EVALUATION OF POTENTIAL PROBIOTIC *Bacillus velezensis* FS26 AGAINST AQUACULTURE PATHOGENS AND ITS ABILITY TO UTILISE PREBIOTICS FROM *Arthrospira platensis* A1

By

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Pathogenic bacteria such as *Vibrio* spp. and *Aeromonas* spp. frequently cause detrimental effects on aquaculture production. Antibiotics were previously utilised to treat infections, but this led to antibiotic-resistant bacteria emerging in the environment. Probiotics and prebiotics were introduced to boost the host's microbiota, disease protection, health condition, growth efficiency, feed consumption, stress response and overall vigour. Synbiotic is another approach that should be evaluated intensively to solve the infections problems. However, probiotic bacteria need to show the ability to utilise prebiotic for synbiotic preparation. Therefore, this study aims to evaluate the potential probiotic of selected bacteria against aquaculture pathogens and capability to use prebiotic from *Arthrospira platensis* A1. *Bacillus velezensis* FS26 (Genome accession number: JAOPEO000000000; 16S rRNA accession number: MZ960133) was isolated from giant freshwater prawn and showed good probiotic properties against pathogens in aquaculture through *in vitro* and *in silico* studies. Agar well diffusion assay exhibited the capability of this bacterium through diameter of inhibition against the pathogenic *Aeromonas hydrophila* LMG13658, *A. veronii* clone DK-*A.veronii*-27, *Vibrio campbellii* PKGL21, V*. alginolyticus* PKS15, *V. parahaemolyticus* PKK24 at 23.7, 25, 30, 13.3 and 12.3 mm, respectively. This bacterium is regarded as harmless based on susceptibility towards 13 antibiotics, γ-haemolytic activity in blood agar and *in silico* prediction for the lack of 96 antibiotic resistance genes, pathogenic genes and virulence factors. Additionally, *B. velezensis* FS26 has shown the capability to utilise commercial prebiotics such as lactulose, raffinose and inulin through *in silico* and *in vitro* investigation. The bacterial genome predicted six enzymes that could hydrolyse glycosidic bonds in prebiotics, such as α-galactosidase, invertase, endo-levanase, β-2,6-fructan-6-levanbiohydrolase and levansucrase. Moreover, using prebiotics from aquaculture sources such as microalgae could enhance the palatability of the synbiotic. Hence, water-soluble nondigestible polysaccharide was extracted from *Arthrospira platensis* A1 as a prebiotic for *B. velezensis*

FS26. The structure of water-soluble polysaccharides demonstrated the presence of α-glycosidic bonds at wavelength 860 nm and water-soluble compounds at wavelength between 300 to 400 nm through FTIR and UV-VIS spectroscopy analysis. In the outcome, water-soluble nondigestible polysaccharide extracted using autoclave exhibited a promising prebiotic activity towards potential probiotic *B. velezensis* FS26 by significantly enhance the bacterial growth on enriched and minimal media at 0.6 x 10^9 CFU/mL and 7.1 x 10^9 CFU/mL, compared to control. In conclusion, the findings demonstrate the capability of probiotic *B. velezensis* FS26 to inhibit the aquaculture pathogens and utilise prebiotic from *A. platensis* A1.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENILAIAN POTENSI PROBIOTIK *Bacillus velezensis* FS26 TERHADAP PATOGEN AKUAKULTUR DAN KEUPAYAANNYA UNTUK MEMANFAATKAN PREBIOTIK DARI *Arthrospira platensis* A1

Oleh

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Bakteria patogen seperti *Vibrio* spp. dan *Aeromonas* spp. sering menyebabkan kesan buruk terhadap pengeluaran akuakultur. Antibiotik sebelum ini digunakan untuk merawat jangkitan, tetapi ini menyebabkan bakteria yang tahan terhadap antibiotik muncul dalam alam sekitar. Probiotik dan prebiotik diperkenalkan untuk meningkatkan mikrobiota hos, perlindungan terhadap penyakit, keadaan kesihatan, kecekapan pertumbuhan, pengambilan makanan, respons tekanan, dan kesegaran keseluruhan. Pendekatan Synbiotic adalah satu lagi pendekatan yang perlu dievaluasi secara intensif untuk menyelesaikan masalah jangkitan. Walau bagaimanapun, bakteria probiotik perlu menunjukkan keupayaan untuk menggunakan prebiotik dalam persediaan synbiotik. Oleh itu, kajian ini bertujuan untuk menilai potensi probiotik bakteria terpilih terhadap patogen akuakultur dan keupayaannya menggunakan prebiotik dari *Arthrospira platensis* A1. *Bacillus velezensis* FS26 (Nombor akses genom: JAOPEO000000000; Nombor akses 16S rRNA: MZ960133) diasingkan dari udang air tawar raksasa dan menunjukkan sifat probiotik yang baik terhadap patogen dalam akuakultur melalui kajian in vitro dan in silico. Ujian penyebaran sumuran agar menunjukkan keupayaan bakteria ini melalui diameter perintangan terhadap *Aeromonas hydrophila patogen* LMG13658, klon *A. veronii* DK-A.veronii-27, *Vibrio campbellii* PKGL21, *V. alginolyticus* PKS15, *V. parahaemolyticus* PKK24 pada 23.7, 25, 30, 13.3, dan 12.3 mm, masing-masing. Bakteria ini dianggap tidak berbahaya berdasarkan kerentanannya terhadap 13 antibiotik, aktiviti γ-hemolitik dalam agar darah, dan ramalan *in siliko* untuk ketiadaan 96 gen rintangan antibiotik, gen patogen, dan faktor virulensi. Tambahan pula, *B. velezensis* FS26 telah menunjukkan keupayaan untuk menggunakan prebiotik komersial seperti laktulosa, rafinosa, dan inulin melalui penyelidikan *in siliko* dan *in vitro*. Genom bakteria meramalkan beberapa enzim yang boleh menghidrolisis ikatan glikosida dalam prebiotik, seperti α-galaktosidase, invertase, endo-levanase, β-2,6-fructan-6-levanbiohydrolase, dan levansucrase. Selain itu,

menggunakan prebiotik dari sumber akuakultur seperti mikroalga dapat meningkatkan kesedapan sinbiotik. Oleh itu, polisakarida tak larut dalam air telah diekstrak dari *Arthrospira platensis* A1 sebagai prebiotik untuk *B. velezensis* FS26. Struktur polisakarida tak larut dalam air menunjukkan kehadiran ikatan α-glikosida pada panjang gelombang 860 nm dan sebatian larut dalam air pada panjang gelombang antara 300 hingga 400 nm melalui analisis spektroskopi FTIR dan UV-VIS. Hasilnya, polisakarida tak larut dalam air yang diekstrak menggunakan autoklaf menunjukkan aktiviti prebiotik yang menjanjikan terhadap pertumbuhan bakteria *B. velezensis* FS26 yang berpotensi pada media yang diperkayakan dan minimal pada 0.6 x 10^9 CFU/mL dan 7.1 x 10^9 CFU/mL, berbanding dengan kawalan. Penemuan ini menunjukkan keupayaan probiotik *B. velezensis* FS26 untuk menghalang patogen akuakultur dan menggunakan prebiotik dari *A. platensis* A1.

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LIST OF ABBREVIATIONS

kg	Kilogram
k	Kilo
g	Gram
bp	Base pair
min	Minute
s	Second
h	Hour
d	Day
˚C	Degree Celsius
sp.	Species (one)
spp.	Species (two or more)
rpm	Rotation per minute
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
PCR	Polymerase chain reaction
CFU	Colony forming unit
OD	Optical density
L	Litter
µL	Microliter
mL	Milliliter
mg	Milligram
USD	United States dollar
UK	United Kingdom
FAO	The Food and Agriculture Organization of the United Nations

%	Percent
mg/mL	Milligram per millilitre
SD	Standard deviation
nm	Nanometer
CAZy	Carbohydrate-active enzymes
antiSMASH	The Antibiotics and Secondary Metabolite Analysis Shell
ANI	Average nucleotide identity
V	Voltage
α	Alpha
β	Beta
γ	Gamma
±	Plus-minus sign
<	Less than
>	More than

CHAPTER 1

INTRODUCTION

1.1 Background

Aquaculture is a critical sector that must be retained for stabilising global food security. This industry has contributed over 179 million metric tonnes of fish production, valued at roughly USD 250 billion. FAO (2022) reported that nearly 156 million metric tonnes of fish are consumed by humans, providing 20.5 kg of aquaculture products per person. Moreover, FAO (2016) and FAO (2018) reported that Malaysia increased aquaculture production by ranking eleventh globally in 2016 as opposed to the fifteenth in 2014. Furthermore, Malaysia made USD 714.1 million in 2017 by exporting significant quantities of prawns and sashimi tuna, according to FAO (2019). Among several types of the aquaculture industry, prawn farming has shown significant growth and new technology has contributed to making production more efficient (El-Sayed et al., 2021; Emerenciano et al., 2022). Nonetheless, the industry frequently faces significant obstacles, such as pathogenic infection, which hinder productivity.

Studies by Zorriehzahra et al. (2016) and Nakayama et al. (2009) stated that pathogenic *Vibrio* is a bacterial genus that caused a massive loss in aquaculture. Tan et al. (2017) reported that *Vibrio parahaemolyticus* affects various types of seafood, especially shellfish. The worst impact of this bacterium is transferable to humans, causing gastrointestinal tract infections (Tan et al., 2017). According to Kavitha et al. (2018), *Aeromonas* spp., *Pseudomonas* spp., *Acinetobacter* spp., and *Streptococcus* spp. are also regarded as pathogens in aquaculture. Study has shown the use of antibiotics, vaccination and bacteriophage as disease management in aquaculture. Even though antibiotics have broad-spectrum activity against pathogens, the emergence of bacteria that are resistant to antibiotics has impacted the environment and exacerbated the problem (Balcazar et al., 2006). Whereas vaccination and bacteriophage are often specific in combat with pathogens. Therefore, probiotics, prebiotics, synbiotics, and postbiotics are proposed as safe and broad activity toward pathogens.

Probiotics are "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (Hill et al., 2014). Suresh (2023) and Biswas et al. (2023) stated that *Bacillus subtilis*, *B. coagulans*, and *B. licheniformis* are among the most studied *Bacillus* species as probiotics. *Bacillus subtilis*, according to Cutting (2011), is a probiotic that can contribute to the host's health benefits. Applying this *Bacillus* species in shrimp cultures has shown promising effects in enhancing the activity of digestive enzymes such as lipase, protease, and amylase. This probiotic also enhances shrimp survivability, according to Buruiană et al. (2014). Similarly, Tank et al. (2018) reported that *Bacillus subtilis* isolated from white-leg shrimp could suppress the growth of pathogenic *Vibrio harveyi*, *V. parahaemolyticus* and *V. vulnificus* in shrimp cultures.

Prebiotics are "substrate that is selectively used by host microorganisms to confer a health benefit" (Gibson et al., 2017). Commercial prebiotic such as lactulose, raffinose and inulin have shown a positive effect towards the growth and disease resistance for aquaculture (Madreseh et al., 2019; Karimi et al., 2020; Ajdari et al., 2022). Moreover, a study by Ramadhani et al. (2019) reported that prebiotics is an effective supplement in aquaculture. Research on microalgae as a prebiotic and an enhancer for probiotic growth is ongoing. *Isochrysis galbana* is an example of marine microalgae that displays promising prebiotic properties due to its ability to boost the growth of lactic acid bacteria and its contents, which comprise highly soluble and insoluble fibre (De Jesus et al., 2016). Lee et al. (2003) reported that *Spirulina (Arthrospira) platensis* contains oligosaccharides as a bioactive compound that improves the prawn's immune system and disease resistance. Additionally, Tayag et al. (2010) demonstrated that *Arthrospira platensis* could produce C-phycocyanin, which improved immunity and disease endurance in the white-leg shrimp.

According to Gibson et al. (2017), synbiotics are "a mixture comprising live microorganisms and substrate(s) selectively utilised by host microorganisms that confers a health benefit on the host". The prebiotic metabolism by probiotics will generate different short-chain fatty acids, which regard as postbiotics, including acetate, lactate, propionate, butyrate, and valerate, which benefits the hosts' health and inhibit the pathogens' growth (Pan et al., 2009; Markowiak-Kope and Liewska, 2020; Holmes et al., 2022). Probiotic bacteria must be able to utilise prebiotics by hydrolysing the glycosidic linkages to create a synbiotic. Using genome sequences to predict glycosidase hydrolyse families with the dbcan2 program is a cutting-edge technology for synbiotic preparation (Zhang et al., 2018). However, due to the limitation of the study, *in vitro* validation is needed to verify the enzyme production.

Apart from synbiotics, probiotics can also produce a variety of postbiotics, including bacteriocin, polymyxin, antibiotics, and lantibiotics, which are efficient antimicrobial agents against infections, according to Chalasani et al. (2015). Postbiotics are the "preparation of inanimate microorganisms and/or their components that confers a health benefit on the host" (Salminen et al., 2021). Nowadays, the postbiotic can be forecast by utilising the genome sequence of the microbes. The Antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) pipeline has emerged as the first tool to identify biosynthetic loci for the complete range of known secondary metabolite compound classes. The antiSMASH website can predict a variety of secondary metabolites, known as postbiotics, in bacterial and fungal genomes, including polyketides, non-ribosomal peptides, terpenes, aminoglycosides, aminocoumarins, indolocarbazoles, lantibiotics, bacteriocins, nucleosides and beta-lactam (Medema et al., 2011).

1.2 Problem statement

Pathogenic bacteria cause infections in aquatic animals and reduce production in the aquaculture industry. Previously, antibiotics had been used to solve this infection problem, but antibiotic-resistant bacteria emerged. A single-used probiotic and prebiotic is another approach but is inadequate to solve the problems, unlike synbiotic. However, probiotic bacteria need to show the ability to utilise prebiotic for synbiotic preparation. Hence, the effectiveness of probiotic bacteria against aquaculture pathogens and ability to utilise prebiotic from microalgae is an approach that should be evaluated intensively to overcome aquaculture diseases.

1.3 Hypothesis

Selected bacterial isolates from the gut of the giant freshwater prawn exhibit potential probiotics. Then, the selected bacterial genome foreseen good probiotic markers for aquaculture together with secondary metabolites genes against aquaculture pathogens. The selected bacterial isolate can utilise prebiotics through *in silico* and *in vitro* approaches. Thus, Microalgae *Arthrospira platensis* A1 exhibit the potential prebiotic and synbiotic with selected bacterial isolates against aquaculture pathogens.

1.4 Objective

The main objective of this study is to evaluate the potential probiotic of selected bacterial isolates against aquaculture pathogens and capability to use prebiotic from *Arthrospira platensis* A1.

The specific objectives of this study are as follows:

1. to isolate and characterise the selected bacteria from the gut of giant freshwater prawns as potential probiotics.
2. to analyse the general genome characteristics and probiotic markers from the selected bacteria with secondary metabolites genes predicted against aquaculture pathogens.
3. to investigate the ability of the potential probiotic of selected bacteria to utilise prebiotics using *in silico* and *in vitro* techniques.
4. to examine the water-soluble nondigestible polysaccharide extracted from *Arthrospira platensis* A1 and its prebiotic effect towards the selected bacteria.

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