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Isolation, Molecular Identification, and Anti-*Staphylococcus aureus* ATCC 25923 Activity Testing of Endosymbiotic Bacteria from *Litopenaeus vannamei*

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Abstract

L. vannamei, one species of the subphylum Crustacea, which is the main commodity of Indonesian seas because it's fast growth, high survival rate, adaptability and high selling price. The presence of endosymbiont microscopic organisms that live and develop in creature tissues by advantageous mutualism, moreover has great capacity and movement as antibacterial specialists. This study aims to isolate, molecularly differentiate, and evaluate the antibacterial activity of S. aureus from endosymbiont microorganisms present in L. vannamei head wastes. Shrimp head waste was immunized on supplement agar media to achieve bacterial containment. The growing bacterial colonies were filtered for atomic differentiating evidence and easily observable, minuscule testing. Using the plate dissemination approach, endosymbiont bacterial confines were tested for antibacterial migration (Kirby-Bauer). The inhibition zone formed was observed. Endosymbiont bacterial colonies that have antibacterial movement were recognized molecularly utilizing 16S rRNA primers followed by analysis using the BLAST method and phylogenetic construction using MEGAX software with UPGMA method. Endosymbiont bacterial separate was gotten which had potential movement shown by the arrangement of an restraint zone against S. aureus ATCC 25923. Auxiliary metabolites of endosymbiont microscopic organisms were aged on supplement broth medium and after that tried for antibacterial movement against S. aureus ATCC 25923. There was antibacterial activity at concentrations of 10%, 15% and 20% of secondary metabolite extracts of endosymbiont bacterial isolates with an average diameter of 11.07 mm, 11.97 mm and 13.91 mm. Molecular identification showed that endosymbiont bacterial isolate was closely related to Staphylococcus gallinarum strain RTE-S1 (LC572265).

I. Introduction

One of the most pressing global health issues is antibiotic resistance. Pathogens develop immunity to antibiotics, causing resistance. The global spread of resistance makes it difficult to treat infectious diseases due to the reduced effectiveness of antibiotics. Around the world, cases of antibiotic resistance need to be addressed through systematic efforts. Indonesia has launched a National Action Plan on Antimicrobial Resistance that includes education, monitoring antimicrobial resistance, reducing the incidence of infection, and optimizing the use of antimicrobials in humans (Biro Komunikasi dan Pelayanan Masyarakat, 2017).

Bacteria are microorganisms that cause infections and lead to antibiotic resistance. One of the bacteria that cause infections is *Staphylococcus aureus*. *S. aureus* is a facultative anaerobic opportunistic pathogen that is part of the normal flora. *S. aureus* is often found in humans and animals and can cause various infections, including skin and soft tissue infections (impetigo, furuncles, carbuncles, cellulitis), mastitis, urinary tract infections (UTIs), osteomyelitis, meningitis, food poisoning, biofilm-associated infections or septicemia, sepsis, endocarditis, osteomyelitis, and pneumonia (Otto, 2013; Tong *et al.*, 2015). *S. aureus* is the primary cause of nosocomial infections, involved in 30% of cases of infective endocarditis (Saeed *et al.*, 2019) and the second most common cause of pneumonia in hospitalized patients (Lesher *et al.*, 2016).

Shrimp is one of Indonesia's most sought-after fishery commodities. One of the most widely cultivated shrimp species in Indonesia is *L. vannamei*. Some of the advantages of vannamei shrimp include broad salinity tolerance and high productivity, making it the top choice for farmers.

This shrimp also has better resistance to several major shrimp diseases, such as white spot syndrome virus (Abdel-Tawwab *et al.*, 2022). Vaname shrimp have significant economic value due to high market demand, even overseas, in the form of processed food by the shrimp freezing industry, including head-on (whole shrimp), headless (shrimp without heads), and peeled (shrimp without heads and shells) (Octovianus *et al.*, 2023).

Shrimp head waste has the potential to be utilized, one of which is chitosan. Chitosan is a polysaccharide obtained from the deacetylation of chitin, which generally comes from animals of the subphylum Crustacea. Chitosan has antibacterial potential because it contains lysozyme and aminopolysaccharide groups. Chitosan is known to have antibacterial activity against *Staphylococcus aureus*, *Salmonella paratyhpi* (Fernandez-Kim, 2004), *Escherichia coli*, *Propionibacterium acnes*, *Pseudomonas aeruginosa*, and *Staphylococcus epidermidis* (Suherman *et al.*, 2018).

Endosymbiotic bacteria are a group of microorganisms that live inside the cells or tissues of their host organisms, forming a symbiotic relationship. This relationship can be mutualism, commensalism, or even parasitism. In mutualistic symbiosis, endosymbiotic bacteria provide specific benefits to their hosts, such as the synthesis of essential nutrients, protection against pathogens, or improved host adaptation to specific environments. In return, the host provides an environment that supports the survival and reproduction of these bacteria (Moran and Bennett, 2014).

Secondary metabolites are organic compounds produced by organisms (Wulandari and Pujiyanto, 2020) that have several activities, including antioxidant, anti-inflammatory, antibiotic, anticancer, antidiabetic, and immunosuppressive activities (Desriani *et al.*, 2014). Secondary metabolites tend to have complex structures and are difficult to synthesize, making them hard to find and giving them high economic value (Mariska, 2013). Based on the above background, the author intends to isolate, molecularly identify, and test the antibacterial activity against *S.aureus* from the endosymbiotic bacteria of *L. vannamei*.

II. Research Method

II.1 Design, place and time

This study was an experimental study conducted at the Microbiology Laboratory, Department of Pharmacy, Health Polytechnic of Surakarta and PT. Genetika Science Indonesia from February to August, 2024.

II.2 Materials and tools

Materials used include *L. vannamei* shrimp obtained from shrimp ponds in Tegal Regency, white tips, yellow tips, sterile gloves (Maxter™), cotton swabs (OneMed®), masks (Sensi®), 1.5 mL PCR tubes, dimethyl sulfoxide (Merck®), nutrient broth (Merck®), chloramphenicol (Oxoid™), Nutrient Agar (Merck®), disc paper (Oxoid™), label paper (Joyko®), Mueller-Hinton Agar (Sigma-Aldrich®), distilled water (OneMed®), 70% alcohol (OneMed®), ethyl acetate (Sigma-Aldrich®); 0.9% NaCl (Otsuka®), Quick-DNATM MagBead Plus Kit (Zymo Research), MyTaqTM HS Red Mix (Bioline, BIO-25048) and 16S rRNA primers (V3–V4).

The equipment used includes Petri dishes (Pyrex®), 250 mL Erlenmeyer flasks (Pyrex®), test tubes (Pyrex®), beakers (Pyrex®), micropipettes (ThermoFisher Scientific®), scalpel blades (Renz®), forceps (Renz®), scissors (Renz®), incubators (Memmert®), 100 mL measuring cups (Pyrex®), inoculation needles, McFarland Equivalence Turbidity Standard (Remel™), Drugalski spatulas (Hammacher Schlemmer®), Bunsen burner, analytical balance (Ohaus®), tweezers (Onemed®), centrifuge (frontierTM5718R), vortex (V–32 Biosan), NovaSeqTM 6000 Sequencing System (Illumina Inc.), NanoDrop spectrophotometer (Implen®), electrophoresis (HU6, SCIE-PLAS), Gel Doc EZ Imager (Bio-Rad®), thermo-shaker (TS-100), autoclave (Equitron®), incubator (Memmert®), and vernier caliper (Vernier®).

II.3 Sterilization of Tools and Materials

Research instruments and media were sterilized in an autoclave for approximately 15 minutes (1 atm) at a temperature of 121ºC. After the sterilization process was complete, the medium was stored in a refrigerator (2-8ºC) and the instruments were stored in a clean place (Sirri *et al.*, 2022).

II.4 Seawater Procurement

The seawater used in the study was obtained from the sampling location of *L. vannamei* shrimp by first filtering it until clear using filter paper, then sterilizing it using an autoclave at a temperature of 121ºC for ± 15 minutes at a pressure of 1 atm (Sirri *et al.*, 2022).

II.5 Preparation of Nutrient Agar Medium

Weigh 3 g of Nutrient Agar, place it in a 250 mL Erlenmeyer flask, then dissolve it in 150 mL of water and heat it on a magnetic stirrer hotplate until it is homogeneous and boiling. The medium is sterilized using an autoclave for ±15 minutes at a temperature of 121°C (Hartono, 2020).

II.6 Isolation of Endosymbiotic Bacteria

L. vannamei head were washed with sterile seawater, then soaked in 70% alcohol for 1 minute, sodium hypochlorite solution for 5 minutes, washed with 70% alcohol, and washed again with sterile distilled water for 2 minutes (Hartono, 2020) to minimize bacterial contamination of the samples. The cleaned vaname shrimp are then cut using a scalpel, their heads are removed and placed in sterilized Nutrient Agar medium (Harmatang, 2014).

II.7 Purification of Endosymbiotic Bacterial Isolates

Purification of endosymbiont bacterial isolates is carried out by transferring different bacterial isolates from the old NA medium to the new NA medium. If macroscopically there are still different bacterial colonies on the medium, reseparation is required until a pure isolate is obtained (Rustini *et al*., 2022).

II.8 Preparation of Suspension and Inoculum of Endosymbiotic Bacterial Isolates

Endosymbiotic bacterial isolates were inoculated at a rate of 4 drops using a round inoculation needle into 20 milliliters of sterile 0.9% NaCl solution. This solution was then homogenized with a vortex and its turbidity was compared to McFarland 0.5 (Somu and White, 2017). The endophytic bacterial isolate suspension was placed in a 20 mL Erlenmeyer flask and then Nutrient Broth (NB) medium was added to a total volume of 200 mL. Next, a rotary shaker was used for 24 hours at a temperature of 30°C and a speed of 200 rpm (Rustini *et. al.*, 2022).

II.9 Fermentation of Endosymbiotic Bacterial Isolates

The shaken endosymbiotic bacterial inoculum was added at a volume of 20 mL to 400 mL of NB medium for each endosymbiotic bacterial isolate. Fermentation was carried out at 37°C for 54 hours using a shaker incubator at a speed of 200 rpm (Octovianus *et al.*, 2023).

II.10 Separation of Biomass and Supernatant

The fermented NB medium was then centrifuged at 5000 rpm for 15 minutes. The bacterial cell biomass separated from the substrate using a micro pipette was then extracted with ethyl acetate (Irwandi, 2018).

II.11 Extraction of Secondary Metabolites from Endosymbiotic Bacterial Isolates

The substrate was macerated with 300 mL of ethyl acetate solvent at a ratio of 1:1 v/v in an Erlenmeyer flask for 24 hours. After that, the solution was transferred to a separating funnel to extract the maceration results by shaking and allowing it to settle until two layers formed. For one day, the lower layer, which is the medium, was macerated again with ethyl acetate solvent at a ratio of 1:1 v/v, and the upper layer, which is the ethyl acetate extract, was evaporated using a rotary evaporator. The concentrated ethyl acetate extract is mixed with 5 mL of methanol and stored at 40°C (Rustini *et al*., 2022).

II.12 Antibacterial Activity Test

Antibacterial activity testing was performed using the Kirby-Bauer disk diffusion method. A suspension of test bacteria (*Staphylococcus aureus* ATCC 25923) standardized to 0.5 McFarland was added 200 µL to 30 mL of sterile Mueller-Hinton Agar (MHA) medium, vortexed, then poured into Petri dishes and allowed to solidify. Antibacterial activity testing was performed on extracts of endosymbiotic bacterial secondary metabolites. The extract concentrations were 10%, 15%, and 20% with DMSO as the solvent. A total of 10 µL of the endosymbiotic bacterial secondary metabolite extract was dripped onto a paper disc, which was then placed on the surface of the solidified MHA and incubated for 24 hours at 37°C. Chloramphenicol 30 µg/disk was used as a positive control, while DMSO solvent was used as a negative control. After incubation, the clear zone around the disc was measured using a caliper (Rustini *et. al.*, 2022).

II.13 Molecular Identification of Endosymbiotic Bacteria

II.13.1 DNA extraction

Active isolates of L. vannamei endosymbiont bacteria were grown for 24 hours on Nutrient Agar medium at 37°C, then extracted using (ZymoResearch, D4082) (B/7.2.1/IKP/009). A total of 400 µL of endosymbiotic bacteria sample was placed in a sterile 1.5 mL microcentrifuge tube containing 400 µL of Quick-DNA™ MagBinding Buffer. The mixture was homogenized by pipetting. Then, 33 µL of MagBinding Beads was added, and the mixture was homogenized again by pipetting. The sample was transferred to a magnetic stand until the magnetic beads separated from the solution, then the supernatant was discarded. The sample was then transferred from the magnetic stand, 500 µL of Quick-DNA™ MagBinding Buffer was added, centrifuged, and incubated again at the same temperature for 10 minutes. The sample is transferred to a magnetic support until the magnetic beads separate from the solution, then the supernatant is separated. Next, 500 µL of DNA Pre-Wash Buffer is added, then centrifuged for 1 minute. The sample is transferred to a magnetic support until the magnetic beads separate from the solution, then separated from the supernatant. The washing process was carried out by adding 900 µL of g-DNA Wash Buffer, then vortexing for 1 minute. The sample was then transferred and incubated at 55ºC for 10 minutes. The elution process was carried out by adding 50 µL of DNA Elution Buffer at room temperature, then centrifuging at the same speed for 5 minutes. The liquid containing DNA collected in microcentrifuge tubes is stored at –4 °C for later use as a PCR template (Nursyam and Prihanto, 2018). Quantitative DNA measurement is performed using nanodrop spectrophotometry (Mollah et. *al.*, 2022).

II.13.2 Amplification of the 16S rRNA gene using the Polymerase Chain Reaction method

Amplification of the 16S rRNA gene for the endosymbiotic bacteria *L. vannamei* was amplified using the MyTaq HS Red Mix, 2X kit (Bioline, BIO-25048) (B/7.2.1/IKP/002), primer pair 27F (5'-

AGAGTTGATCCTGGTCAG-3') and 1429R (5'-GGTTACCTTGTTACGACTT-3'). Thermal conditions were set for pre-denaturation at 95°C for 3 minutes, denaturation of the target DNA at 94°C for 15 seconds, primer annealing at 55°C for 15 seconds, and primer extension at 72°C for 15 seconds, followed by 35 amplification cycles for denaturation, primer annealing, and primer extension. This is followed by post-extension at 72°C for 1 minute and then cooled to 10°C (Rustini *et. al.*, 2022).

II.13.3 Electrophoresis of PCR products

A 0.8% agarose gel is prepared by mixing 1.5 g of agarose powder into 100 mL of 1x TAE Buffer in an Erlenmeyer flask, then heating it in a microwave for 2 minutes until boiling, followed by the addition of 8 µL of Ethidium Bromide. The gel solution is then cooled at room temperature. Once it has cooled slightly, the gel solution is poured into an electrophoresis gel mold using a gel comb with 14 wells. Five microliters of each amplified product is placed into the wells of the 0.8% agarose gel, which is immersed in a tank containing TAE Buffer. Next, electrophoresis is run for 50 minutes at a constant voltage of 100 volts. After 50 minutes, electrophoresis is stopped and the gel is removed for observation under UV light. A positive result is indicated by the presence of only one DNA band (Rustini *et. al.*, 2022).

II.13.4 DNA sequencing

The determination process uses the Dye Terminator method (3'-dye labeled dideoxynucleotide triphosphate), which includes several stages: preparation, template, PCR product purification, electrophoresis with fluorescence scanning, and sequencing reaction. PCR products from samples that showed positive electrophoresis results were sequenced in both directions using the Sanger DNA Sequencing method by using Capillary Electrophoresis through the commercial services of the subcontracted laboratory 1st BASE through PT. Genetika Science (Rustini *et. al.*, 2022).

II.13.5 Phylogenetic analysis

Sequence alignment was performed using Bioinformatics Analysis of Sanger Sequencing results (B/7.2.1/IKP/006) by sequencing the nucleotides from the 16S rRNA sequencing results with the genome database stored in GenBank using the Basic Local Alignment Search Tool (BLAST) program on the website https://www.ncbi.nlm.nih.gov/BLAST, which is used to find similarities between a nucleotide or protein sequence (query sequence) and a database sequence (subject sequence). Phylogenetic construction was performed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (1,000 replications) using the Maximum Likelihood model in the MEGAX software (Wullur, 2023).

III. Results and Discussion

Endosymbiotic bacteria from *L. vannamei* heads were successfully obtained after purification in the form of two isolates. These endosymbiont bacteria began to show growth after *L. vannamei* heads were inoculated on NA medium for 48 hours (Arunachalam, 2010; Jalgaonwala *et. al.*, 2010). To ensure that the bacteria that grew were not contaminants, it was verified that there was no bacterial growth on Petri dishes containing NA medium and the final rinse water from the sterilization process (Kumala and Siswanto, 2008). Based on macroscopic observations of the endosymbiotic bacteria of *L. vannamei* shrimp, there were one isolate of endosymbiotic bacteria that shown in Table 1.

Table 1. Macroscopic observation results of endosymbiotic bacteria in *L. vannamei*

Bacterial Isolate	Macroscopic Observation			
	Shape	Color	Surface	Edge
AW_1	Round	Yellowis-White	Smooth	Smooth

Endosymbiotic bacterial isolates that grew after incubation and purification yielded one pure isolate with yellowish-white with a smooth surface and smooth edges as shown in Figure 1. This is consistent with Bhore and Sathisha (2010), who stated that endosymbiotic bacteria in a single host generally consist of several genera and species.

Genomic DNA was extracted from bacterial isolates using a standard extraction protocol to ensure high purity and integrity suitable for downstream molecular analysis. Briefly, bacterial cells were harvested by centrifugation and subjected to cell lysis using a combination of enzymatic and chemical reagents. The lysate was subsequently purified to remove proteins, lipids, and other contaminants. The concentration and purity of the extracted DNA were determined spectrophotometrically by measuring absorbance at 260/280 nm. High-quality DNA is essential for successful amplification of the 16S rRNA gene and minimisation of PCR inhibition (Green and Sambrook, 2012).

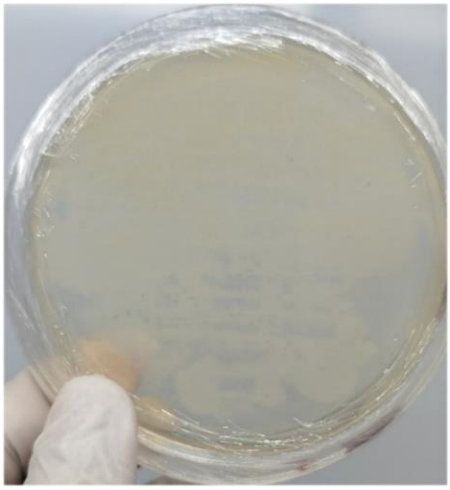

Figure 1. Endosymbiotic Bacteria in *L. vannamei*

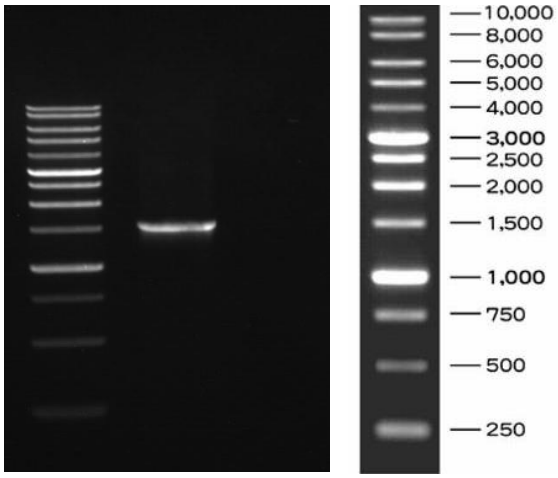

Figure 2. Electrophoresis Image – Amplified Products. 1 µL of amplified product is visualized on a 0.8% agarose gel in TBE buffer. M, 1 kb DNA ladder (in 2.5 µL); NTC: Negative Amplification Control. Sample codes correspond to the serial numbers in the DNA Quantification Results table;

The 16S rRNA gene product plays an important role as one of the components of the small ribosomal subunit in bacteria. Ribosomes function as organelles that synthesize proteins in the cells of organisms. Basically, both prokaryotic and eukaryotic ribosomes are composed only of RNA molecules and proteins. The main difference between the two groups of organisms lies in the size of the rRNA in each part of the ribosome. The large ribosomal subunit is composed of 23S rRNA and 5S rRNA molecules, while the small ribosomal subunit is composed of 16S rRNA molecules (Alzubaidy *et al.*, 2016). The 16S rRNA gene is one of the molecular markers that is still widely used as a standard reference for molecular identification of bacteria and phylogenetic studies among bacterial species. The size of the 16S rRNA gene is approximately 1400 bp (Figure 2), which can be classified into 4 domains. This gene has nine highly variable regions with a size of approximately 30-100 bp that form a secondary structure (Bartram *et al.*, 2011; Sanschagrin and Yergeau, 2014; Dewi *et al.*, 2022)

Table 2. Sequence Assembly Results – Amplification Products of Endosymbiotic Bacteria in *L. vannamei*

Sequence assembly 1425 bp

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   1  TGCAGTCGAG CGAACAGATA AGGAGCTTGC TCCTTTGACG TTAGCGGCGG ACGGGTGAGT
  61  AACACGTGGG TAACCTACCT ATAAGACTGG AATAACTCCG GGAAACCGGG GCTAATGCCG
 121  GATAACATAT AGAACCGCAT GGTTCTATAG TGAAAGATGG TTTTGCTATC ACTTATAGAT
 181  GGACCCGCGC CGTATTAGCT AGTTGGTAAG GTAATGGCTT ACCAAGGCGA CGATACGTAG
 241  CCGACCTGAG AGGGTGATCG GCCACACTGG AACTGAGACA CGGTCCAGAC TCCTACGGGA
 301  GGCAGCAGTA GGGAATCTTC CGCAATGGGC GAAAGCCTGA CGGAGCAACG CCGCGTGAGT
 361  GATGAAGGGT TTCGGCTCGT AAAACTCTGT TATTAGGGAA GAACATATGT GTAAGTAACT
 421  GTGCACATCT TGACGGTACC TAATCAGAAA GCCACGGCTA ACTACGTGCC AGCAGCCGCG
 481  GTAATACGTA GGTGGCAAGC GTTATCCGGA ATTATTGGGC GTAAAGCGCG CGTAGGCGGT
 541  TTCTTAAGTC TGATGTGAAA GCCCACGGCT CAACCGTGGA GGGTCATTGG AAACTGGGAA
 601  ACTTGAGTGC AGAAGAGGAA AGTGGAATTC CATGTGTAGC GGTGAAATGC GCAGAGATAT
 661  GGAGGAACAC CAGTGGCGAA GGCGACTTTC TGGTCTGTAA CTGACGCTGA TGTGCGAAAG
 721  CGTGGGGATC AAACAGGATT AGATACCCTG GTAGTCCACG CCGTAAACGA TGAGTGCTAA
 781  GTGTTAGGGG GTTTCCGCCC CTTAGTGCTG CAGCTAACGC ATTAAGCACT CCGCCTGGGG
 841  AGTACGACCG CAAGGTTGAA ACTCAAAGGA ATTGACGGGG ACCCGCACAA GCGGTGGAGC
 901  ATGTGGTTTA ATTCGAAGCA ACGCGAAGAA CCTTACCAAA TCTTGACATC CTTTGACCAC
 961  TCTAGAGATA GAGCTTTCCC CTTCGGGGGA CAAAGTGACA GGTGGTGCAT GGTTGTCGTC
1021  AGCTCGTGTC GTGAGATGTT GGGTTAAGTC CCGCAACGAG CGCAACCCTT AAGCTTAGTT
1081  GCCATCATTA AGTTGGGCAC TCTAGGTTGA CTGCCGGTGA CAAACCGGAG GAAGGTGGGG
1141  ATGACGTCAA ATCATCATGC CCCTTATGAT TTGGGCTACA CACGTGCTAC AATGGACAAT
1201  ACAAAGGGCA GCTAAACCGC GAGGTCATGC AAATCCCATA AAGTTGTTCT CAGTTCGGAT
1261  TGTAGTCTGC AACTCGACTA CATGAAGCTG GAATCGCTAG TAATCGTAGA TCAGCATGCT
1321  ACGGTGAATA CGTTCCCGGG TCTTGTACAC ACCGCCCGTC ACACCACGAG AGTTTGTAAC
1381  ACCCGAAGCC GGTGGAGTAA CCATTTATGG AGCTAGCCGT CGAAG
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Based on the test results of PT. Genetika Science Indonesia number GSI/GMS-3362 on August 2, 2024, the absorbance values were 1.89 at 260/280 and 1.71 at 260/230. Quantitative DNA measurement was performed using nanodrop spectrophotometry with a comparison of absorbance at 260/280. The 260/280 ratio is used as an indicator of DNA sample purity. Since the optimal value for the 260/280 ratio for pure DNA is 1.8, the percentage of samples for each group with a purity ratio between 1.6 and 2.0 (1.8 ± 0.2) was also determined (Lucena-Aguilar *et al.*, 2016). The working principle of nanodrop spectrophotometry is that pure DNA is able to absorb ultraviolet light due to the presence of purine and pyrimidine bases. Values below 1.8 indicate high levels of protein and polysaccharide contaminants. Conversely, values above two indicate fairly high levels of RNA contaminants in the isolation results (Fatchiyah *et al.*, 2011).

The results of 16S rRNA gene amplification from thermo-lipolytic bacterial isolates tested by electrophoresis are presented in Figure 1. 16S rRNA gene amplification showed a DNA band with a size of ± 1500 bp with relatively the same thickness and the number of DNA bands produced was one. This indicates that the primers used to amplify the DNA were specific for amplifying the 16S rRNA gene, which is approximately 1465 bp in size (Table 2). The use of specific bacterial primers, namely 27F and 1492R, can obtain 16S rRNA gene fragment amplicons with a size of ± 1465 bp. Primers 27F and 1492R are two primers commonly used for the identification of bacterial 16S rRNA genes (Murray *et al.*, 2022)

Analysis of 16S rRNA gene similarity using NCBI BLASTn showed that the AW1 endosymbiotic bacterial isolate was identical to *Staphylococcus gallinarum* strain RTE-S1 (LC572265) with a similarity value of 99.69% that shown in Figure 3. This indicates that the AW1 endosymbiotic bacterial isolate is most likely the same species as *S. gallinarum*. The results of phylogenetic tree reconstruction show that AW1 forms a monoclade with *S. gallinarum*. This phylogenetic tree proves the consistency of the lipolytic activity of this bacterium, which is still rare. *Staphylococcus gallinarum* belongs to the group of coagulase-negative staphylococci (CoNS), which are *Staphylococcus* species that do not produce the coagulase enzyme and generally exhibit lower virulence than *Staphylococcus aureus*. However, recent studies indicate that CoNS can no longer be regarded merely as contaminants, but rather as potential opportunistic pathogens relevant to nosocomial infections and zoonoses (Combar *et al.*, 2026). *S. gallinarum* has been reported to produce the compound Staphyloxanthin, which has strong antioxidant and anticancer activity against four different cancer cell lines, but very low cytotoxicity against normal non-cancer cell lines (Barretto and Vootla, 2018). *S. gallinarum* has been reported to have antimicrobial activity against *S. aureus*, particularly those resistant to antibiotics (Roh *et. al*., 2019).

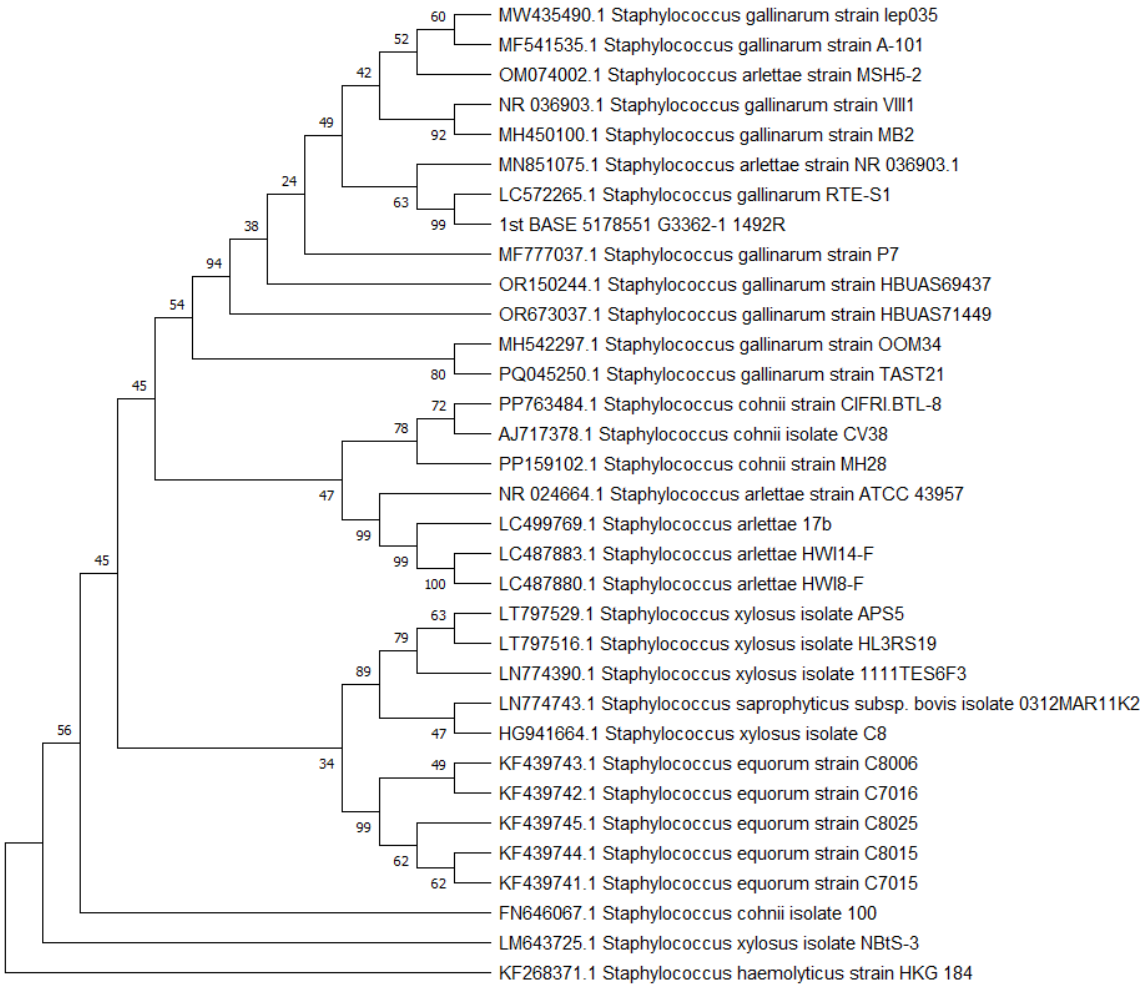

Figure 3. Phylogenetic tree of endosymbiotic bacterial isolate AW1 from *L. vannamei*. Phylogenetic construction used the UPGMA method and bootstrap analysis (1,000 replications). Evolutionary distance was determined using the Maximum Composite Likelihood method. This analysis involved 33 nucleotide sequences with a total of 1,546 positions in the final data set. Phylogenetic analysis was performed using MEGAX software.

This species is phylogenetically closely related to other CoNS such as *Staphylococcus epidermidis* and *Staphylococcus haemolyticus*, which are known to cause opportunistic infections in humans. Phylogenomic studies indicate that the CoNS group possesses high genetic flexibility, enabling adaptation to a variety of environments (Rossi, Ahmad and Giambiagi-deMarval, 2024). Genomic research indicates that *S. gallinarum* possesses sufficient metabolic flexibility to survive under a variety of environmental conditions, including varying osmotic pressures and pH levels. As a CoNS, S. gallinarum does not possess potent toxins like S. aureus, but it still possesses several important virulence factors, including surface adhesins for tissue colonization, the ability to form biofilms, and the production of siderophores (staphyloferrin) to acquire iron. The production of staphyloferrin is known to inhibit the growth of other microorganisms and help the bacteria survive under nutrient-deprived conditions (Dhanya Raj *et al.*, 2023).

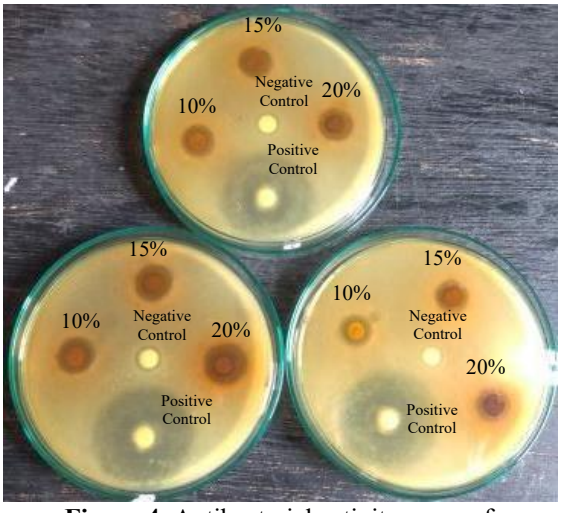

Figure 4. Antibacterial activity assay of endosymbiotic bacterial isolate AW1 against *S. aureus* ATCC 25923

The results of testing the antibacterial activity of endosymbiont bacterial isolates with *S. aureus* ATCC 25923 showed that there was antibacterial activity with an average of 11.07 mm, 11.97 mm, and 13.91 (Figure 4) mm for concentrations of 10%, 15%, and 20% concentrations of secondary metabolite extracts from endosymbiotic bacterial isolates (AW1) and was classified as strong (Setyaningsih, 2008). Fermentation in NB medium with a culture time on the second day using a shaker because the highest number of secondary metabolites occurs at the beginning of the stationary phase or the end of the exponential phase (Djenar, et. al., 2018). Stirring is carried out to maximize secondary metabolite production (Madigan, 2013) and to keep the medium moving so that aeration occurs, which maintains secondary metabolite growth with oxygen distributed evenly throughout the medium. Oxygen content is required by endosymbiotic bacteria to grow and produce secondary metabolites optimally, because the growth medium is rich in organic and inorganic substances, which cause low dissolved oxygen levels (Madigan *et al.*, 2016).

IV. Conclusion

Two isolates of endosymbiotic bacteria have been successfully isolated from *L. vannamei* shrimp. Endosymbiotic bacteria with the code AW1 have the potential to be used as an antibacterial agent, as indicated by the formation of an inhibition zone against *S. aureus* ATCC 25923. Antibacterial activity assay of secondary metabolites from endosymbiotic bacteria against *S. aureus* ATCC 25923 at concentrations of 10%, 15%, and 20% showed inhibition zones of 11.07 mm, 11.97 mm, and 13.91 mm, respectively. Molecular identification showed that the endosymbiotic bacterial isolate AW1 was closely related to *Staphylococcus gallinarum* strain RTE-S1 (LC572265).

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