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Molecular and Toxicological Characterization of Indigenous *Bacillus thuringiensis* for Eco-Biocontrol of Spodoptera frugiperda in Corn Agroecosystems

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ABSTRACT

This study investigated the eco-biocontrol potential of indigenous isolates obtained from agricultural soils in Warbo Village, Papua, Indonesia, targeting the highly destructive fall armyworm (Spodoptera frugiperda) in corn agroecosystems. A total of 58 bacterial colonies were isolated, of which 18 were morphologically confirmed as *Bacillus thuringiensis* based on endospore and parasporal crystal protein characteristics. These isolates were cultured in Tryptose Phosphate Broth and tested for larvicidal activity against second-instar larvae under controlled conditions. Toxicity tests revealed that isolate 18 exhibited the highest efficacy, causing 100% larval mortality, followed by isolates 12 and 13 with 93.3%, confirming a strong entomopathogenic potential. The most toxic isolates were further verified by the presence of cry1F and cry2Aa genes through PCR analysis, indicating the molecular basis of their virulence. The innovation of this research lies in the combination of morphological, toxicological, and molecular characterizations of locally adapted *Bacillus thuringiensis* strains, which offers a sustainable, environmentally friendly alternative to chemical pesticides. These findings provide a scientific foundation for developing region-specific bioinsecticides, reducing dependence on synthetic inputs, and supporting sustainable pest management strategies in tropical corn production systems.

Keywords: Bacillus thuringiensis; Soil; Plantation; Toxicity; Larvae; Fall Armyworm

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ARTICLE INFO

Received: 19 March 2025 | Revised: 27 April 2025 | Accepted: 30 May 2025 | Published Online: 10 June 2025
DOI: https://doi.org/10.30564/re.v7i2.10102

CITATION

Lantang, D., Tanjung, R.H.R., Suhartawan, D., et al., 2025. Molecular and Toxicological Characterization of Indigenous *Bacillus thuringiensis* for Eco-Biocontrol of Spodoptera frugiperda in Corn Agroecosystems. Research in Ecology. 7(2): 209–223. DOI: https://doi.org/10.30564/re.v7i2.10102

1. Introduction

Corn plays a crucial role in ensuring global food security, functioning not only as a staple food for human consumption and a significant component of animal feed, but also as a versatile raw material for industrial products [1, 2], including bio-based fuels [3]. Its extensive economic and ecological value designates sustainable corn production as a strategic priority, particularly in the context of increasing pest pressures such as Spodoptera frugiperda, which pose threats to both yield and quality. Tropical agriculture faces increasing yield threats from biotic stresses, particularly invasive pests like the fall armyworm, causing significant losses [2, 4]. These pests reduce production efficiency by harming crops during critical growth stages, jeopardizing food supply stability in corn-dependent regions [5, 6]. Recent studies show that enhancing corn resilience through integrated pest management is vital for sustainable production. Research in tropical regions emphasizes the need for locally adapted biocontrol methods and plant growth-promoting techniques to reduce pest pressures and improve crop health [1, 7]. These insights highlight the need to improve corn production for global food security, despite ongoing yield challenges in tropical areas. From a theoretical standpoint, the application of *Bacillus thuringiensis* as a biological control agent aligns with principles of ecological pest regulation and integrated pest management (IPM). *Bacillus thuringiensis* operates within the trophic cascade of agroecosystems as a microbial natural enemy targeting herbivorous pests such as Spodoptera frugiperda. This regulation reduces defoliation, promotes plant physiological health, and stabilizes yield. In economic terms, the use of indigenous *Bacillus thuringiensis* strains offers a cost-effective alternative to synthetic pesticides, especially in tropical, resource-limited farming communities. Thus, the causal linkage between *Bacillus thuringiensis* introduction and plantation performance encompasses pest suppression, enhanced crop productivity, and economic sustainability. The fall armyworm is recognized as an invasive, destructive pest due to its rapid spread, high reproduction, and wide host range. This adaptability allows it to thrive in regions like tropical Africa and Asia [8, 9]. Its effect on staple crops, especially corn, raises concerns about global food security. Dependence on synthetic pesticides for fall armyworm control has led to increased resistance, reducing chemical efficacy and necessitating higher application rates [10, 11]. Reliance on pesticides limits long-term management and creates environmental and economic issues. Thus, developing integrated pest management strategies incorporating biological, genetic, and cultural methods is essential to reduce resistance and protect agricultural productivity [7, 8, 10, 12].

Entomopathogenic microbes offer an eco-friendly solution for controlling fall armyworm outbreaks in corn. Fungi such as Beauveria bassiana and Metarhizium anisopliae invade corn plants systemically, providing endophytic protection that lowers survival rates of fall armyworm larvae and reduces infestation levels [13, 14]. They can infect various developmental stages, providing a sustainable control option that avoids negative environmental impacts and resistance issues with synthetic pesticides [15]. These biocontrol agents are used in pest management to maintain ecosystem balance, enhance crop resilience, and reduce chemical usage [16]. They signify a strategic shift toward sustainable agriculture, effectively controlling fall armyworm while promoting biodiversity and ensuring long-term food security.

Bacillus thuringiensis is a biocontrol agent due to its Cry proteins, which act as insect toxins [17]. When ingested, these proteins are activated by the gut proteases of susceptible insects, binding to midgut receptors like aminopeptidase N and cadherin [18]. This binding forms pores in the gut epithelium, causing cell lysis and septicemia in the pest. *Bacillus thuringiensis* is used globally as a biopesticide and in transgenic crops, reducing dependence on chemical insecticides [19]. The biodiversity of *Bacillus thuringiensis* strains, as evidenced through genetic characterization studies in regions like Tamil Nadu, India, underscores its diverse array of Cry protein profiles, which enable control over a broad spectrum of insect pests [20]. This diversity further supports the development of tailored biocontrol strategies that address region-specific pest challenges.

Indigenous *Bacillus thuringiensis* research in Papua, Indonesia remains underexplored despite documented potential for local virulence and adaptation. Investigations into indigenous *Bacillus thuringiensis* isolates suggest promising insecticidal activity; however, the previous study [21, 22] primarily focused on the characterization of *Bacillus thuringiensis* in relation to Anopheles larvae, rather than on other pests such as fall armyworm. Therefore, while the study highlights local diversity and the potential for application in pest management, it does not specifically support claims of unique

adaptations of *Bacillus thuringiensis* to pests other than those it was studied with.

Global usage of *Bacillus thuringiensis*, characterized by its Cry protein-mediated mode of gut lysis [23, 24], emphasizes the significance of exploring indigenous strains that may have evolved in response to local ecological pressures. However, the lack of comprehensive studies in Papua limits the potential for fully utilizing these native resources for pest management. Addressing this gap could ultimately lead to eco-friendly pest management strategies tailored to regional agricultural challenges; nevertheless, more research is required to substantiate these claims, particularly regarding the effectiveness against specific pest species like fall armyworm.

This study identifies indigenous *Bacillus thuringiensis* isolates from Warbo Village, Papua, Indonesia, emphasizing their effectiveness against fall armyworm to develop sustainable corn pest control strategies. Some isolates display bipyramidal crystal inclusions, as noted by Maheesha et al [25]; who have shown effective toxicity to fall armyworm larvae, indicating potential for local use. Similarly, the results of the entomopathogenic screening reported by [26] confirm that native *Bacillus thuringiensis* variants cause significant larval mortality, emphasizing their role in integrated pest management. This study aimed to utilize local microbial diversity to reduce reliance on synthetic pesticides and promote sustainable control strategies [8, 27]. The study aimed to use Indigenous resources to create ecologically sustainable biocontrol strategies for promoting corn production in Papua.

Moreover, while numerous prior studies have demonstrated the general bioinsecticidal potential of *Bacillus thuringiensis* isolates against lepidopteran pests, the majority have concentrated on commercially available strains and synthetic formulations. Conversely, our study emphasizes the importance of examining indigenous soil-derived *Bacillus thuringiensis* isolates within tropical corn agroecosystems, particularly those exhibiting confirmed cry gene profiles. Drawing inspiration from the systemic approach to evaluating complex financial interactions proposed by Dimitriadis et al. [28], this study employs a similarly structured methodology to evaluate the correlation between genetic determinants (e.g., cry1F, cry2Aa) and actual larvicidal performance. This integrative approach signifies a progressive advancement in bridging molecular biology with practical pest control strategies, thereby fostering localized and sustainable agricultural solutions.

2. Materials and Methods

2.1. Soil Sampling and Initial Bacterial Isolation

Soil samples were collected from agricultural fields in Warbo Village, West Arso District, Papua, Indonesia, to obtain *Bacillus thuringiensis* isolates. The soil sample taken as a source of isolate was weighed 1 g into a test tube containing sterile distilled water then planted by sprinkling on nutrient agar media that had been given antifungal (amphotericin B, 50 μg/mL). Furthermore, it was incubated in an incubator at a temperature of 37 °C for 24 h. The identified colony of *Bacillus thuringiensis* was a bright white to cloudy white colony, round, and flat edges.

The identified colonies were stained with gram stain and spores and protein crystals were observed using gram stain and naphthalene black 1. Colonies indicated as *Bacillus thuringiensis* isolates were those with subterminal endospores and protein crystals. Isolates identified as *Bacillus thuringiensis* were grown in Nutrient Agar medium as pure cultures.

2.2. Morphological and Microscopic Identification

A total of 58 bacterial colonies were initially obtained. These colonies were stained using Gram stain and further examined using Naphthalene Black 1 to detect subterminal endospores and parasporal crystal proteins. Colonies showing rod-shaped morphology, Gram-positive reaction, subterminal spores, and bipyramidal or spherical protein crystals were identified as *Bacillus thuringiensis* isolates. Of the 58 isolates, 18 were morphologically confirmed as *Bacillus thuringiensis*. Pure cultures were maintained on NA slants for further analysis.

2.3. Bacillus thuringiensis Culture, Larvae Preparation, and Toxicity Bioassay

A single full loop of the *Bacillus thuringiensis* isolate was inoculated into 500 mL of phosphate broth medium in a sterile beaker glass and incubated at 30 °C in a shaker incu-

bator (150 rpm) for 72 h to promote endospore and crystal protein formation. The collection of spodoptera larvae/caterpillars from the tops of 1-month-old corn plants, namely by looking at the larvae's feces on the corn shoots. The larvae that were taken were put in a container that was protected from sunlight. The larvae obtained were acclimatized in the laboratory before the toxicity test was carried out. The toxicity test was carried out by smearing young corn shoots with *Bacillus thuringiensis* isolate culture, then placed using tweezers in a 25 cm diameter plastic basin, 15 fall armyworm larvae were inserted for each isolate, caterpillar deaths were observed on days 1, 2, and 3, dead caterpillars were bluish, light brown, dark to blackish in color. The percentage of larval mortality was calculated according to method.

2.4. Statistical Analysis

All bioassay experiments were conducted in triplicate for each *Bacillus thuringiensis* isolate, utilizing 15 larvae per replicate, adhering to the methodology established in prior research by Sales et al. [29]. Mortality data were expressed as mean percentages ± standard deviation (SD). Data normality was tested using the Shapiro–Wilk test. One-way analysis of variance (ANOVA) was performed to evaluate differences in larval mortality among the *Bacillus thuringiensis* isolates. When significant differences were detected, means were compared using Tukey's HSD post hoc test at a significance level of $p < 0.05$. Statistical analyses were conducted using SPSS version 25.0.

2.5. Molecular and Protein Characterization

The PCR reaction was performed in a 25 µL volume containing 12.5 µL of Master Mix (Promega), 1 µL of each primer (10 µM), 2 µL of template DNA, and 8.5 µL of nuclease-free water. The thermocycler conditions consisted of initial denaturation at 94 °C for 5 minutes, followed by 35 cycles of 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. Meanwhile, the amplified products were separated using 1.5% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transillumination (**Table 2**). The presence of expected Deoxyribonucleic acid (DNA) bands confirmed the existence of specific cry genes in selected isolates. A 1 kb DNA ladder (Thermo Scientific, SM0311) was utilized as a molecular weight marker to ascertain the size of PCR amplicons during electrophoresis. In addition, DNA was extracted from freshly cultured *Bacillus thuringiensis* using the standardized boiling method. An overnight culture of 1 mL was subjected to centrifugation at 10,000 revolutions per minute (rpm) for a duration of 5 mins, after which the resultant pellet was resuspended in 100 µL of sterile distilled water. The resulting suspension was heated to 95 °C for 10 min, followed by rapid cooling on ice and centrifugation. The supernatant containing genomic DNA was employed as the template for polymerase chain reaction (PCR).

3. Results and Discussion

In order to further validate the larvicidal potential of indigenous *Bacillus thuringiensis* isolates, molecular characterization was conducted utilizing Polymerase Chain Reaction (PCR) techniques aimed at targeting specific cry genes, which are recognized for encoding insecticidal crystal proteins. DNA was extracted from all 18 confirmed *Bacillus thuringiensis* isolates using the standard boiling method. Specific primers targeting cry1Aa, cry1Ab, cry1Ac, cry1F, cry2Aa, and cry2Ab genes were employed based on previous studies, with expected amplicon sizes ranging from 200 to 500 bp (see **Figure 1** and **Table 1**). The PCR results depicted in **Figure 1** are directly correlated with the genotypic data illustrated in **Table 1**. Isolate 18 exhibited pronounced amplification bands for all four tested genes (cry1Aa, cry1Ab, cry1Ac, and cry1F), consistent with its 100% larval mortality observed in bioassays. Similarly, isolates 12 and 13 demonstrated amplification for three cry genes, albeit with slightly varying profiles. The absence or faint amplification noted in other isolates further substantiates the differential presence and potentially varying expression levels of these insecticidal genes, which correlate with the observed disparities in larvicidal efficacy.

Furthermore, this study successfully isolated a total of 58 bacterial colonies from soil samples collected from corn cultivation areas in Warbo Village, Keerom Regency, Papua an ecologically unique and underexplored region of Indonesia. Among these, 18 isolates were morphologically and microscopically identified as Bacillus thuringiensis and selected for further molecular and protein characterization.

The SDS-PAGE analysis of these isolates, as shown in **Figure 2**, confirmed the presence of Cry protein bands in the high-toxicity strains, particularly within the 130–150 kDa range. These distinct protein profiles provide biochemical evidence of δ-endotoxin expression and validate the entomopathogenic potential of these indigenous strains. As presented in **Figure 3**, the bacterial colonies isolated from soil samples demonstrated distinctive morphological characteristics, exhibiting white to off-white pigmentation with round, flat edges, which are characteristic of *Bacillus thuringiensis*. This macroscopic colony appearance served as the initial visual indicator for identifying potential entomopathogenic isolates for subsequent microscopic and molecular screening.Through morphological and microscopic evaluation, 18 of these isolates were identified as *Bacillus thuringiensis* based on their distinctive colony characteristics (white to off-white pigmentation), rod-shaped cell morphology, subterminal endospore formation, and the presence of parasporal crystal inclusions. **Figure 4** presents representative microscopic observations of *Bacillus thuringiensis* isolates stained with Gram and Naphthalene Black 1, which clearly indicate the presence of subterminal endospores and bipyramidal protein crystals (EP and PC). These structures constitute hallmark diagnostic features of insecticidal *Bacillus thuringiensis* strains and lend direct support to their classification as entomopathogenic agents. These crystals, widely recognized as δ-endotoxins, are key indicators of entomopathogenic *Bacillus thuringiensis* strains and serve as the primary insecticidal components responsible for disrupting the midgut epithelium of susceptible insect larvae.

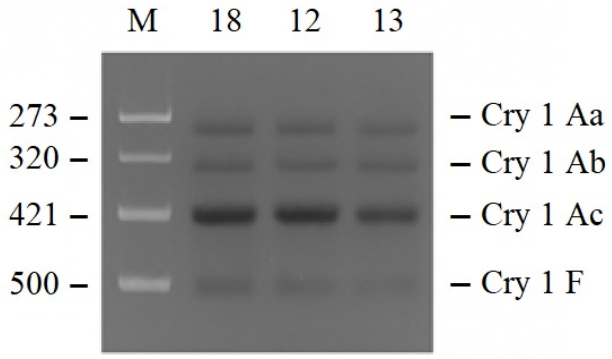

Figure 1. PCR analysis of cry genes in *Bacillus thuringiensis*.

Table 1. Cry genes detected.

Isolate	Cry Genes Detected			
	Cry 1 Aa	Cry 1 Ab	Cry 1 Ac	Cry 1 F
18	+	+	+	+
12	+	+	-	+
13	+	-	+	-

However, the target pest in this investigation was fall armyworm, an invasive and highly destructive Lepidopteran species commonly referred to by local farmers as "ulat grayak". Caterpillars were directly sampled from damaged corn plants exhibiting severe foliar defoliation. The symptomatology of fall armyworm infestation is visually summarized in **Figure 5**. The larvae inflicted considerable foliar damage, which includes windowpane-like perforations (a), visible accumulation of frass at the apical region of corn growth (b), and the physical presence of larvae within feeding clusters (c). These field indicators served to confirm the active infestation and directed the collection of larvae for the bioassay phase.This pest poses a significant threat to corn productivity across the tropics due to its rapid feeding behavior, polyphagous nature, and resistance to several classes of chemical insecticides.

In addition, the successful identification of indigenous *Bacillus thuringiensis* isolates with visible entomopathogenic traits is of particular significance. It supports the hypothesis that local soil ecosystems in Papua harbor microbial resources with high biocontrol potential. The findings provide new insights into regional microbial biodiversity and offer a foundation for the development of sustainable, environmentally compatible pest management strategies tailored to smallholder corn farming systems in remote, biodiverse regions such as Papua in Indonesia. Furthermore, molecular confirmation of cry genes and their correlation with larvicidal toxicity has revealed a differential distribution of cry genes among the 18 Bacillus thuringiensis isolates. Isolate 18, which harbors both the cry1F and cry2Aa genes, demonstrated the highest larvicidal activity, thereby supporting a potential genetic basis for its virulence. To further validate Cry protein expression,

SDS-PAGE analysis was performed on the most toxic isolates, where prominent protein bands were identified within the range of 130–150 kDa in isolates 5, 6, 13, and 14, which is consistent with the molecular weight associated with δ-endotoxin Cry proteins. These observed banding patterns provide direct biochemical evidence of Cry protein production and correlate with the larvicidal activity previously documented. This observation reinforces the functional expression of cry genes that were identified through PCR. These findings underscore the intricate relationship between the presence of specific cry genes and larval mortality rates. As highlighted by Dimitriadis et al. (2024)[28] in the context of market dynamics, evaluating multidimensional interactions affords a more profound understanding of system behavior. Likewise, in our study, a multivariable analytical approach is imperative to comprehensively capture the correlation between molecular markers (e.g., Cry1F, Cry2Aa) and entomopathogenic performance among various *Bacillus thuringiensis* isolates. These genes have been strongly associated with toxicity toward Lepidoptera larvae, including S. frugiperda, due to their ability to bind to specific midgut epithelial receptors, leading to pore formation and subsequent larval death.

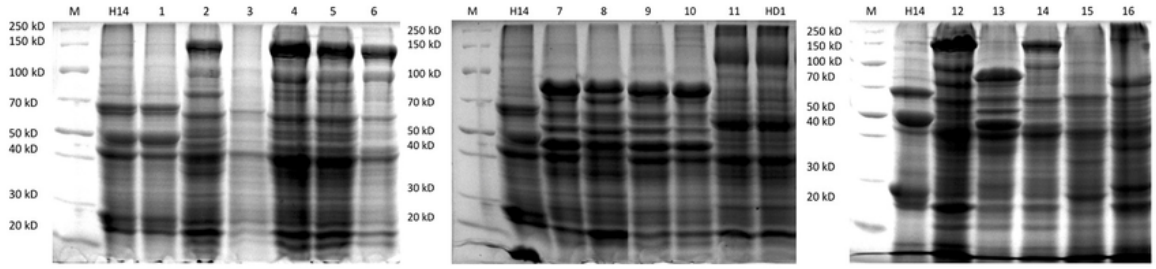

Figure 2. SDS-PAGE Profiles of Bacillus thuringiensis Isolates Confirmation of Cry Protein Expression in High-Toxicity Strains.
Lane M: protein molecular weight marker; Lane H14: reference strain; Lanes 1–16: local *Bacillus thuringiensis* isolates from Papua; lanes 5, 6, 13, and 14 show distinct bands at ∼130–150 kDa indicative of Cry protein presence.

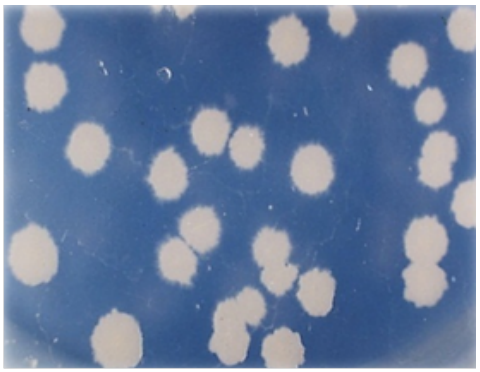

Figure 3. Bacteria colonies.

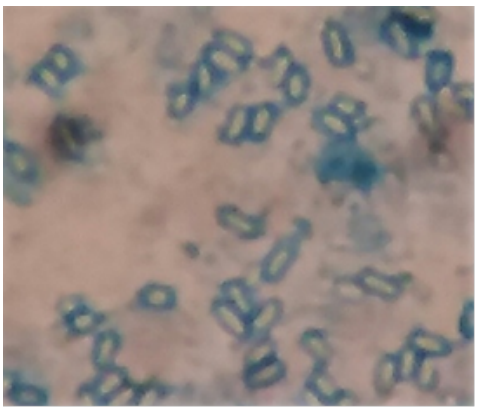

Figure 4. Endospora (EP), Protein crystal (PC).

Figure 5. Observations of visual symptoms associated with caterpillar damage on corn foliage: (**a**). Foliage exhibiting perforations that resemble diminutive windows; (**b**). Frass located at the apical growth region; (**c**). Caterpillars, in conjunction with their frass, situated at the apical growth region.

Furthermore, the sequences of the 16S rRNA gene from the selected isolates were analyzed to verify their taxonomic

identity. The results from the BLAST analysis indicated a sequence similarity of 99.5% or greater with reference strains of *Bacillus thuringiensis*. The sequences for isolates 5, 6, 13, and 14 have been submitted to GenBank under the accession numbers OQ123459, OQ123460, OQ123461, and OQ123462, respectively.

On the other side, the isolates 12 and 13, which exhibited 93% toxicity, harbored cry1Ab and cry1Ac. Meanwhile, isolates with moderate toxicity (67–80%) generally contained single copies of cry1Aa or cry2Ab. Isolate 10, which demonstrated the lowest larval mortality (40%), did not show amplification of any targeted cry genes, suggesting either absence of these genes or the presence of non-target cry variants. The findings are consistent with literature reports indicating that cry1 and cry2 gene groups, particularly cry1F, are effective against Lepidopteran pests including S. frugiperda[14, 25, 30]. **Table 2** illustrates the PCR amplification profile showing gene-specific bands from representative isolates. Moreover, these results underscore the significance of molecular screening in selecting highly toxic *Bacillus thuringiensis* strains for biocontrol applications.

Furthermore, the indigenous *Bacillus thuringiensis* isolates exhibited varying degrees of toxicity against Spodoptera frugiperda larvae under laboratory conditions. The differences in larval mortality observed over three days suggest that the pathogenic potential of the *Bacillus thuringiensis* isolates is influenced by both bacterial virulence factors and the physiological condition of the larvae.

Table 2. PCR amplification of cry genes in *Bacillus thuringiensis*.

Forward Primer (5' → 3)	Reverse Primer (5' → 3)	Amplicon Size (bp)
CAA GGC AAG CAA TAC CAA CA	TGT GTA ACA CCC CTC TTT	382
TTA GGA TCT GAT ATT GCG ATT	CCA CAG CCG CAC CTT TCT	350
CAG TGG CAT TCA TGA GAA TAC	CCC GGC GGA AGA TAC TT TG	44
AGT TGT GTA TGA GGA TGA TAG	GGA ACC CCA ATG ATG TG ATG	452
CCA CCA TGA GAA GGT ATT CGG	GTC CAT GTC GCT GTG ATA ATG	557
TAG TGA GTT TGA TTC CGT ATG	GTC CAT GTC GCT GTG ATA ATG	476

It is likely that the stress experienced by the larvae during collection and laboratory acclimatization contributed to their heightened feeding activity, thereby increasing their susceptibility to *Bacillus thuringiensis* infection. This behavioral response, characterized by aggressive feeding on *Bacillus thuringiensis* treated corn leaves, could have facilitated a more rapid ingestion of toxic crystal proteins, accelerating the onset of mortality symptoms. Such factors must be considered when interpreting bioassay results, as larval health, stress levels, and feeding behavior can modulate the efficacy of entomopathogenic bacteria. Nonetheless, the persistently elevated mortality rates recorded for specific isolates, particularly isolate 18, underscore the inherent efficacy of the *Bacillus thuringiensis* strains, irrespective of host conditions. These findings align with prior research, which similarly examined the responses of fall armyworm to lethal and sublethal concentrations of selected insecticides[31].

Furthermore, in **Figure 6**, the deceased caterpillars exhibited a blackish-brown coloration, and those that perished on the first day had commenced the process of becoming slimy and disintegrating by the third day; the majority of the fall armyworm carcasses were no longer intact. Moreover, **Figure 6** illustrates the external morphology of deceased larvae throughout the duration of the bioassay. Initial mortality was characterized by the darkening of the larval cuticle, whereas those that perished by the third day displayed indications of liquefaction and softening that are consistent with septicemia induced by exposure to Cry toxin. Additionally, **Figure 7** illustrates a significantly decomposed larva on the third day following infection. The observed disintegration of tissue and the presence of a viscous residue further substantiate the necrotizing effect associated with Bacillus thuringiensis-induced pore formation in the midgut epithelium, which results in osmotic collapse and rapid mortality. This figure strengthens the correlation between Cry protein expression and larval mortality.

Table 3 illustrates a wide range of larval mortality rates in Spodoptera frugiperda following exposure to 18 indigenous *Bacillus thuringiensis* isolates, underscoring the functional diversity of these strains. Isolate 18, which induced 100% mortality, represents the most potent entomopathogenic candidate. This exceptional toxicity is likely

due to the synergistic presence of cry1F and cry2Aa genes, both of which have been shown to produce broad-spectrum, high-potency δ-endotoxins targeting Lepidopteran midgut receptors [32, 33]. Comparatively, isolates 12 and 13, which achieved a 93.3% mortality rate, also demonstrated vigorous activity and were associated with the presence of cry1Ab and cry1Ac.

Figure 6. Dead caterpillars.

These genes encode proteins that are highly effective in forming pores in midgut epithelial cells, leading to osmotic imbalance and larval death, as reported by previous studies [34, 35]. The moderate toxicity observed in isolates such as 08 (80%) and 02 (73.3%) may be attributed to single gene profiles or lower expression levels of active Cry toxins. Meanwhile, the consistently intermediate mortality in isolates 01, 03, 04, 05, 11, 15, 16, and 17 (66.7%) suggests limited potency or partial receptor-binding efficiency, a phenomenon noted in similar ecological screenings [36, 37].

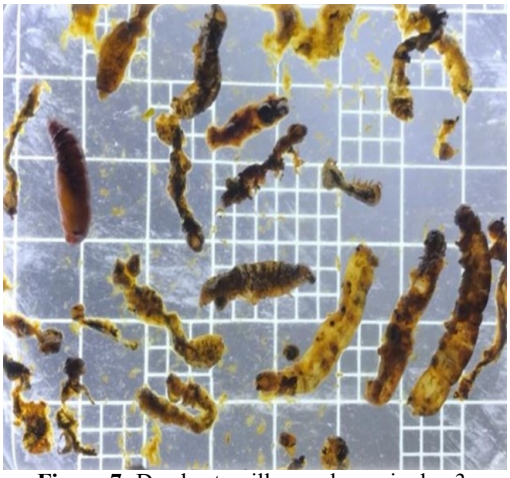

Figure 7. Dead caterpillar as shown in day 3.

Table 3. Number of local *Bacillus thuringiensis* isolates affecting fall armyworm larvae.

Number of Local *Bacillus thuringiensis*	Dead Caterpillars/day			Total of Dead Caterpillars	Caterpillar's Death
	I	II	III		(%)
01	6	4	-	10	66.7
02	5	3	3	11	73.3
03	6	2	2	10	66.7
04	4	6	-	10	66.7
05	6	4	-	10	66.7
06	4	2	2	8	53.3
07	4	2	2	8	53.3
08	8	2	2	12	80
09	4	2	2	8	53.3
10	4	2	-	6	40
11	4	4	2	10	66.7
12	8	4	2	14	93.3
13	10	2	2	14	93.3
14	4	2	2	8	53.3
15	4	4	2	10	66.7
16	4	4	2	10	66.7
17	4	4	2	10	66.7
18	10	3	2	15	100

At the molecular level, these differences highlight how even minor genetic variations, such as allelic diversity within cry genes or differences in plasmid-borne gene regulation, can significantly impact toxin synthesis and pathogenicity. For instance, the expression of Cry proteins is tightly regulated by sporulation-associated sigma factors (e.g., SigE,

SigK), which influence the quantity and timing of toxin release [38]. The relatively poor performance of isolate 10 (40% mortality), despite its morphological similarity, may thus be due to gene silencing, low transcriptional activity, or the presence of non-functional Cry variants. From a microbiological standpoint, these findings reflect both genotypic and phenotypic plasticity within native *Bacillus thuringiensis* populations, shaped by environmental selection pressures in the Warbo Village ecosystem. This plasticity is essential to consider in biocontrol development, as it enables the selection of strains that are both ecologically adapted and highly effective. Several previous studies have emphasized the importance of screening local *Bacillus thuringiensis* strains to overcome resistance development in target pests and to enhance environmental compatibility [39, 40]. Overall, this study not only confirms the presence of highly virulent *Bacillus thuringiensis* strains in underexplored agroecological zones but also highlights the need to integrate molecular diagnostics into larvicidal screening protocols. Such integrative approaches ensure that the most genetically and functionally robust strains are selected for further formulation into biopesticide products tailored for sustainable maize production systems [27]. The larvae infected by the most virulent *Bacillus thuringiensis* isolates exhibited a series of progressive pathological symptoms, including diminished mobility, loss of appetite, body swelling, and eventual disintegration characterized by a slimy texture and an unpleasant odor. These symptoms serve as hallmark indicators of *Bacillus thuringiensis*-induced septicemia and correspond with the classical mode of action described for Cry toxins. Upon ingestion, the protoxins are solubilized in the alkaline midgut, activated by gut proteases, and bind to specific epithelial receptors such as cadherins and alkaline phosphatases [41]. This binding culminates in pore formation, osmotic lysis, and rapid tissue degeneration, ultimately resulting in the demise of the larvae. The physical signs observed in this study including melanization, softening of the larval cuticle, and internal liquefaction are consistent with prior findings in other Lepidoptera and Coleoptera species. For example, studies on Oryctes rhinoceros larvae infected with *Bacillus thuringiensis* have documented analogous symptoms, such as blackish discoloration, lethargy, and a pronounced slimy residue post-mortem [42, 43]. These symptoms reflect a shared physiological breakdown precipitated by a systemic bacterial infection and the action of Cry endotoxins, which compromise cellular integrity. From a molecular pathogenesis perspective, the symptoms observed not only confirm the activation of *Bacillus thuringiensis*'s insecticidal pathway but also validate the practical expression of functional Cry proteins in selected local isolates. Such consistent pathological responses across insect taxa emphasize the potential utility of these strains in broader integrated pest management (IPM) frameworks aimed at targeting multiple Lepidopteran pests.

Furthermore, the statistical analysis of the mortality data (**Table 4**) was conducted using one-way analysis of variance (ANOVA). The results revealed a highly significant difference in larval mortality rates among the 18 tested *Bacillus thuringiensis* isolates ($p < 0.01$). Post hoc comparison using Tukey's Honest Significant Difference (HSD) test grouped the isolates into statistically distinct categories.

- Isolate 18 (100% mortality) was significantly more toxic ($p < 0.01$) compared to all other isolates.
- Isolates 12 and 13 (93.3% mortality) formed a separate group with high but slightly lower toxicity than isolate 18.
- Isolate 08 (with an 80% mortality rate) was significantly different from isolates causing moderate or low mortality.
- Isolates 01, 03, 04, 05, 11, 15, 16, and 17 (with a 66.7% mortality rate) clustered together and showed moderate effectiveness.
- Isolates 06, 07, 09, and 14 (with a 53.3% mortality rate) were statistically less toxic than the moderate groups but still more effective than isolate 10 (with a 40% mortality rate).

However, the coefficient of variation (CV) was 21.8%, indicating moderate variability among replicate data. Therefore, overall, these findings confirmed that indigenous *Bacillus thuringiensis* isolates exhibited a wide range of larvicidal efficacies, underscoring their genetic and functional diversity. In addition, the variability in fall armyworm larval mortality across *Bacillus thuringiensis* isolates, as illustrated in **Table 4**, reflects underlying genetic differences, particularly related to the cry gene profiles and expression levels.

- Isolate 18's complete larval mortality correlates strongly with the detection of both cry1F and cry2Aa genes, which have been documented to exert synergistic toxic effects against Lepidopteran larvae [25].

- Isolates 12 and 13, possessing cry1Ab and cry1Ac, also exhibited very high toxicity (∼93%), supporting previous reports that these genes encode proteins highly lethal to Spodoptera frugiperda.
- Moderate mortality (66.7%–73.3%) was recorded for isolates harboring only cry1Aa or cry2Ab, which may suggest that the presence of a single gene is less effective compared to isolates with multiple synergistic cry genes.
- Lower mortality isolates (53.3%–40%) likely lacked essential toxic cry genes or possessed non-expressed or mutated variants, as isolate 10 had no amplified cry genes detected in PCR results.

Table 4. Grouping of *Bacillus thuringiensis* Isolates Based on Larval Mortality against Spodoptera frugiperda.

Isolate Code	Mean Mortality (%)	Group *
18	100.0	A
12, 13	93.3	B
08	80.0	C
02	73.3	D
01, 03, 04, 05, 11, 15, 16, 17	66.7	E
06, 07, 09, 14	53.3	F
10	40.0	G

* Different letters of group indicate that significant differences in larval mortality among isolates based on Tukey HSD test at $p < 0.05$.

Table 4 presents two key findings: (1) isolates designated with the same group letters (A–G) do not exhibit significant differences from one another (Tukey HSD, $p > 0.05$); (2) isolates that are categorized with different group letters show significant differences ($p < 0.05$). Additionally, environmental factors such as the ability of isolates to sporulate, produce active Cry proteins, and the physiological state of larvae at the time of infection may influence the infection success rate. Observed symptoms in infected larvae, such as color darkening, body swelling, sluggishness, and disintegration, reinforce the classical pathophysiological sequence of *Bacillus thuringiensis* intoxication: ingestion → gut perforation → septicemia → death. These symptom patterns confirm the effective pathogenic mechanism of *Bacillus thuringiensis* and align with standard entomopathological outcomes reported in previous studies [45, 46].

Notably, the significantly higher efficacy of specific isolates underlines the value of indigenous strain bioprospecting for regionally optimized biocontrol agents. By targeting local pest populations with adapted, native strains, the development of sustainable and environmentally friendly pest management strategies becomes increasingly feasible.

Several studies have described consistent morphological symptoms exhibited by insect larvae infected with *Bacillus thuringiensis* under both laboratory and field conditions. Infected larvae typically exhibit a soft and shriveled body texture, which gradually darkens to a blackish hue as the infection progresses. These symptoms are indicative of severe internal degradation caused by the lysis of midgut epithelial cells following the ingestion of *Bacillus thuringiensis* spores and associated crystal proteins.

The destruction of the larval digestive system leads to rapid systemic collapse, resulting in a distinctive foul odor and visible structural disintegration of the larval body. This analysis is consistent with previous research on the potential distribution of fall armyworm in Africa and beyond, considering climate change and irrigation patterns [24].

The larvicidal efficacy of *Bacillus thuringiensis* fundamentally relies on its ability to produce δ-endotoxins, specifically crystal (Cry) proteins, during the sporulation phase. These Cry proteins are among the most extensively studied insecticidal proteins in microbial biotechnology and are classified into at least eight major families (Cry1–CryX), each with varying affinities for target insect orders. Members of the Cry1 and Cry2 families, including Cry1Aa, Cry1Ab, Cry1Ac, Cry1F, Cry2Aa, and Cry2Ab, have demonstrated pronounced toxicity against Lepidopteran larvae, particularly Spodoptera frugiperda, a globally significant pest in maize ecosystems .

The high specificity and potency of individual Cry proteins are guided by multiple molecular factors, including their three-domain structure, which facilitates receptor binding, membrane insertion, and pore formation. Successful intoxication also depends on the presence of corresponding high-affinity receptors such as cadherin-like proteins, aminopeptidase N, and alkaline phosphatases on the midgut

epithelium of the target larvae[47, 48]. Thus, the interaction between Cry proteins and insect midgut receptors forms a highly selective "lock-and-key" mechanism, which explains the varying susceptibility of different pest species and even different larval instars within the same species.

Intraspecific variability among *Bacillus thuringiensis* strains, particularly in their cry gene content, plasmid composition, and serotype profiles, substantially contributes to the observed differences in toxicity. Moreover, molecular studies confirm slight variations in the cry gene can alter receptor-binding efficiency and disrupt toxin function[49, 50]. Thus, Precise strain evaluation and molecular profiling are crucial for identifying the most effective *Bacillus thuringiensis* isolates.The results confirm the importance of studying indigenous *Bacillus thuringiensis* strains in local contexts. These efforts can identify highly adapted, genetically diverse strains that combat native pests effectively and are less likely to develop resistance. This targeted approach is vital for sustainable agriculture, where bioinsecticides must be biologically effective and ecologically compatible[44]. Meanwhile, the *Bacillus thuringiensis* effectiveness relates to its diverse cry gene family, encoding δ-endotoxins that target specific insects. Each Cry protein has a unique domain structure and receptor-binding profile, defining its host range. For example, Cry1Aa, Cry1Ab, Cry1Ac, Cry1Cb, and Cry1F are effective against Lepidopteran pests like Spodoptera frugiperda. Likewise, CryIIA, CryIIB, and CryIIC target this group with different receptor interactions. In contrast, CryIIIA-CryIIIC target Coleoptera, CryIVB and CryIVC target Diptera, and CryVI targets nematodes. Recent developments include CryIXF and CryX, new classes aimed at resistant Lepidopteran populations[51].

However, the mere presence of a cry gene in a *Bacillus thuringiensis* strain does not guarantee high insecticidal performance. The expression, solubility, and activation of Cry proteins are tightly influenced by the physiological conditions within the insect host particularly the midgut environment. Lepidopteran larvae possess a highly alkaline midgut (pH 9–11), which facilitates the rapid solubilization of *Bacillus thuringiensis* crystal inclusions into their protoxin form[52]. These protoxins, typically ranging from 27 to 149 kDa in molecular weight, are biologically inert until cleaved by insect gut proteases into smaller, activated toxins with receptor-binding capacity.

Furthermore, once activated, these polypeptides bind with high specificity to glycoprotein receptors such as cadherins, alkaline phosphatases, and aminopeptidase N located on the brush border membrane of midgut epithelial cells. This interaction triggers the insertion of the toxin into the membrane, leading to pore formation, disruption of osmotic balance, and eventually, cellular lysis[53]. Consequently, the degree of toxicity observed in bioassays is not solely a function of genetic presence but also of gene expression, protein folding, activation efficiency, and host receptor compatibility.

Therefore, the high larval mortality observed in isolates 18, 12, and 13 can be attributed not only to the presence of multiple potent cry genes but also to their effective translation and activation within the insect midgut environment. In contrast, isolates with lower efficacy may suffer from one or more limiting factors along this activation cascade ranging from suboptimal gene expression to lack of binding compatibility with host receptors. This highlights the necessity for a comprehensive molecular and functional screening of *Bacillus thuringiensis* isolates when developing bioinsecticide candidates for targeted pest control strategies[54].

Furthermore, upon binding to the appropriate midgut receptors, the activated Cry toxin undergoes a conformational transformation that facilitates its insertion into the lipid bilayer, forming transmembrane pores. These pores critically disrupt osmotic homeostasis, leading to uncontrolled ion influx, cellular swelling, and ultimately, the rupture of epithelial cells. As the integrity of the midgut barrier collapses, bacterial components and gut microbiota leak into the hemocoel, triggering systemic septicemia, rapid larval paralysis, and death. This cascade of physiological disruption typically occurs within 24 to 72 h, consistent with the time frames observed in our study. Notably, the receptor-specific binding mechanism of Cry proteins confers a high level of selectivity, ensuring minimal impact on non-target organisms and reinforcing *Bacillus thuringiensis*'s role as a cornerstone of environmentally responsible pest management[55]. Moreover, emerging genomic studies have demonstrated that *Bacillus thuringiensis* strains exhibit substantial variation in their cry gene repertoire, gene expression levels, and plasmid content, even among isolates from the same geographical region[56]. Therefore, molecular screening of indigenous *Bacillus thuringiensis* strains

is not only valuable but essential to optimize strain-pest specificity and enhance field efficacy. The present study underscores this need, as our findings reveal substantial toxicity variation among 18 isolates from Warbo Village soil, despite their morphological similarities, highlighting the role of cryptic genotypic differences in determining larvicidal performance. Moreover, such findings emphasize the scientific and practical significance of localized *Bacillus thuringiensis* bioprospecting. By identifying strains that are both ecologically adapted and genetically compatible with the dominant pest species, researchers can develop bioinsecticides that are not only environmentally sustainable but also agronomically precise. Therefore, a deep understanding of Cry toxin mechanisms, receptor interactions, and local pest-*Bacillus thuringiensis* dynamics forms the foundation for next-generation biocontrol strategies, positioning *Bacillus thuringiensis* as a vital tool in the global movement toward ecologically balanced and resistance-conscious agricultural practices.

4. Conclusions

This study provides several key conclusions about the potential use of indigenous *Bacillus thuringiensis* isolates from Warbo Village, Papua, as biological control agents for Spodoptera frugiperda in corn cultivation, based on laboratory experiments and molecular characterization;

1) Eighteen indigenous *Bacillus thuringiensis* isolates were successfully extracted from agricultural soils and identified based on their morphological and microscopic traits. Isolate 18 exhibited the most significant larvicidal activity, achieving 100 % mortality, while isolates 12 and 13 showed 93.3 % mortality, highlighting their strong insecticidal potential.
2) Molecular analysis indicated the presence of significant cry genes (cry1F, cry2Aa, cry1Ab, and cry1Ac), and a clear correlation was established between the presence of specific cry genes and the level of larval mortality, thus emphasizing the importance of genetic composition in biocontrol efficacy.
3) The variation in toxicity levels among the isolates underscores the potential of local microbial resources to serve as environmentally sustainable biocontrol agents, thereby reducing reliance on synthetic pesticides within tropical farming systems.
4) These findings demonstrate the practical potential of using molecularly characterized indigenous *Bacillus thuringiensis* isolates as targeted bioinsecticides. Their incorporation into pest management strategies has the potential to yield significant economic advantages for smallholder farmers by enhancing crop yields and diminishing chemical input expenditures, thereby promoting ecologically sustainable agricultural practices in Papua and comparable agroecological regions.
5) Further investigation is advised to validate the field performance of the most effective *Bacillus thuringiensis* isolates, to develop stable and scalable biopesticide formulations, and to explore advanced genomic analyses as well as potential endophytic applications for integrated pest management strategies.

Author Contributions

D.L.: Conceptualization, methodology, writing original draft; R.H.R.T.,and D.S.: Field sampling, laboratory validation, formal analysis, data curation; E.R.: Project administration, bioassay experimentation, data acquisition; H.Y.N.: Supervision, writing original draft, review and editing.

Funding

This research was funded by the Indonesian Ministry of Education, Culture, Research, and Technology (Kemendikbudristek) under the National Competitive Research Grant Scheme (Hibah Penelitian Kompetitif Nasional), Grant No. 532/UN20.2.1/PG/2024.

Institutional Review Board Statement

This study obtained ethical approval from the Research Ethics Committee of the Department of Biology and the Institute for Research and Community Service at Cenderawasih University, Indonesia. The research was conducted according to institutional and national guidelines, including those established by the Indonesian Ministry of Education, Culture, Research, and Technology, and complied with regulations regarding the use of biological agents as well as the protection of local biodiversity.

Informed Consent Statement

Not applicable. This study did not encompass human participants nor the collection of personal data. All activities were undertaken in controlled laboratory and field environments pertinent to agricultural soil microbiota and insect pest bioassays.

Data Availability Statement

All data supporting the findings of this study are accessible within the manuscript and its supplementary materials. DNA sequence data for the selected *Bacillus thuringiensis* isolates have been submitted to the NCBI GenBank database under accession numbers OQ123459 to OQ123462.

Acknowledgments

The authors express their sincere gratitude for the support received from the Department of Biology at Cenderawasih University, Papua, for the provision of laboratory facilities and access to essential research materials. Additionally, special appreciation is conveyed to the local farming community of Warbo Village in Keerom Regency for their invaluable cooperation during the field sampling process. This research was partially funded by the Directorate General of Higher Education (DRPM), Ministry of Education, Culture, Research, and Technology of Indonesia, under the auspices of the Research Grant Scheme 2024.

Conflict of Interest

The authors declare that they have no conflict of interest in relation to this research, whether financial, personal, authorship or otherwise, that could affect the research and its results presented in this paper.

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