

Original Research Article

New records of an insect species from decomposed animal feces that carry pathogenic bacteria on human

ABSTRACT

Insects are introduced as the largest group of animals in the animal kingdom. There is always a risk of disease outbreaks from insects that greatly affects people's health. Hundreds of thousands of species have been determined, whereas many others remained unnamed and unidentified. This study was carried out to identify the insect species obtained in Mo Cay Bac district, Ben Tre province, Vietnam, and identify the human pathogenic bacteria that parasitize on this insect. The study results showed that the insect carried *Bacillus cereus* and *Micrococcus luteus*, two strains of bacteria capable of causing festering wounds; whereas, the antibiotic *Gentamicin* (10 µg/mL) was shown to be capable of inhibiting *Bacillus cereus* and *Micrococcus luteus*. Based on morphological characteristics and DNA fragment of COII gene, the insect species has been identified as a species belonging to the Scatopsidae family, which belongs to complete metamorphosis. Its life cycle lasts 7-8 days and goes through 4 stages, including egg (1 day), larva (3-4 days), pupa (2-3 days), and adult. This insect species carry the biological characteristics of an entirely new insect species.

Keywords: Bacillus cereus, Ben Tre, insect, Micrococcus luteus, Scatopsidae, Vietnam

1. INTRODUCTION

Vietnam is a tropical monsoon country with a hot and humid climate. The conditions are quite ideal for the development of insects, including many species that carry dangerous diseases for humans. In some communes of Mo Cay Bac district, Ben Tre province, Vietnam there is an insect with small size, black color, and wings that can cling to the scratched wounds of the human's body, causing inflammation and fester. This insect appears particularly a lot in areas where livestock and poultry are raised and on decomposing animal feces. These kind of insect species are might at risk of outbreaks, causing many impacts on people's health. Pathogens parasitic on insects transmit and can even live for several days if it is along with food into the human stomach and intestines. Pathogens from the insect can transmitt directly through food, water, air, hands, etc. Insect mainly transmit intestinal infections and some skin diseases (Sarwar, 2015).

Entomological data of many insect species are still limited (Higley et al., 2001; Dekeirsschieter et al., 2013; Didham et al., 2020). Many insect species remain undetermined, partly due to the difficulties in cultivating under laboratory conditions. To provide more scientific data to identify insect species that carry bacteria causing human disease. Simultaneously determine antibiotic resistance and identify pathogenic bacteria.

The authors isolated and identified bacteria capable of causing festering on wounds and identified and surveyed the life cycle and biological characteristics of the insect species collected in Mo Cay Bac district, Ben Tre province, Vietnam, under laboratory conditions. The research result will create a scientific basis to help people in livestock and poultry raising areas to have environmentally friendly solutions, avoiding an outbreak in the quantity of these insect species.

2. MATERIAL AND METHODS

Sample collection and characteristic identification

The insect species living in decomposed pig manure were collected in June – July 2021, in Mo Cay Bac district, Ben Tre province, Vietnam, then taken to the laboratory for experiments. Adult insects were reared in a prepared medium (consisting of soil manure collected from autoclaved media), allowed to mate for 24 h, and removed the parent insects. Observed daily the growth lifecycle day by day and recorded the results. New insects that have just been collected from decomposed pig manure were used to isolate strains of bacteria that cause disease in humans. Insects used in identifying traits for classification was 5th generation. The experiment was conducted with five replicates.

Observation of morphological features

The adult insects were collected and anesthetized with CO₂. They were then cleaned, dissected, and mounted on microscope slides. Specimens were examined, measured, and illustrated with pictures. Measurements were in millimeters.

Identification of insect by molecular biology method

DNA extraction: Insect DNA was extracted by a phenol-chloroform method using iVAaDNA Extraction Kit P. The cytochrome oxidase gene (COII) gene is used to identify collected insect species. COII F (5'-AATATGGCAGATTAGTGCA-3') and COII R (5'-GTTAAGAGACCAGTACTTG-3') primers (Marinho et al., 2017) were used to amplify and sequence the gene COII fragment. Components of a 25 µL PCR reaction include 12,5 µL master mix; 0.5 µL of both primers, 5 µL of DNA; and 7 µL of water. PCR products were checked using agarose gel electrophoresis 1.5% and sequenced by the Sanger method on an automated gene sequencing machine (Sanger et al., 1977), made by Macrogen Trading Company (Seoul, Korea). Sequences were analyzed by SnapGene software and compared with gene data bank of NCBI using the BlastN tool.

Isolation and identification of parasitic bacteria on insect

Bacterial isolation

Bacterial samples were isolated from insects collected from decomposing pig manure. Used a cotton swab to soak the insect's body, then put the swab into a sterile tube. The sample was labeled and stored in a refrigerator. The sample was diluted and spread on a petri dish containing and blood agar (Blood Agar Base). Plates were allowed to dry naturally and incubated at 37°C for 24 to 48 h. Colonies in the plate were inoculated to a new plate to obtain discrete colonies. The colonies were separated on a petri dish containing MRS and fresh blood agar. Bacterial strains were kept in the tubes containing NB (Nutrient Broth) medium at -80°C for further experiments.

Antibiotic resistance testing

Bacterial strains were isolated to evaluate antibiotic resistance. The tested antibiotics and concentration were as Kanamycin (30 µg/mL), Clindamycin (2 µg/mL), Penicillin (10 µg/mL), Ciprofloxacin (5 µg/mL), and Gentamicin (10 µg/mL). The antibiotic resistance of bacteria was determined by the disc diffusion method according to Bauer et al. (1966) and CLSI

(Clinical and Laboratory Standards Institute) according to Cockerill, (2011). The antibacterial ability of antibiotics were determined based on the diameter of the inhibition zone.

Bacterial strain identification

DNA extraction of bacteria: The DNA extraction procedure was performed according to Breugelmans and Uyttebroek (2004) method. After DNA purification, PCR reaction was performed with 16S RNA primers designed according to Lane et al. (1985) with the following sequence: 27F: 5' AGAGTTTGATCCTGG CTCAG 3'; 1492R: 5' GGTTAC CTTGTTACGACTT 3'. Selected PCR products were samples with bold and clear lines on 1.5% agarose gel and then sent for DNA sequencing at Macrogen company (Korea). These sequences were compared with those in the NCBI gene bank using the BlastN tool from the sequencing results.

3. RESULTS AND DISCUSSION

The life cycle of the insect

Insects with a monogamous metabolism undergo a complete metamorphosis from larvae to adults. The life cycle of the insect goes through four stages, including egg, larva, pupa, and adult.

Table 1. Duration of development stages of collected insect

Time of the development stages (day)				Life cycle (day)
Egg	Larva	Pupa	Adult	
1.00±0.00	3.6±0.52	2.3±0.47	1.00±0.00	7.9±0.74

Note: Data presented are the mean of 5 replicates ± standard deviation

Under laboratory conditions ($T^{\circ}\text{C} = 28 - 31$, $\text{H}\% = 74 - 86$), the development time from egg to adult ranged from 7 to 8 days, with an average of 7.9 ± 0.74 days. In which the average length of the egg stage was 1.0 ± 0.00 days, the average development time of the larva was 3.6 ± 0.52 days, the pupal stage was 2.3 ± 0.47 days, and the time for the pre-laying stage was 1.00 ± 0.00 days. The insect's life cycle is shown in Table 1 and Figure 1.

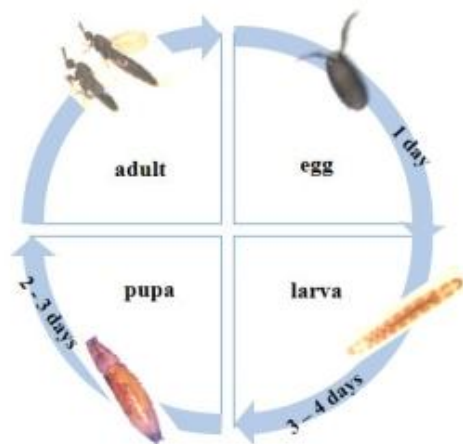


Figure 1. The development life cycle of collected insect

Biological characteristics of the development stages of collected insect

The features of the insect development stages are presented in Table 2.

Egg: Eggs were laid sporadically. They appeared after 24 hours of laying. The egg was 0.502 ± 0.019 mm long, 0.158 ± 0.002 mm wide, opaque white, and oval.

Larva: Larva was 3.50 ± 0.019 mm long, 0.494 ± 0.005 mm wide, cylindrical, legless, clearly segmented with abdominal segments of similar size. It was yellow-brown and crawled to the surface of the manure medium. This stage was when the larva absorbed and accumulated the necessary nutrients and proteins for the body to prepare for the next stage. Therefore, during this period, the larva ate well and developed quickly.

Pupa: The pupa was about 2.256 ± 0.044 mm long and 0.426 ± 0.005 mm wide. It was initially soft and yellow-brown, then gradually turned brown and dark, and its outer covering became hard and clinging firmly to the soil surface. The pupal stage was the development and formation of adult organs from the germinal disc (Griffiths et al., 2000). After 2-3 days of pupation, the pupa emerged into a adult insect. Female insects were larger than male insects.

Table 2. Insect dimension through development stages

Stages	Length (mm)	Width (mm)
Egg	0.502 ± 0.019	0.158 ± 0.002
Larva	3.502 ± 0.019	0.494 ± 0.005
Pupa	2.256 ± 0.044	0.426 ± 0.005
Male adult	1.768 ± 0.043	0.410 ± 0.004
Female adult	2.256 ± 0.044	0.509 ± 0.004

Note: Data presented are the mean of 5 replicates \pm standard deviation

An adult insect was dark brown or black. The color of the insect body could be formed by pigments present in the skin wall of the body: the purine-based pigment and melanin usually present in the exocuticle. This color was called a chemical color. The formation of pigments in insects was often genetic. These pigments could be influenced by environmental factors. Insects could synthesize some pigments, but most pigments were formed through food absorbed by insects (Cai et al., 2020).

Insects share common arthropod features, such as having an exoskeleton covered with a cuticle (a product of dermal tissue). The body of a insect consists of three parts, including the head, thorax, and abdomen (Figure 2).



Figure 2. Structure of the insect body

Characteristics of the male insect

The characteristics to distinguish male and female insects are presented in Table 3 and Figure 3. The male body length was about 1.768 ± 0.043 mm; the entire head was black. Black compound eyes were located close together and hairy. The compound eye consisted of many ocelli, which were units of light called ommatidium. Each ommatidium consisted of a transparent horny membrane that was hexagonal on the outside; underneath was the conical crystalline lens, both of which formed the lenticular part of the ommatidium (Chapman et al., 1998). Thanks to their compound eyes, insects could distinguish between

shapes, colors, movements, distances, and even polarized light (Figure 3a). The antennae were black with a flagellum length of 0.47 mm (Figure 3c). They were in the form of a serrated antenna with many stiff hairs, consisting of 10 segments protruding to one side as a serrated tooth, flagellum I - IX had bigger width than length, flagellum X was elongated. The basic antennae structure consisted of the scape, the pedicel, and the flagellum. The antennae could be moved easily thanks to the muscular system that controls the operation of the antennae located at the scape and pedicel segments. The main function of the antennae was the senses of touch and smell. The shape and size of the antennae varied greatly depending on the type of insect.

Thorax: black, divided into three segments: the prothorax, mesothorax, and metathorax. Each thoracic segment had a pair of legs, while the middle and posterior thoracic segments had wings. The middle thorax was the largest part of the body containing the muscular system that powered the wings (Markow et al., 2006; Demerec et al., 2008).

Wing: (Figure 3b) greyish-white, brown front vein, dense gill. The wing was about 2.43 mm long and 0.86 mm wide; Costa (C) vein was 0.72 mm long; Radius (R) vein divided into two branches, including SR1 and SR2.

Leg: (Figure 3d) was similar in structure to other species in the Drosophilidae group. The pectoral foot of the insect was a jumping leg with a developed thigh segment. The tibial segment was long and tubular. The femur and tibia were similar; the anterior femur was dark brown, whereas the middle and posterior femur was slightly brown. The tip of the tibia and the trunk segments had sharp spines; The I body of all legs was longer than the following segments; tarsomere IV in all legs was shorter than the others; claws were sharp with well-developed claw cushion. The thoracic feet of insects were mostly used for walking and clinging. Due to different living situations and habits in many species, the shape and size of the thorax have been deformed to suit different functions. The shape and number of metatarsal segments were commonly used in classification.

Abdomen: black; consisting of 7 abdominal segments; and bigger length than width. It has a rounded and non-ripple posterior segment.

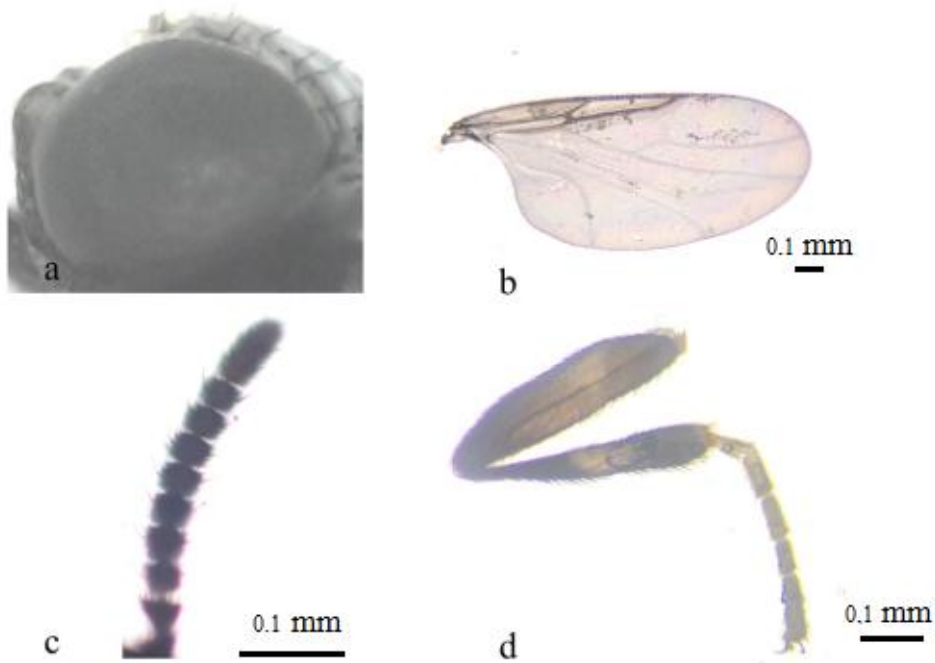


Figure 3. Morphological characteristics of collected insect (a) eye; (b) wing; (c) antennae; (d) leg

Characteristics of the female insect

Similar to males, the body of the female insect was 2.256 ± 0.044 long and 0.509 ± 0.004 mm wide. Its head was dark brown. Eyes were made up of compound eyes, black but located far apart and hairy. The antennae were black; the flagellum was about 0.52 mm, longer than the male.

Thorax: Thorax was dark brown; its length was bigger than width. The wing was about 2.48 mm long, 0.87 mm wide, and grey. The Costa (C) vein was 0.74 mm long, the Radius (R) vein was also divided into SR1 and SR2 branches. The abdomen was dark brown, also included 7 abdominal muscle segments, but the segments were longer than the male.

Table 3. Distinguishing characteristics of female and male fruit insects

Characteristic	Female insect	Male insect
Size	Big (about 2.256mm)	Small (about 1.768mm)
Color	Dark brown	Dark brown (dark color)
Head	Small (about 0.217mm)	Big (about 0.268mm)
Swing	Grey, big	White-grey, small
Last abdominal segment	Big	Small
Morphological characteristics	Two long flagella outside	There were two flagella and two pincer-shaped hardpoints



Figure 4. Adult insect, female (right) and male (left)

Identification of insects by molecular biology

PCR results of the COII gene fragment showed that the size of the amplified gene fragment was about 800 bp in length (Figure 5). This PCR product was purified and sequenced.

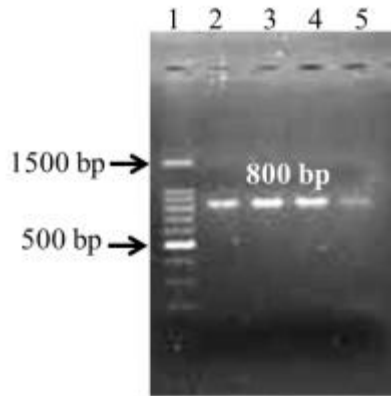


Figure 5. Gel electrophoresis of PCR product from the collected insect on 1.5% agarose gel. Lane 1: ladder 100 bp (Invitrogen); lane 2-5: PCR product from whole body insect

Sequencing results showed that when using the COII F forward primer (5'-AATATGGCAGATTAGTGCA-3'), the gene fragment was 680 bp, and when using the COII R reverse primer (5'-GTTTAAGAGACCAGTACTTG-3'), sequencing results in a 660 bp gene fragment (Figure 6).

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TTACCGGCCCTAAGAATTATTGATAAATTCATTAAATAAATGATTATAGATGTTCTT
TCAATTACGATTGGTATAAACTGTGGTTTGCCTCCACAATTCGGAACATGTCCAAA
AAATAAGCCTGGGCGGTTTATAAAAAATTGTTTGATTAAATCGTCCAGGAGTCCAT
CAATTTTACTCTAGGGCTGGAAACAGTTCATGAGTGAATACATCTGTGCTGTTACT
AAGACTCGAATTTGGGTGTTTATGGGTAAGATAGTTCGATTGTCAACATCAGTAGTC
GAAATTCGTTGTATTAATGAATAGTTGGAATATATAAGAATCAAATTCATAATTA
AAAATCGAATATTCATATCTCAGTATCATTGATGACCAATTGCTTTAGAGTAATTGA
TGGAGAATTAATTCATCTAATAAATAATAATCGAAGAGAAGGGAAGCAATAAAT
AATAGGATAATTCAGCTAAATTTCAATAATTTCAATAAGTTGCTCTGATAGTAA
AAATCGATTGATATATTGTATAAAATAATAATTTAAATACCCAATAAGGGTA
ATTATAATAAAATTAAGAGAATGATCGTGAAAAAAATTAATTGTTCTATTAAGGG
AGAATTAATTTGTAGATTAGTTGATCAGGTAGCCATTTTCTAATAAAAGAGT
TTCCTTATATATGGGGGTTTAAGTCCAT
GAGTGTGCCGTGCACTAGAATTTTGATAATTCATTAAATAAATGATTATAG
ATGTTCTTTCAATTACGATTGGTATAAACTGTGGTTTGCCTCCACAATTCCTG
AACATTTGCCAAAAATAAGCCTGGGCGGTTTATAAAAAATTGTTTGATT
AATCGTCCAGGAGTCCATCAATTTTACTCTAGGGCTGGAAACAGTTCATGA
GTGAATTCATCTGTTGCTGTTACTAAGACTCGAATTTGGGTGTTTATGGGTAA
GATAGTTCGATTGTCAACATCAGTAGTCGAAATTCGTTGTATTAATGAATTA
GTGGGAATATATAAGAATCAAATCTAAATTAATAAATCTGAATATCAATCT
TCAGTATCATTGATGACCAATTGCTTTAGAGTAATGATGGAGAATTAATTC
ATCTAATAAATAATAATCGAAGAGAAGGGAAGCAATAAATAAGGATAAT
TGCAGGTAAATTTGTTCAATAATTTCAATAAGTTGCTCTGATAGTAA
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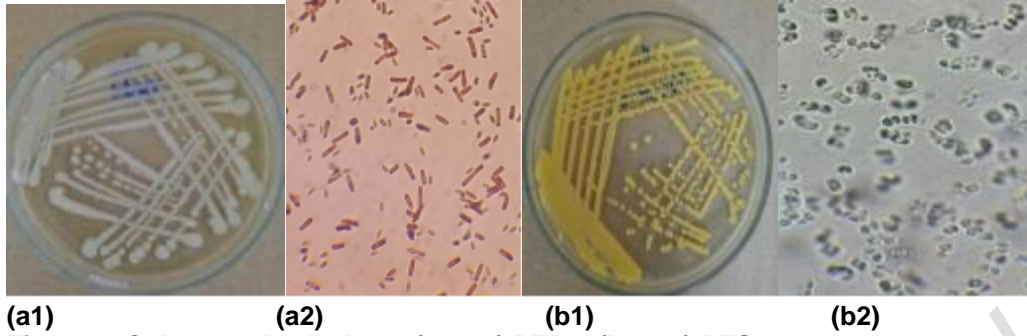
Figure 6. The results of sequencing the COII gene fragment from insect was obtained. Left: using forward primer, right: using reverse primer

Comparison results on the gene bank using the BlastN tool for gene segments sequenced by forwarding primer showed that there are 100 gene sequences in the gene bank (NCBI homepage) that are similar to COII gene fragment from insect obtained in Ben Tre province. The results of examining the external morphological characteristics from 100 species with similar sequences showed no species with similar shapes to the insects collected in this study. Similar results were also obtained for the sequence of gene using a reverse primer of the COII gene. From that, it is possible that this insect has not yet uploaded the COII gene sequence to the NCBI gene database. Based on the external morphological features, and compared with the characteristics of the insect group of the Scaptosidae family, it is possible the insect collected on the decomposing pig manure in this study belong to the family Scaptosidae. Further studies on examining in more detail the external morphological characteristics, matK gene sequencing needs to be performed to have a sufficient scientific basis for identifying this insect.

Isolation and identification of bacteria from collected insect

Bacterial isolation

Two bacterial strains were isolated from the insect leg sample on Blood Agar Base (Infusion Agar) and there were named as BTS1 and BTP1.



(a1) (a2) (b1) (b2)
Figure 7. Colony and cell shape (a1, a2) BTP1; (b1, b2) BTS1

The isolated bacterial strains had the common characteristic on Blood Agar Base without sheep blood added after 48 h. The colonies of two strains of bacteria were yellow and milky with 1-2 mm size (Figure 7). The results of the biochemical characterization survey shown in Table 4 presented that the bacteria isolated from insects belonging to the Scaptosidae family had the same shape and biochemical characteristics as that of *B. cereus* (Duport et al., 2016) and *Micrococcus luteus* (Li et al., 2021); thereby proving that insects were the vectors that carried pathogens from contaminated environments to the wound and made the wound become worse (Table 4).

Table 4. Colony and morphological characteristics of bacterial strains on Blood Agar Base (Infusion Agar) after 48 h of culture

Strain	Colony				Bacteria		
	Form	Color	Size (mm)	Shape	Biochemical characteristics		
					Gram	Catalase	Oxidase
BTS1	round, entire edge, raised	Yellow	1-1.5	Spherical	+	+	-
BTP1	round, entire edge, flat	Opaque white	2-3	Rod-shaped	+	+	-

Note: (+) positive, (-) negative

Antibiotic resistance assay

The survey results presented in Table 5 show that all five antibiotics used in the study were resistant to two isolated strains. According to the criteria for *Lactobacillus* of Georgieva et al. (1966), the diameter of antibacterial zone < 7 mm is resistance bacteria strain, weak sensitive strain is from 7 – 16 mm, moderate sensitive strain is from 16 – 25 mm, and strong sensitive > 25 mm. According to Table 5 shown BTP1 and BTS1 are resistance to Clindamycin. Gentamicin had moderate sensitivity to two bacteria strains. Penicillin, Ciprofloxacin, and Kanamycin gave weakly sensitive results. The research results were consistent with the study of Jackson et al. (1971) that antibiotics were resistant to the growth of some bacteria causing festering wounds but suggested using at low doses. Therefore, Clindamycin could be used during treatment, but considered using at a low dose of 3 mg/mL to ensure antibiotic resistance.

Table 5. Sterile ring diameter (mm) and antibiotic susceptibility of bacterial strains

Bacterial strains	Diameter of antibacterial zone (mm)				
	KN	GE	PN	CI	CL

BTP1	12.00	22.33	8.33	8.32	5.38
BTS1	11.67	22.67	7.67	7.02	6.55

Note: KN: Kanamycin (30 µg/mL); CL: Clindamycin (2 µg/mL); PN: Penicillin (10 µg/mL); Cl: Ciprofloxacin (5 µg/mL); GE: Gentamicin (10 µg/mL).

Bacterial strain identification

The surveying results concluded that the BTS1 and BTP1 bacterial strains were capable of causing wound festering to perform PCR reaction with primer pair 27F-1492R, amplifying the 16S rRNA region of the bacteria. The PCR product has a size of 1500 bp. Gene sequencing is performed, and BlastN software is used to compare with the DNA sequences of the bacterial strains contained in the NCBI data bank. The results showed that the sequence of the BTP1 strain was similar to the 16S rRNA gene of *Micrococcus luteus* with 100% similarity and 100% coverage. Therefore, the BTP1 strain was identified as *Micrococcus luteus*. Similarly, the BTS1 strain was also identified as *Bacillus cereus* species with 100% similarity and 100% coverage, and BTS1 strain was identified as *Bacillus cereus*.

The analysis results are consistent with the study of Messelhäußer and Ehling-Schulz (2018), who suggested that some species of group *B. cereus* are capable of causing festering wounds in humans with more severity. In addition, Ehling-Schulz et al. (2004) also suggested that the group of bacteria *B. cereus* and *Micrococcus luteus* are capable of causing more serious wound festering. In addition, the results are consistent with the study of Monodane et al. (1997), suggesting that with high bacterial densities, they have virulent activities related to inducing septic shock, septic arthritis, endocarditis, meningitis, and possibly pneumonia.

4. CONCLUSION

The research results concluded that the insect species collected belonged to the Scaptosidae family. It also recorded the presence of *Micrococcus luteus* and *Bacillus cereus*, the two strains of bacteria that can cause festering wounds on the human body in the insect. Further studies are needed to understand the biology and reproductive behavior of the species to prevent the transmission of pathogenic bacteria in humans. Besides that, other studies examining the external morphological characteristics in more detail, matK gene sequencing needs to be performed to have a sufficient scientific basis for identifying this insect.

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