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**EVALUATION OF ANTIMICROBIAL PROPERTIES OF PRONUTRI+
AGAINST *A. HYDROPHILA* AND *V. ALGINOLYTICUS*.**

By

WONG CHIN HUI

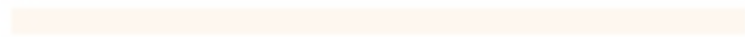
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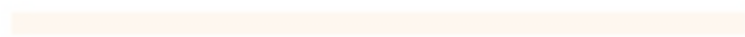
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ABSTRACT

The bacterium, *Aeromonas hydrophila*, is a pathogen that can cause Epizootic Ulcerative Syndrome and Motile Aeromonas Septicemia (MAS) in freshwater fish, including ornamental fish, tilapia, and catfish. Meanwhile, *Vibrio alginolyticus* is a high mortality, gram negative bacteria found in brackish and marine environments. Both pathogens are zoonotic and would result in gastroenteritis, septicemia and subcutaneous bruising in humans. The colonization of these bacteria could be controlled by oxytetracycline. However, it was reported that these bacteria began to develop resistance towards oxytetracycline. To aid this situation, products such as ProNutri + were marketed. This product initiates the use of phytotherapy via organic compounds as an immune booster and to inhibit colonization of aquatic pathogens. The purpose of this study was to determine whether ProNutri+, as an organic substitute for oxytetracycline, has a larger zone of growth inhibition against *A. hydrophila* and *V. alginolyticus* at varying dilutions. Other than that, this study was also conducted to determine the antimicrobial components of ProNutri+ which are efficient for controlling *A. hydrophila* and *V. alginolyticus* infection. Both *A. hydrophila* and *V. alginolyticus* samples were obtained from previous research projects. These bacteria samples were isolated from tilapia and sea bass from Kelantan, Malaysia. The samples were then used to conduct Agar Well Diffusion Test (AWDT) and Disc Diffusion Test (DDT) for Antimicrobial Sensitivity Test (AST) with oxytetracycline diffusion disc as a control. Meanwhile, the ProNutri + solution was also used for GC-MS qualitative analysis. Based on the result, ProNutri + shows no inhibitory effect towards both *A. hydrophila* and *V. alginolyticus*. From the GC-MS result, this product contains phytochemicals such as cyclotrisiloxane, isopropyl myristate, naphthalene, cyclobutane, 1,1'-(1,2-cyclobutanediyl), benzophenone, methylene chloride, arsenous acid which have a broad spectrum antimicrobial effect. However, these components are not proven effective to inhibit colonization of *A. hydrophila* and *V. alginolyticus*.

Keywords: *Aeromonas hydrophila*, *Vibrio alginolyticus*, ProNutri +, Agar Well Diffusion Test, GC-MS

ABSTRAK

Bakteria *Aeromonas hydrophila* merupakan patogen yang boleh menyebabkan Epizootic Ulcerative Syndrome dan Motile *Aeromonas* Septicemia (MAS) pada ikan air tawar, termasuk ikan hiasan, tilapia, dan ikan keli. Selain itu, *Vibrio alginolyticus* merupakan bakteria Gram negatif yang mempunyai kadar kematian yang tinggi dan dijumpai di persekitaran payau dan marin. Kedua-dua patogen ini akan menyebabkan penyakit zoonotik dan mengakibatkan gastroenteritis, septisemia dan lebam subkutaneus pada manusia. Penjajahan bakteria ini boleh dikawal menggunakan oxytetracycline. Walau bagaimanapun, bakteria ini telah dilaporkan mempunyai daya tahan terhadap Oxytetracycline. Untuk membantu keadaan ini, produk seperti ProNutri + telah dipasarkan dengan penggunaan fitoterapi melalui sebatian organik sebagai penggalak imun untuk menghalang penjajahan patogen akuatik. Tujuan kajian ini adalah untuk menentukan sama ada ProNutri+, sebagai pengganti organik untuk oxytetracycline, mempunyai zon perencatan pertumbuhan yang lebih besar terhadap *A. hydrophila* dan *V. alginolyticus* pada pencairan yang berbeza-beza. Selain itu, kajian ini juga dijalankan untuk menentukan komponen antimikrob ProNutri+ yang berkesan untuk mengawal jangkitan *A. hydrophila* dan *V. alginolyticus*. Sampel *A. hydrophila* dan *V. alginolyticus* telah diperolehi daripada projek penyelidikan terdahulu. Sampel bakteria ini telah diasingkan daripada tilapia dan siakap dari Kelantan, Malaysia. Sampel tersebut kemudiannya digunakan untuk menjalankan Agar Well Diffusion Test (AWDT) dan Disc Diffusion Test (DDT) untuk Antimicrobial Sensitivity Test (AST) dengan cakera resapan oxytetracycline sebagai kawalan. Sementara itu, penyelesaian ProNutri+ juga telah digunakan untuk menjalankan analisis kualitatif GC-MS. Daripada hasilnya, ProNutri + tidak menunjukkan kesan perencatan terhadap kedua-dua *A. hydrophila* dan *V. alginolyticus*. Dikesan oleh GC-MS, produk ini mengandungi fitokimia seperti cyclotrisiloxane, isopropyl myristate, naphthalene, cyclobutane, 1,1'-(1,2-cyclobutanediyl), benzophenone, methylene chloride, arsenous acid yang mempunyai kesan antimikrob spektrum luas. Tetapi, komponen ini tidak terbukti berkesan untuk menghalang penjajahan *A. hydrophila* dan *V. alginolyticus*.

Kata kunci: *Aeromonas hydrophila*, *Vibrio alginolyticus*, ProNutri +, Agar Well Diffusion Test, GC-MS

CERTIFICATION

This is to certify that we have read this research paper entitled '*Evaluation of antimicrobial properties of ProNutri+ against A. hydrophila and V. alginolyticus*' by **Wong Chin Hui**, and in our opinion, it is satisfactory in terms of scope, quality, and presentation as partial fulfillment of the requirements for the course DVT 55204 – Research Project.

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DEDICATIONS

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Figure 4: Methods for Agar Well Diffusion Test.

LIST OF ABBREVIATIONS

API	-	Analytical Profile Index
AST	-	Antimicrobial Sensitivity Test
AWDT	-	Agar Well Diffusion Test
DDT	-	Disc Diffusion Test
GC-MS	-	Gas Chromatography Mass Spectrophotometry
MAS	-	Motile Aeromonas Septicemia
MHA	-	Mueller Hinton Agar
OTC	-	Oxytetracycline
PCC	-	Per Capita Consumption
ROS	-	Reactive Oxygen Species
SSR	-	Self Sufficiency Level
TCBS	-	Thiosulfate citrate bile salts
TSA	-	Tryptic Soy Agar
TSB	-	Tryptic Soy Broth

CHAPTER 1

1.0 INTRODUCTION

From 2017 to 2022, Malaysia's fisheries self-sufficiency ratio (SSR) has decreased from 92.7 to 90.2 % (Department of Fisheries Malaysia, 2022). However, Per Capita Consumption (PCC) of fish in Malaysia remains as high as 46.22 % due to the acknowledgement of fish as a healthier choice of source of protein. The Balance of Trade (BOT) of fishes in Malaysia is RM - 3.13 billion in 2022, indicating the importation of fish is greater than exportation (Department of Fisheries Malaysia, 2022). The main resource of fisheries was obtained from catching (69.2%) while another from aquaculture (30.4%) (Department of Fisheries Malaysia, 2022). Other than fish for consuming purposes, ornamental fish aquaculture is another sector which is important because Malaysia is currently the 8th massive world producer of ornamental fish and exports 70% of the fishes results Malaysia to devote 9% to global ornamental fish trade (Anjur et al., 2021). Therefore, to boost the SSR of the fisheries industry and minimize importation of fish, we must explore and search for a solution for the main problems that arose among marine fisheries and freshwater aquaculture. The main problems include water source pollution, shortage of quality fish seed and disease infections. Common bacterial infection in Malaysia's brackish water fish is *Vibrio spp.*. Meanwhile, the freshwater fishes were commonly infected with *Aeromonas hydrophila*, *Pseudomonas* and *Edwardsiella spp.* (Anjur et al., 2021).

Aeromonas hydrophila is a gram negative, rod shaped, motile, positive oxidase and catalase, facultative anaerobe and glucose fermenting bacteria. Commonly found in freshwaters, this bacterium leads to a disease known as Motile Aeromonas Septicemia (Anurag et al., 2023). The aerolysin cytotoxic enterotoxin of *A. hydrophila* results in

destruction of tissues from shallow to hemorrhagic gray lesion and progresses to skin and muscle necrosis. With a prevalence percentage up to 31.6% (Hala et al., 2024) and average mortality rate as high as 30% (Olga et al., 2023)., this bacterium also causes skin ulceration, abscessation of internal organs, renal tubule necrosis and erythrolysis. It may also lead to tail rot, fin rot or gills and eye lesions.

Other bacteria commonly found in marine environments include *Vibrio alginolyticus*. *Vibrio alginolyticus* is a gram negative, rod shaped, highly motile, positive oxidase and catalase, facultative anaerobe and sucrose fermenting bacteria which grows on Thiosulfate citrate bile salts (TCBS) (Burak et al., 2015). Vibriosis in fish will be observed with signs such as lethargy, loss of appetite, changes in body color, increased eye opacity, skin and fin ulceration (Tilusha et al., 2023). Malaysia's Asian Seabass *Lates Calcarifer* infected with *V. alginolyticus* will have a mortality rate of 100% (Nurliyana et al., 2019).

Due to the high mortality rate in fishes, Oxytetracyclines, quinolones and other broad-spectrum antibiotics were administered to the fishes suspected to be infected with *A. hydrophila* while waiting for the bacteria culture and antibiotic susceptibility test result (Reham, 2023). Other medications suggested to be provided include immunostimulants , phytobiotics, probiotics, vaccinations and supplements to minimize the mortality rate in fishes (Salah et al., 2023) (Anjur et al., 2021). Oxytetracycline, quinolones and trimethoprim were widely used to control and treat *V. alginolyticus* (Nurliyana et al., 2019). Meanwhile, every *Vibrio spp.* in Malaysia was shown to be highly resistant to Ampicillin (Nurliyana et al., 2019). Due to slowly progressing antibiotic resistance in fishes to treat *A. hydrophila* and *V. alginolyticus*, new therapeutic strategies had to be developed with less side effect on fish

products, less impact to other aquatic animals and animals in the wild as well as to prevent mortality of fishes and reduce the loss in aquaculture and catching fisheries sector.

Ecojubilee Aquaculture Conglomerate Resources is a company originated from Klang, Malaysia which is providing consultancy services to aquaculture farms delivered by their expert in aquaculture and fish diseases. Other than that, the company is also actively developing high-quality fish supplements which aims to enhance growth by boosting immunity and nutrient absorption. One of the most renowned products is ProNutri+ which claims to be an immunostimulant to boost the health and immunity of fishes and improves feeding and growth performances in fishes. Hence, these effects could be effective to inhibit *Aeromonas spp.* and *Vibrio spp.* infection. This product contains nucleotides, glutamic acids, peptides, functional nutrients from yeast, fruit extract, zinc and proteinate. Therefore, this study aims to assess the antimicrobial properties of ProNutri + against *A. hydrophila* and *V. alginolyticus* infection.

1.1 RESEARCH PROBLEM STATEMENT

It is a key to discover alternative antimicrobials against *A. hydrophila* and *V. alginolyticus* due to the high mortality rate caused by the disease and progression of antibiotic resistance by the bacteria. Moreover, due to the fear of antibiotic residue in fish products, impact to other aquatic animals or wildlife animals, it is crucial to develop a product which is organic and does not contain harmful chemicals. It was reported that fish fed with diets supplemented with nucleotides and yeast extract were able to gain better protection against *Aeromonas spp.* and *Vibrio spp.* infection (David et al., 2017).

1.2 RESEARCH QUESTIONS

1.2.1 What is the inhibitory effect of ProNutri+ at different dilutions as an antimicrobial against *A. hydrophila* and *V. alginolyticus* compared to Oxytetracycline (OTC)?

1.2.2 Does ProNutri + contain antimicrobial components which are efficient against control and prevention of *A. hydrophila* and *V. alginolyticus* infection?

1.3 RESEARCH HYPOTHESIS

1.3.1 There is no difference in mean zone of growth inhibition between ProNutri+ with different dilutions and Oxytetracycline (OTC) against *A. hydrophila* and *V. alginolyticus*.

1.3.2 ProNutri+ contains antimicrobial components which are efficient for control of *A. hydrophila* and *V. alginolyticus* infection.

1.4 RESEARCH OBJECTIVES

1.4.1 To investigate whether ProNutri+ with different dilutions has a greater zone of growth inhibition towards *A. hydrophila* and *V. alginolyticus* for it to be an organic alternative to Oxytetracycline.

1.4.2 To determine the antimicrobial components of ProNutri+ which are efficient for controlling *A. hydrophila* and *V. alginolyticus* infection.

CHAPTER 2

2.0 LITERATURE REVIEW

2.1 *Aeromonas spp.* infection in freshwater fish.

Aeromonas spp. was once classified under Vibrionaceae, hence, they are closely related and similar to *Vibrio spp.* such as having similar biochemical features and living in similar aquatic ecosystems (Liu, 2015). *Aeromonas spp.* is a gram negative, facultative anaerobic rod, non-spore forming, motile, single polar flagellated and glucose fermenting bacteria. It is commonly isolated from freshwater and marine fish, infecting both homeothermic and poikilothermic fishes, such as catfish, tilapia and other ornamental fishes (Anjur et al., 2021).

The *Aeromonas spp.* could be identified by Analytical Profile Index (API) 20E to identify Gram negative enteric bacteria and MicroScan W/A to quickly identify aerobic bacteria (Olga et al., 2023). Among the *Aeromonas spp.* identified from an aquarium in Malaysia, 60% of them are infected with *Aeromonas hydrophila* (Musa et al., 2008).

This ubiquitous bacterium pathologically affects the fishes when they are in contact with affected fish under heavily stressful or polluted aquatic environments (Carl & Lluís, 2016). This includes overcrowding, lack of dissolved oxygen and changes in aquatic temperature. As the corticosteroid level spikes in stressed fishes, there will be inhibition of lymphocytes to produce immunoglobulins or antibodies on their cell surface along with suppression of leukocytosis, resulting in immunosuppression (Carl & Lluís, 2016). In stressed conditions, bacteria's virulence factors such as structural component and secretion system allows them to

adhere and invade host cells as well as escape from host immune defenses, compete for nutrients and cause host cell damage and infection (Carl & Lluís, 2016).

Most prominent clinical signs observed in fish infected with *A. hydrophila* include pale gills, abnormal swimming patterns, ascites, exophthalmos, septic skin ulceration and hemorrhagic septicemia (Bashir et al., 2023). *Aeromonas hydrophila* will result in Motile Aeromonas Septicemia (MAS) and Epizootic Ulcerative Syndrome (Anjur et al., 2021). The average mortality rate for *A. hydrophila* is as high as 30% (Olga et al., 2023). Oxytetracycline and terramycin (2.5 - 3.75 g/100 lb fishes added in aquatic feed per day for 10 days) were most commonly used antibiotics for *Aeromonas spp.* (Anurag et al., 2023)

2.2 *Vibrio spp.* infection in brackishwater / marine fish.

Vibrio spp. is a gram negative, positive oxidase, polar flagellated, rod-shaped bacteria. It is ubiquitous in brackish and marine environments. It could be identified through bacteria isolation using Trypticase Soy Agar (TSA) containing 1% of sodium chloride. This is because the *Vibrio spp.* strains highly dependent on water salinity of maximum 0.5% to 1.5 % along with maximum 30°C for survival. Meanwhile, the specific species of *Vibrio spp.* can be identified using the API 20E. It was stated that the prevalence of *Vibrio vulnificus* among groupers in Malaysia were as high as 33% from the total *Vibrio spp.* isolated. Meanwhile, the groupers were also highly infected with *Vibrio alginolyticus* up to 24% (Nor Zulkipli et al., 2019). Both *Vibrio spp.* was highly infectious and pathogenic to fishes in Malaysia.

These *Vibrio spp.* contains capsular polysaccharides, loose slime as an adhesive factor and flagella which aids them to adhere to the host tissues and starts to form biofilm for colonization. Beginning by colonizing the mouth, gills and skins, *V. alginolyticus* progresses

to colonize the intestine. *V. alginolyticus* adheres and penetrates the intestinal mucosa more severely compared to the skin (Tilusha et al., 2023). Lacking M cells and Peyer patches like other mammals, the bacteria were phagocytized by the enterocytes before engulfed by the neutrophils (Tilusha et al., 2023). Translocation of *V. alginolyticus* happens intracellularly through penetration of adjacent enterocytes or paracellularly via loosening of cellular junction (Finn, 2013).

Clinical signs of fishes infected with *V. alginolyticus* include dark coloured skin, exophthalmia, cloudy corneal and skin surface ulceration (Esraa et al., 2024). There would also be presence of peritoneal fluids and hemorrhagic livers. In experimental conditions, Malaysia's Asian Seabass *Lates Calcarifer* infected with *V. alginolyticus* revealed a mortality rate of 100% (Nurliyana et al., 2019). Other than vaccination via Outer membrane protein K (OmpK) vaccine (Santha et al., 2020), the bacteria could be prevented in freshwater environments and below 26°C (Olga et al., 2023).

2.3 Zoonotic effect of *Aeromonas spp.* and *Vibrio spp.*

Aeromonas spp. could be transmitted to humans via open wound or ingestion of contaminated water, food or feces. A day after the bacteria gains entry to the skin from the wounds, the infected wound will be observed to be having purulent discharges and edematous (Olga et al., 2023). If the wound was left untreated the infection will progress and result in necrotizing fasciitis, myositis and osteomyelitis. Apart from that, *Aeromonas spp.* infection in humans also leads to gastroenteritis and subcutaneous bruising (Princeton University, 2022). Meanwhile, humans would get infected with *V. alginolyticus* due to consumption of brackish water or marine fishes. These bacteria would trigger the inflammatory response in humans

via inflammasome and also lead to gastroenteritis, septicemia and otitis in humans (Wang et al., 2021).

2.4 Active ingredients in ProNutri +.

ProNutri + contains nucleotides, glutamic acids, peptides, functional nutrients from yeast, fruit extract (orange, apple, banana, grapefruit, lemon and avocado), zinc and proteinate. Nucleotides function for DNA and RNA synthesis for cellular metabolism such as ATP, cAMP and NAD production. Therefore, nucleotides are a semi essential supplement for fishes to enhance fish innate immune response through boosting their lysozyme activity, enhancing healthy gastrointestinal system by increasing the intestinal fold and microvilli height (Dina, 2017). Meanwhile, glutamic acid, also known as glutamate, is an amino acid which functions to improve gastrointestinal performances and as an immunostimulant (Seerengaraj et al., 2023). Antimicrobial peptides were also used as supplements for boosting the immunity related genes in the gastrointestinal system while enhancing the antioxidant and digestive enzymatic activity (Wang et al., 2022). Other than that, the yeast nutrients within the product also function as antioxidants as well as enhance gastrointestinal immunity and physical barriers by promoting the gene for tight junction protein of the enterocytes (Cai et al., 2023). Meanwhile, zinc oxides supplementation has 70 -80 % ability to minimize the biofilm activity of *Aeromonas spp.*, hence, with the breaching of biofilm the efficacy of antibiotic reactions will increase and the *Aeromonas spp.* could be eliminated (Gopi et al., 2021).

2.5 Alleviating *Aeromonas spp.* and *Vibrio spp.* via organic compounds.

Compared to chemical drugs, phytotherapy using organic compounds is environmentally friendly, more affordable and more efficient to minimize the pathogenic

effect of pathogens (Anjur *et al.*, 2021). Many aquatic supplements contained organic compounds as immunostimulants as these organic compounds not only function to boost immunoglobulin levels and phagocytic activity but they are also efficient in stimulating appetite, relieving stress and good in promoting growth (Anurag *et al.*, 2023). Apart from prevention, these organic compounds also function to increase the survival percentage of post *Aeromonas spp.* infected juveniles whose kidney function has already been damaged. Examples of plants involved in immunostimulating fishes for *A. hydrophila* include *Citrus medica* which has an antifungal and antibacterial effect, paper flowers which functions to increase the post infection survival rate of *Cyprinus carpio* to 67.74% (Sib *et al.*, 2023) and ginger for *Oncorhynchus mykiss* which could reduce the mortality rate of *A. hydrophila* fishes to 0% (Nya & Austin, 2009). Meanwhile, the mortality rate of *Oreochromis mossambicus* infected with *Vibrio spp.* were observed to be reduced to 63 to 80% after feeding with golden apple extract (*Aegle marmelos*) (Immanuel *et al.*, 2009).

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 ETHICAL CONSIDERATIONS

No ethics application required for this study, as the *Aeromonas hydrophila* and *Vibrio alginolyticus* samples were obtained from archive samples from a previous research project.

3.2 SAMPLE COLLECTION AND PREPARATION

Aeromonas hydrophila and *Vibrio alginolyticus* samples were obtained from previous archived samples isolated from tilapia (*Oreochromis niloticus*) and seabass (*Lates calcarifer*) from Kelantan, Malaysia. In this study, the archived bacteria samples were used for detecting antimicrobial activity and properties of ProNutri+ using Antimicrobial Sensitivity Test which includes Agar Well Diffusion Test and Disc Diffusion Test.

3.3 Preparation of Tryptic Soy Agar (TSA).

Based on the instruction from OXOID, Tryptic Soy Agar was prepared by adding 40 g of TSA into 1 L of distilled water. For *V. alginolyticus*, 1.5 g NaCl was added to TSA. Then, the medium was boiled until all the powder had completely dissolved. The medium was autoclaved for 15 minutes at 121°C. After that, the medium was poured in the petri dishes (Figure 1).

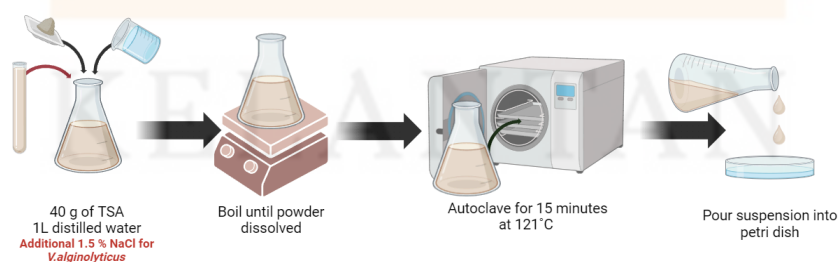


Figure 1: *Methods of preparation of Tryptic Soy Agar (TSA).*

3.4 Preparation of Tryptic Soy Broth (TSB).

Based on the instructions from OXOID, the TSB solution was prepared by mixing 30g of dehydrated media and 1.5 g NaCl were added into 1 litre distilled water. The solution was then autoclaved for 15 minutes at 121°C before pouring into sterile glass vials.

3.5 Preparation of Mueller Hinton Agar (MHA).

Based on the instructions from OXOID, MHA was prepared by adding 38 g of MHA to 1 L of distilled water. For *V. alginolyticus*, 1.5 g NaCl was added to MHA. Then, the medium was boiled until all the powder had completely dissolved. The medium was then autoclaved for 15 minutes at 121°C before pouring into the petri dishes (**Figure 2**).

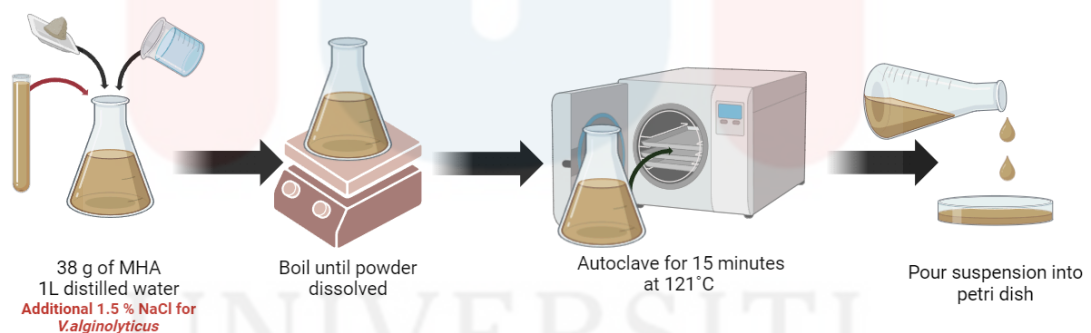


Figure 2: *Methods of preparation of Mueller Hinton Agar (MHA).*

3.6 *Aeromonas hydrophila* and *Vibrio alginolyticus* inoculum revival and collection.

Aeromonas hydrophila and *V. alginolyticus* were retrieved from the archived samples stored in the Zoonotic Laboratory, Faculty of Veterinary Medicine, Universiti Malaysia Kelantan. The archived samples were kept in -80°C in the Trypticase Soy Broth (TSB) along with 50% glycerol. To revive the *A. hydrophila* sample, the sample was streaked onto the Tryptic Soy Agar (TSA). After 24 hours, Then, a loop full of inoculum was retrieved and

inoculated into 2 - 3 ml of physiological saline solution. The inoculum was then compared with 0.5 McFarland Standard solution before utilization for the antimicrobial sensitivity test.

Meanwhile, to revive *V. alginolyticus*, a loop full of samples was added into TSB with an additional 1.5% NaCl. This follows by incubation for 24 hours at 30°C. After 24 hours, a loop full of samples was retrieved and streaked onto Chrom agar and incubated for 24 hours at 30°C. Then, a loop full of cream colored colonies, indicative of *V. alginolyticus*, was retrieved and inoculated into 2 - 3 ml of physiological saline solution (Anja, 2022). The inoculum was then compared with 0.5 McFarland Standard solution before utilization for the antimicrobial sensitivity test (**Figure 3**).

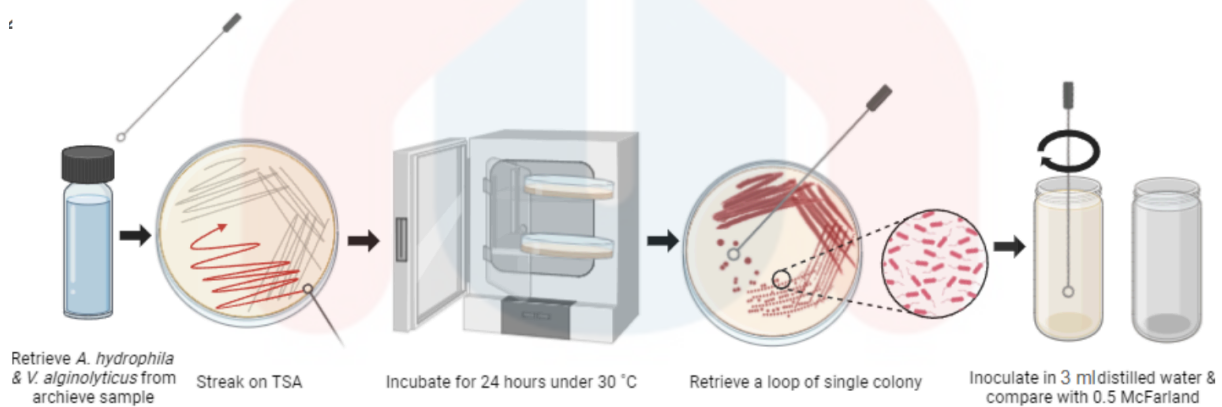


Figure 3: Methods to revive *Aeromonas hydrophila* and *Vibrio alginolyticus*.

3.7 Control antibiotics.

Being the most common antibiotic used to treat aquatic diseases, Oxytetracycline (OTC 30 µg, Oxoid) was used as control antibiotics in this project (Jarod, 2020).

3.8 Agar Well Diffusion Test for *Aeromonas hydrophila* and *Vibrio alginolyticus*.

The *A. hydrophila* isolates were spreaded onto a Mueller-Hinton agar (MHA) plate. Meanwhile, *Vibrio alginolyticus*, a sterile cotton swab, was streaked evenly onto MHA with

an additional 1.5 % of NaCl. Then, a sterile cork borer with diameter of 6 mm was used to bore 4 wells on each agar plate (Marina et. al., 2020). After that, 50 μ l of ProNutri + with different dilutions (25%, 50%, 75% and 100%) were pipetted into the respective wells. Meanwhile, an Oxytetracycline diffusion disc was placed at the center of the MHA plate, surrounded by 4 wells. The plate was then kept in the incubator at 37°C for 24 hours. After 24 hours of incubation, the diameter of the zone of inhibition (mm) for each well was measured by a calliper (**Figure 4**).

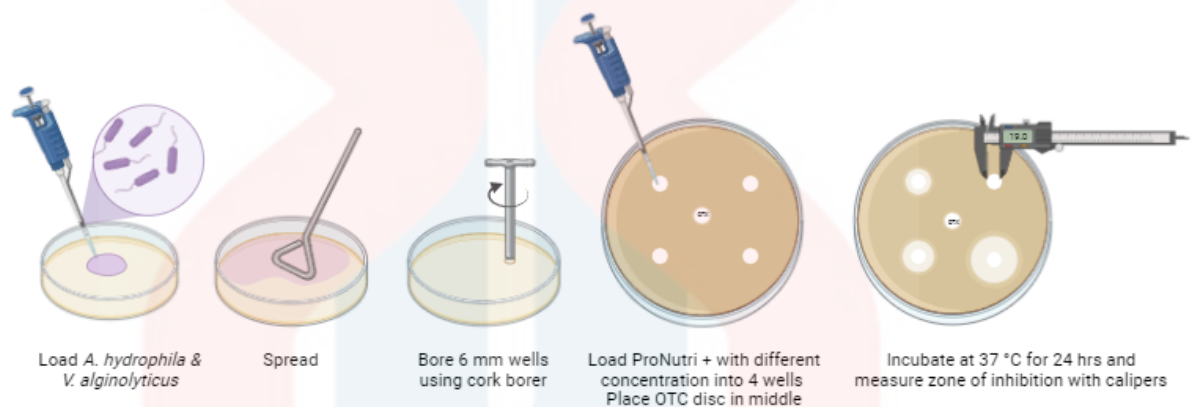


Figure 4: Methods for Agar Well Diffusion Test.

3.9 Disc Diffusion Test for *Aeromonas hydrophila* and *Vibrio alginolyticus*.

Diffusion discs were made by punching holes on diffusion paper using a hole puncher. The discs were then kept in a glass vial and autoclaved for 15 minutes at 121°C. The discs were then soaked into 4 centrifuge tubes containing different dilutions (25%, 50%, 75% and 100%) of Pro Nutri + for 10 minutes (FWD AMR, 2022). The *A. hydrophila* isolates were streaked onto a Mueller-Hinton agar (MHA) plate. Meanwhile, *Vibrio alginolyticus* was streaked evenly onto MHA with an additional 1.5 % NaCl. After that, the discs were retrieved using sterile forceps and placed onto each MHA plate at 4 different locations surrounding the OTC diffusion disc. After 24 hours of incubation, the diameter of the zone of inhibition (mm) for each disc was measured by a calliper.

3.10 Gas Chromatography Mass Spectrophotometry (GC-MS) Profiling.

Gas Chromatography Mass Spectrophotometry (GC-MS) was used to determine the antimicrobial compounds of ProNutri+. The crude extracts of ProNutri+ were separated by using HP5-MS fused capillary GC column (30 m x 0.32 mm (ID) x 0.25 μ m film thickness) along with Agilent GC system which includes Agilent 600 MS (Harish *et.al.*, 2024). Helium gas with a flow rate of 1.0 mL/min was utilized as a carrier. Following that, the temperature of the injection port was set as 280°C, and detector temperature was set as 300°C (Harish *et.al.*, 2024). Meanwhile, the GC temperature profile was set with an initial temperature of 60 °C for 2 minutes, elevated at 10°C min⁻¹ before gradually increasing till 300°C for 6 minutes (Harish *et.al.*, 2024). After GC setting, 1 μ L of ProNutri+ was injected into the splitless mode injection port, which has a solvent delay time of 3 minutes. SCAN mode of GC functions to detect the mass spectral data detected in a specific interval which is related to the voltage. Therefore, SCAN mode was selected during MS condition, with a source temperature of 240°C, transfer line temperature at 240°C, ionisation mode electron impact at 70 eV, scan period of 0.2 seconds and scan interval of 0.1 second (Harish *et.al.*, 2024). The ProNutri+ antimicrobial compounds were recorded based on the GC-MS result obtained.

CHAPTER 4

4.0 RESULTS

4.1 Antimicrobial Sensitivity Test

The antimicrobial sensitivity tests for both *A. hydrophila* and *V. alginolyticus* were repeated thrice by using the Agar Well Diffusion and Disc Diffusion method. Unfortunately, none of the concentrations (25%, 50%, 75% and 100%) showed a zone of inhibition compared to oxytetracycline (29.7 ± 1.25 mm).

4.2 Compounds found in ProNutri+ solution.

Based on the detection of chemical compounds in ProNutri+ solution from the GCMS method, ProNutri+ contains cyclotrisiloxane, hexamethyl ; isopropyl myristate, 1,3-butadiene, 1,4-diphenyl-, (E,E) ; naphthalene, 1-(1-cyclohexen-1-yl) ; naphthalene, 1,2,3,4-tetrahydro-1-phenyl ; cyclobutane, 1,3-diphenyl ; 2,6 - diisopropyl naphthalene ; [2,2] paracyclophane ; benzene, 1,1'-(1,2-cyclobutanediyl)bis-, cis ; benzophenone ; methylene chloride ; arsenous acid, tris(trimethylsilyl) ester.

CHAPTER 5

5.0 DISCUSSION

ProNutri+ contains nucleotides, glutamic acids, peptides, functional nutrients from yeast, fruit extract (orange, apple, banana, grapefruit, lemon and avocado), zinc and proteinate. Although ProNutri+ shows no inhibition effect towards both *A. hydrophila* and *V. alginolyticus*, there were few types of bioactive compounds extracted from GC-MS qualitative analysis which contain certain antimicrobial effects.

Cyclotrisiloxane could be found on peels of fruits such as orange, grapefruit, apple and avocado. Cyclotrisiloxane has a broad-spectrum antimicrobial, antifungal and antioxidant effect (Ismail et al., 2020). This phytochemical was proven to have an anti-biofilm effect towards 13 types of bacteria excluding *Aeromonas sp.* and *Vibrio sp.* (Dilek et al., 2012). This includes *Staphylococcus sp.*, *Bacillus sp.*, *Escherichia coli*, *Enterobacter sp.*, *Pseudomonas sp.*, *Klebsiella sp.* and *Candida sp.* Meanwhile, it is stated that isopropyl myristate extracted from *M. oleum* and *L. romanae* has an inhibition effect towards *Aeromonas hydrophila* and *Vibrio anguillarum* (da Cunha et al., 2018). However, this synthetic ester only functions to aid in antimicrobial effect by disrupting the bacterial wall and enhancing the penetration of other substances (Nandini et al., 2009).

Other chemical components found, such as Naphthalene and Cyclobutane, 1,3-diphenyl, could also disrupt the cell wall of *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Streptococcus faecalis* (Anastasia et al., 2008) (Rokade & Sayyed, 2009). Derivatives of Naphthalene will be degraded by *Vibrio cholerae*

(Abarian et al., 2018). Therefore, a high concentration of Naphthalene was required as a toxic dose to *Vibrio sp.* which is having a high reactive oxygen species (ROS) that could cause oxidative stress and cellular damage to *Vibrio sp.*

As a benzene derivative, 1,1'-(1,2-cyclobutanediyl) bis-, cis has the function to interfere with transcription of bacteria, resulting in inhibition of bacterial growth such as *Staphylococcus epidermidis*. It could also alter the biofilm formation of *Vibrio parahaemolyticus* (Yang et al., 2024). Meanwhile, benzophenone functions as an antimicrobial against both gram positive and negative bacteria by damaging the cell wall which leads to protein and cytoplasm leakage (Martha Isabel et al., 2019). Apart from that, ProNutri+ also contains methylene chloride which could alter the cell membrane structure to enter the cell and depletes the adenosine triphosphate pool by altering the enzymatic active site. However, methylene chloride is ineffective on *Bacillus cereus*, *Vibrio cholerae*, *Salmonella choleraesuis* and *Klebsiella pneumoniae* (Amal et al., 2013).

Arsenous acid, tris(trimethylsilyl) ester was proven to have an antimicrobial properties towards *Klebsiella aerogenes*, *K.pneumoniae*, *Staphylococcus epidermidis* and *Escherichia coli* (Barathikannan et al., 2016). The arsenic acids cross the cell membrane into the cell via transporter aquaglyceroporins will suppress activity of enzyme pyruvate dehydrogenase causing disability of pyruvate oxidation to acetyl-CoA resulting in energy disruption of the cell and apoptosis (Palacios and Capdevila, 2013).

GC-MS could detect trace amounts of phytochemicals in the sample solution. Most of the chemical compounds found in ProNutri+ solution have an indirect broad spectrum

antimicrobial effect. However, they were not proven to be able to inhibit the colonization of both *Aeromonas hydrophila* and *Vibrio alginolyticus*. This is because most of the compounds only disrupts the cell wall or organisms and aids in penetration of antimicrobials instead of directly causing a bacteriostatic or bactericidal effect (Anastasia et al., 2008). Therefore, they require synergistic effects with other antimicrobials to prevent colonization of bacteria.

Certain compounds, such as isopropyl myristate and naphthalene, were detected by GC-MS even in trace amounts. However, they could only inhibit colonization of *Aeromonas sp.* and *Vibrio sp.* in DDT at high concentrations which are highly toxic to aquatic animals. A high concentration of naphthalene could cause tissue damage, necrosis, cellular damage and ulceration resulting in retardation of growth, reproduction and physiological functions in fishes (Sukhendu, 2024).

Meanwhile, antioxidant components in ProNutri+ such as cyclotrisiloxane could minimize the oxidative stress in fishes resulting in improved fish health and growth. Most importantly, antioxidants could prevent apoptosis and improve in intestinal tight-junction complexes to prevent *Aeromonas hydrophila* infection (Yauheni & Wolf, 2024).

CHAPTER 6

6.0 CONCLUSION AND RECOMMENDATION

In conclusion, ProNutri+ does not contain antimicrobial properties against both *Aeromonas hydrophila* and *Vibrio alginolyticus*. The antimicrobial sensitivity test via Agar Well Diffusion Test (AWDT) and Disc Diffusion Test (DDT) reveals no inhibitory effect of ProNutri+ towards both *Aeromonas hydrophila* and *Vibrio alginolyticus* regardless of its concentration. However, Oxytetracycline (OTC) was proven to be effective in inhibiting colonization of both *Aeromonas hydrophila* and *Vibrio alginolyticus*. Even though ProNutri+ could not inhibit both *Aeromonas hydrophila* and *Vibrio alginolyticus* specifically, it is possible for this product to be used as antimicrobial for other bacteria such as *Bacillus cereus*, *Vibrio cholerae*, *Salmonella choleraesuis* and *Klebsiella pneumoniae*. The antioxidant compounds detected from GCMS qualitative analysis could boost the immunity of fishes by protecting their cells against oxidative damage. This study serves as a valuable reference and guidelines for future research regarding usage of phytochemicals as an antimicrobial alternative for *Aeromonas hydrophila* and *Vibrio alginolyticus*. Therefore, to achieve this, further studies and research such as *in vivo* research could be conducted.

7.0 APPENDICES

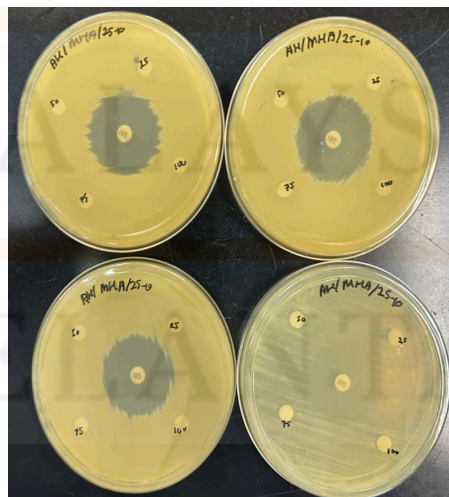
Appendix 1 Agar preparation.



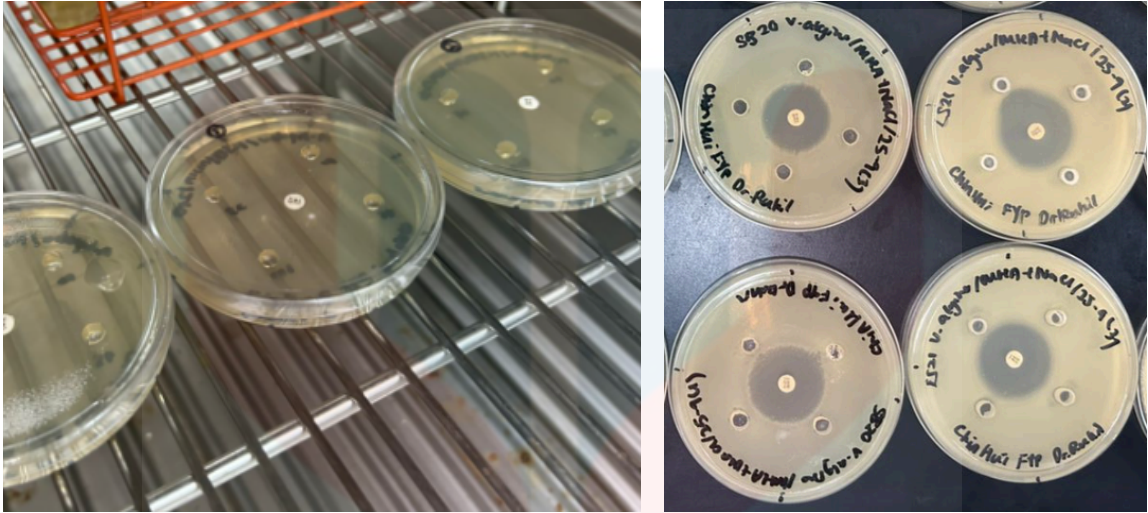
Appendix 2 Materials and equipment used.



Appendix 3 Disc Diffusion Test.



Appendix 4 Agar Well Diffusion Test.



FYP FPV



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