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Evaluation of *Omphisa fuscidentalis* Larvae as a Virulence Model Injected with Bacteria from Healthy Individuals and Hospitalized Patients

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ABSTRACT

Background and Aim: One of the animal models for bacterial virulence testing is *Omphisa (O.) fuscidentalis*. The purpose of this study was to evaluate *O. fuscidentalis* larvae as an animal model for bacterial virulence testing in healthy individuals and hospitalized patients.

Materials and Methods: The design of this study was experimental. Bacterial samples were obtained from Mitra Keluarga Hospital, Bekasi, Indonesia. Molecular identification of bacteria was confirmed using partial *16S rRNA* gene sequencing. Identification and susceptibility testing of bacteria was performed using Vitek 2 compact. The dose treatment of bacterial isolates *Staphylococcus (S.) haemolyticus*, *Enterococcus (E.) faecalis*, and *Klebsiella (K.) pneumoniae* (10^3, 10^4, 10^5) was carried out on survivability of *O. fuscidentalis* larvae over 24 hr post-injection. Giemsa staining was used to find hemocytes in the hemolymph of *O. fuscidentalis*. Hemocytes were also evaluated through microscopic observation.

Results: *Klebsiella pneumoniae* strains were resistant to more than five classes of antibiotics. The lowest survival percentage was obtained in *O. fuscidentalis* larvae injected with *K. pneumoniae.* Microscopic observation of dead *O. fuscidentalis* larvae showed hemocytes from melanized larvae exhibited swelling.

Conclusion: Although this research is still basic, *O. fuscidentalis* larvae showed to be a probable promising alternative model to assess bacterial virulence. However, comparative studies with established test animals are needed.

Keywords: Virulence, *Omphisa fuscidentalis*, Animal Model, Multi-drug Resistant, Hemocytes, *Galleria mellonella*

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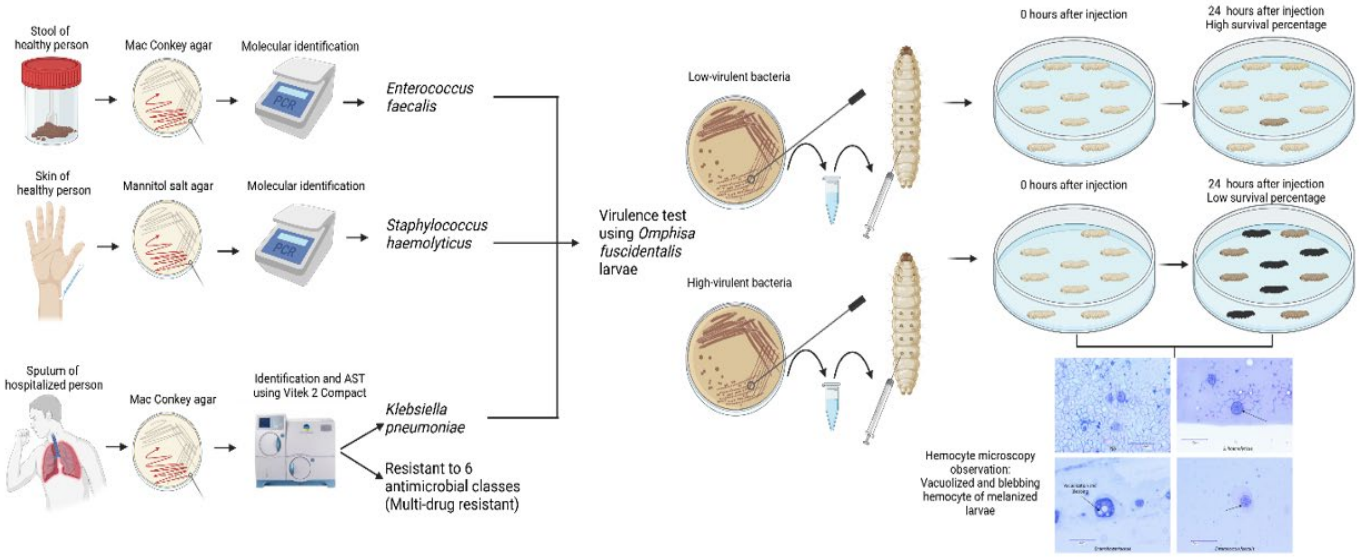
Graphical Abstract



(Designed by Authors, 2025)

1. Introduction

Animals can be used at various stages of testing, including development, manufacturing, and quality control. Animal testing is crucial as it provides information on the resistance of animals to pathogenic bacteria under investigation. The first use of experimental animals was conducted by Robert Koch, who utilized mice. However, the use of mouse models has declined due to high costs and the need for complex ethical approvals **(1, 2)**. Over time, researchers have increasingly shifted from mice to other model organisms such as murines, zebrafish, nematodes, *Caenorhabditis elegans*, and insect larvae. Various types of larvae have been employed in research to generate pharmacokinetic and pharmacodynamic data, one of which is *Galleria (G.) mellonella* larvae **(3-6)**.

These larvae have been widely used in virulence and antimicrobial efficacy studies due to several advantages, including their ability to incubate at 37°C, which is crucial as temperature influences the expression of microbial virulence factors. Additionally, *G. mellonella* larvae have a short lifespan, are easy to handle, allow for precise dosage administration, are not subject to ethical considerations, are cost-effective to maintain as they do not require specialized equipment, and lack an adaptive immune system **(5, 7, 8)**.

Despite lacking an adaptive immune system, *G. mellonella* larvae possess an immune system similar to that of humans **(8-10)**, making them a suitable model for assessing microbial pathogen virulence and generating results comparable to those obtained from mammalian systems. This characteristic makes *G. mellonella* larvae particularly valuable for studying early host-pathogen interactions and infections that do not involve an adaptive immune response. However, *G. mellonella* larvae are pests that infest honeycombs, making them difficult to obtain and commercially unavailable in Indonesia. Moreover, this insect has a challenging way to be reared **(11)**. As an alternative, this study utilizes *Omphisa (O.) fuscidentalis* larvae as experimental models. These larvae belong to the same order as *G. mellonella* but are more abundant in Indonesia. *O. fuscidentalis* larvae are commonly used in Indonesia as feed for birds, fish, and reptiles. Researchers opted for this species over other insects due to its prolonged larval diapause period, which allows it to adapt to its natural habitat. Diapause is a period of suspended or arrested development in an insect life cycle.

One advantage of using *O. fuscidentalis* larvae is their large population in Indonesia, as they require relatively lower maintenance costs compared to *G. mellonella* larvae. To the best of our knowledge, only a few studies have investigated the use of *O. fuscidentalis* larvae as experimental models for bacterial infections. However, previous studies on these larvae have demonstrated that they can distinguish bacterial virulence levels based on larval mortality following bacterial injection at varying concentrations **(12-15)**. This research is essential for exploring alternative bacterial infection models, particularly using *O. fuscidentalis* larvae.

To evaluate the response of *O. fuscidentalis* larvae to infection by low- and high-virulence bacteria, we initially isolated bacteria from the stool and skin of healthy individuals. We compared them to multidrug-

resistant (MDR) bacterial pathogens isolated from the sputum of hospitalized patients. We then observed larval survival rates, melanization occurrence, and the hemocyte response in melanized larvae.

2. Materials and Methods

2.1 Bacterial Isolate

Bacterial isolates were obtained directly from Mitra Keluarga Hospital, Bekasi, Indonesia. The samples were isolated from the stool and skin of patients and healthy individuals. All the standards for handling MDR bacteria in the Mitra Keluarga Health Science and Technology Laboratory included BSL-2 biosafety equipment, personal protective equipment, and operational procedures (sample handling and analysis, decontamination and disposal, autoclaving and cleaning, and incident handling). Preparation procedures included ensuring proper calibration of the BSC and HEPA, disinfection with 1:10 bleach and 70% ethanol, and the availability of containers for sharps and disposable items. Sample handling and analysis procedures were carried out in the BSC, avoiding aerosols. If contamination occurred due to spills, cleaning was carried out with 10% bleach and 70% alcohol. Destruction was carried out by autoclaving all media, cups, pipettes, and biological waste (121°C, 15 minutes).

2.2 Molecular Identification Using Partial *16S rRNA* Gene Sequencing

The dominant single colony from each medium was further analyzed for the molecular identification based on partial *16S rRNA* gene sequencing using the polymerase chain reaction (PCR) method. Genomic DNA from the selected colony was extracted using the Genomic DNA Promega extraction kit (Promega, Madison, WI, USA). The concentration and purity of the extracted genomic DNA were measured using a Nanodrop UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The partial *16S rRNA* gene was amplified using primers 1387r (5'GGGCGGWGTGTACAAGGC3') and 63f (5'CAGGCCTAACACATGCAAGTC3') **(16)**. The total PCR reaction volume was 50 µL, consisting of 25 µL of GoTaq Green PCR Master Mix (Promega, Madison, WI, USA), 5 µL of 10 pmol 1387r primer, 5 µL of 10 pmol 63f primer, 4 µL of genomic DNA, and 11 µL of nuclease-free water. The PCR conditions were as follows: 30 cycles, pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 sec, annealing at 55°C for 4 sec, and elongation at 72°C for 10 min. PCR products were separated using electrophoresis on a 1.5% agarose gel (Himedia, Mumbai, India) for 30 min at 100V. The agarose gel was stained with GelRed solution (Biotium, San Francisco, USA) and visualized using a UV transilluminator (Optima, Tokyo, Japan). The PCR product size was approximately 1300 bp. The amplicons were sequenced at 1st Base Genetika Science, Indonesia, using the Sanger sequencing method. The obtained partial *16S rRNA* sequences were trimmed using MEGA 11 software and subjected to BLASTn analysis in the NCBI database. The closest match, including strain and species names, was recorded in the results.

2.3 Bacterial Identification and Susceptibility Testing of Bacteria Using *Vitek 2 Compact*

Bacteria isolated from hospitalized patients' sputum were biochemically identified using Vitek 2 Compact system (bioMérieux, Marcy-l'Étoile, France). Bacterial colonies grown overnight on *MacConkey* agar (MCA) were used for this test. The colonies were collected using a sterile cotton bud and suspended in 3 mL of sterile saline (0.9% NaCl) in a glass tube. The bacterial suspension was measured using the DensiCHEK Plus device to ensure an acceptable density range (0.5–0.63 McFarland). If the turbidity was below the range, additional bacterial colonies were added, while if it exceeded the range, more sterile saline was added. The adjusted bacterial suspension (145 µL) was aliquoted into a Gram-negative cassette for both identification (ID) and antibiotic susceptibility testing (AST). The results were obtained after at least 24 hr of incubation.

2.4 Virulence Assay

The virulence assay using *O. fuscidentalis* larvae was conducted following the method of Ilsan, Yunita **(14)**. Healthy larvae weighing 200–300 mg were selected and stored for two days without food before the experiment. One day before the experiment, the larvae were acclimated at 37°C. Bacterial cultures were prepared in 5 mL of Mueller-Hinton Broth (MHB) and incubated for 16 hr with shaking. A 1 mL aliquot of each bacterial culture was centrifuged at 10,000 ×g for 7 min. The supernatant was discarded, and the bacterial pellet was re-suspended in 1 mL of phosphate-buffered saline (PBS). The bacterial suspension was then diluted in PBS and adjusted to an OD600 of 1 using a Genesys 10S UV-Vis Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), corresponding to approximately 1×10^9 colony-forming units per mL (CFU/mL). A 10 µL bacterial suspension was injected through the top right proleg using a 26- gauge microsyringe. The final bacterial concentrations were prepared as follows: 10^7 CFU/mL (10^5 CFU/larva), 10^6 CFU/mL (10^4 CFU/larva), and 10^5 CFU/mL (10^3 CFU/larva). Groups of 10 larvae were topically disinfected on the last left proleg using a tissue paper soaked in 70% ethanol. For each experimental group, larvae injected with 10 µL of PBS served as a control group to ensure that the

injection procedure itself did not cause mortality. The larvae were incubated at 37°C in the dark without food and were considered dead when they were unresponsive to touch and displayed a dark brown to black (melanized) color. Survival was monitored at 0, 4, 12 and 24 hr post-injection. The hemolymph of melanized larvae was compared to that of control larvae (un-injected) under a digital microscope (Keyence Digital Microscope VHX 6000, Japan). Larvae were dissected at the abdominal region using a sterilized razor blade, and the hemolymph was extracted by gently squeezing the larvae.

2.5 Giemsa staining and microscopic evaluation

Hemocytes representation in the hemolymph of *O. fuscidentalis* was shown by Giemsa staining. The hemocytes were also observed microscopically.

2.6 Statistical Analysis

All experiments were analyzed in tables and diagrams, with results expressed as mean±SD. All statistical analyses were performed using GraphPad Prism v 6.00 (one-way ANOVA; larval survivability). Differences were considered significant at $P<0.05$.

3. Results

Molecular identification of bacteria from the skin and stool of the healthy individual's revealed majority *S. haemolyticus* and *E. faecalis*, respectively (Table 1). The bacteria from the skin exhibited 99.53% identity to *S. haemolyticus* strain WY-8, with a *16S rRNA* gene sequence length of 1,425 bp. Meanwhile, the bacteria from the stool exhibited 99.76% identity to *E. faecalis* NBRC 100481 in the NCBI database, with a sequence length of 1,426 bp.

A putative high-virulence bacterium was represented by a bacterial isolate obtained from the sputum of a hospitalized patient. We got the isolate from our laboratory of our affiliated hospital. This isolate was identified and subjected to antimicrobial susceptibility testing using Vitek 2 Compact automated system. The bacterial species was identified as *Klebsiella (K.) pneumoniae* based on biochemical analysis. This *K. pneumoniae* strain was categorized as extensively drug-resistant (XDR) since it exhibited resistance to more than five antimicrobial classes (Table 2).

The virulence of bacteria isolated from a healthy individual was compared to that of the putatively highly virulent *K. pneumoniae* isolate from a hospitalized patient's sputum. *O. fuscidentalis* larvae were used as an *in vivo* model for virulence testing. The survival rate of larvae injected with bacteria from the healthy individual, including *S. haemolyticus* from the skin, and *E. faecalis* from the stool, remained at 90% regardless of whether they were injected with 10^3 or 10^5 CFU/larvae. Interestingly, the survival rate of larvae injected with *K. pneumoniae* was significantly lower, with 20%, 10%, and 0% survival at doses of 10^3, 10^4, and 10^5 CFU/larvae, respectively, after 24 hr of incubation (Figure 1).

Omphisa fuscidentalis larvae that died due to bacterial injection appeared black due to melanization. Larvae injected with *K. pneumoniae* strain 122 had the lowest survival rate, reaching 0% at a dose of 10^5 CFU/larvae within 24 hr post-injection. This finding contrasts sharply with the other three isolates, which exhibited 90% survival rate at 24 hr (Figure 2). Hemolymph samples were collected from larvae exhibiting melanization and stained using the Diff-Quik method. Microscopic analysis using both light and 3D microscopy revealed that hemocytes in melanized larvae exhibited vacuolization and were larger compared to the control group. In contrast, normal hemocytes showed no signs of vacuolization (Figures 3 and 4).

Table 1. BLASTn results of bacteria isolated from the skin (SA) and stool (V) of a healthy individual. The PCR amplicon corresponds to a partial *16S rRNA* gene sequence used for molecular identification

Isolate	Species	Max Score	Total Score	Query Cover	E-value	Per. Ident	Accession Length	Accession Number
SA	S. haemolyticus strain WY-8	2329	2329	100%	0.0	99.53%	1425	MH930438.1
V	E. faecalis strain NBRC 100481	2276	2276	100%	0.0	99.76%	1426	NR_113902.1

Table 2. Results of the antimicrobial susceptibility test for *K. pneumoniae* isolated from the sputum of a hospitalized patient using Vitek 2 Compact automated system. R = Resistant, I = Intermediate, S = Sensitive.

CD4 (Mean ± S.E)	LSD	P-Value
Penicillins	Ampicillin	R
β-lactam combination	Ampicillin sulbactam	I
β-lactam combination	Piperacillin tazobactam	S
Cephems	Cefazolin	R
Cephems	Ceftazidime	R
Cephems	Ceftriaxone	R
Cephems	Cefepime	S
Monobactam	Aztreonam	R
Carbapenem	Ertapenem	S
Carbapenem	Meropenem	S
Aminoglycosides	Amikacin	S
Quinolones	Gentamicin	S
Aminoglycosides	Ciprofloxacin	R
Gyycylcycline	Tigecylcine	S
Sulfonamides	Trimethoprim/Sulfamethoxazole	R
Fosfomycins	Fosfomycin	S
β-lactam combination	Ceftazidime avibactam	S

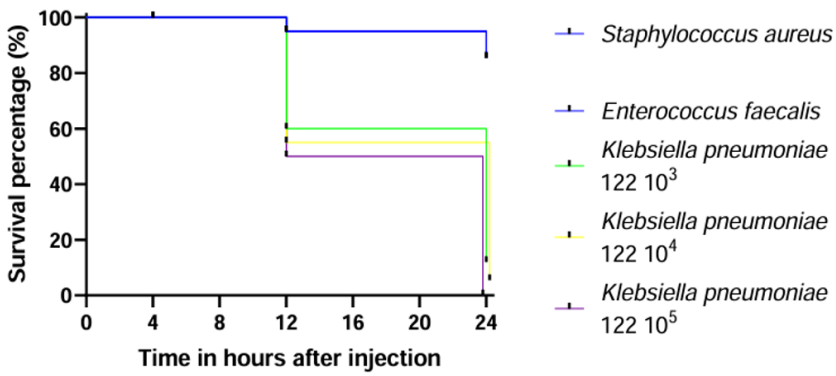


Figure 1. The Kaplan-Meier curve shows the survival percentage of *O. fuscidentalis* larvae after 24 hr injection of pathogenic bacteria. The lowest survival percentage was found in *O. fuscidentalis* larvae injected with *K. pneumoniae* bacteria. The Log Rank test showed significant difference between treatment groups (P<0.0001). **(Designed by Authors, 2025)**.

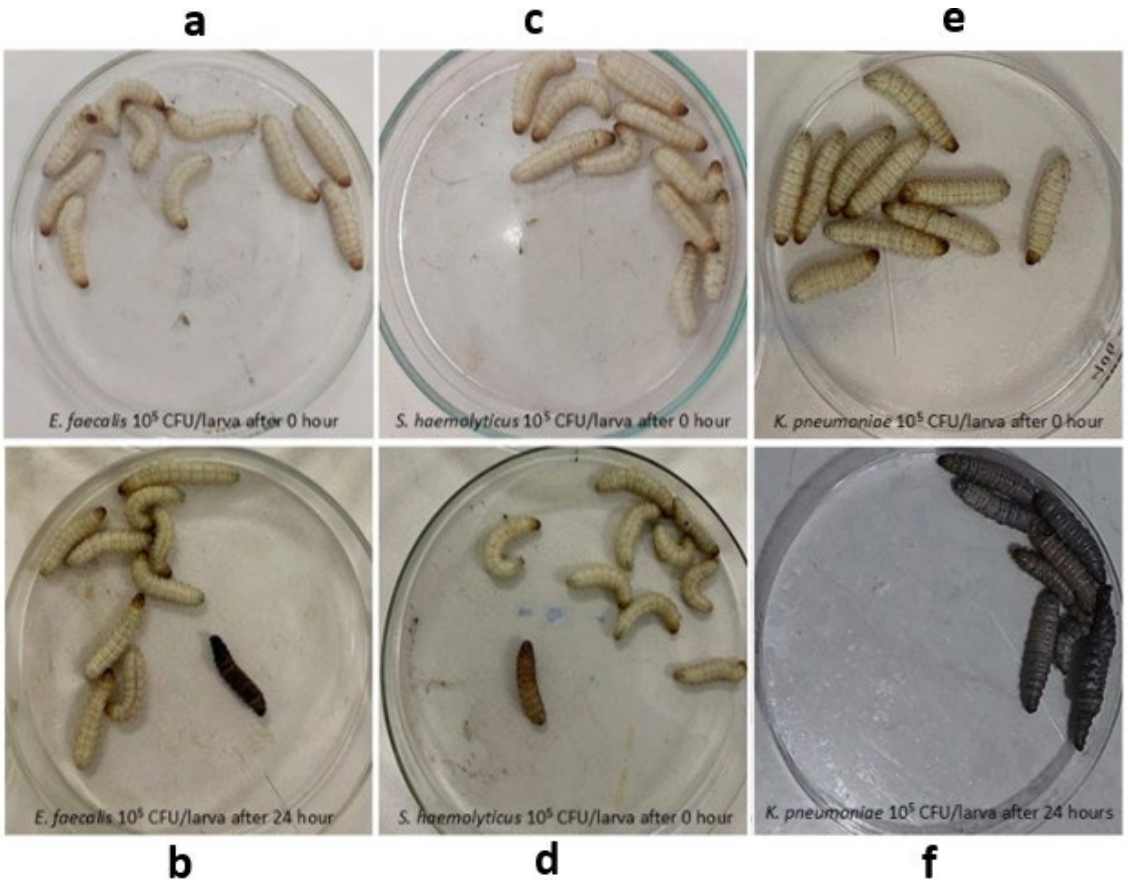


Figure 2. Virulence test in *O. fuscidentalis* larvae injected with pathogenic bacteria at a dose of 10^5 for 0 hr and 24 hr. Dark colored show melanized dead larvae. (a-b) *E. faecalis.* (c-d) *S. haemolyticus*. (e-f) *K. pneumoniae*. **(Designed by Authors, 2025)**.

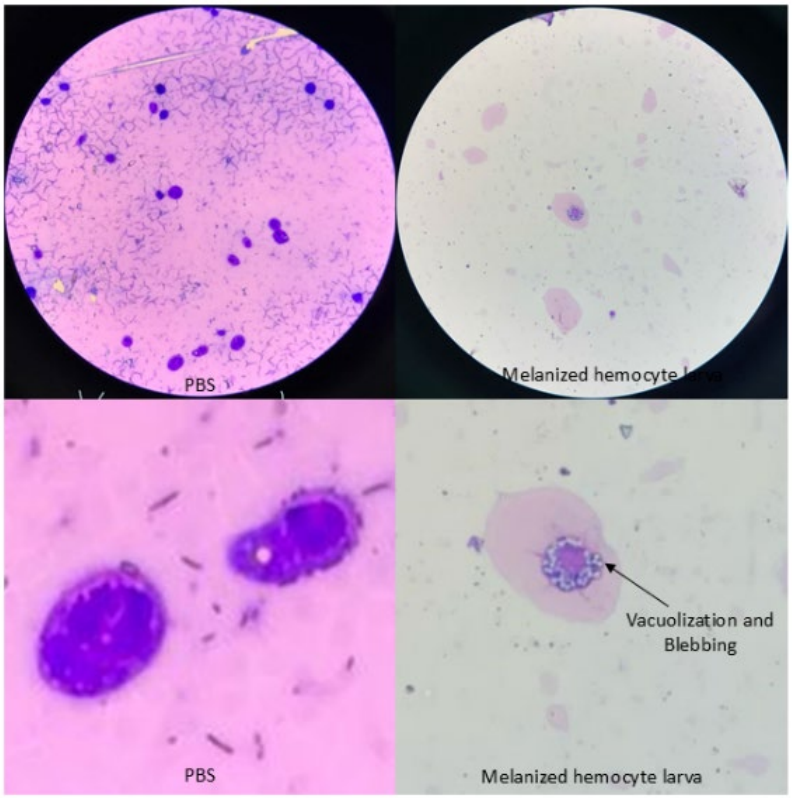


Figure 3. Hemocyte representation found in the hemolymph of *O. fuscidentalis* after Giemsa staining. (a & c) Control group hematocytes (PBS) did not vacuolate and blebbed. (b & d) Hematocytes in the melanization treatment group showed vacuolation and blebbing **(Designed by Authors, 2025)**.

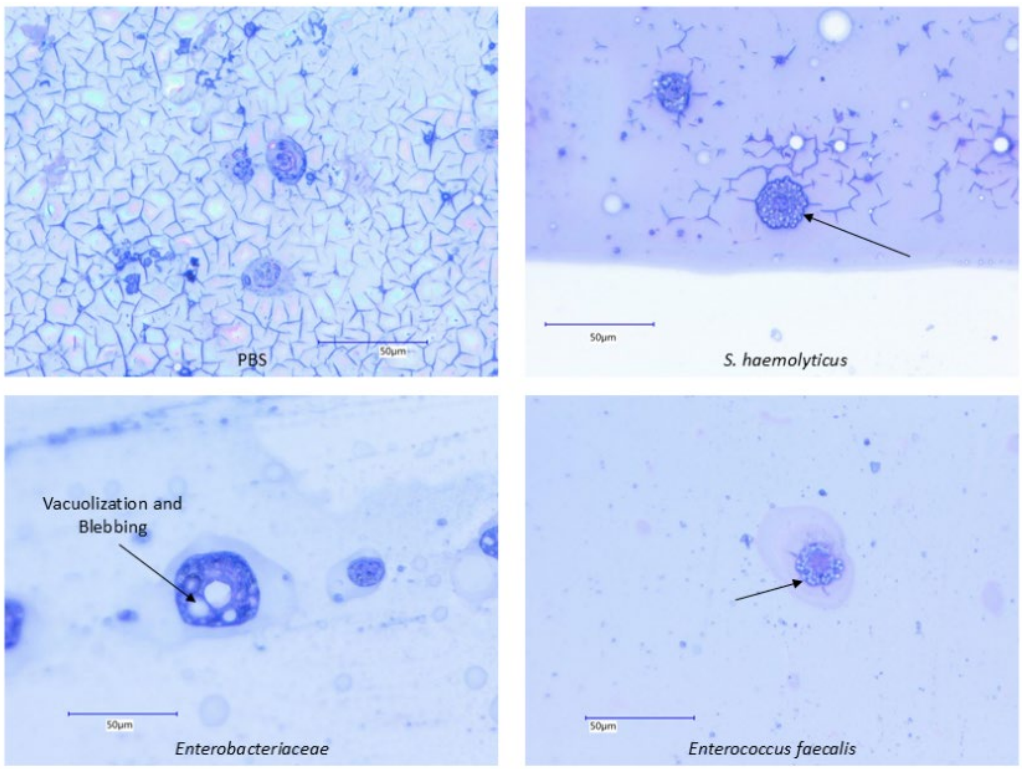


Figure 4. 3D microscopic observation of hemocytes from melanized larvae compared to control larvae injected with PBS **(Designed by Authors, 2025)**.

4. Discussion

Infections caused by human bacterial pathogens have been increasing worldwide in recent years. Moreover, the inappropriate use and misuse of antimicrobial therapy have led to difficulties in treatment due to MDR and XDR bacteria. In experimental research, virulence testing of human clinical bacteria has become increasingly necessary to measure bacterial virulence levels. Various animal models have been used for virulence testing, including mice, zebrafish, guinea pigs, the nematode *Caenorhabditis elegans*, and the insect larvae. *Galleria mellonella* has several advantages as a model for bacterial virulence testing, such as ease of injection and handling, as well as a shorter infection evaluation period. The progression of infection in *G. mellonella* is relatively fast, typically ranging from 12 hr to 3 days. Additionally, maintaining *G. mellonella* larvae requires a lower budget compared to other animal models **(17)**. However, *G. mellonella* is not naturally found in Indonesia in all regions. Therefore, we propose an alternative animal model for bacterial virulence testing. *O. fuscidentalis* larvae are readily available in Indonesia and are even sold freely as fish bait. Since *O. fuscidentalis* belongs to the same order as *G. mellonella*, it shares similar physiological defense mechanisms. Several studies have been conducted using *O. fuscidentalis* as an alternative to *G. mellonella* in bacterial pathogen virulence testing **(14)**.

Researchers tested a MDR *Escherichia fergusonii* strain isolated from the surface of a toilet bowl in a medical university using *O. fuscidentalis* larvae **(14)**. Additionally, virulence testing with *O. fuscidentalis* larvae demonstrated that clinical *K. pneumoniae* exhibited higher virulence **(12)** *O. fuscidentalis* has also been used as an animal model for virulence testing of *Aeromonas spp.* isolated from kitchen sponges, showing relatively low virulence **(6)**. Finally, *O. fuscidentalis* has been utilized for the virulence testing of MDR *Ralstonia insidiosa* isolated from drinking water, which also exhibited low virulence **(8)**.

Omphisa fuscidentalis larvae have been tested for virulence assessment when injected with both low-virulence and high-virulence bacteria. In this study, *S. haemolyticus* and *E. faecalis*, isolated from healthy individuals, were categorized as low-virulence bacteria, as indicated by a 90% survival rate of *O. fuscidentalis* larvae 24 hr after injection. In contrast, *K. pneumoniae* strain 122, isolated from the sputum of a hospitalized patient, was categorized as a highly virulent bacterium due to 0% survival rate of *O. fuscidentalis* larvae 12 hr after injection. A study using *O. fuscidentalis* larvae injected with *E. fergusonii* from a toilet bowl in a medical university showed low virulence, with a 75% survival rate at 24 hr for a dosage of 10^6 CFU/larvae **(14)**. The *K. pneumoniae* strain 122 used in this study was categorized as XDR, as it was non-susceptible to more than five antibiotic classes. These classes included penicillins, β-lactam combinations, cephems, monobactams, quinolones, and sulfonamides. The *Enterobacteriaceae* group, including *K. pneumoniae,* has been found in Morocco, containing *mcr-1,* which is responsible for colistin resistance **(18)**. Moreover, *K. pneumoniae* also contained several virulence factors, such as hemolysin, protease, and lipase **(19)**. According to Magiorakos and Srinivasan **(20)** bacteria are classified as MDR if they are non-susceptible to more than three antibiotic classes. In comparison, XDR bacteria are non-susceptible to more than five antibiotic classes.

Highly virulent bacteria caused *O. fuscidentalis* larvae to turn black and ultimately die. This symptom is similar to that of *G. mellonella* larvae infected with highly virulent bacteria. The blackening of larvae following bacterial injection indicates melanization. In insects, melanization is a marker of immune response due to microbial infections or injury **(21)**. This reaction is similar to the immune response in mammals, which involves recognizing foreign substances. Pattern recognition receptors (PRRs) identify bacteria through cell wall components such as peptidoglycans and lipopolysaccharides (LPS) **(22)**. This recognition triggers the pro-phenoloxidase cascade in insects, leading to production of melanin, which accumulates around bacterial organs and damaged tissues, preventing bacterial spread throughout the tissues and organs **(21)**.

The study conducted by Quansah and Ramoji **(23)** demonstrated changes in *G. mellonella* larvae after injection with *E. faecalis*, including melanization, nodule formation, increased phagocytic activity of hemocytes, and vacuolization. In this study, *E. faecalis* and *S. haemolyticus* were categorized as low-virulence bacteria. We evaluated the hemocytes of unhealthy and black-melanized larvae. The hemocytes of *O. fuscidentalis* larvae injected with these bacteria exhibited vacuolization and increased cell size compared to those injected with phosphate-buffered saline as control. Vacuolization is a well-known natural phenomenon that occurs when bacteria infect a suitable host, ultimately leading to cell death **(24)**. Injection of *G. mellonella* with *Pseudomonas (P.) aeruginosa* has been reported to induce hemocyte vacuolization, membrane blebbing, cell swelling (increased size), and chromatin condensation **(24)**. Similarly, Kazek and Kaczmarek **(25)** demonstrated that *G. mellonella* hemocytes underwent vacuolization within 48 hr of infection with *Conidiobolus coronatus*. This phenomenon was followed by hemocyte destruction, degranulation, and the formation of hemocyte microaggregates. In this

study, *S. haemolyticus* was isolated from healthy human skin. *S. haemolyticus* is primarily recognized as a component of the normal human skin microbiota and is classified as a coagulase-negative *Staphylococcus* (CoNS), which makes it less virulent than coagulase-positive Staphylococcus species, unlike *S. aureus* **(26)**. However, recent studies have reported *S. haemolyticus* as a causative agent of various infections, including bloodstream infections **(27)**, eye infections **(28)**, nosocomial infections **(29)**, male infertility **(30)**, chronic prostatitis, as well as community-acquired and soft tissue infections **(26)**.

The insect immune response to pathogenic bacteria involves both cellular and humoral responses. Changes in hemocyte numbers generally determine it. Several dominant hemocyte types in the hemolymph of Lepidoptera order include granulocytes (48%), plasmatocytes (47%), spherulocytes (3%), and oenocytoids (1%). Increased hemocyte numbers lead to the release of the enzyme phenoloxidase (PO), which catalyzes the melanization process **(21)**.

One common type of insect hematocyte that plays an essential role in resisting bacterial infections is the granulocyte. The formation of cytoplasmic vacuolation is likely due to Staphylococcus, Enterococcus, and Klebsiella infection in granulocytes and plasmatocytes through the mechanism of autophagocytosis **(31)**. This process plays a vital role during the interaction between host and *O. fuscidentalis*.

Autophagy refers to the mechanism of cytoplasmic components degradation by lysosomes, selectively or non-selectively. In general, autophagy occurs in the cell cycle. However, autophagy imbalance due to bacterial infection causes cell death, which is characterized by the formation of vacuoles (bubbling) in the cytoplasm of hematocyte larvae of *O. fuscidentalis* **(32)**. Previous research using *Tenebrio molitor* larvae reported that hemocyte and phenoloxidase levels tended to increase in larvae injected with high doses of pathogenic microorganisms. Therefore, changes in hemocyte and phenoloxidase levels are indicators of Tenebrio molitor larvae cytotoxicity due to high-dose pathogenic microorganism injection **(33)**.

The limitations of this study are the lack of histological stains, measurements of swollen hematocyte diameter, and immune response parameters such as hemocyte levels and phenoloxidase activity. Therefore, further research is needed on immune response of *O. fuscidentalis* larvae to pathogenic bacterial infections, as well as the reaction of drug testing to pathogenic bacterial infections in *O. fuscidentalis* larvae. In addition, further research is needed on the comparison of *G. mellonella* with *O. fuscidentalis* in a wider range of bacteria such as *P. aeruginosa, Acinetobacter baumannii* so that the differences in the immune responses of the two test animals after being injected with pathogenic bacteria are clearly visible.

5. Conclusion

Based on this research, we propose *O. fuscidentalis* larvae as a promising alternative model for assessing bacterial virulence. However, this research is still very basic. Thus, comparative studies with established test animals are needed, such as comparison of immune response, hematocyte histology size, measurement of changes in hemocyte levels, and phenoloxidase activity before and after injection of pathogenic microorganisms.

6. Declarations

6.1 Acknowledgment

The authors are thankful to the staff of the Department of Medical Laboratory Technology, STIKes Mitra Keluarga, Indonesia, and ILab in the National Research and Innovation Agency, Indonesia.

6.2 Ethical Considerations

All methods and experiments were approved by the Ethics Committee of Universitas Muhammadiyah Purwokerto, Indonesia, with registration number KEPK/UMP/107/III/2025. All participants agreed to be involved in this study.

6.3 Authors' Contributions

All authors contributed to the study conception and design. NAI, NPRAK, and MI conceived the idea and designed the experiments. NAI, NPRAK, MI, JAP, FAHH, ODW, SRS, NSR performed the experiments. NAI and NPRAK validated the results. NAI, RZA, and FAHH drafted the manuscript. RZA and NAI finalized the manuscript. All authors have read and approved the final manuscript.

6.4 Conflict of Interests

The authors have no conflicts of interest.

6.5 Financial Support and Sponsorship

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6.6 Using Artificial Intelligence Tools (AI Tools)

All authors declare that there is no use of AI Tools in this study, including the writing of this manuscript.

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