IIAJ INTERNATIONAL INTEGRATED AGRICULTURAL JOURNAL
Volume 02 Number 02
November 2026
e-ISSN: 3124-8179
p-ISSN: 3124-7865

Isolation and Preliminary Characterization of Culturable Gut Bacteria from White Grub (*Lepidiota stigma*) Larvae

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Abstract:
The white grub *Lepidiota stigma* (Coleoptera: Scarabaeidae) is a major root-feeding pest of sugarcane in Indonesia, and its digestive tract may harbor bacteria with useful metabolic functions. This study aimed to isolate culturable bacteria from the digestive tract of *L. stigma* larvae and to characterize them based on colony morphology. Third-instar larvae were collected from three nests in a sugarcane plantation in Gayasan Village, Jember Regency, in June–July 2026. The digestive tract was removed aseptically after surface sterilization, homogenized in sterile saline, serially diluted, and plated on nutrient agar. Morphologically distinct colonies were purified and described by shape, margin, surface, and color, and morphotype diversity was calculated. Twenty-five isolates (A1–A25) were obtained. Most colonies were irregular (68%), had undulate margins (56%), were shiny (64%), and were white (44%). The 25 isolates formed 17 distinct morphotypes, 11 of which were represented by a single isolate, giving a Shannon index of 2.73, an evenness of 0.96, and a Simpson diversity of 0.97. Entire margins occurred only in circular colonies. These results show that the culturable gut bacteria of L. stigma are phenotypically diverse and provide a set of purified isolates for further identification and functional screening.

Keywords:
colony morphology, gut bacteria, *Lepidiota stigma,* morphotype diversity, white grub

Article info:
Submitted:
12-09-2026
Revised:
12-11-2026
Accepted:
13-11-2026
Published:
21-11-2026

https://doi.org/10.53713/iiaj.v2i2.859

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INTRODUCTION

White grubs, the soil-dwelling larvae of scarab beetles (Coleoptera: Scarabaeidae), are among the most damaging below-ground pests of crops worldwide. In Indonesia, *Lepidiota stigma* is a key pest of sugarcane, particularly in sandy soils, where its larvae destroy the root system, causing plants to wilt and die (Purnomo et al., 2021; Siswanto et al., 2025). Because the larvae live in the soil, they are difficult to control, and farmers still rely heavily on chemical insecticides. Cultural practices, such as plastic mulching, and biological agents, such as entomopathogenic nematodes and fungi, have been tested as alternatives (Sunarto and Subiyakto, 2020; Siswanto et al., 2025). Understanding the biology of this pest, including its associated microorganisms, may open further options for its management and for the use of its microbial resources.

The digestive tract of scarab larvae is a specialized habitat for microorganisms. The hindgut is enlarged into a fermentation chamber that houses dense microbial communities that help the larvae digest the cellulose-rich roots and other organic matter on which they feed. In *Holotrichia parallela*, a root-feeding white grub, both aerobic and anaerobic bacteria were cultured from the fermentation

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e-ISSN: 3124-8179
p-ISSN: 3124-7865

chamber, and the cultivable fraction was dominated by Proteobacteria and Clostridia (Zhang et al., 2018). In *Protaetia brevitarsis*, gut bacteria contribute to the degradation of lignocellulose (Wang et al., 2022); the larval frass contains bacteria with plant growth-promoting and disease-suppressing potential (Xuan et al., 2022); and the composition of the hindgut microbiota differs among larvae from different environments (Min et al., 2024). Insect gut bacteria are also known to provide nutrients, degrade toxic compounds, and protect their hosts against pathogens (Geng et al., 2022).

These functions make insect gut bacteria a valuable source of microorganisms for biotechnology and agriculture. Cellulolytic bacteria have been isolated from the guts of termites such as *Macrotermes gilvus* and *Heterotermes indicola* (Oktiarni et al., 2022; Azhar et al., 2024), and culturable bacteria from the black soldier fly *Hermetia illucens* showed a broad range of hydrolytic activities (Callegari et al., 2020). Termite-associated bacteria have also been shown to suppress plant pathogens, including *Colletotrichum* sp. (Dinata et al., 2026). By comparison, the gut bacteria of *L. stigma* have received little attention, even though this species is abundant in sugarcane fields in East Java and feeds on lignocellulose-rich roots.

Sugarcane is a strategic crop in Indonesia, and East Java is the largest sugarcane-producing province. In this region, white grub infestation is a recurring problem, especially where sugarcane is grown on dry, sandy land rather than on irrigated paddy fields. Third-instar larvae are the most destructive stage because they consume large amounts of root tissue over a long period, and the life cycle of *L. stigma* lasts about one year (Purnomo et al., 2021). The large quantity of root material processed by the larvae suggests that their digestive tract may contain bacteria adapted to the degradation of cellulose and other plant polymers, as has been demonstrated for other root-feeding scarabs (Zhang et al., 2018). These bacteria have not yet been explored in *L. stigma* populations in Jember Regency, one of the main sugarcane-growing areas of East Java.

Isolation and preliminary characterization are the first steps in exploring a new microbial source. Culture-dependent isolation provides living isolates that can later be identified and tested for useful activities, which is not possible with sequencing-based methods alone. Colony morphology, including shape, margin, surface, and color, is a simple and inexpensive way to distinguish isolates, to avoid the repeated purification of identical colonies, and to group isolates before microscopic, biochemical, and molecular analyses (Janatiningrum et al., 2022; Ananda et al., 2023; Aisah and Zul, 2026). However, few studies have quantified the morphological diversity of isolates obtained from insect guts, and information on the culturable gut bacteria of *L. stigma* from the sugarcane plantations of Jember Regency is lacking.

Therefore, this study aimed to isolate culturable bacteria from the digestive tract of *L. stigma* larvae collected from a sugarcane plantation in Gayasan Village, Jenggawah District, Jember Regency, and to characterize the isolates based on colony morphology. Specifically, the study (i) described the macroscopic characteristics of each isolate, (ii) determined the frequency of each characteristic, and (iii) grouped the isolates into morphotypes and quantified their diversity. The results are intended to provide a characterized collection of isolates for further identification and functional screening.

METHOD

The study was conducted from June to July 2026. White grub larvae were collected from a sugarcane plantation in Gayasan Village, Jenggawah District, Jember Regency, East Java, Indonesia. Bacterial isolation, purification, and characterization were carried out at the Plant Protection Laboratory, Department of Agricultural Production, Politeknik Negeri Jember. The study

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e-ISSN: 3124-8179
p-ISSN: 3124-7865

consisted of four stages: larval sampling, bacterial isolation, purification, and macroscopic characterization.

Larvae were collected from three nests (sampling points) apart within the plantation, which was planted with sugarcane aged one month. At each point, the soil around the root zone of damaged plants was dug to a depth of 0–30 cm, and third-instar larvae were collected by hand. Larvae were identified as *L. stigma* on the basis of their morphology, raster pattern, and head capsule width. Healthy, active larvae were placed individually in labeled plastic containers filled with soil from the sampling site and transported to the laboratory, where they were processed within 24 h of collection.

Nutrient agar was prepared according to the manufacturer's instructions and sterilized in an autoclave at 121 °C and 1 atm for 15 min. Normal saline (0.85% NaCl) and distilled water used for rinsing and dilution were sterilized in the same way. Dissecting tools, glassware, and pestles were sterilized by autoclaving or by dry heat before use, and all work involving open cultures was performed in a laminar airflow cabinet that had been cleaned with 70% ethanol and exposed to UV light for 15 min before use. Media were poured into sterile Petri dishes 9 cm in diameter and allowed to solidify before inoculation.

Bacteria were isolated from the digestive tract following the method of Yusnia et al. (2019) with modifications. The surface of each larva was sterilized by immersion in 70% ethanol for 1 min and rinsed twice with sterile distilled water. The larvae were then dissected aseptically with a sterile scalpel in a laminar airflow cabinet, and the entire digestive tract was removed. The tract was washed in sterile distilled water to remove adhering tissue, placed in a tube containing 1 mL of sterile normal saline (0.85% NaCl), and homogenized with a sterile pestle. The homogenate was serially diluted tenfold in sterile saline to 10^{-6}, and 0.1 mL of the 10^{-4} to 10^{-6} dilutions were spread onto nutrient agar (NA) plates. The plates were incubated at 28 ± 2 °C for 24–48 h until colonies developed. Isolation was repeated three times, once for the larvae from each nest. To confirm that the surface sterilization was effective and that the isolates originated from inside the digestive tract, 0.1 mL of the final rinse water was spread on NA as a sterility control, and an uninoculated NA plate was incubated alongside each batch as a medium control. Plates from the dilutions that produced well-separated colonies were used for colony selection.

Colonies that differed in appearance were selected from the isolation plates and subcultured onto fresh NA using the quadrant streak method under aseptic conditions. Plates were incubated for 24 h, and the transfer was repeated until each culture showed colonies with uniform characteristics (Handoko et al., 2020). Purified isolates were coded A1–A25 and maintained on NA slants at 4 °C for further study. Colony morphology was observed on pure cultures grown on NA for 48 h at 28 ± 2 °C. Four characteristics were recorded for each isolate: colony shape, colony margin, surface appearance, and color (Table 1). Observations were made with the naked eye and, where necessary, with a stereo microscope under consistent lighting. Descriptions followed standard terminology for bacterial colony morphology (Janatiningrum et al., 2022; Dissasa et al., 2022). Each observation was checked by two observers to reduce subjective bias in the description of color and surface.

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Table 1. Colony characteristics observed and categories used for description

Characteristic	Categories recorded	Description
Shape	Circular; irregular	Overall outline of the colony viewed from above
Margin	Entire; undulate; lobate	Form of the colony edge: smooth, wavy, or lobed
Surface	Shiny; dull	Light reflection of the colony surface
Color	White; clear white; milky white; cream; yellow	Pigmentation of the colony under reflected light

Note: Observations were made on pure cultures grown on nutrient agar.

RESULTS

Bacterial colonies grew on NA plates inoculated with gut homogenates from the larvae of all three nests. Based on differences in colony appearance, 25 colonies were selected and purified, and they were coded A1–A25. All 25 isolates grew well on NA after repeated subculturing and produced uniform colonies, indicating that pure cultures were obtained. These isolates represent the culturable bacteria able to grow aerobically on NA under the conditions used in this study. The 25 isolates differed in colony shape, margin, surface, and color (Table 2). Two colony shapes, three margin types, two surface types, and five colors were observed.

Table 2. Colony morphology of bacterial isolates from the digestive tract of *L. stigma* larvae

Isolate	Shape	Margin	Surface	Color	Morphotype
A1	Irregular	Lobate	Shiny	Yellow	M1
A2	Irregular	Undulate	Shiny	White	M2
A3	Irregular	Undulate	Dull	Cream	M3
A4	Circular	Undulate	Shiny	Yellow	M4
A5	Irregular	Undulate	Shiny	Cream	M5
A6	Irregular	Undulate	Shiny	Cream	M5
A7	Irregular	Undulate	Dull	White	M6
A8	Circular	Entire	Dull	White	M7
A9	Irregular	Undulate	Shiny	Yellow	M8
A10	Circular	Undulate	Shiny	White	M9
A11	Irregular	Undulate	Dull	White	M6
A12	Irregular	Lobate	Shiny	White	M10
A13	Circular	Entire	Shiny	White	M11
A14	Circular	Entire	Shiny	Yellow	M12
A15	Circular	Entire	Shiny	White	M11
A16	Circular	Entire	Shiny	Yellow	M12
A17	Irregular	Lobate	Shiny	Milky white	M13
A18	Irregular	Undulate	Dull	Clear white	M14
A19	Circular	Lobate	Dull	Milky white	M15
A20	Irregular	Undulate	Shiny	White	M2
A21	Irregular	Undulate	Shiny	White	M2
A22	Irregular	Undulate	Dull	White	M6
A23	Irregular	Lobate	Dull	Cream	M16
A24	Irregular	Undulate	Dull	Milky white	M17
A25	Irregular	Lobate	Shiny	Milky white	M13

Note: Morphotype = group of isolates with an identical combination of the four colony characteristics (M1–M17).

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Volume 02 Number 02
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e-ISSN: 3124-8179
p-ISSN: 3124-7865

Table 3. Frequency of colony characteristics among the 25 bacterial isolates

Characteristic	Category	Number of isolates	Percentage (%)	Isolates
Shape	Irregular	17	68	–
	Circular	8	32	A4, A8, A10, A13–A16, A19
Margin	Undulate	14	56	–
	Lobate	6	24	A1, A12, A17, A19, A23, A25
	Entire	5	20	A8, A13–A16
Surface	Shiny	16	64	–
	Dull	9	36	A3, A7, A8, A11, A18, A19, A22–A24
Color	White	11	44	A2, A7, A8, A10–A13, A15, A20–A22
	Yellow	5	20	A1, A4, A9, A14, A16
	Cream	4	16	A3, A5, A6, A23
	Milky white	4	16	A17, A19, A24, A25
	Clear white	1	4	A18

Note: n = 25 isolates. – = remaining isolates in that characteristic.

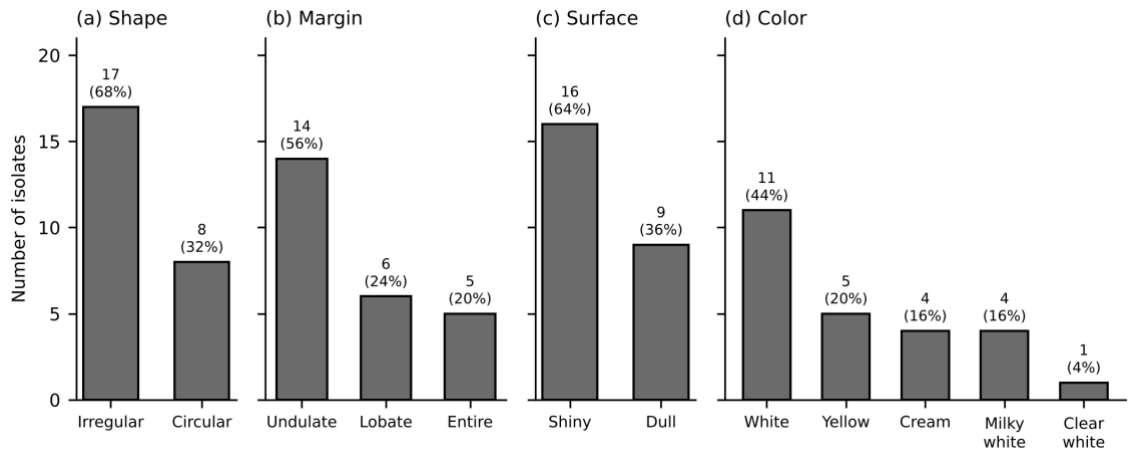


Figure 1. Frequency (number of isolates) of colony characteristics among the 25 bacterial isolates from the digestive tract of *L. stigma* larvae: (a) shape, (b) margin, (c) surface, and (d) color

When the four characteristics were combined, the 25 isolates formed 17 distinct morphotypes (Table 4). Eleven morphotypes were represented by a single isolate, and six morphotypes contained two or three isolates. The largest groups were irregular, undulate, white colonies with either a shiny surface (A2, A20, and A21) or a dull surface (A7, A11, and A22). The other shared morphotypes were irregular, undulate, shiny, cream colonies (A5 and A6); circular, entire, shiny, white colonies (A13 and A15); circular, entire, shiny, yellow colonies (A14 and A16); and irregular, lobate, shiny, milky-white colonies (A17 and A25).

Table 4. Morphotypes shared by two or more isolates

Morphotype	Shape / margin / surface / color	Number of isolates	Isolates
M2	Irregular / undulate / shiny / white	3	A2, A20, A21
M6	Irregular / undulate / dull / white	3	A7, A11, A22
M5	Irregular / undulate / shiny / cream	2	A5, A6
M11	Circular / entire / shiny / white	2	A13, A15
M12	Circular / entire / shiny / yellow	2	A14, A16
M13	Irregular / lobate / shiny / milky white	2	A17, A25

Note: The remaining 11 isolates (A1, A3, A4, A8, A9, A10, A12, A18, A19, A23, A24) each formed a unique morphotype.

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e-ISSN: 3124-8179
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The diversity indices showed that the isolates were distributed almost evenly among morphotypes (Table 5). The Shannon index was 2.73, close to its maximum possible value of 2.83 for 17 morphotypes, and evenness was 0.96. Simpson's diversity index was 0.97, meaning that two isolates selected at random had a 97% probability of belonging to different morphotypes. Cross-tabulation of shape and margin showed a clear association: entire margins occurred only in circular colonies, whereas 12 of the 17 irregular colonies had undulate margins and the remaining five had lobate margins (Table 5).

Table 5. Summary of morphotype diversity and association between colony shape and margin

Parameter	Value
Number of isolates (N)	25
Number of morphotypes (S)	17
Morphotypes represented by one isolate	11 (64.7% of morphotypes)
Morphotypes represented by two or three isolates	6 (14 isolates)
Shannon diversity index (H′)	2.73 (maximum ln S = 2.83)
Pielou's evenness index (E)	0.96
Simpson's diversity index (1 − D)	0.97
Circular colonies: entire / undulate / lobate margin	5 / 2 / 1
Irregular colonies: entire / undulate / lobate margin	0 / 12 / 5

Note: Indices were calculated from the morphotype frequencies in Table 2 and describe phenotypic, not taxonomic, diversity.

DISCUSSION

This study obtained 25 culturable bacterial isolates from the digestive tract of *L. stigma* larvae and showed that they differed considerably in colony morphology. The isolates formed 17 morphotypes, most of which were represented by only one isolate, and the diversity indices were high. To our knowledge, this is the first report to describe and quantify the morphological diversity of culturable gut bacteria from *L. stigma* collected in sugarcane fields in Jember Regency. The results show that the gut of this pest contains a phenotypically diverse culturable bacterial fraction that can be recovered with simple culture methods.

The recovery of a diverse set of isolates is consistent with what is known about scarab larvae. Their hindgut acts as a fermentation chamber with dense microbial populations that help the larvae digest plant material (Zhang et al., 2018; Wang et al., 2022). In *H. parallela*, culturable aerobic bacteria were dominated by γ-Proteobacteria, and the culturable fraction accounted for only a small proportion of total bacterial cells (Zhang et al., 2018). Gut communities of scarab larvae also vary with the environment in which the larvae live (Min et al., 2024). Because *L. stigma* feeds on sugarcane roots and soil organic matter, its gut is likely to receive a continuous supply of soil- and root-associated bacteria, which may partly explain the variety of colony types observed in this study.

Colony shape and margin were useful first-level characteristics. Irregular colonies with undulate margins were the most common type, suggesting that many isolates spread unevenly across the agar surface. Such growth patterns are often associated with motile or spreading bacteria, although this cannot be confirmed from colony morphology alone. An interesting finding was that entire margins occurred only in circular colonies, while irregular colonies always had undulate or lobate margins. This association is expected, because colonies that grow evenly in all directions tend to form smooth, regular edges, whereas uneven growth produces both an irregular outline and a wavy or lobed margin. Colony shape and margin therefore partly describe the same growth pattern, and their combination with other characteristics is needed to effectively separate isolates. Similar

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e-ISSN: 3124-8179
p-ISSN: 3124-7865

descriptors have been used to differentiate isolates from environmental and clinical samples before biochemical or molecular identification (Dissasa et al., 2022; Radhakrishnan and Krishnasamy, 2024).

Surface appearance and color increased the ability to distinguish isolates. Most isolates were shiny, which may indicate the production of extracellular polysaccharides or a moist colony surface, whereas dull colonies may have drier or rougher surfaces. Color was the most variable characteristic, with five categories. Yellow pigmentation, observed in five isolates, is produced by several groups of bacteria that synthesize carotenoid or flavonoid pigments, while white and cream colonies are common among many unrelated genera. Pigmentation has been widely used to distinguish bacterial morphotypes during isolation (Azhar et al., 2024; Jiru and Ayanaw, 2024). The value of color was clear in this study: isolates A13–A16 were all circular, entire, and shiny, but they could be separated into two morphotypes by color. Nevertheless, colony surface and color can vary with medium composition, incubation time, and temperature, so they should be considered descriptive rather than diagnostic characteristics.

Grouping the isolates into morphotypes provided a more informative picture than examining each characteristic separately. The 17 morphotypes and the high Shannon, evenness, and Simpson values show that the isolates were not dominated by a few colony types. This information has practical value. Isolates belonging to the same morphotype, such as A2, A20, and A21, or A7, A11, and A22, may represent the same strain recovered from different larvae or nests, and one representative from each group could be prioritized for identification to save time and cost. Conversely, the 11 singleton morphotypes may represent bacteria that are less common in the culturable fraction and deserve particular attention in later functional screening. Morphotype grouping is routinely used for this purpose in microbial isolation studies (Radhakrishnan and Krishnasamy, 2024; Aisah and Zul, 2026). However, morphotype diversity should not be interpreted as species diversity, because different species can produce similar colonies, and a single species can produce different colony types under different conditions.

The isolates obtained in this study provide a starting point for exploring the functional potential of the *L. stigma* gut microbiota. Bacteria from insect guts have been shown to produce cellulases and other hydrolytic enzymes (Callegari et al., 2020; Oktiarni et al., 2022; Azhar et al., 2024), and bacteria isolated from scarab larvae and their frass include strains with plant growth-promoting and antagonistic activities (Xuan et al., 2022). Termite symbiotic bacteria have also been developed as biological control agents against plant pathogens (Dinata et al., 2026). Because *L. stigma* feeds on lignocellulose-rich roots, its gut isolates are promising candidates for screening for cellulolytic activity. In addition, knowledge of this pest's gut bacteria may support the development of control methods that target its digestive symbionts or combine entomopathogens with an understanding of the larval microbiota (Purnomo et al., 2021; Siswanto et al., 2025).

The approach used in this study also has methodological strengths that support the reliability of the isolates obtained. Surface sterilization of the larvae before dissection reduced the risk of recovering bacteria from the cuticle or the surrounding soil, and repeated subculturing ensured that each isolate was derived from a single colony type. Isolation was repeated with larvae from three nests, increasing the likelihood of recovering bacteria that occur across different individuals and microhabitats within the plantation. The occurrence of identical morphotypes among isolates selected in different isolation rounds, such as the two groups of irregular, undulate, white colonies, suggests that some bacteria may be consistently associated with the gut of *L. stigma* in this plantation. Recording the nest of origin for each isolate would allow this pattern to be tested formally

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e-ISSN: 3124-8179
p-ISSN: 3124-7865

and would help to distinguish resident gut bacteria from transient bacteria ingested with soil and roots (Min et al., 2024).

Several limitations should be considered. First, only aerobic bacteria capable of growing on NA were recovered. The scarab hindgut is largely anoxic, and anaerobic bacteria can dominate the culturable fraction under anaerobic culture conditions (Zhang et al., 2018). Many gut bacteria also require specific nutrients or host-derived factors and cannot be cultured on general media. The 25 isolates therefore represent only a fraction of the gut community, as also emphasized in other culture-dependent studies of insect gut bacteria (Callegari et al., 2020). Second, the whole digestive tract was homogenized, so the isolates could not be assigned to the foregut, midgut, or hindgut. Third, the characterization was limited to colony morphology, which cannot establish the identity of the isolates. Fourth, larvae were collected from a single plantation over a single season, so the results may not represent *L. stigma* populations at other locations or times.

Future studies should therefore include Gram staining and cell morphology, biochemical tests, and molecular identification based on 16S rRNA gene sequencing, which have been used successfully to identify cellulolytic bacteria from various sources (Ananda et al., 2023; Yarashima et al., 2024). The use of selective media, anaerobic culture, and separation of gut compartments would increase the range of bacteria recovered. Culture-independent methods, such as 16S rRNA amplicon sequencing, would show how well the culturable isolates represent the entire gut community (Geng et al., 2022; Min et al., 2024). Finally, the isolates should be screened for cellulolytic, proteolytic, and antagonistic activities to determine their potential for agricultural and biotechnological use.

CONCLUSION

Twenty-five culturable bacterial isolates (A1–A25) were obtained from the digestive tract of third-instar *Lepidiota stigma* larvae collected from a sugarcane plantation in Gayasan Village, Jember Regency. The isolates varied in colony shape (mostly irregular, 68%), margin (mostly undulate, 56%), surface (mostly shiny, 64%), and color (mostly white, 44%), and they formed 17 morphotypes with high diversity (Shannon index 2.73; Simpson index 0.97). These results show that the culturable gut bacteria of *L. stigma* are phenotypically diverse, and the purified and characterized isolates provide a basis for molecular identification and screening of their functional potential.

ACKNOWLEDGEMENT

The authors would like to express their sincere gratitude to the Directorate of Learning and Student Affairs (Direktorat Pembelajaran dan Kemahasiswaan), Ministry of Higher Education, Science, and Technology of the Republic of Indonesia, for providing financial support for this research through the 2026 Student Creativity Program (Program Kreativitas Mahasiswa 2026). The authors also acknowledge the support and facilities provided by Politeknik Negeri Jember, particularly the Plant Protection Laboratory.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this research, its authorship, or the publication of this manuscript.

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e-ISSN: 3124-8179
p-ISSN: 3124-7865

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November 2026
e-ISSN: 3124-8179
p-ISSN: 3124-7865

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