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Streptomyces sp. MUM 195J: A Promising Probiotic for Controlling *Vibrio parahaemolyticus* Infection in Aquaculture

Joanna Xuan Hui Goh[1], Loh Teng-Hern Tan[2,3], Jodi Woan-Fei Law[2,4], Giva Kuppusamy[5], Janaranjani M[5], Kooi-Yeong Khaw[6], Wei Hsum Yap[7,8], Kok-Gan Chan[1,2,9], Vengadesh Letchumanan[1,10*], Learn-Han Lee[1,2*], Bey-Hing Goh[6,11,12*]

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[1]Novel Bacteria and Drug Discovery Research Group (NBDD), Microbiome and Bioresource Research Strength (MBRS), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia; joanna.vetpharm@gmail.com (JXHG)

[2]Microbiome Research Group, Research Centre for Life Science and Healthcare, Nottingham Ningbo China Beacons of Excellence Research and Innovation Institute (CBI), University of Nottingham Ningbo China, Ningbo 315000, China; loh-teng-hern.tan@nottingham.edu.cn (LT-HT); jodi-woan-fei.law@nottingham.edu.cn (JW-FL)

[3]Innovative Bioprospection Development Research Group (InBioD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia

[4]Next-Generation Precision Medicine and Therapeutics Research Group (NMeT), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia

[5]GK Aqua Sdn Bhd, Lot 5602-5603, Jalan 100 Ekar, Bukit Pelanduk, Port Dickson, Negeri Sembilan 71950, Malaysia; giva@gkaqua.com.my (GK); jana@gkaqua.com.my (JM)

[6]Biofunctional Molecule Exploratory (BMEX) Research Group, School of Pharmacy, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia; khaw.kooiyeong@monash.edu (K-YK)

[7]School of Biosciences, Taylor's University, 47500, Subang Jaya, Selangor, Malaysia; weihsum.yap@taylors.edu.my (WSY)

[8]Centre for Drug Discovery and Molecular Pharmacology, Faculty of Medical and Health Sciences, Taylor's University, Subang Jaya, 47500, Selangor, Malaysia

[9]Division of Genetics and Molecular Biology, Institute of Biological Sciences, Faculty of Science, University of Malaya, Kuala Lumpur 50603, Malaysia; kokgan@um.edu.my (K-GC)

[10]Pathogen Resistome Virulome and Diagnostic Research Group (PathRiD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia

[11]Sunway Biofunctional Molecules Discovery Centre (SBMDC), School of Medical and Life Sciences, Sunway University, Sunway City, Selangor, Malaysia

[12]Faculty of Health, Australian Research Centre in Complementary and Integrative Medicine, University of Technology Sydney, Ultimo 2007, NSW, Australia

*Corresponding author: Bey-Hing Goh; Sunway Biofunctional Molecules Discovery Centre (SBMDC), School of Medical and Life Sciences, Sunway University, Sunway City, Selangor, Malaysia; beyhingg@sunway.edu.my (B-HG); Vengadesh Letchumanan; Pathogen Resistome Virulome and Diagnostic Research Group (PathRiD), Jeffrey Cheah School of Medicine and Health Sciences, Monash University Malaysia, Bandar Sunway 47500, Selangor, Malaysia; vengadesh.letchumanan1@monash.edu (VL); Learn-Han Lee; Microbiome Research Group, Research Centre for Life Science and Healthcare, Nottingham Ningbo China Beacons of Excellence Research and Innovation Institute (CBI), University of Nottingham Ningbo China, Ningbo 315000, China; learn-han.lee@nottingham.edu.cn (L-HL)

Abstract: Aquaculture is gaining prominence in meeting the increasing global food demand. However, persistent episodes of pathogenic infections have greatly affected production and incurred substantial financial losses to the industry. Regrettably, there is a lack of effective contemporary therapeutic measures to control infectious diseases in aquaculture. The repercussions of antimicrobial resistance have underscored the drawbacks of the contemporary practice of relying solely on antibiotics in disease control. The aquaculture industry needs a safer, environmentally viable, and economically efficient means for disease management. In this regard, this study aims to investigate the effectiveness of mangrove-derived *Streptomyces* sp. probiotics in controlling *Vibrio parahaemolyticus* infections. *In vitro* screenings were undertaken to evaluate the inhibitory activity of five *Streptomyces* sp. isolates. Subsequently, a series of *in vivo* trials was conducted, with the Malaysian giant freshwater prawn, *Macrobrachium rosenbergii* larvae as the animal model. Following that, molecular analyses were employed to examine the changes in gene expression. In essence, *Streptomyces* sp. MUM 195J emerges as a promising probiotic strain that demonstrates a strong inhibitory effect against *V. parahaemolyticus*. Its application as a feed additive elevates the survival rate of *M. rosenbergii* threefold, thus demonstrating efficacy at par with florfenicol antibiotic when challenged with *the V. parahaemolyticus* pathogen. Besides, *Streptomyces* sp. MUM 195J elevated the growth rate of *M. rosenbergii* by 17%. Real-time quantitative polymerase chain reaction (RT-qPCR) analysis revealed that probiotic supplementation elevated the immune function of the animal. Additionally, *Streptomyces* sp. MUM 195J demonstrates the potential to ameliorate the quality of the rearing water.

Keywords: Aquaculture; *Streptomyces*; mangrove; probiotic; *Vibrio parahaemolyticus*; prawn; SDG 3 Good health and well-being

1. Introduction

Aquaculture, the practice of cultivating aquatic organisms for food, is deeply rooted in human history, with its recorded inception dating back as far as 8000 years ago [1]. Today, it has evolved into a thriving global industry, with more than 600 species cultivated

worldwide [2-4]. However, despite its growth and potential, the aquaculture industry grapples with a significant bottleneck stemming from a complex web of factors. Among the many challenges, the recurrent scourge of infectious diseases stands out as a formidable hurdle, accounting for nearly half of the decline in total production [5]. One poignant example of such a challenge is Acute Hepatopancreatic Necrosis Disease (AHPND), caused by the bacteria *Vibrio parahaemolyticus* [6, 7]. *V. parahaemolyticus* is a Gram-negative halophilic bacterium found in aquatic surroundings, particularly in tropical and temperate marine coastal water and estuaries [8-11]. The pathogen has become a dire threat to multiple commercially valuable shrimp species, including *Litopeneaus vannamei*, *Penaeus monodon*, *Metapenaeus monoceros* and *Macrobrachium rosenbergii* [12-14]. Since its initial occurrence in China in 2009, this disease can now be classified as a pandemic, persistently haunting shrimp farms across different regions worldwide with recurring outbreaks [15]. The menacing nature of this disease is primarily attributed to its rapid and acute transmission, capable of inducing mass mortality up to 100% within an alarmingly short time frame. Reports have underscored the devastating impact of AHPND on shrimp production, revealing an average decline of 60% in affected regions. This disruption to the delicate supply chain translates to a collective loss exceeding 43 billion USD in Asia and Mexico, which constitutes a heavy financial burden for the stakeholders and local farmers [13, 16].

Notably, the repercussions of infectious diseases in aquaculture extend far beyond the immediate financial implications. The prevalent occurrences of bacterial infections in aquaculture prompted the frequent usage of antibiotics, which led to a cascade of other issues [17-19]. Chiefly, the inappropriate and indiscriminate application of antibiotics precipitated the proliferation of antimicrobial-resistant isolates [20-24]. With antimicrobial resistance (AMR) progressively emerging as a crucial topic in public discourse, the continuous use of antibiotics in aquaculture has sparked increasing controversy [25, 26]. At the heart of this issue was the mounting evidence that links AMR in aquaculture to clinically relevant isolates [27-34]. On top of this, the AMR problem is further exacerbated by the implications of global warming, which places nations vulnerable to climate change at risk [35].

The challenge of infectious diseases in aquaculture underscores the critical need for effective disease prevention and management strategies to safeguard the sustainability and economic stability of the industry. In this light, this research aims to explore the potential of introducing mangrove-derived *Streptomyces* sp. as probiotics in aquaculture usage. Probiotics have been harnessed for a broad spectrum of advantages to the host, including improving overall well-being, resisting diseases, enhancing immunity, increasing endurance against stressors, stimulating growth and ameliorating water quality [36-38]. Nonetheless, there remain constraints in their current application. Chiefly, the efficacy of probiotics varies among different isolates and ensuring compatibility with the specific culture system is a crucial consideration. Selecting an appropriate probiotic strain tailored to a particular species with specific indications also poses a challenge [39]. Considering the growing instances of bacterial infections affecting aquaculture systems, it is imperative to devote proper research attention to promising probiotics.

Streptomyces sp. stands out in its promising antibacterial potential among the numerous probiotic candidates [40-45]. It is a genus from the *Streptomycetaceae* family within the actinobacteria phylum [46-48]. This Gram-positive spore-forming bacterium usually resides in a variety of underexplored environments, including mangrove soils [46, 49]. It is worth noting that *Streptomyces* sp. is renowned for its prominent antimicrobial properties, with over 80% of actinomycete antibiotics being derived from this genus [50-52]. Moreover, *Streptomyces* sp. holds clinical significance as it is widely recognised for its prolific production of functional metabolites, including antibiotics and a range of other highly relevant pharmaceuticals [44, 53-56].

This study can be viewed as one of the pioneering endeavours that delve into the potential of mangrove-derived *Streptomyces* sp. within this nation for use in aquaculture [57-59]. Its primary goals encompass verifying the identity of the putative *Streptomyces* sp. isolates obtained from the mangrove soil and their antagonistic activity against *V. parahaemolyticus*. Furthermore, it seeks to identify the most suitable probiotic strain through a series of *in vitro* and *in vivo* trials evaluating its impact on survival and growth rate, gene expression modulation, and water quality. This research also aims to significantly contribute to creating a systematic framework for identifying, formulating, and implementing native probiotics that can effectively combat specific pathogens across various aquaculture species. The ultimate objective is to establish a holistic experimental model that can serve as a technology platform to expedite the practical application of efficient and cost-effective probiotics tailored for specific uses in aquaculture. The potential revelation of disease-inhibitory attributes in *Streptomyces* sp. probiotics holds the promise of unlocking a series of meaningful discoveries that could benefit the aquaculture industry.

2. Materials and Methods

2.1. Collection of soil sample

The soil sample was collected at a depth of 20 cm beneath the mangrove floor from three different sites KTTAS 1 (1°41’48.57” N 110°11’15.30” E), KTTAS 4 (1°41’48.48” N 110°11’13.40” E) and KTTAS 7 (1°41’48.08” N 110°11’15.14” E) of the mangrove forest located in Kampung Trombol, Telaga Air, Kuching, Sarawak in June 2015.

2.2. Isolation and purification of putative Streptomyces sp. isolates

The bacterial isolates were obtained and purified based on the culture-dependent approach described by Law et al. [60]. Briefly, the soil sample collected was air-dried for seven days. Following that, the soil samples were mixed thoroughly with mortar and pestle before being subjected to wet heat treatment at 50 °C for 15 minutes. The sample was diluted 10,000 times with sterilized water before being spread on an International *Streptomyces* Project (ISP 2) agar plate containing 20 mg/L of nalidixic acid and 50 mg/L of cycloheximide. The plate was incubated at 28 °C for seven to 28 days to allow growth. Five colonies exhibiting *Streptomyces*-like morphological characteristics, such as those showing prominent mycelium

features, were isolated and streaked for purification on fresh ISP 2 agar plates and incubated at 28 °C for seven to 28 days to allow growth. The purified isolates were maintained in individual ISP 2 agar slants and stored at 28 °C until further analysis.

2.3. DNA extraction, PCR amplification of 16S rRNA gene and phylogenetic analysis

The five putative *Streptomyces*-like isolates were subjected to molecular identification to confirm the isolate identity. The genomic DNA (gDNA) of each isolate was extracted for 16S rRNA gene sequencing based on the protocol described by Hong et al. [61] using the soil community DNA extraction kit, Fast DNA®SPIN Kit (Q-BIOgene, USA). Following that, the extracted gDNA was subjected to PCR amplification on the 16S rRNA gene using the 27F-1492R universal primer pair, which comprised of the forward primer (5′-AGAGTTTGATCCTGGCTCA-3′) and reverse primer (5′-GGTTACCTTGTTACGACTT-3′) according to the procedures described by Lee et al. [62]. Briefly, the gDNA extracted from each isolate was subjected to PCR using TurboCycler 2 (Blue-Ray Biotech, Taipei, Taiwan), with highQu Taq DNA polymerase (Kraichtal, Germany), in reaction mixtures of 20 µL in volume. The PCR was conducted under the cycling conditions set as follows: (i) pre-denaturation of DNA at 95°C for 5 min; (ii) 35 cycles of denaturation at 94°C for 50 s, (iii) annealing at 55°C for 1 min; (iv) extension at 72°C for 1 min and 30 s; (v) final elongation at 72°C for 8 min. The PCR amplification products were separated via electrophoresis in 1.5% agarose gel (Promega, USA) stained with ethidium bromide (0.5 μg/mL) and visualised using the gel documentation system (ChemiDoc™ XRS, Bio-Rad, USA). The amplified samples were sequenced with the Sanger Deoxy method to determine the 16S rRNA gene bases.

The 16S rRNA gene sequences of each isolate were utilized to build a phylogenetic tree to decipher its genetic relatedness with (i) its respective closest 40 deposited strains in the database and (ii) each other and their respective top seven closest hits (after removing duplicates) in the database with reference to the protocol devised by Lee et al. [62] and Law et al. [60]. Briefly, the sequenced 16S rRNA gene for each isolate was manually trimmed with the BioEdit Sequence Alignment Editor Software [63] before being submitted to the EzBioCloud database for matching [64]. The gene sequences of the 40 closest strains to each were retrieved from the database and aligned using the CLUSTAL-X software, followed by the manual adjustment and verification of the alignment (Thompson et al., 1997). MEGA 7.0 software was employed for molecular evolutionary genetic analysis to infer the phylogenetic tree based on the neighbour-joining algorithm [65]. The evolutionary pairwise distances between sequences were computed based on Kimura's two-parameter model [66]. The stability of the tree topology was enumerated using the bootstrap resampling method based on the 1000 replicates [67], and the sequence similarities were calculated using the EzBioCloud server [64].

2.4. Cross-streak assay

The antagonistic activity of each isolate against *V. parahaemolyticus* pathogen, VP20/2016, obtained from a blood cockle (*Tegillarca granosa*) was assessed through a modified cross-streak assay adapted from Das et al. [68]. Basically, 500 µL seed media of each of the five isolates were individually inoculated into 50 mL Falcon tubes containing 15 mL of tryptic soy broth (TSB) each and incubated at 28 °C for seven days in a shaker incubator set to 200 rpm. After which, 100 µL of the seven-day culture was streaked across the centre of the Mueller Hilton Agar (MHA) plate and incubated for another seven days at 28 °C. Meanwhile, 100 µL of the *V. parahaemolyticus* VP20/2016 isolate was inoculated and revived in 4 mL of TSB with 2% NaCl prior to incubation at 37 °C for 18 hours in a shaker incubator set to 200 rpm. Following that, 20 µL of the 18-hour culture of *V. parahaemolyticus* was streaked perpendicular to the *Streptomyces* sp. colonies on the MHA plate, and the same step was repeated for the other side. After 18 hours of incubation at 37 °C, the length of the inhibition zone was used to define the inhibiting effect of the isolate against the *V. parahaemolyticus* pathogen. The inhibitory activity was arbitrarily categorised into three groups: no inhibition (-), moderate inhibition (+), and strong inhibition (++). Amongst the five isolates, the isolate exhibiting the strongest antagonistic capability against *V. parahaemolyticus* was selected as the candidate strain for this project.

2.5. Phenotypic characterization of the candidate strain

2.5.1. Scanning Electron Microscopy (SEM)

The SEM slide for the candidate strain was prepared based on the alcohol dehydration protocol improvised internally in our laboratory. Briefly, the seven-day colony grew on MHA was gently washed with 100 µL of phosphate-buffered saline (PBS) twice at five-minute intervals and left to dry in the fume hood before being fixated with 200 µL of 2.5% (v/v) glutaraldehyde in phosphate buffer and incubated for 30 minutes in the fume hood protected from light. The sample was then serially dehydrated with 20%, 40%, 60%, 80% and 100% (v/v) of ethanol at ten-minute intervals. The dehydration with 100% ethanol was repeated thrice at three-minute intervals. The sample was left to dry in the fume hood for 16 hours before being placed on an aluminium stub and gold-coated with a sputter coater (Quorum, Q150R S) in a vacuum at 0.05 mbar pressure to achieve a thickness of around 6 nm. The specimen was viewed with a variable-pressure scanning electron microscope (VPSEM) (Hitachi, S-3400N). The electron micrographs of the sample were taken with the same device.

2.5.2. Growth in different culture mediums

The candidate strain was streaked and grown in 13 different culture mediums, namely the actinomycetes isolation agar (AIA), Luria broth agar (LBA), Muller Hilton Agar (MHA), nutrient agar (NA), *Streptomyces* agar (SA), starch casein agar (SCA), tryptic soy agar (TSA), International *Streptomyces* Project (ISP) 2, ISP 3, ISP 4, ISP 5, ISP 6, ISP 7 agar plates using the four-quadrant technique. The plate was then sealed with parafilm and

incubated for seven days in a static inverted position in an incubator set to 28 °C. The growth of the strain was reported on a scale of four as "no growth (-)", "low growth (+)", "good growth (++)", and "very good growth (+++)". The colour of colony mycelium from both aerial and substrate views and the colour of the diffusing pigment was reported according to the Intersociety Colour Council - National Bureau of Standards (ISCC- NBS) colour chart [69].

2.5.3. Growth at different temperatures

The candidate strain was streaked and grown on seven ISP 2 agar plates incubated at seven different temperatures, viz. 4 °C, 26 °C, 28 °C, 32 °C, 37 °C, 40 °C and 50 °C respectively, in a static inverted position for seven days. After seven days, the growth of the strain was reported on a scale of four as "no growth (-)", "low growth (+)", "good growth (++)", and "very good growth (+++)". The presence of diffusing pigment on the agar plates was also observed and recorded.

2.5.4. Growth at different pH levels

The candidate strain was inoculated in six TSB cultures with differing pH adjusted to pH 2, 4, 6, 7, 8, and 10, respectively. The inoculated tubes were incubated at 28 °C for seven days in a shaker incubator set to 200 rpm. After seven days, the growth in each tube was observed and recorded.

2.5.5. Growth at different salinities

The candidate strain was inoculated in six different TSB cultures supplemented with 0%, 2%, 4%, 6%, 8%, and 10% (w/v) NaCl, respectively. The inoculated tubes were incubated at 28 °C for seven days in a shaker incubator set to 200 rpm. After seven days, the growth in each tube was observed and recorded.

2.5.6. Chemical profiling of the candidate strain

2.5.6.1. GC-MS analysis of the methanolic extract of FM3 ferment

The candidate strain was inoculated and cultured in FM3 broth [61] under shaking conditions at 200 rpm for 14 days at 28 °C. The fermented culture was centrifuged at 3000 × *g* (Refrigerated Microcentrifuge, Eppendorf 5702R) for 10 min to separate the supernatant from the biomass. The resulting supernatant was filtered using filter paper (Whatman Filter Paper, No. 3 Pore Size: 6µm) and frozen at -80 °C before being subjected to the freeze-drying process using ScanVac Freeze Dryer, Labconco. The freeze-dried sample was extracted with 150 mL of methanol, and this process was repeated thrice. The final extract was concentrated using the rotary evaporator (IKA, RV10) set to 40 °C. The final concentration was suspended in methanol before being subjected to GC-MS for chemical profiling. GC-MS analysis was conducted based on the protocol developed by Supriady et al. [70]. The Agilent 6980N Network Gas Chromatograph (GC) supported by Agilent 5979 Mass Selective Detector

(MSD) with 30 m × 250 μm × 0.25 μm 5%-phenyl-methylpolysiloxane phase (HP-5MS) capillary column was employed for the GC-MS analysis. The MSD was set to run at 70eV with helium at an influx rate of 1 mL per minute as the carrier gas. The initial column temperature was set to 40 °C for 10 minutes before being programmed for a temperature increment of 3 °C per minute to reach 250 °C, which was isothermally maintained for 5 minutes. The mass spectral data of the detected chemical constituents were compared against the standards in the National Institute of Standards and Technology (NIST) mass spectral reference library [71].

2.5.6.2. GC-MS analysis of the methanolic extract of culture on MHA agar

The candidate strain was streaked at 1 cm width across an MHA agar plate and incubated for 14 days at 28 °C. The MHA agar from the immediate areas around the border of the colony growth was cut with a sterilized scalpel and subjected to solid-liquid methanolic extraction. The agar pieces were immersed in the methanol for 3 days, followed by another 2 rounds of extraction with fresh methanol for a day. The methanolic extract was collected, filtered, and concentrated via a rotary evaporator before GC-MS analysis as described in Section 2.5.6.1.

2.5.7. In vitro probiotic properties assessment of the candidate strain

2.5.7.1. Co-culture assay

The co-culture assay was conducted based on the modified protocol devised by Yang et al. [72]. Briefly, 10^4 CFU of *V. parahaemolyticus* from the overnight culture of *V. parahaemolyticus* VP20/2016 was added into three *Streptomyces* cultures of different concentrations, namely 0% (w/v), 0.5% (w/v) and 1% (w/v). All three tubes were incubated under shaking conditions at 200 rpm, 28 °C for 24 hours. After the stipulated incubation period, the content in each tube was diluted to a range of presumptive readable dilutions and plated on the selective HiCrome agar plate (HiMedia, India) in six replicates. The HiCrome agar plates were incubated at 37 °C for 18 hours in a static inverted position. The colonies on the HiCrome plates were then enumerated, and the viable *Vibrio* sp. count in each tube was calculated and reported in CFU/mL. The *Vibrio* sp. count in the control group, 0% (w/v), was assumed to be 100% viable. The percentage of viable *Vibrio* sp. in the treatment groups was calculated using the formula:

$$\text{Viable } Vibrio \text{ sp. } (\%) = \left(\frac{\text{Vt}}{\text{V0}}\right) \text{ x } 100\%$$

where $V0$ is the viable *Vibrio* sp. count in the control group; Vt is the viable *Vibrio* sp. count in the treatment group.

2.5.7.2. Congo red binding assay

The Congo red binding assay was conducted based on the modified protocol adapted from Ambalam et al. [73]. Briefly, seven different supernatant concentrations of the seventh-day culture of the candidate strain in TSB were created through one-half serial dilution using

PBS. 1.5 mL microcentrifuge tubes containing 400 µL of each supernatant concentration (0%, 1.56%, 3.13%, 6.25%, 12.5%, 25%, 50%) were created in three replicates. 400 µL of the overnight culture (18 hours of incubation) of *V. parahaemolyticus* VP20/2016 adjusted to 10^5CFU/mL was added to each microcentrifuge tube. The content in the microcentrifuge tube was mixed thoroughly by vertexing for two seconds and incubated at 28 °C for 24 hours. After incubation, the microcentrifuge tubes were centrifuged at 3000 × *g* (Heraeus Pico 17 Centrifuge, ThermoScientific) for five minutes. The supernatant was removed. The biomass was washed with PBS buffer. PBS and Congo red (100 µg/mL) were added to each microcentrifuge tube at a 1:1 ratio, with 100 µL each. The content in each tube was vortexed for two seconds for thorough mixing. The microcentrifuge tubes were left to stand still for 20 minutes. Next, the microcentrifuge tubes were centrifuged at 3000 × *g* (Heraeus Pico 17 Centrifuge, ThermoScientific) for five minutes. 100 µL of supernatant was pipetted from each tube into a 96-well plate. The amount of Congo red left in the supernatant was quantified by measuring the absorbance of the supernatant using the microplate reader (Eon Biotek) at OD 480 nm. The reading of the three replicates was averaged to produce the final reading for each group. The percentage of Congo red binding was calculated using the formula:

$$\text{CR bound (\%)} = 100 - \left(\frac{\text{Cf x } 100}{\text{C0}}\right)$$

*C*f is the absorbance of the residual Congo red in the supernatant of the test sample, whereas *C*0 is the initial absorbance of the Congo red used in the experiment.

2.6. Probiotic formulation

Mass culture of the candidate strain *Streptomyces* sp. MUM 195J probiotic was initiated at least a week prior to the feeding trial (Section 2.7). The probiotic was mass cultured using the protocol optimised in our lab. Briefly, ten healthy *Streptomyces* sp. MUM 195J colonies grown on tryptic soy agar (TSA) plates were inoculated into the three 1 L conical flasks containing 600 mL of freshly prepared TSB each. The inoculated flask was secured with aluminium foil and sealed using parafilm before being incubated under shaking at 200 rpm, at 28 °C for seven days. After the incubation period, the content in the flask was centrifuged at 3000 × *g* (Refrigerated Microcentrifuge, Eppendorf 5702R) for 10 min to separate the supernatant from the biomass. The supernatant was discarded, and the biomass was harvested and packed into tubes containing fresh TSB. The prepared tubes were refrigerated at 2 °C to 8 °C until use. These preparation steps were repeated weekly to ensure a continuous supply of probiotics throughout the feeding trial.

2.7. Feeding trial

The *in vivo* testing was conducted using the *M. rosenbergii* larvae at GK Aqua, Sdn. Bhd., a hatchery with Bionexus status, located at Bukit Pelanduk, Port Dickson. Animal

ethics approval was sought from Monash University's animal ethics committee (Project identification number assigned: 23910), and permission was granted prior to the initiation of the *in vivo* test.

Two cement tanks, labelled "-" (control tank) and "+" (with *Streptomyces* sp. MUM 195J 1% (w/v)), were sterilised with chlorine before being filled with one tonne of 15 ppt of brackish water (freshwater from tap mixed with seawater treated via aeration with chlorine) each. A proper aeration system was fixed for each tank to ensure adequate oxygen supply. Newly hatched *M. rosenbergii* were stocked at a density of 90 larvae per litre, summing up to a total of 90,000 larvae in each cement tank [74]. The cement tanks were covered with an opaque shade for temperature and light intensity regulation. The larvae in both cement tanks were subjected to a 12-hour light and 12-hour dark photoperiod cycle daily by adjusting the position of the opaque shade.

The rearing water was maintained at 15 ppt salinity from day 1 to day 21. The salinity was lowered and maintained at 12 ppt from day 22 to day 29. Briefly, 50% of the water was exchanged daily. Water quality parameters, including salinity, temperature, pH, ammonia, nitrite, and nitrate levels, were closely monitored to ensure these parameters lie within the range that supports the optimum growth of the animals. The well-being of the animal was closely monitored throughout the experimental period. Any abnormal physical appearance or clinical signs were recorded in the monitoring sheet if present.

Throughout the experiment, an equal amount of feed was supplied to both tanks simultaneously. The larvae were fed with artemia *ad libitum* during the whole length of the investigation. From day 6 onwards, egg custard was added to the diet. The egg custard meal was prepared fresh on-site every alternate day according to the modified preparation adopted as the hatchery's proprietary recipe. Briefly, the egg was cooked via continuous stirring, crushed into small pieces over a sieve, and diluted into a paste form. The formulated feed was kept in the refrigerator at 2 to 8 °C until use. The egg custard was given at an average amount of 400 mL twice a day. From day ten onwards, blood worms were supplemented to the diet and fed *ad libitum*.

The feeding trial was initiated on day 8 and lasted 21 days until day 28. *Streptomyces* sp. MUM 195J probiotic was supplemented to the egg custard diet through physical mixing at 1% (w/v) of the total wet weight of the egg custard feed. *Streptomyces* sp. MUM 195J probiotic treatment was introduced twice daily to the tank labelled with "+", concurrent with the egg custard feeding, in which 400 mL was given on average for every feeding session. An equal volume of TSB (as the placebo for the *Streptomyces* sp. MUM 195J probiotic treatment) was added to the egg custard feed for the "-" group.

2.8. Growth performance assessment

The weight of the animals was recorded on days 21 and 28 of the *in vivo* trial. The pooled weight of ten randomly sampled shrimps was measured using an electronic balance in ten replicate samplings.

2.9. Challenge test with V. parahaemolyticus

The challenge test was conducted on day 28 of the experiment following the three-week feeding trial. Fifteen plastic tanks of 2.5 L in capacity with dimensions of 21 cm × 13 cm × 11.5 cm (L × W × H) were filled with 1 L of rearing water adjusted to 15 ppt salinity each. An aeration pump was fixed in each tank to ensure constant aeration for the animal subjects.

The experiment was conducted in five different groupings, namely (i) control, (ii) control + *Vibrio*, (iii) *Streptomyces* sp. MUM 195J 1% (w/v), (iv) *Streptomyces* sp. MUM 195J 1% (w/v) + *Vibrio* and (v) Florfenicol + *Vibrio* with three replicates each. 10% florfenicol antibiotic was added to achieve a concentration of 12 mg/L for the Florfenicol + *Vibrio* group.

Thirty larvae from the respective cement tank were transferred into each plastic tank. The shrimps were acclimatised in the plastic tanks for two hours before the commencement of the challenge test. After the acclimatization period, the *V. parahaemolyticus* cultured at 37 °C at 200 rpm for 18 hours before the experiment was inoculated to the respective tanks labelled "+ *Vibrio*" as the infectious agent. The concentration of the *V. parahaemolyticus* was adjusted to achieve a concentration of 1.2×10^6 CFU/mL in tanks while the same volume of TSB was added to the groups without "+ *Vibrio*".

The animals were fed with artemia *ad libitum* throughout the experiment. The survival rate of the larvae in each tank was recorded at 24-hour intervals. The moribund shrimps that do not respond to mechanical stimuli were considered dead. The dead larvae typically turn bright red and sink to the bottom of the tank. The dead larvae were removed from the tank daily after taking the readings and carefully disposed of as biohazard waste. The final results were presented as the survival rate (SR) of the animal calculated based on the formula:

$$\text{SR (\%)} = \left(\frac{\text{Number of surviving animal}}{\text{Total initial number of animal stocked}}\right) \text{x } 100\%$$

At the end of the challenge test, the remaining animals were sacrificed by euthanasia (adding crushed ice to render the shrimps insensible) and carefully disposed of as biohazard waste. The culture water was treated with sodium hypochlorite at a 5 g/L concentration to inactivate the pathogen.

2.10. Water amelioration assessment

Sixteen plastic tanks of 2.5 L in capacity with dimensions of 21 cm × 13 cm × 11.5 cm (L × W × H) were filed with 1.5 L of rearing water obtained from a shrimp culture tank with the last water exchanged 24 hours ago at 50%. The baseline value of the ammonia

(NH_3/NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) levels of the rearing water were measured using the respective water quality test kits (API). An aeration pump was fixed in each tank to ensure constant aeration to sustain the system. *Streptomyces* sp. MUM 195J probiotic were added to four different groups of tanks to achieve a concentration of (i) 0% (w/v) [control], (ii) 0.0001% (w/v), (iii) 0.001% (w/v), and (iv) 0.01% (w/v) with four replicates for each grouping. The water quality parameters were reassessed after 48 hours without water exchange.

2.11. RT-qPCR assay

At the end of the three-week feeding trial, thirty larvae from each cement tank were sampled on day 28. The larvae were sacrificed by euthanasia through immersion in ice water. The animal samples were immediately transported to the lab on dry ice for RT-qPCR analysis to evaluate gene expression. The samples collected were washed twice with PBS. Five shrimps were pooled in a 1.5 mL Eppendorf microcentrifuge tube to create six replicates. Following that, the samples were subjected to total RNA extraction using TRIzol reagent, according to the modified protocol from a previous study [75]. Briefly, 300 μL of TRIzol reagent was thawed and added into each microcentrifuge tube. Chloroform (60 μL) was added to the tube and vortexed for homogeneous mixing. The sample was put aside for three minutes before centrifugation at 13,500 rpm (Centrifuge 5424 R, Eppendorf) at 4 °C for 15 minutes. The upper layer, which appeared as the colourless aqueous phase, was carefully extracted and transferred to a new microcentrifuge tube. The tubes were kept on ice throughout the process. Briefly, 150 μL of 100% (v/v) isopropyl alcohol was added to each tube, and the content was mixed gently and left aside for ten minutes prior to centrifugation at 13,500 rpm at 4 °C for 15 minutes. The supernatant was carefully discarded. Briefly, 200 μL of 75% (v/v) ethanol was mixed thoroughly with the remaining product prior to centrifugation at 10,500 rpm at 4 °C for five minutes. This step was again repeated. Finally, the product (total RNA extracted) was air-dried before 10 μL of Diethylpyrocarbonate (DEPC)-treated water was added to the sample. The total RNA was kept at -150 °C until use. The quality and quantity of the extracted RNA were assessed using a NanoPhotometer (N60, Implen, Germany). The quality of the RNA is acceptable if the RNA absorbance at A260/A280 falls within the range of 1.9 to 2.0.

Next, the RNA was converted into cDNA using a synthesis kit (High capacity cDNA reverse transcription kit, Applied Biosystems) in accordance with the product's user manual. Every sample was prepared in a volume of 20 μL, each containing 2 μL of 10× reverse transcription (RT) buffer, 0.8 μL of 25× deoxynucleotide triphosphate (dNTP), 2 μL of 10× RT random primers, 1 μL of Multiscribe reverse transcriptase, 0.9 μL of RNase inhibitor and 13.3 μL of template containing 500 ng of total RNA. The samples were subjected to thermal cycling in a Thermal Cycle (T100, Bio-Rad), according to the cycle setting of denaturation at 25 °C for ten minutes, annealing at 37 °C for 120 minutes, elongation at 85 °C for five minutes and holding at 4 °C until infinity.

The cDNA synthesized was used for the RT-qPCR analysis according to the protocol described by Goh et al. [76]. A total volume of 10 μL of RT-qPCR reaction mixture comprised 1 μL of the cDNA, 5 μL of the SYBR Green PCR master mix, 0.2 μL of the forward primer, 0.2 μL of the reverse primer and 3.6 μL of DEPC-treated water was prepared for each sample.

ß-actin was selected as the housekeeping gene. Heat Shock Protein (HSP70), Dorsal, Relish, Anti-lipopolysaccharide Factor (ALF5), and Crustin were chosen as the target genes to indicate inflammatory and stress response. Lipopolysaccharide and β-1,3-glucan-binding protein (LGBP), Lectin, Prophenoloxidase (ProPO), Peroxinectin, α-2-Macroglobulin (α2M) and Lysozyme were the target genes for immune response indication. Gene expression of Crustacean Hyperglycemic Hormone (CHH) was assessed to provide insights into the growth response of the shrimps.

The gene expression was studied using the RT-qPCR instrument (StepOnePlus, Thermo Fisher Scientific). The thermal cycling profile was set to 10 seconds of holding time at 95 °C, followed by 40 cycles of 95 °C for 15 seconds, at melting temperature (Table S1) for 60 seconds and 95 °C for 15 seconds, after which the reading of the mean fluorescence threshold value (C_T) of each sample was captured. At least two technical replicates for each biological sample were conducted. The primer specificity was confirmed by assessing the respective melt curve peak. The housekeeping gene, β-actin expression, was used to normalise the expression levels of all target genes. Gene expression levels were calculated using the $2^{-\Delta\Delta CT}$ method, whereby the $\Delta\Delta C$T values were determined using the formula:

$$\Delta\Delta C_T = \Delta C_T \text{ reference} - \Delta C_T \text{ target}$$

2.12. Statistical Analysis

The results from the replicates were expressed as mean ± standard deviation. Data analysis was performed using IBM SPSS statistics (version 26). One-way analysis of variance (ANOVA) and Tukey's *post-hoc* analysis were performed to determine the statistical difference between experimental groups. The statistical significance was declared at $p < 0.05$.

3. Results

3.1. Isolation of five putative Streptomyces-like isolates

The addition of cycloheximide and nalidixic acid effectively inhibited the growth of fungi and undesired Gram-negative bacteria. Five bacterial isolates, namely MUM 2J, MUM 58J, MUM 134J, MUM 180J, and MUM 195J, were purified, isolated, and selected for subsequent testing (Table S2).

3.2. All five isolates are Streptomyces sp.

The 16S rRNA gene sequences of the five isolates used in this study range between 1255 and 1349 base pairs. The search results from the 16S rRNA gene sequence blast in the

EzBioCloud database revealed that the 40 closest strains to each of the five isolates are streptomycetes. The high percentage similarities (> 98.56%) of these strains with the *Streptomyces* sp. type strains in the EzBioCloud database confirmed that all five isolates, namely, MUM 2J, MUM 58J, MUM 134J, MUM 180J, and MUM 195J belong to the genus *Streptomyces.* The evolutionary relationship between each isolate and the related type strains is illustrated in the phylogenetic trees (Figure 1-5).

Blast results from the EzBioCloud database revealed that *Streptomyces* sp. MUM 2J demonstrated the highest similarity to *Streptomyces leeuwenhoekii* C34^T (LN831790) (98.81%). Figure 1 confirms the short evolutionary distance between both strains and further revealed that both of the strains along with *Streptomyces thermocoprophilus* B19^T (AJ007402) (98.2%), *Streptomyces griseosporeus* NBRC13458^T (AB184419) (98.28%), *Streptomyces coeruleoprunus* NBRC15400^T (AB184651) (97.98%) and *Streptomyces somaliensis* DSM40738^T (AJ007403) (98.13%) formed a distinct clade at bootstrap value 96%. Figure 2 shows that *Streptomyces* sp. MUM 58J is closely related to *Streptomyces xylanilyticus* SR2-123^T (LC128341) and *Streptomyces spectabilis* NBRC13424^T (AB184393), where the percentage similarities between the strains are 98.13% and 97.99%, respectively. Figure 3 shows that *Streptomyces* sp. MUM 134J formed a distinct clade with *Streptomyces fuscigenes* JBL-20^T (AB980255) (97.83%) and *Streptomyces pathocidini* NBRC 13812^T (AB184501) (97.76%) at bootstrap value 61%. The phylogenetic tree in Figure 4 shows that *Streptomyces* sp. MUM 180J formed a distinct clade with *Streptomyces abikoensis* NBRC13860^T (AB184537) (97.99%), *Streptomyces somaliensis* DSM40738^T (AJ007403) (98.07%), *Streptomyces coeruleoprunus* NBRC15400^T (AB184651) (98.54%) and *Streptomyces fradiae* DSM40063^T (MIFZ01000280) (98.38%).

Interestingly, the EzBioCloud blast results of *Streptomyces* sp. MUM 195J showed significant similarities with *Streptomyces* sp. MUM 180J. In fact, the top seven hits for both isolates are similar—both *Streptomyces* sp. MUM 180J and MUM 195J are likely sister strains. This is confirmed by the highly identical phylogenetic tree in Figure 5. Like *Streptomyces* sp. MUM 180J, *Streptomyces* sp. MUM 195J formed a distinct clade with *Streptomyces abikoensis* NBRC13860^T (AB184537) (97.99%), *Streptomyces somaliensis* DSM40738^T (AJ007403) (98%), *Streptomyces coeruleoprunus* NBRC15400^T (AB184651) (98.48%) and *Streptomyces fradiae* DSM40063^T (MIFZ01000280) (98.48%).

The evolutionary relationship between the five *Streptomyces* sp. isolates and their respective closely related strains is depicted in Figure 6. The tree indicates that *Streptomyces* sp. MUM 2J and *Streptomyces* sp. MUM 58J are highly related, as both form a monophyletic clade with a bootstrap value of 99%. The two isolates exhibited a close evolutionary relationship with *Streptomyces xylanilyticus* SR2-123^T (LC128341) and *Streptomyces flavofungini* NBRC13371^T (AB14359). Similarly, *Streptomyces* sp. MUM 180J and *Streptomyces* sp. MUM 195J also form a monophyletic clade with a bootstrap value of 99%. *Streptomyces* sp. MUM 134J forms a distinct clade with *Streptomyces lichensis* LCR 6-01^T (LC360144) and *Streptomyces nanningensis* YIM 33096^T (AY222320).

3.3. Streptomyces sp. MUM 195J is selected as the candidate probiotic strain

The cross-streak assay results (Table 1) revealed that *Streptomyces* sp. MUM 58J, MUM 180J and MUM 195J demonstrated a strong inhibitory effect against *V. parahaemolyticus*, as no visible colony of *V. parahaemolyticus* is noted on these plates. In contrast, *Streptomyces* sp. MUM 2J and MUM 134J showed no significant inhibition against the growth of *V. parahaemolyticus*. Among the three isolates that exhibited a strong inhibition, *Streptomyces* sp. MUM 195J was chosen as the candidate strain due to a significantly better growth rate.

Table 1. The inhibitory effect of each isolate against *V. parahaemolyticus.*

Bacterial isolates	**MUM 2J**	**MUM 58J**	**MUM 134J**	**MUM 180J**	**MUM 195J**
Inhibitory effect against *V. parahaemolyticus*	-	++	-	++	++

(-) no inhibition; **(+)** moderate inhibition; **(++)** strong inhibition.

3.4. SEM micrograph of Streptomyces sp. MUM 195J

The mycelium and distinct thread-like filamentous morphology of *Streptomyces* sp. MUM 195J, distinctive to the species [77, 78], is clearly visible in the SEM micrographs (Figure 7).

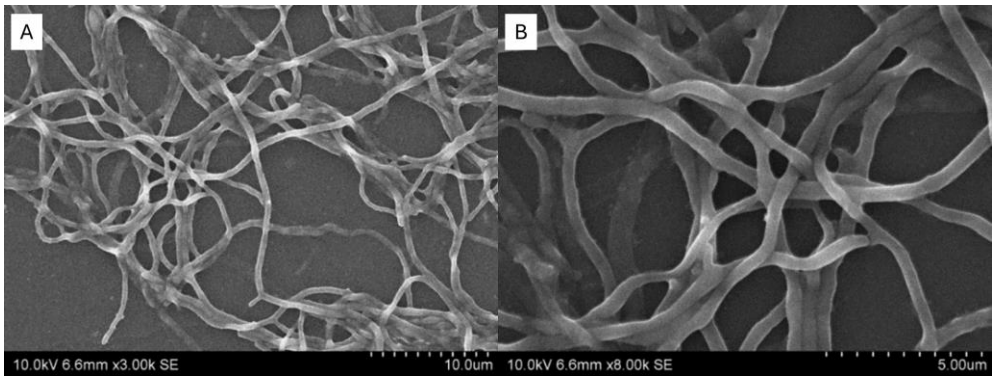

Figure 7. VPSEM of *Streptomyces* sp. MUM 195J at 10.0kV, under (A) ×3000 and (B) ×8000 magnification.

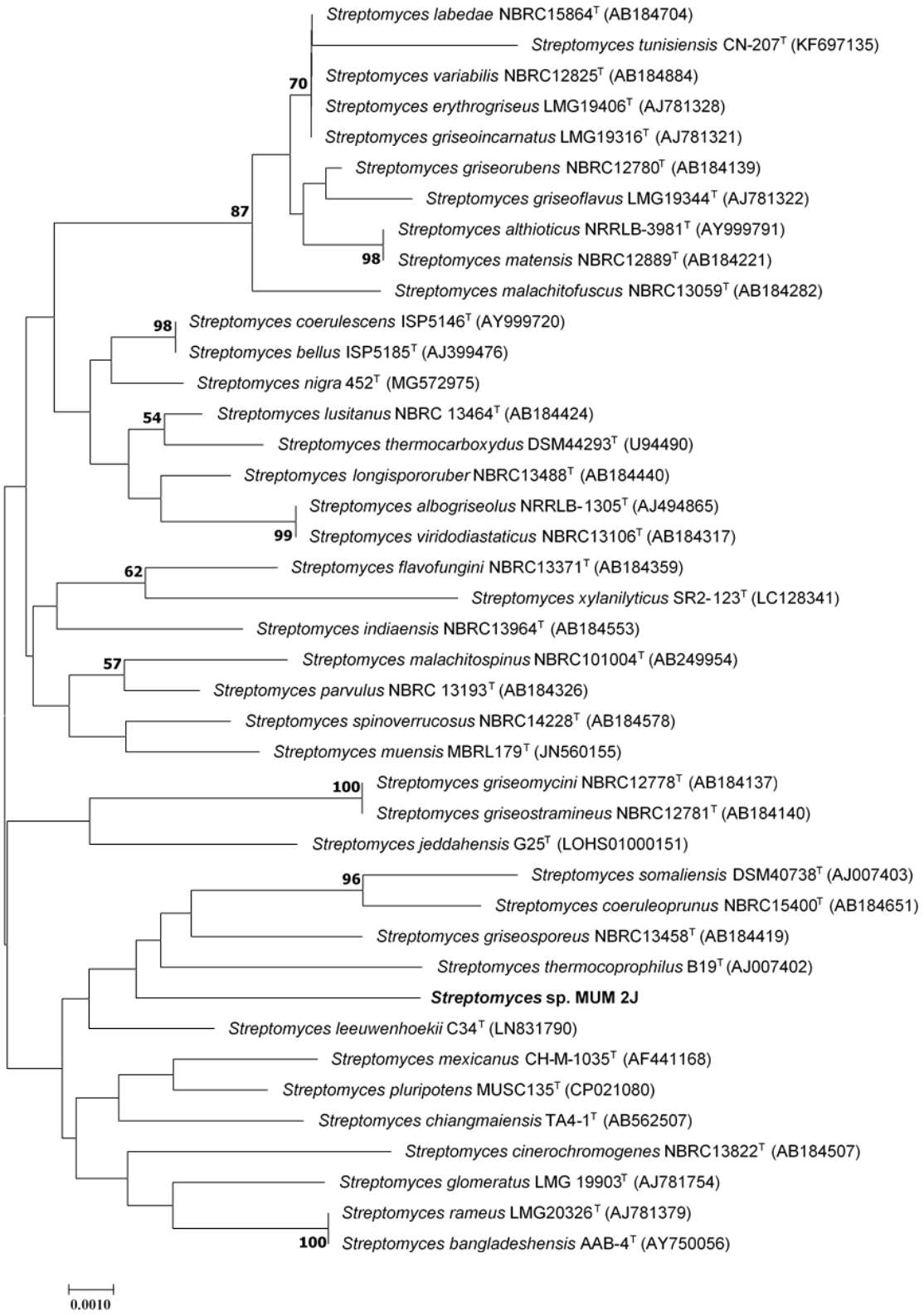

Figure 1. Neighbour-joining phylogenetic tree based on 1344 nucleotides of 16S rRNA gene sequences showing the relationship between *Streptomyces* sp. MUM 2J and representatives of related taxa. The numbers at nodes indicate the percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.001 substitutions per site.

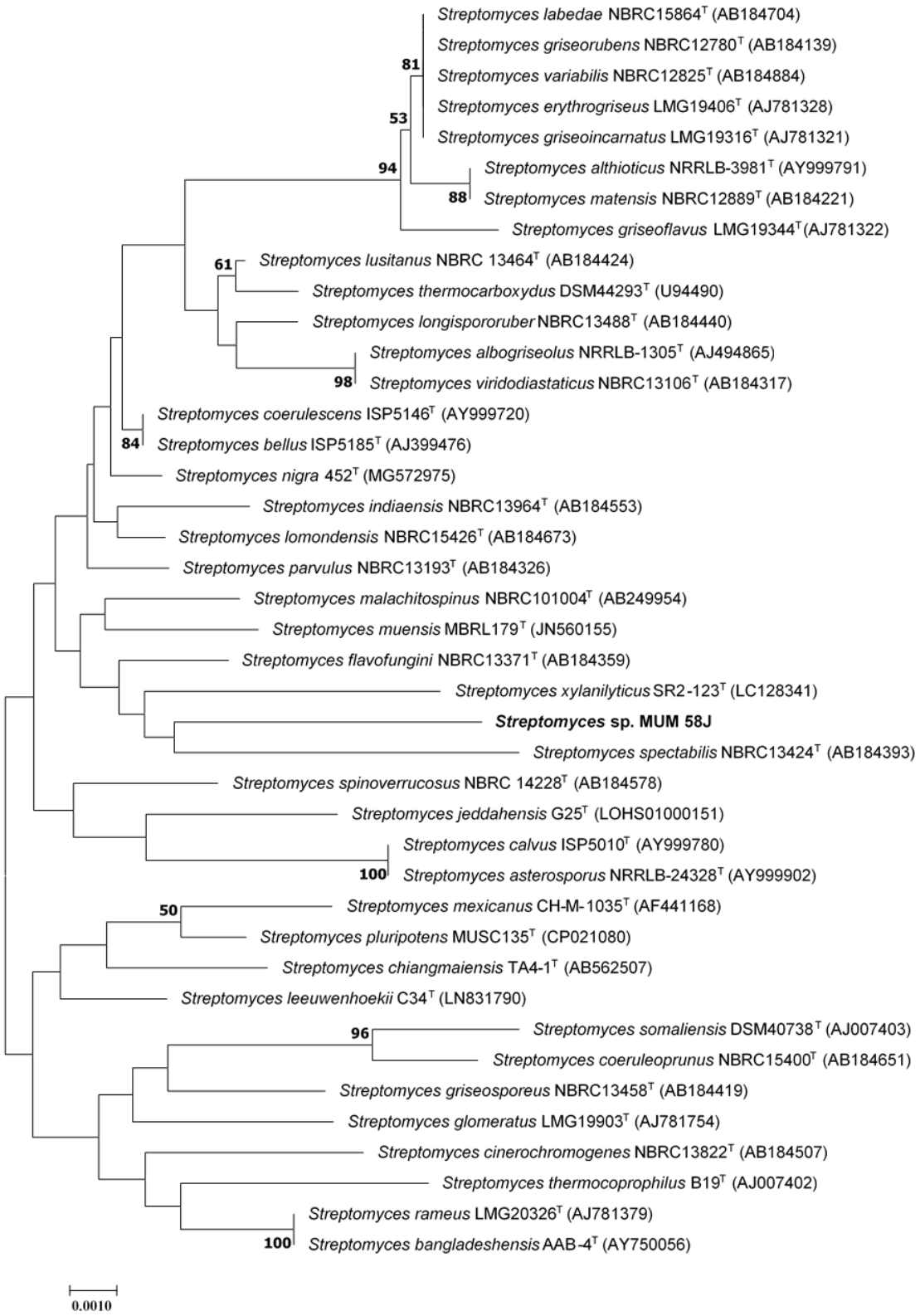

Figure 2. Neighbour-joining phylogenetic tree based on 1343 nucleotides of 16S rRNA gene sequence showing the relationship between strain MUM 58J and representatives of related taxa. Numbers at nodes indicate percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.001 substitutions per site.

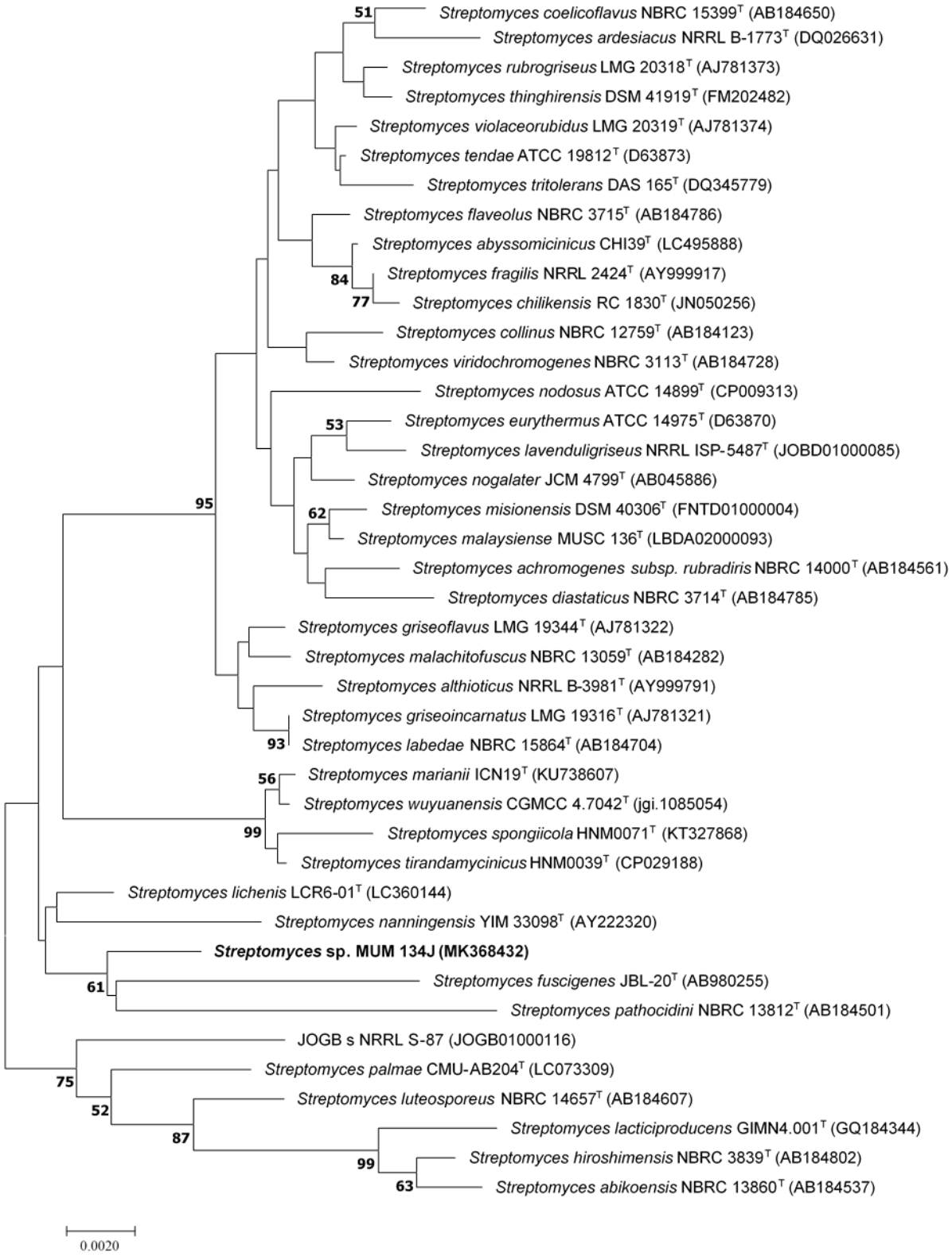

Figure 3. Neighbour-joining phylogenetic tree based on 1349 nucleotides of 16S rRNA gene sequence showing the relationship between *Streptomyces sp.* MUM 134J and representatives of related taxa. Numbers at nodes indicate percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.002 substitutions per site.

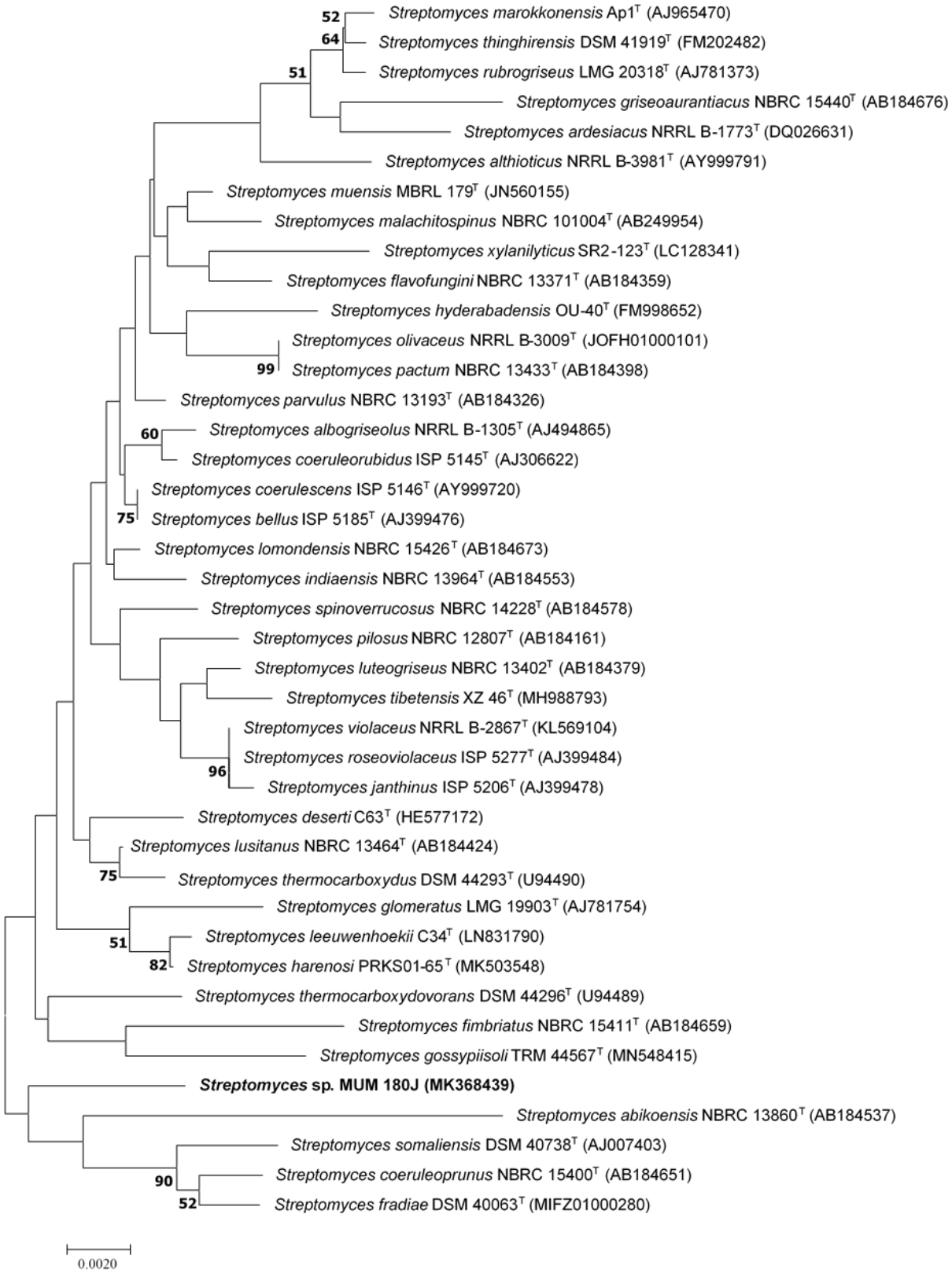

Figure 4. Neighbour-joining phylogenetic tree based on 1302 nucleotides of 16S rRNA gene sequence showing the relationship between *Streptomyces* sp. MUM 180J and representatives of related taxa. Numbers at nodes indicate percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.002 substitutions per site.

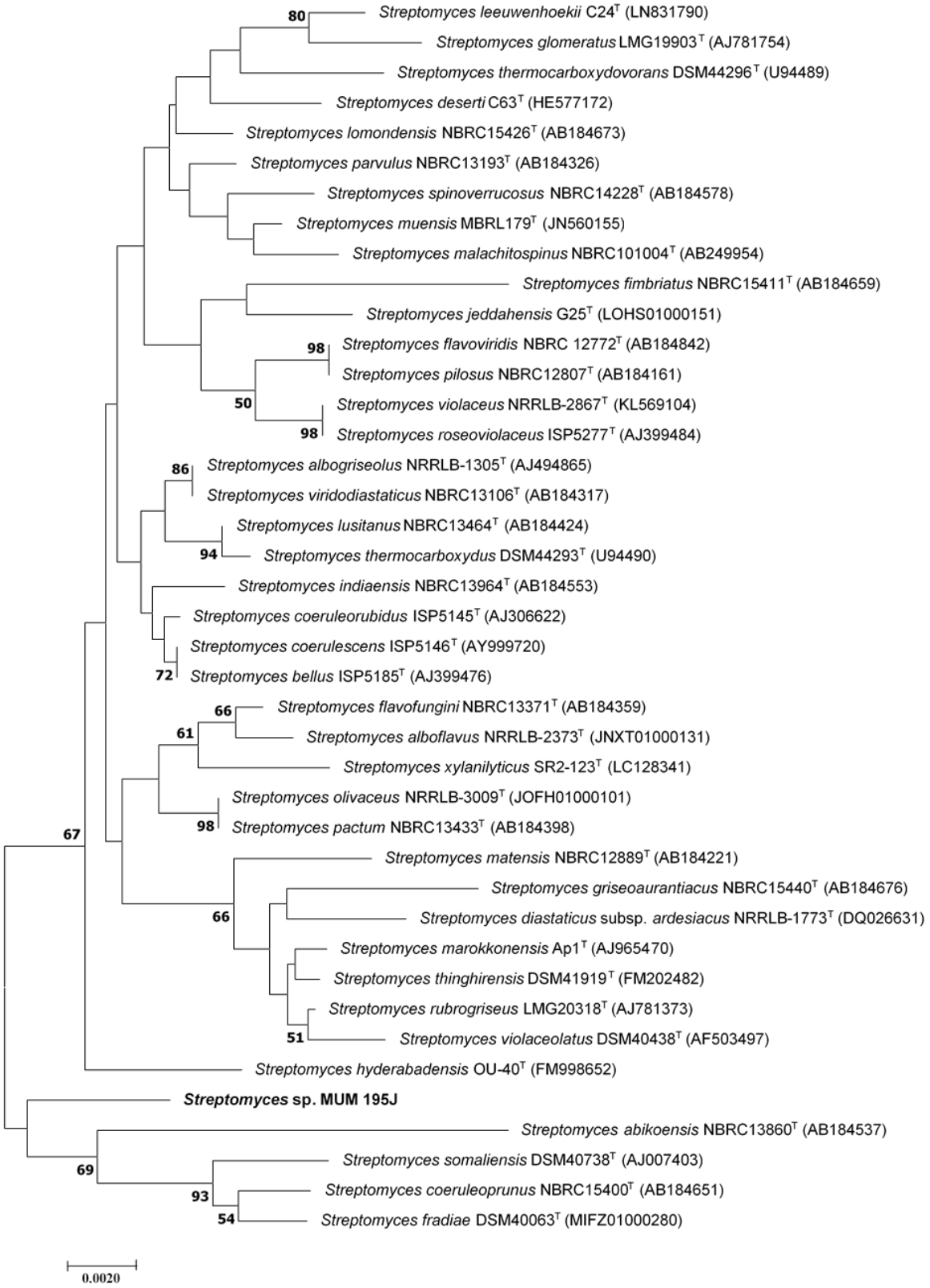

Figure 5: Neighbour-joining phylogenetic tree based on 1255 nucleotides of 16S rRNA gene sequence showing the relationship between *Streptomyces* sp. MUM 195J and representatives of related taxa. Numbers at nodes indicate percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.002 substitutions per site.

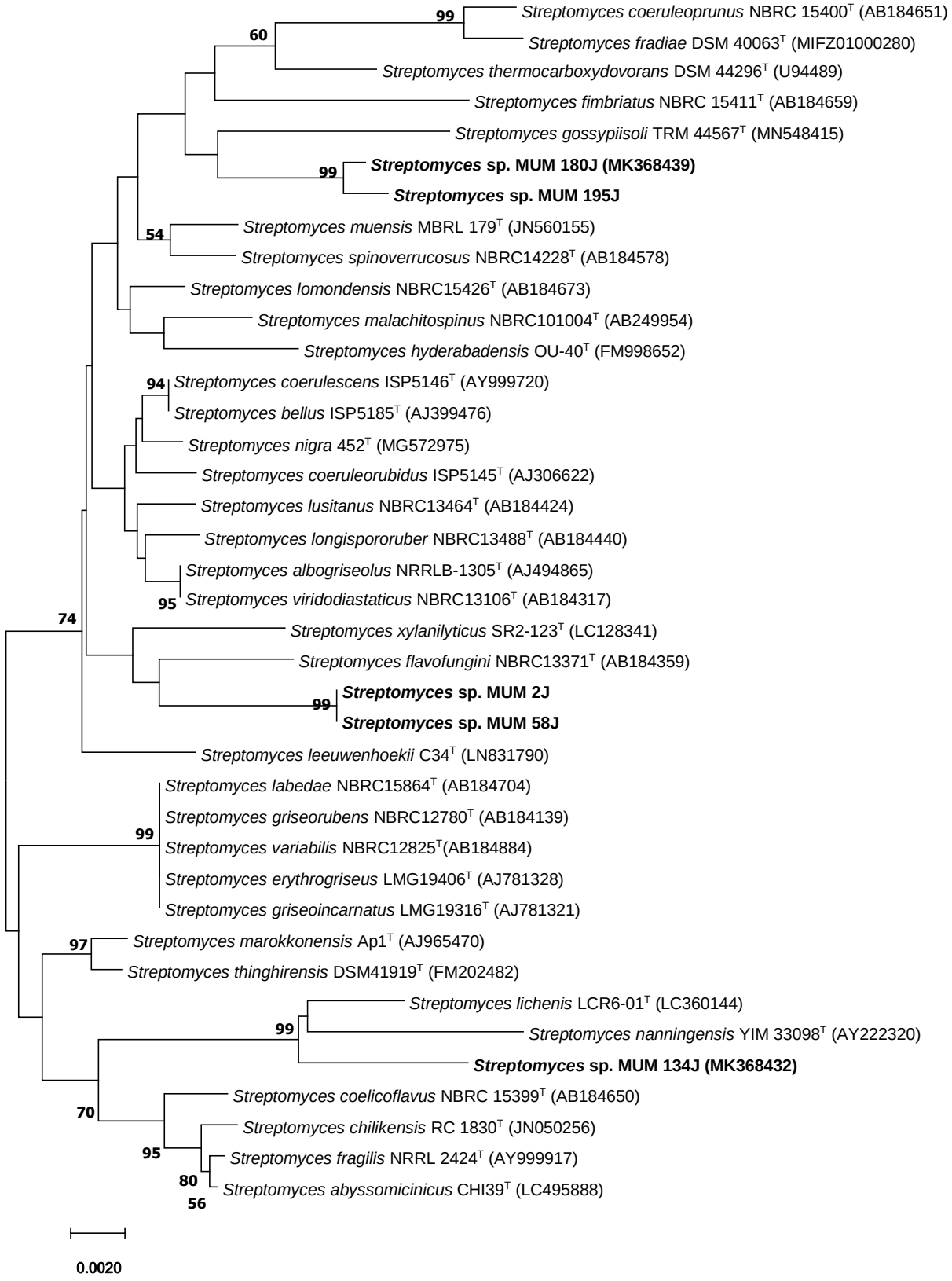

Figure 6. Neighbour-joining phylogenetic tree constructed based on 16S rRNA gene sequences inferring the evolutionary relationship between the five *Streptomyces* sp. isolates and their closely related type strains. Numbers at nodes indicate percentages (> 50%) of 1000 bootstrap re-sampling. Bar, 0.002 substitutions per site.

3.5. Phenotypic characteristics of Streptomyces sp. MUM 195J

Streptomyces sp. MUM 195J exhibits different growth patterns when cultured in different culture mediums (Table 2). Notably, *Streptomyces* sp. MUM 195J exhibits an excellent growth rate (+++) in Luria broth agar (LBA), Muller Hilton Agar (MHA), nutrient agar (NA), *Streptomyces* agar (SA) and tryptic soy agar (TSA). A good growth rate (++) was observed on ISP 2 and ISP 6. The growth rate was rated as low (+) in actinomycetes isolation agar (AIA), starch casein agar (SCA) and ISP 7. ISP 3 and ISP 4 do not support the growth of *Streptomyces* sp. MUM 195J. Diffusing pigments were noted in almost all plates except on AIA, ISP 3, ISP 4, ISP 5 and ISP 7 agar plates.

Table 2. The growth condition, the colour of colonies mycelium and diffusing pigments of *Streptomyces* sp. MUM 195J in different culture media.

Media	**Growth**	**Colour of colony mycelium**		**Colour of diffusing pigment**
		Aerial	**Substrate**	
AIA	+	Yellowish white	Yellowish white	nil
LBA	+++	Pale yellow	Light olive brown	Light olive brown
MHA	+++	Yellowish white	Strong yellow	Strong yellow
NA	+++	Pale yellow	Moderate yellow	Deep yellow
SA	+++	Pale yellow	Moderate yellow	Moderate yellow
SCA	+	Pale yellow	Pale yellow	Light yellow
ISP 2	++	Pale yellow	Greyish yellow	Moderate yellow
ISP 3	-	nil	nil	nil
ISP 4	-	nil	nil	nil
ISP 5	+	Yellowish white	Yellowish white	nil
ISP 6	++	Greyish greenish yellow	Dark greyish yellow	Dark olive
ISP 7	+	Yellowish white	Yellowish white	nil
TSA	+++	Pale orange yellow	Strong yellow	Strong yellow

AIA: actinomycetes isolation agar; **LBA:** Luria broth agar: **MHA:** Muller Hilton Agar; **NA:** nutrient agar; **SA:** *Streptomyces* agar; **SCA:** starch casein agar; **ISP:** International *Streptomyces* project; **TSA:** Tryptic soy agar; **-:** no growth; **+:** low growth; **++:** good growth; **+++:** very good growth; **nil:** no colony or no diffusing pigment.

The growth pattern of *Streptomyces* sp. MUM 195J changes with the incubation temperature (Table 3). *Streptomyces* sp. MUM 195J exhibits desirable growth rates between 26 °C and 37 °C. In contrast to the general optimum temperature at 28°C that supports *Streptomyces* sp. growth, *Streptomyces* sp. MUM 195J grows best at a slightly higher temperature of 37°C.

Table 3. The growth pattern of *Streptomyces* sp. MUM 195J on ISP 2 agar plates at 4 °C to 50 °C.

Temperature	Growth	Presence of diffusing pigments
4 °C	-	No
26 °C	++	Yes (Dark greyish yellow)
28 °C	++	Yes (Light olive brown)
32 °C	++	No
37 °C	+++	No
40 °C	-	No
50 °C	-	No

-: no growth; **+:** low growth; **++:** good growth; **+++:** very good growth.

At the extremes of pH 2, pH 4, and pH 10, the growth of *Streptomyces* sp. MUM 195J was inhibited entirely. No colony was visible to the naked eye. *Streptomyces* sp. MUM 195J exhibited a favourable growth rate within the pH range of 6 to 8. A remarkably higher growth rate was noted at pH 7.

In terms of salinity, *Streptomyces* sp. MUM 195J remains viable within a wide salinity range of 0% to 4% (w/v) NaCl. *Streptomyces* sp. MUM 195J exhibited an excellent growth rate at 0% (w/v) NaCl. TSB supplement with 2% (w/v) NaCl provides the most conducive environment for *Streptomyces* sp. MUM 195J to thrive. Hence, the highest growth rate was observed. A slight compromise in growth rate was noted when the salinity was increased to 4% (w/v) NaCl. On the other hand, further increments of the salinity to 6%, 8% and 10% (w/v) NaCl caused a total inhibition of the growth of *Streptomyces* sp. MUM 195J.

3.6. GC-MS analysis

GC-MS chromatogram analysis helped identify eight chemical compounds (Table 4; Figure 8) in the FM3 ferment.

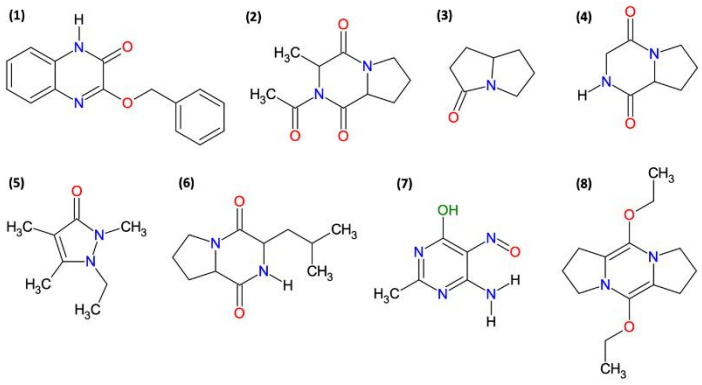

Figure 8. Molecular structures of the chemical compounds identified from the methanolic extract of *Streptomyces* sp. MUM 195J fermented in FM3 from GC-MS analysis.

Similarly, GC-MS chromatogram analysis of the metabolites in the methanolic extract of MHA agar cultured with *Streptomyces* sp. MUM 195J also detected eight chemical compounds (Table 5; Figure 9), whilst five compounds were similar to those identified from the methanolic extract of FM3 ferment.

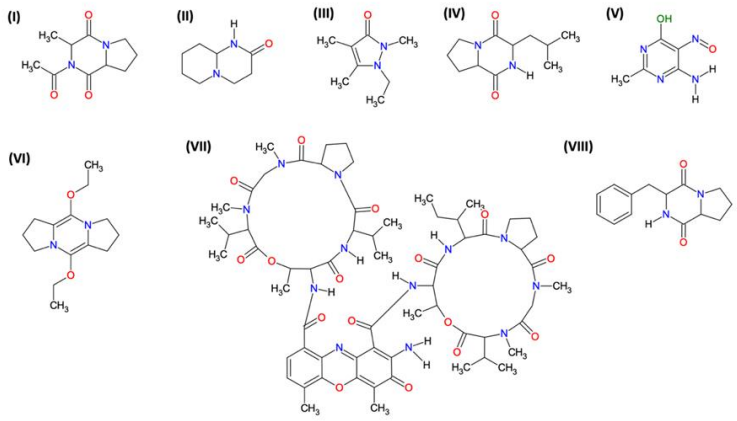

Figure 9. Molecular structures of the chemical compounds identified from the methanolic extract of *Streptomyces* sp. MUM 195J fermented in MHA from GC-MS analysis.

3.7. Co-culture assay

The co-culture assay results demonstrated that introducing *Streptomyces* sp. MUM 195J significantly reduces the viability of *V. parahaemolyticus* in the system (Figure 10). The *Vibrio* sp. viability in all treatment groups was significantly different ($p < 0.05$). Only 72.0 ± 0.9% of the *V. parahaemolyticus* remained viable when 0.5% (w/v) of *Streptomyces* sp. MUM 195J was introduced. The percentage of *Vibrio* sp. viability was further reduced to 64.8 ± 1.8% when 1.0% (w/v) of *Streptomyces* sp. MUM 195J was introduced. In other words, the co-culture test demonstrates that treatment with 0.5% (w/v) *Streptomyces* sp. MUM 195J diminishes *V. parahaemolyticus* viability by 28% while increasing the dosage to 1.0% (w/v) *Streptomyces* sp. MUM 195J successfully suppresses *V. parahaemolyticus* viability by 35%.

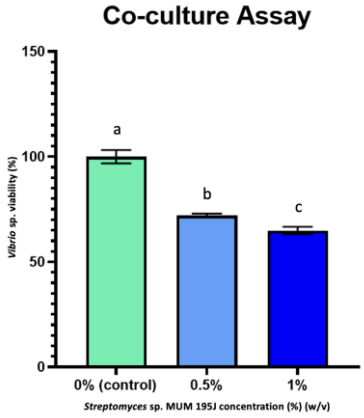

Figure 10. The percentage of *Vibrio* sp. when co-cultured with *Streptomyces* sp. MUM 195J at different concentrations. The error bars show the standard deviations of the replicates (n = 6). The different letters ($^{a-c}$) above each bar indicate significant differences between the groups ($p < 0.05$).

Table 4. Chemical compounds identified in the methanolic extract of *Streptomyces* sp. MUM 195J fermented in FM3 through GC-MS analysis.

No.	Retention time (min)	Compound	Chemical formula	Molecular weight	Similarity (%)	Similar sources identified	Activity
1	34.158	3-Benzyloxy-1,2-dihydro-2-oxoquinoxaline	$C_{15}H_{12}N_2O_2$	252	97.7	Plant *Albizia adianthifolia* [79]	Yet to be determined
2	52.258	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione,N-acetyl-	$C_{10}H_{14}N_2O_3$	210	96.7	Actinobacteria [80] *Bacillus* spp. [81, 82]	Yet to be determined
3	53.173	Hexahydropyrrolizin-3-one	$C_7H_{11}NO$	125	98.2	*Streptomyces* sp. [83] Polyherbal extract of *Terminalia chebula*, *Phyllanthus emblica* and *Dimocarpus longan* [84]	Antimicrobial, Anti-inflammatory [84]
4	53.898	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-	$C_7H_{10}N_2O_2$	154	95.2	*Bacillus* spp. [82, 85, 86] *Streptomyces* spp. [87, 88] Shewanella sp. [89]	Antimicrobial, Antifungal, Algicidal, Antioxidant Anti-inflammatory, Anti-cancer, Antihypertensive [82, 85, 86, 89, 90]
5	55.868	2-Ethyl-1,3,4-trimethyl-3-pyrazolin-5-one	$C_8H_{14}N_2O$	154	97.1	Food *tteok* [91]	Yet to be determined
6	59.067	Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-	$C_{11}H_{18}N_2O_2$	210	92.2	*Bacillus* sp. [82] *Streptomyces* sp. [92-94]	Antimicrobial, Algicidal [92], Antifungal [82]
7	59.697	4(1H)-Pyrimidinone, 6-amino-2-methyl-5-nitroso-	$C_5H_6N_4O_2$	154	92.7	*Bacillus* sp. [95, 96]	Antimicrobial [96]
8	59.822	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-α:1',2'-δ]pyrazine	$C_{14}H_{22}N_2O_2$	250	86.5	*Bacillus* sp. [82] *Lactobacillus* sp. [97]	Antimicrobial [82], Antifungal [97]

Table 5. Chemical compounds identified in the methanolic extract of MHA agar cultured with *Streptomyces* sp. MUM 195J through GC-MS analysis.

No.	Retention time (min)	Compound	Chemical formula	Molecular weight	Similarity (%)	Similar sources identified	Activity
I*	52.192	3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione,N-acetyl-	$C_{10}H_{14}N_2O_3$	210	96.7	Actinobacteria [80] *Bacillus* spp. [81, 82]	Yet to be determined
II	55.897	Octahydro-2H-pyrido(1,2-α)pyrimidin-2-one	$C_8H_{14}N_2O$	154	97.8	*Streptomyces* sp. [92] Fungus *Colletotrichum gloeosporioides* [98]	Yet to be determined
III*	56.747	2-Ethyl-1,3,4-trimethyl-3-pyrazolin-5-one	$C_8H_{14}N_2O$	154	97.1	Food *tteok* [91]	Yet to be determined
IV*	59.133	Pyrrolo[1,2-α]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)	$C_{11}H_{18}N_2O_2$	210	92.2	*Bacillus* sp. [82] *Streptomyces* sp. [92, 99, 100]	Antimicrobial, Algicidal [92], Antifungal [82]
V*	59.763	4(1H)-Pyrimidinone, 6-amino-2-methyl-5-nitroso-	$C_5H_6N_4O_2$	154	92.7	*Bacillus* sp. [95, 96]	Antimicrobial [96]
VI*	59.858	5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-α:1',2'-δ]pyrazine	$C_{14}H_{22}N_2O_2$	250	86.5	*Bacillus* sp. [82] *Lactobacillus* sp. [97]	Antimicrobial [82], Antifungal [97]
VII	60.027	Actinomycin C2	$C_{63}H_{88}N_{12}O_{16}$	1268	92.5	*Streptomyces* sp.[101]	Antimicrobial, Antioxidant, Anticancer [102, 103]
VIII	72.723	Pyrrolo[1,2-α]pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)-	$C_{14}H_{16}N_2O_2$	244	99.0	*Streptomyces* sp. [104, 105] fungus *Mortierella alpina* [106]	Antimicrobial [104, 106]

* The compounds are similarly identified in the methanolic extract of FM3 ferment (Table 4).

3.8. Congo red binding assay

The Congo red binding assay results indicate that the introduction of *Streptomyces* sp. MUM 195J supernatant reduced the percentage of Congo red binding (Figure 11). However, the reduction was insignificant when the 7-day *Streptomyces* sp. MUM 195J supernatant concentration was less than 12.5% (v/v). At 25% (v/v) supernatant concentration, the percentage of Congo red binding was reported at 47.1 ± 3.6%. These results imply that the cell surface hydrophobicity of *V. parahaemolyticus* was significantly reduced by 18% ($p < 0.05$). When 50% (v/v) supernatant concentration was applied, the percentage of Congo red binding was recorded at 31.5 ± 2.7%, signifying a reduction of the cell surface hydrophobicity of *V. parahaemolyticus* by 45% ($p < 0.05$). Based on this result, it could be surmised that *Streptomyces* sp. MUM 195J would lower the colonizing capacity of *V. parahaemolyticus* in the gastrointestinal tract of the subjects, thus lowering the risk of infectious disease.

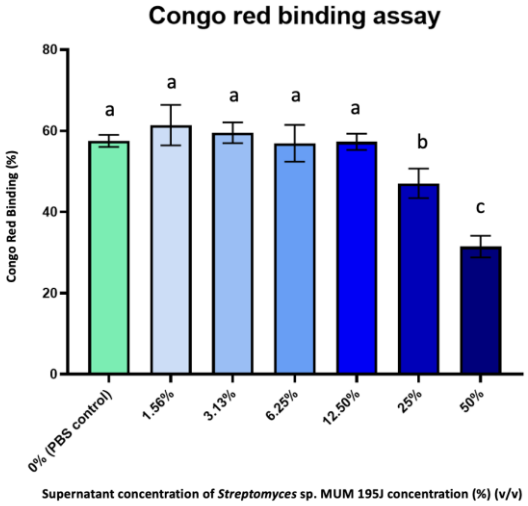

Figure 11. The Congo red binding percentage when the supernatant of *Streptomyces* sp. MUM 195J was introduced at different concentrations. The error bars show the standard deviations of the replicates (n = 3). The letters ([a-c]) above the bars indicate the significance and non-significance of data ($p < 0.05$). [a] signifies no significant difference with the other groups labelled letter [a]. The letter [b] indicates a significant difference from the groups labelled with the letter [a] and [c]. The letter [c] shows a significant difference from groups labelled with the letter [a] and [b].

3.9. Growth performance of M. rosenbergii

The growth performance of *M. rosenbergii* assessed on day 21 revealed that the average weight of the shrimp in the "+" treatment group (3.53 ± 0.62 mg) was higher than that in the "-" control group (3.09 ± 0.33 mg) (Figure 12). However, non-parametric unpaired t-test results showed that the differences between both groups were not statistically significant. When the growth data was reassessed on day 28, the average weight of the shrimp in the "+" treatment tank (5.62 ± 0.83 mg) became significantly higher ($p < 0.05$) than the average weight of shrimps in the "-" control tank (4.81 ± 0.31 mg) by 16.8%.

3.10. Survival rate of M. rosenbergii

Upon inoculation of the pathogen, the survival rate of *M. rosenbergii* (Figure 13) decreased substantially over time and was recorded as less than 25% at the 48th hour. On the other hand, the survival rate of the shrimps in both the "non-infected" groups remained at 98.0 ± 2.0% after 48 hours. The survival rate of the shrimps in the "*Streptomyces* sp. MUM 195J 1% (w/v) + *Vibrio*" group was recorded at 17.3 ± 5.8%, which is significantly ($p < 0.05$) higher than that of the "control + *Vibrio*" group, with survival rate recorded at 6.0 ± 3.5%, by approximately threefold. The survival rate of the shrimps in the "*Streptomyces* sp. MUM 195J 1% (w/v) + *Vibrio* " group (17.3 ± 5.8%) was not significantly different from that of the "Florfenicol + *Vibrio*" group (23.3 ± 5.0%). In other words, the efficacy of the prophylactic treatment of *Streptomyces* sp. MUM 195J probiotic as a feed additive was comparable to the treatment with the florfenicol antibiotic. This encouraging result suggests that *Streptomyces* sp. MUM 195J offers a promising potential to be an antibiotic alternative in mitigating *V. parahaemolyticus* infections.

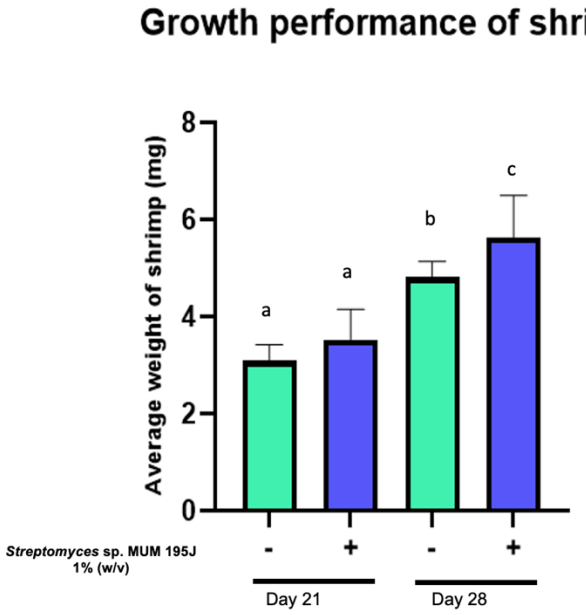

Figure 12. Growth performance of *M. rosenbergii* with and without probiotic supplementation with *Streptomyces* sp. MUM 195J at 1% (w/v) concentration on day 21 and day 28. The error bars show the standard deviations of the replicates (n = 100). The letters ($^{a-c}$) above the bars indicate the significance and non-significance of data ($p < 0.05$). ^{a} signifies no significant difference with the other groups labelled letter ^{a}. The letter ^{b} indicates a significant difference from the groups labelled with the letter ^{a} and ^{c}. The letter ^{c} shows a significant difference from groups labelled with the letter ^{a} and ^{b}.

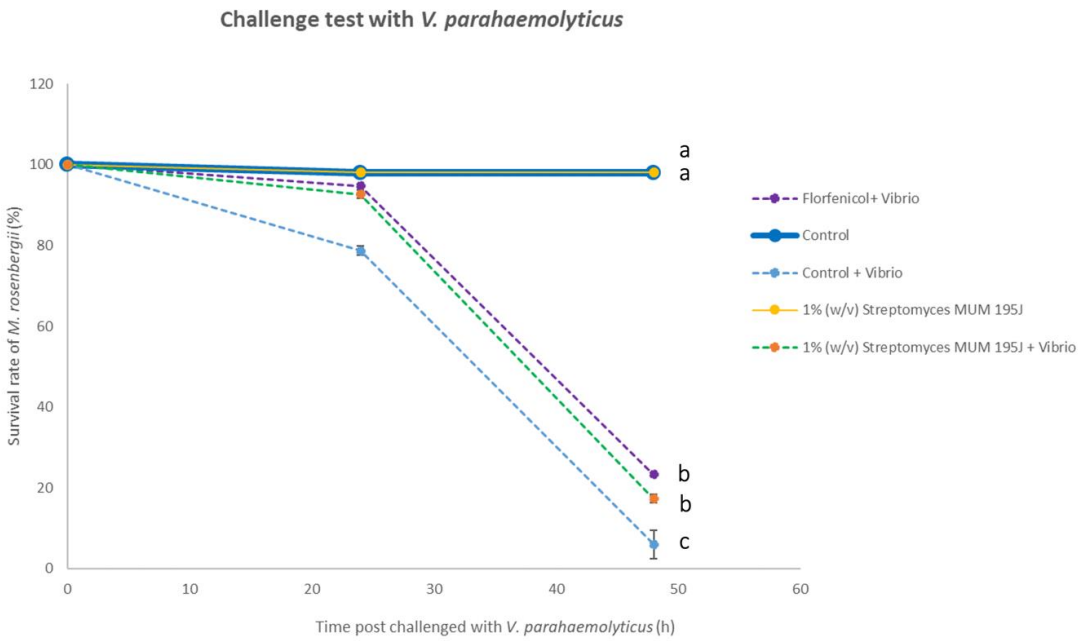

Figure 13. The survival rate of *M. rosenbergii* post-challenged with *V. parahaemolyticus*. The error bars show the standard deviations of the replicates (n = 3). The small letters ($^{a-c}$) beside the lines indicate the significance and non-significance of data ($p < 0.05$). ^{a} signifies no significant difference with the other groups labelled with the letter ^{a} beside the line. The letter ^{b} indicates no significant difference from the other groups labelled with the letter b, but it is significantly different from those labelled with the letter ^{a} and ^{c}. The letter ^{c} shows a significant difference from groups labelled with the letter ^{a} and ^{b}.

3.11. Water ameliorative effect

In this experiment, the water ameliorative effect of *Streptomyces* sp. MUM 195J probiotic was studied without water exchange for three days. The baseline values of ammonia, nitrite and nitrate levels were recorded at 4, 0 and 20 mg/L, respectively, before the initiation of the experiment. No changes in the ammonia level were reflected in any of the groups.

The probiotic introduced at the concentration of 0.01% (w/v) successfully lowered the nitrate concentration by 50% compared to the untreated group. In contrast, no significant changes in nitrate concentration were observed in groups treated with lower probiotic concentrations (Figure 14).

Besides, it was noted that 0.01% (w/v) of *Streptomyces* sp. MUM 195J helped maintain the nitrite concentration at 0 mg/L, whereas the nitrite levels in other groups, which include the non-treatment group and the groups with lower concentrations of *Streptomyces* sp. MUM 195J supplementation was recorded at 0.25 mg/L.

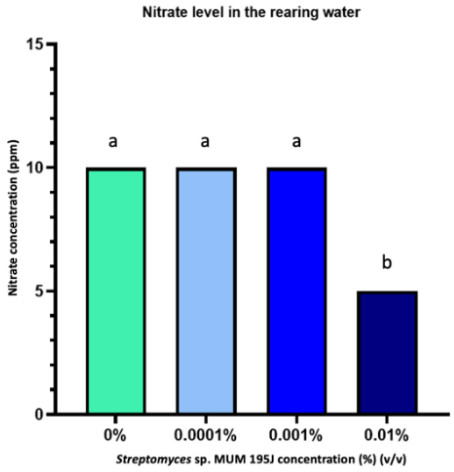

Figure 14. Nitrate level in the rearing water with different concentrations of *Streptomyces* sp. MUM 195J probiotic treatments (n = 4). The small letters ([a] and [b]) above the bars indicate the significance and non-significance of data ($p < 0.05$). The similar letter above the bars indicates no significant difference between the groups labelled. [b] signifies a significant difference from the groups labelled with the letter [a].

3.12. RT-qPCR assay

Analysis of the RT-qPCR assay (Table 6) demonstrates that HSP70 expression (Figure 15A) was significantly lowered in the treatment group by 56.1%. The differences in gene expressions of Dorsal (Figure 15B), Relish (Figure 15C), ALF5 (Figure 15D), and Crustin (Figure 15E) levels in both groups were not statistically significant. The RT-qPCR assay showed that the ProPO gene expression (Figure 15F) in the treated group was 2.44 folds higher than in the untreated group. Interestingly, the gene regulating the expression of upstream molecules in the ProPO immune system, particularly the pattern recognition proteins (PRPs) such as LGBP (Figure 15G) and lectin (Figure 15H), were down-regulated. In addition, our results showed that the α2M (Figure 15I) gene expression increased by 3.44 folds in the treated group. On the contrary, Peroxinectin expression was lowered in the treatment group (Figure 15J). The study also revealed that CHH expression in the treatment group increased significantly by sevenfold (Figure 15K).

Table 6. Gene expression changes of *M. rosenbergii* treated with 1% (w/v) *Streptomyce*s sp. MUM 195J.

Gene	Gene expression
Heat Shock Protein (HSP70)	- *
Dorsal	-
Relish	+
Anti-lipopolysaccharide Factor (ALF5)	-
Crustin	+
Prophenoloxidase (proPO)	+ *
Lipopolysaccharide and β-1,3-glucan-binding protein (LGBP)	- *
Lectin	-
α-2-Macroglobulin (α2M)	+ *
Peroxinectin (PE)	-
Crustacean Hyperglycemic Hormone (CHH)	+ *

-: gene expression lowered; +: gene expression elevated; *: significant ($p < 0.05$).

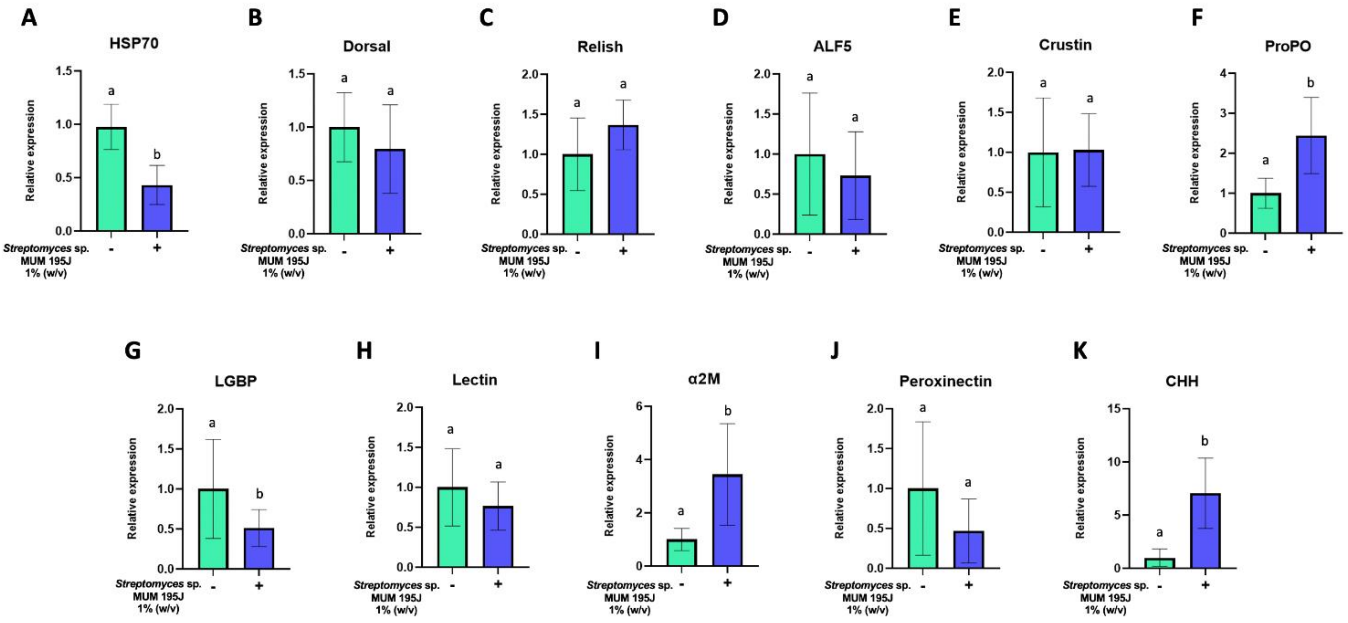

Figure 15. Gene expression in *M. rosenbergii* sampled from the treatment groups. A) The relative expression of HSP70 (n = 10); B) The relative expression of Dorsal (n = 11); C) The relative expression of Relish (n = 4); D) The relative expression of ALF5 (n = 10); E) The relative expression of Crustin (n = 11); F) The relative expression of ProPO (n = 5); G) The relative expression of LGBP (n=10); H) The relative expression of Lectin (n = 10); I) The relative expression of α2M (n = 5); J) The relative expression of Peroxinectin (n = 10); K) The relative expression of CHH (n = 4). The small letters (a and b) above the bars indicate the significance and non-significance of data ($p < 0.05$). The similar letter above the bars indicates no significant difference between the groups. Labelling with different letters, a and b, signifies a significant difference between both groups.

4. Discussion

Phylogenetic analysis of the highly-conserved 16S rRNA gene confirmed the identity of the five putative isolates as streptomycetes. The phylogenetic tree illustrates that the isolates obtained from the same site are closely related. It is interesting to note that both *Streptomyces* sp. MUM 180J and MUM 195J demonstrate a high percentage similarity to *Streptomyces fradiae*, which has been introduced as a feed supplement and water additive to *P. monodon* with positive outcomes [107].

Interestingly, both *Streptomyces* sp. MUM 2J and MUM 58J displayed distinct activities and characteristics despite being obtained from the same site and are closely related isolates. This suggests that despite their common origin, these isolates exhibited notable differences in their properties. The finding also implies that the activity of *Streptomyces* sp. is specific to each isolate. Therefore, a comprehensive screening process is necessary to identify isolates with the desired activities for exploitation.

Streptomyces sp. MUM 195J was selected as the candidate strain and deposited in the China General Microbiological Culture Collection Centre (CGMCC) due to its potent

antagonistic activity against *V. parahaemolyticus* observed in the cross-streak assay and its exceptional growth rate. The high growth rate and abundant pigment production observed in most of the culture mediums provide additional insights into the high metabolic capacity of *Streptomyces* sp. MUM 195J.

It is reassuring to observe that the temperature and salinity range that supports the optimum growth of *Streptomyces* sp. MUM 195J falls within the optimal temperature range for the development of *M. rosenbergii*, which is reported to be between 27°C and 31°C [108, 109]. Moreover, it is notable that *Streptomyces* sp. MUM 195J demonstrated a desirable development rate within the pH range of 6 to 8, as this range aligns with the typical pH range of 6 to 7.5, which is common to the hatchery, nursery and grow-out ponds for *M. rosenbergii* [110]. These characteristics increase the chances of the probiotic colonizing the aquaculture system and maintaining its performance even when confronted by the fluctuating parameters within the aquaculture system.

GC-MS analysis of the methanolic extract of *Streptomyces* sp. MUM 195J culture revealed that the isolate secretes a range of antimicrobial metabolites, including Hexahydropyrrolizin-3-one, Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-, Pyrrolo[1,2-α]pyrazine-1,4-dione,hexahydro-3-(2-methylpropyl)-, 4(1H)-Pyrimidinone, 6-amino-2 -methyl-5-nitroso- and 5,10-Diethoxy-2,3,7,8-tetrahydro-1H,6H-dipyrrolo[1,2-α:1',2'-δ]pyrazine [82, 84, 92, 96]. These metabolites may be responsible for the inhibitory effect against *V. parahaemolyticus*. However, the direct contribution of the chemical compounds on the inhibitory mechanisms of *Streptomyces* sp. against *V. parahaemolyticus* is yet to be elucidated.

Despite having a relatively slower growth rate than *V. parahaemolyticus*, the presence of *Streptomyces* sp. MUM 195J consistently depressed the growth of *V. parahaemolyticus* in the co-culture test [111, 112]. This result corroborates a previous study, where the viable count of *V. parahaemolyticus* was effectively reduced when co-cultured with *Streptomyces* sp. RL8 [113]. Similarly, *Streptomyces* sp. S073 was reported to depress the proliferation of *V. parahaemolyticus* by 31% [72]. Another study demonstrated that co-culturing *Streptomyces* sp. M56 bio granules with *V. parahaemolyticus* significantly eliminate 60% of the pathogens in 24 hours. The activity led to the elimination of 80% of the pathogens by 48 hours. All 100% of the *V. parahaemolyticus* was inhibited after 72 hours of treatment [114]. The antagonistic activity of *Streptomyces* sp. against *V. parahaemolyticus* could be attributed to competitive exclusion or the secretion of inhibitory compounds such as siderophore [72, 115].

The modified Congo red binding used the percentage of Congo red binding as a surrogate marker for hydrophobicity, a pathogenicity feature for *V. parahaemolyticus.* The observed lower Congo red binding could indicate biofilm disruption, which is essential for pathogen colonization at mucosal surfaces [73]. It can be inferred that *Streptomyces* sp. may influence factors such as protease activity, pH level, or ionic properties within the system, which are key determinants affecting the percentage of Congo red binding. However, further

assessment can be conducted to define the exact mechanism of action if the *in vivo* testing shows promising results.

In general, infections related to *Vibrio* sp., which can lead to mass mortality, are more common during the early life stages of shrimps, particularly in hatcheries. The peak of infection typically occurs between day 20 and day 30 post-stocking in grow-out ponds [116-118]. The rationale behind introducing probiotics at an earlier stage of life proffered a higher likelihood of achieving better outcomes. This approach contrasts with previous studies that primarily assessed *Streptomyces* sp. efficacy in post-larval stages, which are the most commonly assessed stages in *in vivo* evaluations [68, 107, 119-121].

The immune response is an important parameter to be assessed concerning the usage of probiotics in disease control. The ability to resist pathogens is directly correlated with the immunological function of the animal [122]. In recent years, there has been increasing evidence supporting the immune regulating effect of probiotics on both the cellular and humoral immunity of crustaceans [123, 124]. However, only a few studies looked into the immunological impact of *Streptomyces* sp. probiotics supplementation on shrimps [125-127]. Although these findings garnered positive results, the data supporting the immunological effect of *Streptomyces* sp. is considerably less when compared to other probiotics like *lactobacillus* and *bacillus*. Our RT-qPCR assay offered a glimpse into the immune priming and growth-promoting effect of *Streptomyces* sp. MUM 195J. This effort offers some insights into the likely mechanism of action of the probiotic—the immune modulatory effect of *Streptomyces* sp. MUM 195J probiotic was established when the RT-qPCR assay showed that the ProPO and α2M gene expression was significantly higher than the untreated group. Different aspects of the ProPO systems have been investigated in black tiger shrimp (*Penaeus monodon*) [128, 129], brown shrimps (*Penaeus californiensis*) [130], Chinese shrimp (*Fenneropenaeus chinensis*) [131], Kuruma prawn *(Penaeus japonicus*) [132], ridge-tail white prawn (*Exopalaemon carinicauda*) [133] and white shrimp (*Litopenaeus vannamei*) [134-136]. Shrimps treated with these probiotics generally demonstrate higher immunological indexes such as haemocyte counts, phagocytic rate and phenoloxidase activity [124, 137].

On the other hand, there is limited data comparing the efficacy of probiotics and antibiotics in controlling *Vibrio* spp. infection. To the best of our knowledge, our experiment is the first trial that compared the effectiveness of *Streptomyces* sp. probiotic with florfenicol antibiotic in mitigating *V. parahaemolyticus* infection in shrimp. Our findings corroborate a previous report that compared the efficacy of 5×10^9 to 5×10^{11} CFU/kg *Bacillus subtilis* probiotic with 0.3% of florfenicol [138]. The treatment with 0.3% of florfenicol recorded a significantly higher relative percentage survival of *L. vannamei* than probiotic treatment groups in the fourth week but exhibited no significant difference with the probiotic efficacy in the eighth week.

Comparing the growth profile obtained at different time points of the study noted that the growth-promoting effect becomes more prominent with increasing duration of the treatment. The preliminary data of our study suggested that a treatment duration of more than

two weeks is necessary to achieve a significant growth promotion effect. A similar trend was also observed in a previous finding, which showed that the weight improvement of *L. vannamei* was not significant on day 42 but was significantly elevated by 20% on day 87 following the initiation of the feeding trial [139]. However, our findings showed that the growth promotion effect became evident within 21 days of daily treatment with *Streptomyces* sp. MUM 195J probiotic, which is a relatively shorter time frame compared to the previous report. This rapid growth promotion effect is advantageous for the *M. rosenbergii* hatcheries as the post-larvae are usually sold to farmers for rearing in grow-out ponds around day 30 after hatching.

The growth promotion effect of *Streptomyces* sp. probiotics could be attributed to various factors, including modulation of the gut morphology and microbial composition, elevation of the enzymatic activity, stimulation of the appetite, and enhancement of immune function [140]. Our preliminary studies showed that CHH expression in the probiotic treatment group increased significantly. Upregulation of this gene regulating carbohydrate metabolism may be one of the contributing mechanisms for growth [141]. However, growth regulation is multifactorial [140]. An in-depth analysis of other genes involved in the metabolic process is needed to decipher a more precise mechanism of activity.

The beneficial effects of introducing growth-promoting probiotics are evident from both the practical and economic perspectives. In recent years, there has been an increasing dependence on the utilization of low-quality agricultural by-products and waste as alternative feed sources for aquaculture due to the escalating commercial feed costs [142, 143]. This feed often lacks the complete nutritional content to support the optimum growth of the aquatic livestock, thus negatively impacting the overall quality of the production systems. This idea of utilizing probiotics as a feed additive to promote animal growth is particularly helpful and can act as a safe and natural means to enhance production.

Routine water exchange is a fundamental husbandry practice to maintain optimum water quality for shrimp growth. However, frequent water exchanges demand high water resource input, risk animal escape, and increase the impact of waste on the environment [144]. The release of improperly untreated water from an open system leads to eutrophication. At the same time, the accumulation of organic particulates and depletion of essential nutrients is an issue for a closed circulatory system [145, 146]. In this regard, our results show that the effectiveness of probiotics in nitrate and nitrite level amelioration is concentration-dependent. A minimum of 0.01% (w/v) is required for the probiotic to demonstrate its efficacy.

5. Conclusions

Streptomyces sp. MUM 195J was selected as the candidate probiotic strain for this study, attributed to its strong inhibitory potential against the *V. parahaemolyticus* pathogen. The cross-streak assay constitutes a rapid and cost-effective method that enables the rapid cognisance of the inhibitory potential of the isolates against *Vibrio* sp. Additionally, the co-culture assay and Congo red binding assay effectively demonstrated the probiotic properties of the candidate strain. Moreover, the anti-bacterial properties of *Streptomyces* sp. MUM 195J was preliminarily established with the identification of bioactive compounds via GC-MS analysis.

The challenge test results showed that treatment with *Streptomyces* sp. MUM 195J probiotic significantly increased the survival rate of *M. rosenbergii* by threefold, achieving an efficacy comparable to the treatment with 12 mg/L of florfenicol antibiotic. In particular, the significant antagonistic effect against *V. parahaemolyticus* and effective augmentation of the survival rate of the post-larvae during infection suggests that *Streptomyces* sp. MUM 195J could be an excellent alternative to antibiotics in disease prophylaxis. This finding could revolutionize the aquaculture industry and mitigate its contribution to AMR development. Moreover, RT-qPCR analysis demonstrated the suitability for *Streptomyces* sp. MUM 195J to be introduced as a natural agent to prime the immune function of the shrimps to enhance their resilience towards *Vibrio* sp. infections, particularly at the early stages of cultivation when the infection risk is the highest.

Besides the remarkable *Vibrio* sp. infection control property, the *in vivo* feeding trial also witnessed a significant growth promotion effect of 16.8% following the three-week-long treatment with *Streptomyces* sp. MUM 195J probiotic. This suggests that *Streptomyces* sp. MUM 195J can also be promoted as a growth-promoting supplement. Last but not least, *Streptomyces* sp. MUM 195J probiotics could be deliberately harnessed to regulate water quality, which can indirectly help reduce aquaculture water usage and partly solve the effluent pollution issue. Figure 16 shows a graphical abstract of the potential use of mangrove-derived *Streptomyces* sp. as probiotics against *V. parahaemolyticus* infection in aquaculture farming.

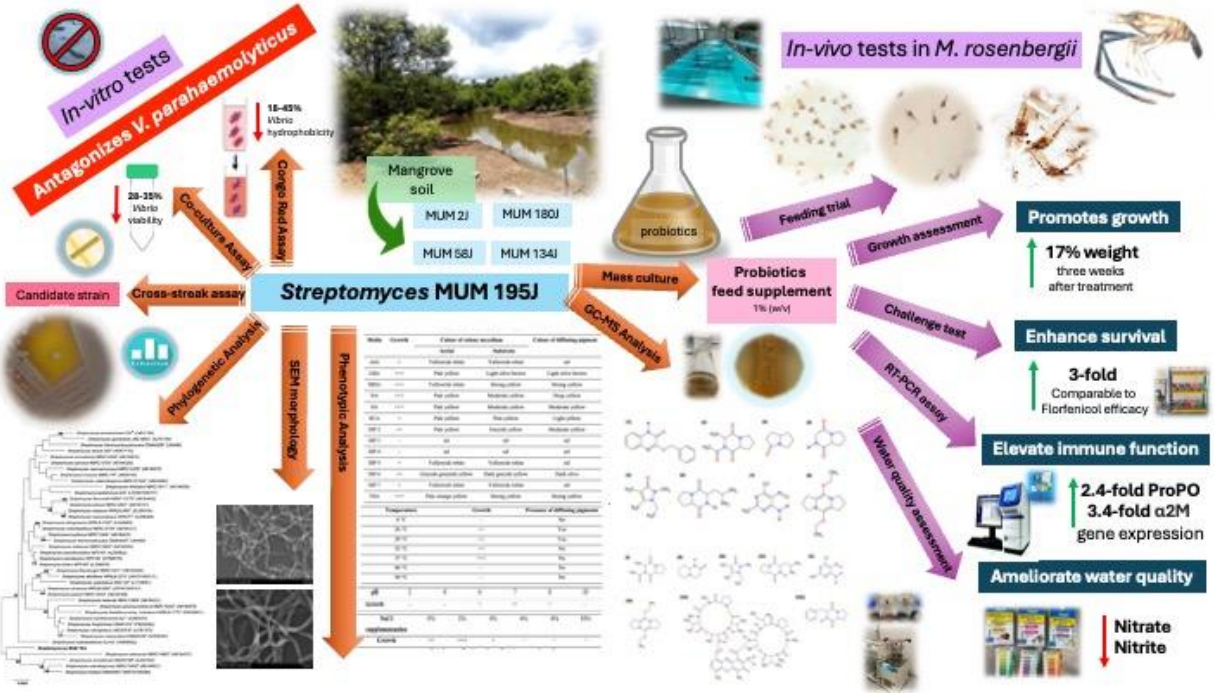

Figure 16. Graphical abstract: The potential use of mangrove-derived *Streptomyces* sp. as probiotics against *V. parahaemolyticus* infection in aquaculture farming. *Streptomyces* sp. MUM 195J has exhibited promising and potential probiotic effects in *in vitro* and *in vivo* tests.

Being one of the pioneer studies that aims at investigating the potential of mangrove-derived *Streptomyces* sp. in aquaculture applications in Malaysia, this study sought to make a meaningful contribution towards developing a systematic framework that expedites the identification, formulation, and implementation of indigenous probiotics, specifically designed to combat specific pathogens affecting different aquaculture species. The goal is to develop effective functional feed additives that local farmers can readily adopt to realize the potential of self-sustaining aquacultural production. On a broader scope, this study aims to contribute to mitigating antimicrobial resistance by proposing an alternative to antibiotics in controlling bacterial infection. This holistic approach is crucial for realizing the full and sustainable potential of aquaculture as a cornerstone of global food security.

Author Contributions: Conceptualization, JGXH, LT-HT, VL, L-HL, B-HG; formal analysis, JGXH, LT-TH; data curation, JGXH, LT-HT; writing-original draft preparation, JGXH; writing-review, editing and proof, JGXH, LT-HT, JW-FL, GK, JM, K-YK, WHY, K-GC, VL, L-HL, B-HG.

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Conflicts of Interest: The authors declare no conflict of interest.

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