx.0.2g of each organ samples were weighed separately by using an electronic weighing scale. Then the tissue samples were homogenized using sterilized mortar and pestle. Each of the tissue homogenates was mixed with 0.4 ml sterilized peptone water. Then, 0.4 ml of the homogenate was diluted in a series of two fold dilutions viz. 2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}, and 2^{-5}. One drop of the mixture (0.1ml) from each serial dilution was spread onto the marine TSA agar by using sterilized bended glass rod. All the plates were then sealed by using parafilm and incubated for 24 hours at 30°C to allow bacterial growth. The bacterial load quantification formulae adapted from Maturin and Peeler (2001) is as follows:

Bacterial Load Quantification Formula

$$\text{CFU/mL} = \text{cfu/ml} = \frac{\text{(no. of colonies x dilution factor)}}{\text{Volume of inoculum}}$$

4.0 RESULTS

4.1 Fish Samples

The 10 fish samples were divided into i) clinically infected (Group A) and ii) apparently healthy (Group B) fish. Three out of ten fish samples namely AS1, AS6 and AS10 showed gross lesions of scales sloughing off and haemorrhagic ulcerations on the lateral part of the body. The three fishes with clinical signs were grouped together in Group A. While the rest of the fish (AS2, AS3, AS4, AS5, AS7, AS8 and AS9) were grouped in Group B for apparently healthy fish.

Figure 3: Group A AS1 with gross signs of dermal ulceration and the body weight is 100g and the body length is 18.5cm.

Fish Id	Weight (g)	Standard body length SBL (cm)	Total body length TBL (cm)
AS1	100.0	17.0	18.5
AS2	150.0	17.5	20.0
AS3	200.0	18.0	20.5
AS4	150.0	15.0	17.0
AS5	160.0	17.0	19.5

AS6	150.0	18.5	21.0
AS7	160.0	17.0	19.5
AS8	200.0	19.0	21.5
AS9	100.0	15.5	17.5
AS10	100.0	17.0	19.0

Table 1: Showing the body weight (g), standard body length (cm) and total body length (cm) of ten sampled Asian seabass.

The average weight for the clinically infected fish (Group A; n=3) was 116.67g, while the average SBL and TBL were 17.5cm and 19.5cm, respectively. The average weight for the apparently healthy fish (Group B; n=7) was 160g, while the average SBL and TBL were 17.0 cm and 19.36 cm, respectively. The average weight for all ten fish samples was 147g, with the average SBL and TBL at 17.15 cm and 19.4 cm respectively.

4.2 Farm Management and Water Quality

The water samples for water parameter quality determination were taken at the front (bow) and back (stern) part of the floating net-cages. The water quality was determined by using the water analysis device and equipment where we submerged the device detection probe 1 meter deep into the water and the reading from the analysed data was recorded. The physicochemical of water samples indicated that all the parameters were within the normal recommended range (Table 2).

Parameter	Farm Water Sample Site		Normal Range
	Bow	Stern	
pH	7.9	7.91	6.5 – 9.0 (BFAR, 2007)
Dissolved Oxygen (D.O)	91.4% / 5.67ppm	80.3%/5.00 ppm	3.0 – 7.0 (BFAR, 2007)
Conductivity	53.11 µS/cm	52.09 µS/cm	
Total Dissolved Solids (TDS)	26.56 ppt tds	26.05 ppt tds	
Salinity	34.86 psu	34.11 psu	25 – 40 psu
Temperature	30.13	30.16	25 - 30°C (BFAR, 2007)
Atmospheric Pressure	100.98kpa	100.97kpa	
Nitrite (mg/ml)	0	0	0.4 max (BFAR, 2007)
Nitrite (mg/ml)	5	5	7.0 max (BFAR, 2007)
Ammonia (ppm)	0.25	0.5	<0.05 (Lowson, T. B., 1955)

Table 2: The physicochemical of the water samples taken from the marine fish farm at Pulau Ketam, Selangor taken at 13:30 hours.

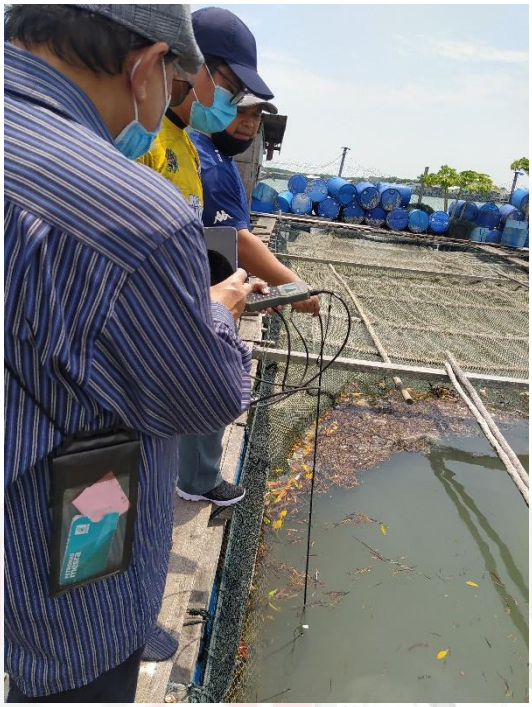

Figure 4: Water quality testing at the back (stern) part of the floating net-cages by using water analysis equipment.

4.3 Bacterial Isolation and Identification

The growth of the first colony on the marine TSA and TCBS agar, incubated at temperature of 30°C was recorded in more or less than 24 hours. Presence of swarming bacterial colonies could be detected by observing the zonal growth formation of creamy colonies on some of the marine TSA, an indicator for motility that was further confirmed by motility test by using the hanging drop method.

From the results of bacteria isolation from all the fish samples revealed a total number of 86 bacterial isolates, where seven isolates were gram-positive cocci (6.02%), 21 isolates were gram-positive bacillus (18.06%), five isolates were gram-negative cocci (4.3%) and 53 isolates were gram-negative bacillus (45.58%). Out of the 53 gram-negative bacillus isolates, 29 gram-negative bacterial isolates were successfully identified as *Photobacterium damselae*, *Stenotrophomonas maltophilia*, *Shewanella*

putrefaciens, and *Vibrio alginolyticus* based on cell morphologies, biochemical tests and API20E test results.

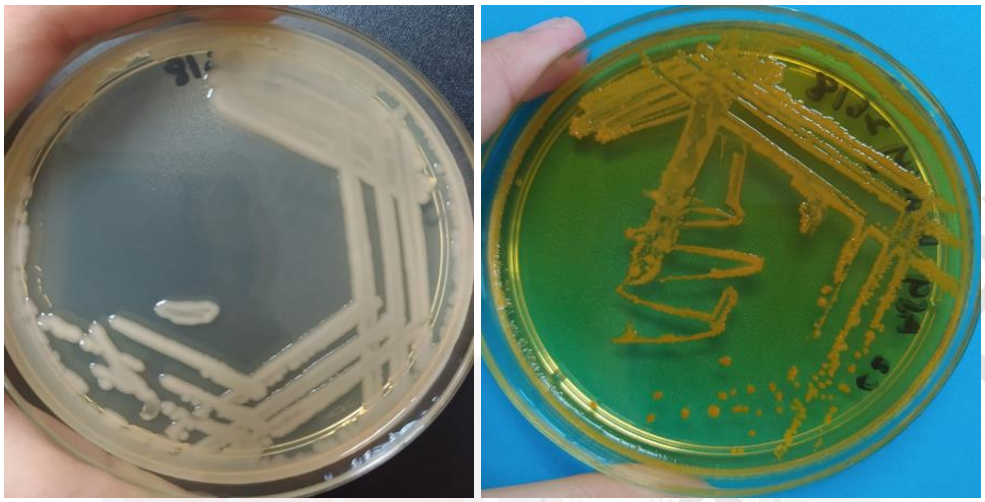

Figure 5: Colony of *Vibrio alginolyticus* on marine TSA (A) and TCBS agar (B) (yellow colonies).

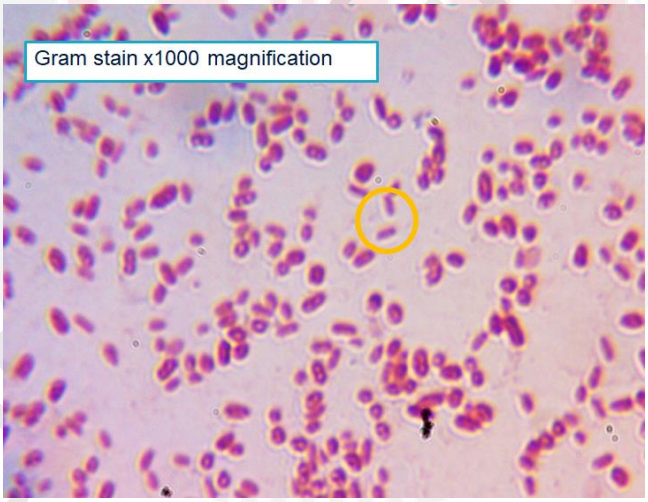

Figure 6: Gram's staining of *Vibrio alginolyticus*, showing Gram-negative, single large rod-shaped cells.

On the marine TSA the colonies appeared as large creamy mucoid colonies, slightly convex with circular and regular edges, while on TCBS, the colonies were large, yellowish, slightly convex with circular and smooth edges.

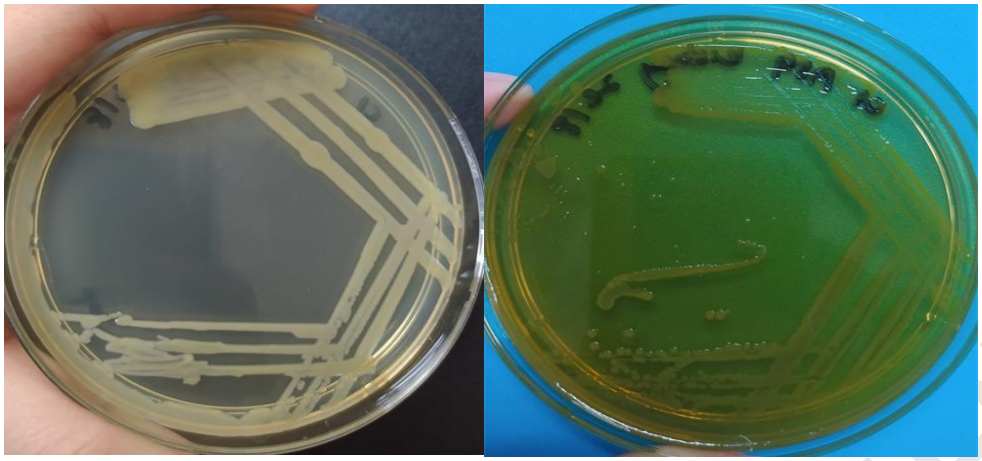

Figure 7: Colonies of *Stenotrophomonas maltophilia* on marine TSA (A) and TCBS agar (B) (yellow colonies).

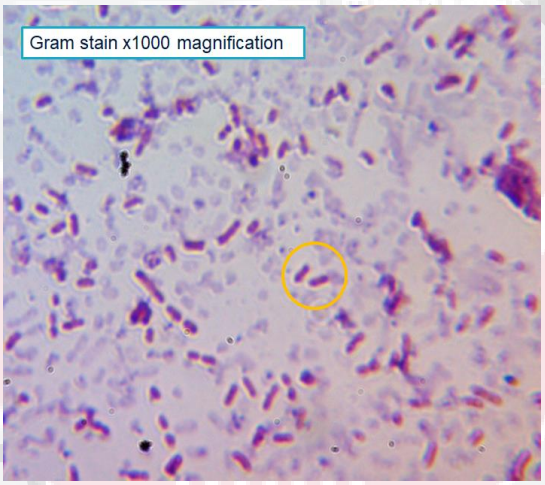

Figure 8: Gram's stain of *Stenotrophomonas maltophilia* showing rod-shaped bacilli.

Stenotrophomonas maltophilia is a Gram negative, single rod shaped that grows on the marine TSA as large white mucoid colonies, slightly convex with circular and regular edges while on TCBS agar are large yellow mucoid colonies, slightly convex with circular and regular edges.

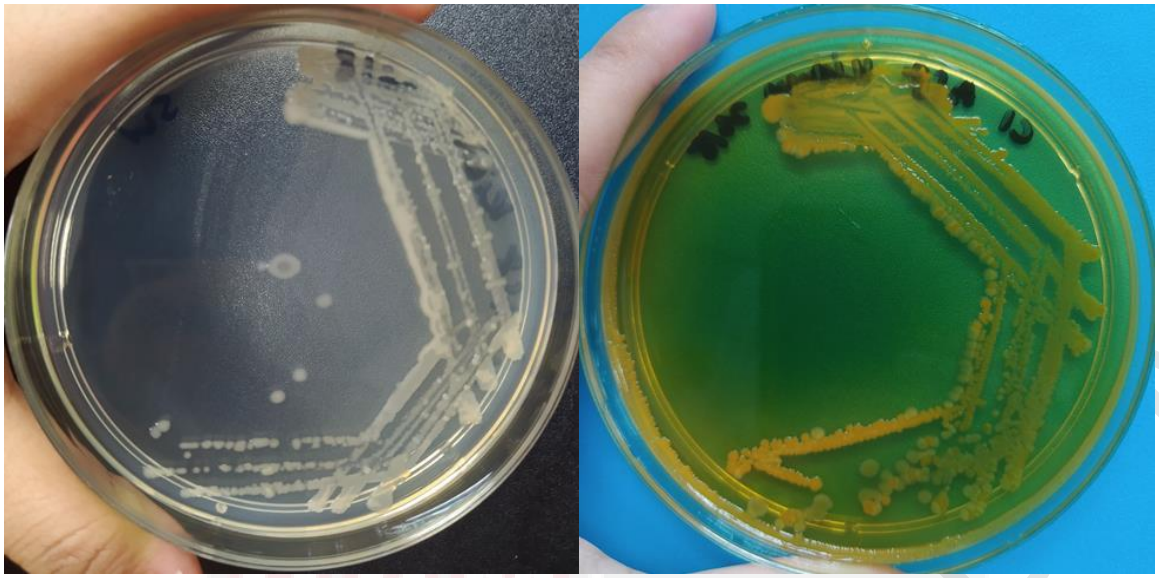

Figure 9: *Photobacterium damselae* on marine TSA (A) (creamy colonies) and TCBS agar (B) (yellow colonies).

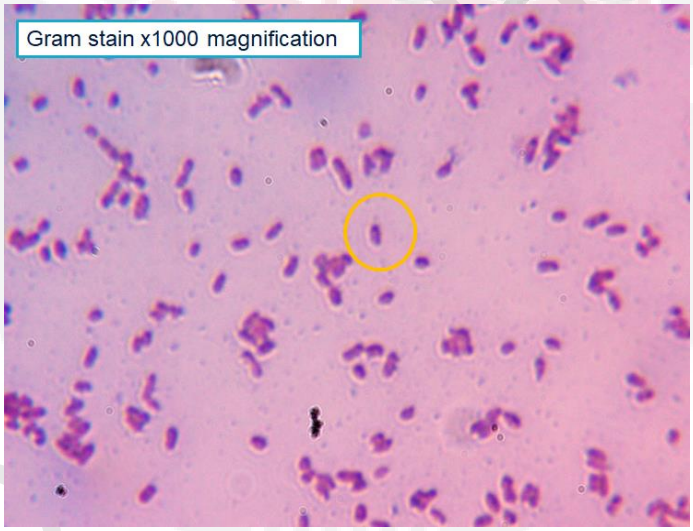

Figure 10: Gram stain of *Photobacterium damselae* showing stumpy rod-shaped, gram negative bacilli.

Photobacterium damselae is a Gram negative, single rod-shaped that grew on the marine TSA as large white mucoid colonies, slightly convex with circular and smooth edges while on TCBS agar they were large yellow colonies, slightly convex with circular and smooth edges.

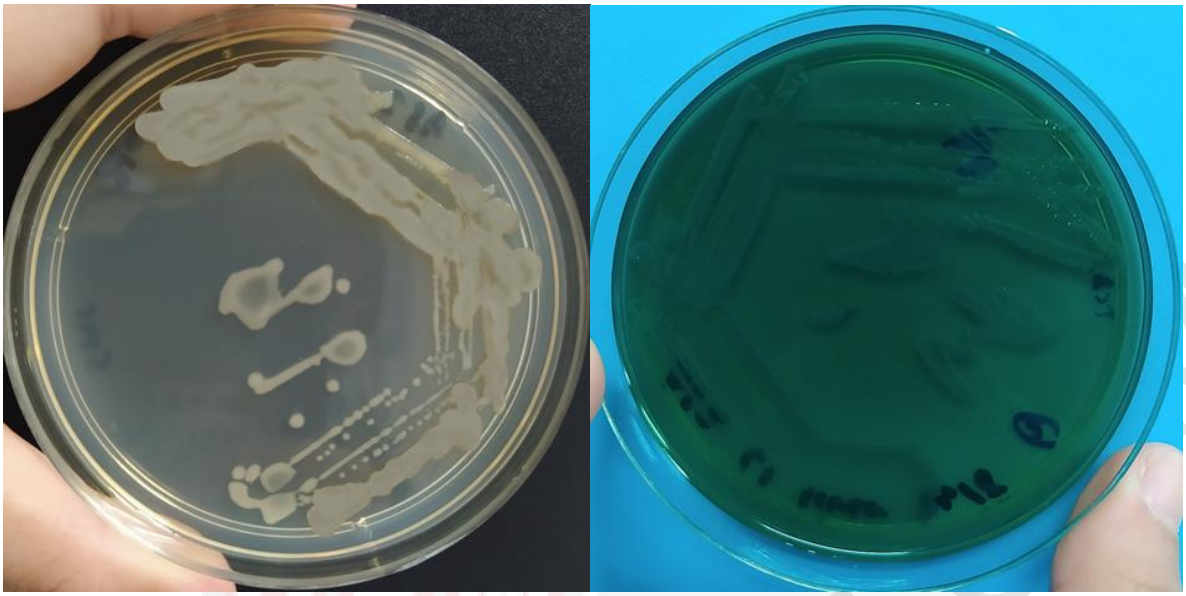

Figure 11: *Shewanella putrefaciens* on marine TSA (A) and TCBS agar (B) (green colonies).

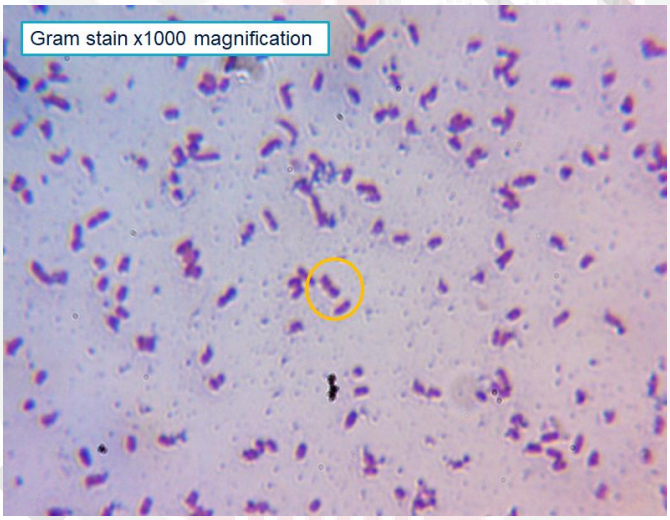

Figure 12: Gram stain of *Shewanella putrefaciens* showing short rod-shaped Gram negative bacteria in pairs.

Shewanella putrefaciens is a Gram negative, single rod-shaped that grew on the marine TSA as large white mucoid colonies, slightly convex with circular and regular edges while on TCBS agar they were large green mucoid colonies, slightly convex with circular and irregular edges.

Table 3 indicates that the highest prevalence among the isolated bacteria species was *Vibrio alginolyticus* (41.38%), followed by *Stenotrophomonas maltophilia* (34.48%), *Photobacterium damselae* (20.69%) and *Shewanella putrefaciens* (6.9%).

	Photobacterium damselae		*Stenotrophomonas maltophilia*		*Shewanella putrefaciens*		*Vibrio alginolyticus*	
	No. of fish infected	%	No. of fish infected	%	No. of fish infected	%	No. of fish infected	%
Group A n=3 (Clinically Infected)	1	33.33	0	0	2	66.67	3	100
Group B n=7 (Apparently Healthy)	4	57.14	2	28.57	4	57.14	6	85.71

Table 3: Prevalence of bacteria species in Group A and Group B fishes.

Furthermore, results showed that the percentage of fishes infected by pathogenic bacteria in Group A were 100%. All clinically infected fishes were infected by *V. alginolyticus*, 66.67% by *S. maltophilia*, and 33.33% by *P. damselae* , while no *S.putrefaciens* were identified in this group. On the otherhand, in Group B, *V. alginolyticus* exhibited 85.71% of the infection in the apparent healthy fishes and 57.14% were by both *S. maltophilia* and *P. damselae*, and 28.57% were by *S. putrefaciens.* The total prevalence for the selected bacteria species in organs of Group A were 10.34% for both liver and ulcers (abnormal lesions), 6.9% for kidney and 3.45% for intestines. The total prevalence for the bacteria species in organs of Group B were 27.59% in liver, 24.13% in intestine and 17.24% in kidney.

Biochemical Tests	Isolated Bacteria Species			
	Vibrio alginolyticus	*Stenotrophomonas maltophilia*	*Photobacterium damselae*	*Shewanella putrefaciens*
Oxidase	+	-	+	+
Catalase	+	+	weak	+
Triple Sugar Iron (slant/ butt)	acid/ acid	alkaline/ acid	acid/ acid	alkaline/ acid
Motility Test	+	+	+	+
Presence of β-galactosidase (ONPG)	-	+	-	-
Arginine Dihydrolase (ADH)	-	-	+	-
Lysine Decarboxylase (LDC)	+	+	-	-
Ornithine Decarboxylase (ODC)	+	-	-	+
Citrate (CIT)	-	+	-	+
Hydrogen sulfide (H2S)	-	+	-	+
Urease (URE)	-	-	+	-
Tryptophan Deaminase (TDA)	-	-	-	-

Indole production (IND)	+	-	-	-
Voges-Proskauer test for Acetoin Production (VP)	+	-	+	-
Gelatinase production (GEL)	+	+	-	+
Glucose Fermentation (GLU)	+	+	+	-
Mannose Fermentation (MAN)	+	+	-	-
Inositol Fermentation (INO)	-	-	-	-
Sorbitol Fermentation (SOR)	-	-	-	-
Rhamnose Fermentation (RHA)	-	-	-	-
Sucrose Fermentation (SUC)	+	+	-	-
Melibiose Fermentation (MEL)	-	-	-	-
Amygdalin Fermentation (AMY)	+	-	-	-

Arabinose Fermentation (ARA)	-	-	-	-

Table 4: The biochemical tests results for all the bacteria species identified.

Sample	Organ	*Vibrio alginolyticus*		*Stenotrophomonas maltophilia*		*Photobacterium damselae*		*Shewanella putrefaciens*		Total	
		No.	%	No.	%	No.	%	No.	%	No.	%
Group A n=3 (Clinically Infected)	Kidney	1	8.33	1	10	0	0	0	0	2	6.9
	Intestine	0	0	0	0	1	16.67	0	0	1	3.45
	Liver	2	16.66	1	10	0	0	0	0	3	10.34
	Ulcers	1	8.33	2	20	0	0	0	0	3	10.34
Group B n=7 (Apparently Healthy)	Kidney	1	8.33	2	20	3	50	0	0	5	17.24
	Intestine	4	33.33	3	30	0	0	0	0	7	24.13
	Liver	3	25	1	10	2	33.33	2	100	8	27.59
Total		12	41.38	10	34.48	6	20.69	2	6.9	29	100

Table 5: The prevalence of the bacteria species identified in the organs and lesions samples

4.4 Bacterial Loads

The total colony forming unit (CFU) from the serial dilution of 2^{-3} for Group A (clinically infected group) were 3.8 x 10^3/ml in kidney, 3.7 x 10^3/ml in liver, and too numerous to count (TNTC) in the intestine. Meanwhile, the CFU from the serial dilution of 10^{-3} for the Group B (apparently healthy) fishes were 4.9 x 10^3/ml in the kidney, in the liver ranged from 1.8 x 10^3/ml to 3.2 x 10^4/ml, and TNTC in the intestine. The CFU in the serial dilution of 2^{-4} for Group A for the kidney was negligible (NG), too few to count (TFTC) in the liver and TNTC in the intestine. CFU in the serial dilution of 2^{-4} in Group B were 1.7 x 10^4/ml in the kidney, 7.4 x 10^3/ml in the liver and 1.5 x 10^4/ml in the intestine. The CFU in the serial dilution of 2^{-5} in Group A for the kidney was NG, TFTC in the liver and 4.7 x 10^4/ml in the intestine while the CFU in the serial dilution of 2^{-5} in Group B ranged from 2.9 x 10^3/ml to 1.9 x 10^4/ml in kidney, TFTC in liver and in intestine was 2.1 x 10^4/ml, respectively.

Fish Sample	Organ	**Serial Dilution**		
		2^{-3}	2^{-4}	2^{-5}
Group A n=3 (Clinically Infected)	Kidney	3.8 x 10^3/ml	NG	NG
	Liver	3.7 x 10^3/ml	TFTC	TFTC
	Intestine	TNTC	TNTC	47 x 10^3/ml
Group B n=7 (Apparently Healthy)	Kidney	4.9 x 10^3/ml	1.7 x 10^4/ml	2.9 x 10^3/ml - 1.9 x 10^4/ml
	Liver	1.8 x 10^3/ml- 3.2 x 10^4/ml	7.4 x 10^3/ml	TFTC
	Intestine	TNTC	1.5 x 10^4/ml	2.1 x 10^4/ml

Table 6: The bacterial load quantification (cfu/g) in the organs sample of both Group A and Group B.

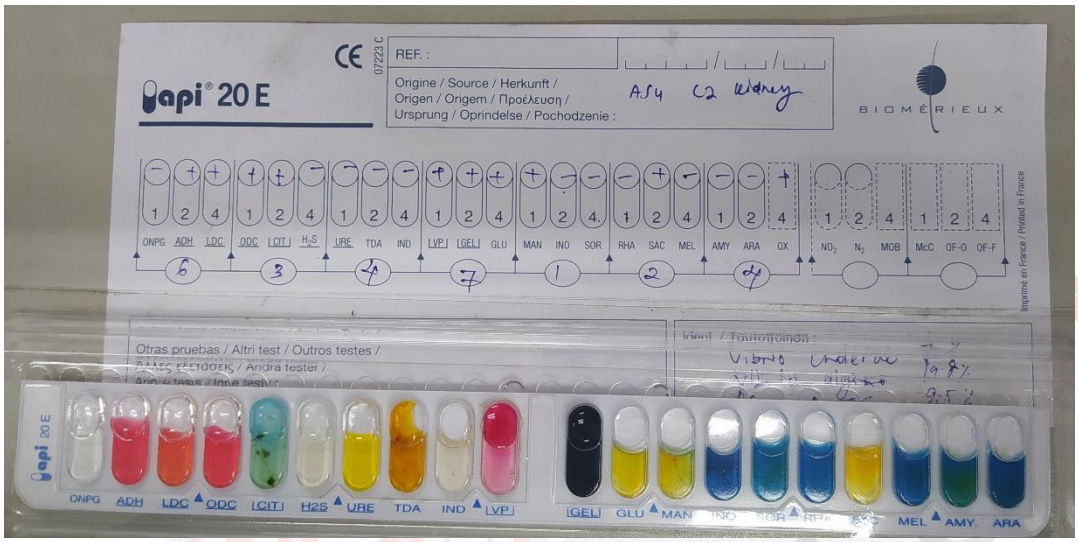

Figure 13: API20E test results for the kidney sample Group B AS4.

5.0: DISCUSSION

The sloughing off of scales and haemorrhagic ulcerations on the flank or lateral side of the body in the clinically infected seabass were similar to earlier reports by Tendecia (2002), Austin (2019) and Ina-Salwany et al. (2020) whom stated that the clinical signs of bacterial infection includes haemorrhagic dermal ulceration located on the surface of the integument or the oral cavity. The external lesions caused the breakdown of the physical barriers that protect the subcutaneous layers which then allowed the environmental and opportunistic pathogen to enter and cause infection. The haemorrhages suggested that there was rupture of blood vessels thus may introduce any pathogen into the blood circulation and will lead to bacteraemia and eventually will cause septicaemia and septic shock to the fish.

The water quality parameters that were taken at the bow and stern of the net-cages were all within the normal range which suggested that poor water quality was not one of the stressors that causes bacterial diseases in the sampled Asian seabass. However, daily feeding of trash fish diets might be the contributing factor to the cause of bacterial infection.

Sample from the selected organ and abnormal lesions were inoculated and incubated 24 hours in a control temperature of 30°C. This was relevant to the report made by Austin (2019) that *Ph. damselae subsp. piscicida* need to be incubated longer than 24 hours and temperatures below 37°C were more suitable to culture fish pathogens. Besides that, growth of creamy swarming colony on the marine TSA indicative of motile bacteria which was similar to Sairi et al. (2015) whom reported that *Aeromonadaceae* and *Vibrionaceae* family which exhibited swarming motility on marine agar. The API20E are used specifically to detect Gram-negative rods shaped from the *Enterobacteriaceae* family and other non-fastidious bacteria. Current study focused more on Gram-negative bacteria because compared to Gram-positive bacteria, most Gram-negative bacteria are

pathogenic and tend to become Multi Drugs Resistant Organisms (MDRO). Brooke (2012) reported that Gram-negative bacteria such as *Ps. aeruginosa* and *St.maltophilia* are more prone to turn into MDRO due to their unique and virulence characteristics. Hence, it is also more cost effective to use the API20E biochemical test to detect Gram-negative bacteria.

Bacterial species with the highest prevalence found in the sampled of Asian seabass were *V. alginolyticus* (41.38%), followed by *St. maltophilia* (34.48%), *Ph. damselae* (20.69%) and *Sh. putrefaciens* (6.9%). The results obtained were in agreement with Al-sunaiher (2010), Ezzat et al. (2018) and Chiew et al. (2019) that stated that one of the highest prevalence bacteria in aquatic fish was from *Vibrio sp.* other than *Aeromonas sp.* and *Pseudomonas sp.* Thus the alternative hypothesis was accepted because there there a distinctive bacterial species found in the Asian seabass samples. Moreover, this finding suggested that there was presence of co-infections between different bacterial species like *V. alginolyticus*, *St. maltophilia*, *Ph. damselae* and *Sh. putrefaciens* in Asian seabass. However, this depends also on the individual fish susceptibility towards infection by opportunistic pathogens. Stress factors such as extreme changes in the water's physicochemical properties and frequent used of trash fish diet in the marine fish farm may contribute to causing immunosuppressive state in the fishes.

Vibrio alginolyticus has the highest prevalence among the bacteria species identified in the Asian seabass in the current study. It is an environmental Gram-negative bacillus bacteria that causes vibriosis and initiates clinical signs such as sloughing off scales and hemorrhagic ulceration in infected fishes. Mohd-Nor et al., (2019) reported that the production cost in farms that were affected by endemic vibriosis increases by 7.8%. In addition, *V. alginolyticus* could lead to gastroenteritis, wound infections and sepsis in humans (Lafisca et al., 2008; Chiew et al., 2019).

Photobacterium damselae is a Gram-negative bacillus bacteria that was formerly known as *V. damselae* and recently considered as an emerging pathogen in marine aquaculture (Labella et al., 2011). The common symptoms of fish photobacteriosis are presence of haemorrhages on the body surface and dermal ulceration (Rivas et al., 2013). It is also of economic important as it causes wound infection and haemorrhagic septicaemia in fishes that might affect the production cost per cycle (Nurliyana et al., 2018).

Stenotrophomonas maltophilia is a ubiquitous Gram-negative bacillus bacteria that was previously known as *Ps. maltophilia.* Abraham et al., (2016) described the clinical signs from the infection of the bacteria in fishes such as fin or tail rot, focal cutaneous hemorrhage and distended abdomen. In humans, this bacteria species led to nosocomial infection that was related to respiratory infection (Victor et al., 1994) and it is now known as a global emerging MDRO (Brooke, 2012).

Shewanella putrefaciens is a Gram-negative bacillus bacteria that is also an opportunistic pathogen that causes disease in immunosuppressed fishes (Kozinska and Pekala, 2004). This bacteria species has never been isolated in Asian seabass, *Lates calcarifer*. Instead, it had only been isolated and reported in European seabass, *Dicentrarchus labrax* (Korun et al., 2009).

The bacterial load quantification (CFU) gives more information on the health status of the fish in addition to the prevalence of the bacterial disease. The highest total bacterial loads (CFU/g) in the organ samples taken from Asian seabass was 10^4 per gram which was below the standard level of microbial load limitation in raw fish samples as stated in the Malaysia's Food Regulation (1985) where the TPC at 37°C for 48 hours for food fish should not exceed 10^6 per g. This insinuate that the fish flesh was safe to be consume as long as the fish was not contaminated by the visceral organs of fish that were showing clinical signs of bacterial diseases and had subclinical infection.

The total CFU was higher in the intestine for both groups as compared to the kidney and the liver. This is in agreement with Olugbojo et al. (2015) which stated that it was normal for the gastrointestinal tract to have a higher bacterial burden as it contains a huge normal flora or microbiota communities. Moreover, the total CFU for both kidney and liver in Group B (apparently healthy) was higher than the total CFU in Group A (clinically infected). This finding suggested that there was a presence of subclinical infection in Group B (apparently healthy) that can be exacerbated with stress. Besides that, this also indicative that the total CFU in Group A might be affected by the virulency of pathogenic bacteria that might predominate the bacterial communities in the visceral organs instead of the normal microbiota.

6.0 CONCLUSIONS

The common types of marine bacteria species in Asian seabass (*Lates calcarifer*, Bloch 1790) cultured in floating net-cages at Pulau Ketam, Selangor, Malaysia are *V. alginolyticus, St. maltophilia, Ph. damselae and Sh. putrefaciens.* There was a low bacterial load, CFU/g (below 10^6) in the samples of kidney, liver and intestines taken from Asian seabass whereby the total CFU in the intestine was higher compared to the total CFU of the kidney and liver in both groups (A and B).

7.0 RECOMMENDATION

For future research, it is recommended to increase the sample size to more than 10 samples to obtain an accurate average or mean calculated and it will also help to identify the outliers. The actual estimated sample size is 382 fish samples for fish population size of 60,000 with 95% confidence level. Next, it is better to balance out the numbers of clinically infected and apparently healthy in their respective group with the ratio of 1:1 to acquire a greater comparison between those two groups. Besides that, it is advisable to retrieve the fish samples from distinctive locations and at different times and seasons because these variables will affect the presence of common bacteria as well as the bacterial load in the fish samples. Moreover, the hygiene of the equipment and the surroundings need to be taken care of to prevent contamination of the fish samples that may affect the data collection and analysis.

Furthermore, a recommendation suggested to the fish farmers in order to control disease outbreak is to practice good farm management by properly cleaning the net-cages especially before starting a new rearing cycle and prevent from feeding the trash fish diet too frequently to the fishes. Consumers on the other hand should clean and cook the fish properly before consuming the fish meat. Farmers also need to appropriately clean and cover up any open wound before handling fishes to prevent wound infection by zoonotic bacteria. In addition, the policy makers and the authorities need to promote sustainable aquaculture practice by encouraging everyone particularly the farmers to practice the use of vaccines by helping them to establish their own vaccination programme.

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APPENDIX A

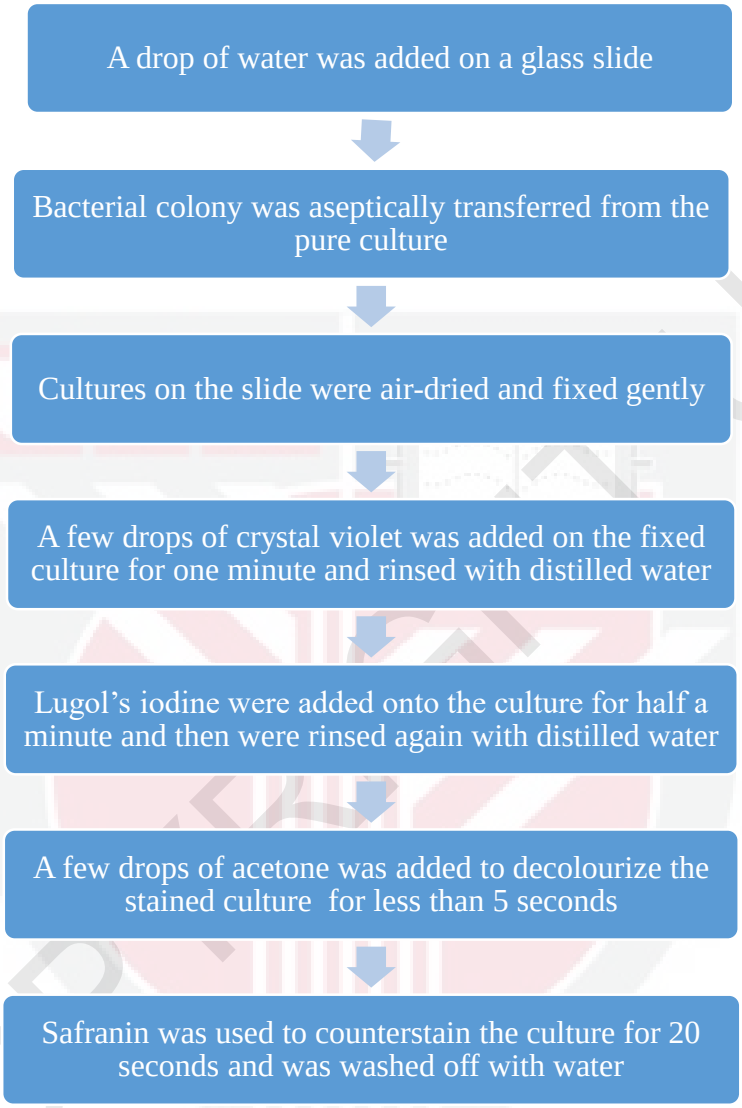

Figure 14: Gram staining steps on the bacteria inoculum for cell morphology examination.

APPENDIX B

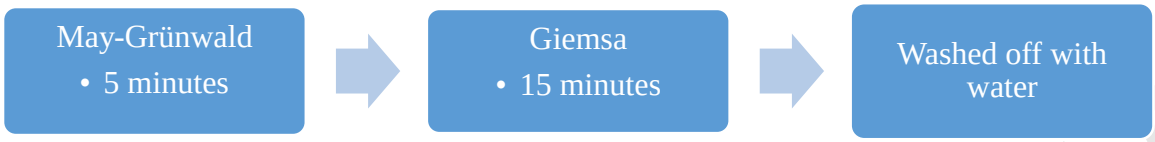

Figure 15: May-Grünwald + Giemsa staining steps for the squashed smear of the organ samples.