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Efficacy of *Bacillus thuringiensis* subsp. *aizawai* against the Greater Wax Moth, *Galleria mellonella* (Lepidoptera: Pyralidae) in Libya: A Bioassay Approach for Sustainable Wax Moth Control

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فعالية البكتيريا الباسيلية الثرينجنسية إيزاواي (*Bacillus thuringiensis* subsp. *aizawai*) في مكافحة دودة الشمع الكبرى (*Galleria mellonella*) في ليبيا: دراسة حيوية لتطوير استراتيجية مستدامة لمكافحة افة الشمع

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Abstract:
The greater wax moth belongs to the order Lepidoptera, family Wax Moth. It is a serious and destructive pest in honeybee farming, causing significant losses to honeycomb throughout North Africa. Due to its severity, most beekeepers in the region resort to dangerous chemical pesticides such as paradichlorobenzene (Paradex), leading to its accumulation in bee products and posing an environmental risk. This study aims to evaluate the effectiveness of a microbial pesticide (*Bacillus thuringiensis genus aizawai)* on the larvae and its mechanism of action in controlling them. The larval stages of the greater wax moth were obtained from infested bee colonies and reared in a nutrient-rich medium. Tests were conducted at different concentrations of the microbial pesticide: 1.0%, 1.8%, 3.3%, and 6.0%. The results of a one-way ANOVA analysis showed a dose-dependent difference in larval mortality. At a concentration of 6.0%, larval mortality was 100%. The mean standard deviations clearly and statistically significant ($p < 0.05$) confirm the effectiveness of the higher concentrations (6% and 3.3%) of the pesticide, indicating the efficacy of its active ingredient, B. thuringiensis. The alkaline environment within the midgut causes the bacteria's toxic crystals to rupture the tissue and lyse the epithelial cells, leading to septicemia and larval death. (Midgut of the larva.) Spraying stored wax combs with a 3.3% microbial pesticide in the fall protects the combs from infestation by this pest, thus contributing to reducing or avoiding chemical control, which ensures that honeybee products meet health standards and are exported abroad.

Keywords: Galleria Mellonella; Bacillus Thuringiensis Subsp. Aizawai; Biological Control, Apiculture, Libya.

الملخص
تُعد دودة الشمع الكبرى (Galleria mellonella) حرشفية الأجنحة: (Pyralidae) من الآفات الخطيرة والمدمرة في تربية نحل العسل، حيث تسبب خسائر كبيرة في الأقراص الشمعية في مناطق واسعة من شمال أفريقيا. ونظرًا لشدة ضررها، يعتمد العديد من النحالين على المبيدات الكيميائية، مثل الباراديكلوروبنزين (Paradex)، والتي قد تتراكم في منتجات النحل وتشكل مخاطر بيئية وصحية. هدفت هذه الدراسة إلى تقييم فعالية المبيد الحيوي الميكروبي Bacillus

thuringiensis subsp. aizawai ضد يرقات دودة الشمع الكبرى، ودراسة آلية تأثيره في المكافحة. تم جمع يرقات دودة الشمع الكبرى من خلايا نحل مصابة وتربيتها في وسط غذائي صناعي غني بالعناصر الغذائية. أُجريت الاختبارات الحيوية باستخدام أربعة تراكيز من المبيد الميكروبي هي: 1.0%، 1.8%، 3.3%، و6.0%. أظهر تحليل التباين الأحادي (One-way ANOVA) وجود تأثير معنوي يعتمد على الجرعة في نسب نفوق اليرقات ($p < 0.05$) . وقد حقق التركيز الأعلى (6.0%) نسبة نفوق بلغت 100%، بينما أظهر التركيز 3.3% فعالية عالية في القضاء على اليرقات. وتتمثل آلية التأثير في أن البيئة القلوية داخل الأمعاء الوسطى لليرقة تؤدي إلى إذابة بروتينات البلورات السامة (Cry toxins) وتنشيطها، مما يسبب ارتباطها بخلايا الظهارة المعوية وحدوث تحلل للخلايا واضطراب في نسيج الأمعاء، يليه تسمم دموي وموت اليرقة. وتشير نتائج الدراسة إلى أن رش الأقراص الشمعية المخزنة بمعلق Bacillus thuringiensis subsp. aizawai بتركيز 3.3% قبل التخزين يمكن أن يمثل استراتيجية فعالة للحد من الإصابة بدودة الشمع الكبرى. ويساهم هذا الأسلوب الحيوي في تقليل الاعتماد على المبيدات الكيميائية، ودعم إنتاج منتجات نحل عسل أكثر توافقًا مع معايير السلامة الصحية الدولية.

الكلمات المفتاحية: عثة الشمع الكبرى، البكتريا الباسيلية، المكافحة البيولوجية، تربية النحل، ليبيا

Introduction

Apicultural practices represent a cornerstone of sustainable agriculture, seamlessly bridging macro-economic viability with indispensable socio-ecological ecosystem services. Globally, the apicultural sector exhibits robust expansion, with its worldwide valuation projected to reach approximately 14.04 billion [1]. While direct hive commodities—including honey, Propolis, royal jelly, and beeswax—generate substantial market revenue, the broader ecological dividends of managed honey bee Apis mellifera L. populations far surpass direct harvest yields [1, 2]. These pollinators function as the primary biotic vectors for cross-pollination, thereby sustaining wild plant biodiversity and securing international food networks. Quantitative ecological assessments indicate that insect-mediated pollination services contribute over 200 billion annually to global agricultural infrastructure, making managed apiaries fundamentally indispensable for terrestrial conservation and agro-ecosystem resilience [2, 3].

Locally, within North Africa and specifically the semi-arid Mediterranean coastal belt of Libya e.g., the Zawia region, beekeeping serves as a critical socioeconomic driver that sustains rural livelihoods, enhances smallholder financial autonomy, and preserves indigenous floral biodiversity [4]. Recent field diagnostics revealed that the regional apicultural infrastructure underpins over 2,200 managed colonies across the coastal lowlands, providing vital supplementary income for approximately 73% of local practitioners who balance apiculture alongside public sector employment [5]. However, the long-term sustainability of local apicultural infrastructure is increasingly threatened. The convergence of climate-driven foraging shifts, habitat fragmentation, and the escalating virulence of endemic diseases and pests compromises colony homeostasis, causing substantial annual economic deficits and forcing regional beekeepers to confront unprecedented colony mortality rates [4, 5].

Among the biotic constraints limiting apiary productivity, the greater wax moth, Galleria mellonella L. Lepidoptera: Pyralidae, is recognized as one of the most ubiquitous and destructive pests of honeycomb architecture. This invasive pest has achieved a cosmopolitan distribution through anthropogenic channels, driven primarily by the global commercialization of unsterilized honey bee packages, contaminated hive equipment, and migratory apiculture networks [6]. Currently, G. mellonella has successfully colonized almost every continent, demonstrating high ecological plasticity across temperate, subtropical, and tropical zones [6, 7]. Interestingly, beyond its status as a severe apicultural pest, G. mellonella has recently emerged as an invaluable in vivo model organism in advanced scientific research [8]. Owing to its complex innate immune system, which utilizes specialized defense cells called hemocytes that share structural and functional homologies with vertebrate Defences, the larvae have become a critical screening tool for toxicological assays and antimicrobial drug discovery, effectively supporting the 3 Rs principle" replace, reduce, and refine in animal testing [8, 9]. Furthermore, recent bio-environmental screenings have revealed another evolutionary capability of these larvae; they possess specialized saliva enzymes and intestinal microbiota capable of executing plastic biodegradation, efficiently breaking down complex polymers like low-density polyethylene LDPE and polystyrene [8].

The invasive and destructive efficacy of G. mellonella in apiaries is fundamentally driven by its highly specialized thermoregulatory adaptations and high reproductive capacity. The insect passes through four distinct ontogenetic stages: egg, larva, pre-pupa/pupa, and adult moth [8]. In regions characterized by semi-arid Mediterranean climates, such as coastal Libya, environmental conditions remain highly optimized for the pest throughout most of the annual cycle. The total absence of prolonged winter freezes allows G. mellonella to sustain up to six overlapping generations annually [4]. During the voracious larval stage, which can persist for several weeks, the caterpillars reach up to 3 cm in size, burrowing extensive silk-lined tunnels through the comb matrix to consume beeswax, pollen, and honey [8]. This uninterrupted voltinism leads to devastating structural collapses in vulnerable apiaries and severe degradation of stored comb assets.

For decades, the containment of G. mellonella within Libyan apiaries and wax-storage facilities relied heavily on conventional synthetic chemical tools. Broad-spectrum organophosphate sprays, pyrethroids applications, and volatile chemical fumigants, such as paradichlorobenzene PDB and naphthalene, were deployed indiscriminately to suppress larval populations. Field surveys in the Zawia district indicated that up to 71% of local beekeepers routinely utilize PDB crystallized formulations due to the lack of regulated bio-rational alternatives [5]. Although synthetics offer rapid knockdown efficacy, their chronic use has triggered a cascade of systemic issues. These include lipophilic chemical accumulation within honey and honeycomb matrices exceeding international safety thresholds >0.01mg/kg, the accelerated emergence of resistant G. mellonella cohorts, and acute neurotoxic stress on non-target A. mellifera workers. Consequently, modern entomological frameworks require an immediate paradigm shift away from traditional agrochemical dependence toward integrated, bio-rational management systems.

To address these critical challenges within the contemporary Libyan apicultural context, this study synthesizes and updates long-term entomological datasets, integrating them with recent advancements up to 2026. We evaluate the updated efficacy of Bacillus thuringiensis subsp. aizawai (Bt), a highly specialized biopesticides that synchronizes the production of ellipsoidal endospores and bipyramid parasporal delta-endotoxin crystals [12]. When ingested by target pyralid larvae, these proteins induce midgut cell membrane lysis and rapid mortality [12, 13]. By bridging historical colony baseline data with comprehensive, multi-generational life-table assessments and modern multi-dose bioassays calibrated for 2026 climate and management profiles, this study establishes a robust, ecologically sound framework. This integrated synthesis aims to define an economically viable, updated threshold for target pyralid suppression, providing Libyan beekeepers with a validated, residue-free alternative to chemical fumigation in the current apicultural landscape.

Material and methods

Laboratory Mass-Rearing and Colony Synchronization

The laboratory mass-rearing of the greater wax moth, *Galleria mellonella* L., was established at the Department of Biology, Faculty of Education, University of Zawia, Libya, to secure a continuous, standardized supply of specific life stages for subsequent bio-assays. The initial parental stock was isolated from heavily infested managed hives of *Apis mellifera* L. in the coastal agricultural belt of Zawia, Libya 32∘45′ N, 12∘43′ E. To establish a synchronized laboratory strain, colonies were maintained inside a precision environmental incubator under optimized micro-climatic settings. The micro-climatic profile consisted of a constant thermal regime maintained at 32±1∘C, with the relative humidity stabilized at 75±5%. The photoperiod was set to a continuous dark phase 24-h Dark to closely mimic the natural ecological conditions within a dark honey bee hive matrix, following standardized protocols established in previous pyralid colonization studies [4, 5]. The insect colony was maintained under these controlled conditions using a structurally optimized, multi-grain semi-synthetic diet. The synchronized development of *G. mellonella* was systematically monitored across all developmental phases **Figure 1**.

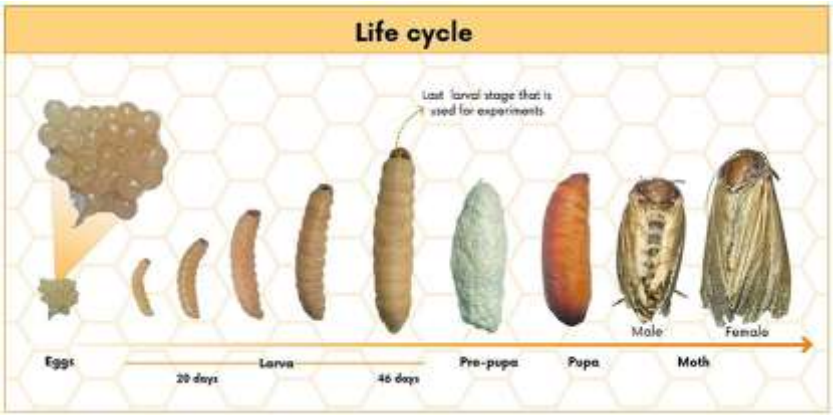

Figure 1. Stages of *Galleria mellonella* L. life cycle: egg, larva, pre-pupa, pupa, and adult moth [6].

Nutritional Composition of the Modified Beck's Artificial Diet

Larvae were reared on a customized, semi-synthetic dietary matrix adapted from the foundational formulation of Beck [1], incorporating structural modifications to optimize nutritional conversion efficiency and larval biomass accumulation as described by Jorjão et al. [9]. The homogenous dietary paste was prepared using precise biochemical and physical proportions. The solid phase comprised a homogenized carbohydrate-protein base

totaling 420 g, which consisted of an equal-proportion 1:1:1:1, 105 g each multi-grain pulverized matrix of ground barley flour, maize flour, whole wheat flour, and standard commercial wheat bran. This base was supplemented with 150 g of a commercial multi-grain Cerelac formula to supply vital zinc, iron, and B-complex vitamins.

To stimulate natural mastication and provide metabolic lipids, 20 g of a finely grated, pristine natural yellow beeswax was integrated as a phagostimulant and cohesive agent. The probiotic fermentation agent consisted of 10 g of active dry baker's yeast *Saccharomyces cerevisiae* powder to supply essential amino acids and sterols. The liquid phase and humectant matrix were composed of 150 mL of pure, unpasteurized natural honey, 150 mL of chemically pure pharmaceutical-grade glycerine 99.5% purity to regulate water activity, and 30 mL of sterile double-distilled water. The ingredients were mechanically blended until a uniform, moist, yet non-sticky crumbly diet ure was attained, minimizing lipid peroxidation and preventing larval entrapment within the rearing substrate.

Rearing Infrastructure and Cohort Separation Protocols

For group colonization and mass rearing, large-scale stock populations were maintained concurrently within five high-density wire-mesh cubic cages 45 times 45 times 45, fitted with sealed, escape-proof canvas sleeves. Fresh batches of the modified Beck's diet were replenished every 48 hours within sterile glass Petri dishes to prevent starvation-induced cannibalism, providing a robust biological reservoir of all life stages [12].

For individual life-table assays, to precisely chart the development, pupation, and adult eclosion dynamics without density-dependent interference, a cohort of 20 ultimate-instar mature larvae exhibiting a homogenous body length of approximately 2.5 cm and uniform body mass was meticulously isolated from the stock colony. Each larva was placed individually into a specialized ventilated plastic bio-assay vessel 15 times 10 times 5 cm featuring a micro-perforated lid 0.2 mm mesh to balance internal gas exchange and moisture retention under identical ambient laboratory conditions ranging from 25°C to 30°C at 75% RH.

Daily developmental milestones were monitored using high-resolution hand-held optical lenses to record early-instar transitions. Larvae were handled non-destructively using soft entomological camel-hair brushes and blunt forceps. Biomass changes were tracked using a calibrated digital analytical balance with an accuracy of pm0.1 mg. To stimulate natural oviposition, standardized fan-shaped accordion-pleated waxed paper strips were inserted into adult containers. Parallel morphological characterizations and life-stage verifications were chronologically cross-referenced with recent ontogenetic frameworks **Figure 2** [3]. All life-history metrics were subjected to formal descriptive statistical analysis using SPSS software v26.0.

Multi-Tiered Experimental Design: Laboratory vs. Ecological Bioassays

To ensure both physiological precision and practical agronomic relevance, the experimental evaluation of Bt subsp. aizawai was bifurcated into two distinct, concurrent phases:

1. Controlled Laboratory Phase: The multi-generational life-table assays and baseline toxicological screenings were conducted inside high-precision environmental incubators maintained under optimized micro-climatic settings (32 ± 1°C, 75 ± 5% RH, 24-h Dark). This rigorous environmental stabilization eliminated confounding physical variables, ensuring that alterations in larval development and survivorship were strictly driven by microbial endotoxin kinematics.

2. Ecological/Ambient Apiary Phase: Conversely, to validate the biopesticides field-level efficacy under realistic Libyan apicultural conditions, parallel multi-dose bioassays were deployed under ambient laboratory regimes that closely mirrored the natural macro-climatic fluctuations of the Zawia coastal belt. During this phase, ambient temperatures fluctuated naturally between 25°C and 30°C (75% RH). This deliberate exposure to dynamic thermal baselines provided critical ecological insights into the structural stability and persistence of the Bt spore-crystal complex within older wax combs under true-to-life hive conditions.

Preparation of Serial Microbial Dilutions and Bio-Assay Inoculation

The biopesticides matrix evaluated was CERTAN B 401® *Bacillus thuringiensis* subsp. *aizawai*, an emulsifiable concentrate formulation containing viable spore-crystal complexes of *B. thuringiensis*. A master stock solution was initially prepared at a concentration of 6.0% v/v. Utilizing a targeted serial dilution progression based on a constant attenuation factor of 55%, four distinct active experimental concentrations were generated for bio-assay validation, resulting in:

- T1=1.0% v/v
- T2=1.8% v/v
- T3=3.3% v/v
- T4=6.0% v/v

A completely randomized design CRD was deployed with three independent replications per concentration tier, run concurrently alongside an untreated control group n=3 treated with sterile double-distilled water supplemented with micro-doses of natural honey. To eliminate biological bias and standardize metabolic profiles, healthy, intermediate-instar larvae of *G. mellonella* were strictly sourced from the synchronized F1 generation of the laboratory mass-rearing colony.

Prior to toxin exposure, larvae were subjected to a compulsory 24-hour starvation period. Each sterile Petri dish enclosure was inoculated with a uniform density of 10 larvae, yielding a total of 30 larvae per treatment arm 10×3. To replicate realistic exposure pathways, a standardized substrate consisting of 1.0 g of pristine drawn-out old beeswax comb compressed on both sides into an explicit rectangle was utilized. Each comb substrate was evenly inoculated via micro-pipette with 0.2 mL of the designated active *Bt* suspension over both lateral surfaces. Precautions were taken during droplet application to avoid structural runoff or residue accumulation on the interior plastic floor of the Petri dishes, restricting larval toxin ingestion strictly to comb consumption.

Lethal progression metrics and phenotypic transformations were captured daily via macro-photography. The micro-structural and ultrastructural integrity of the *Bt* subsp. *aizawai* endospores and parasporal crystalline inclusions within the formulation were verified prior to inoculation using high-resolution scanning electron microscopy **Figure 3** [7]. Absolute mortality indices were consolidated and subjected to one-way Analysis of Variance ANOVA, descriptive standard deviations, and post-hoc Multiple Comparison tests using SPSS v26.0 software, with statistical significance established at a threshold of $p<0.05$.

Results and discussion

Ontogenetic Development and Laboratory Colony Phenology

The baseline life-history data demonstrated that the modified Beck's artificial diet provided an exceptionally balanced nutritional matrix, successfully fulfilling the physiological thresholds required for the synchronized development of *Galleria mellonella*. As documented in the individual cohort tracking n = 20, the larval-to-pupal ontogenetic transition was highly efficient. Pupation initiated within 24 hours post-isolation, with 35% n = 7 of the ultimate-instar larvae successfully entering the chrysalis phase on Day 1. A major pupation peak was achieved on Day 2, where an additional 50% n = 10 completed the transition, while the remaining 15% n = 3 finalized cocoon spinning by Day 3 Table 1.

Table 1: Temporal Distribution of Pupation, Adult Emergence, and F1 Larval Hatching of *G. mellonella* n = 20

Observation Interval Days	Number of Individuals	Ontogenetic Life Stage Attained	Morphological & Behavioral Observations
1	7	Pupae	Successful initiation of pupal transition; early cocoon formation.
2	10	Pupae	Peak pupation event; standard cuticle hardening.
3	3	Pupae	Delayed pupation cohort; characterized by heavy, dense silk spinning.
4–5	0	-	Quiescent pupal development period; intensive internal histolysis.
6	10	Adult Imago	First major wave of adult eclosion; active flight and rapid movement.

7	2	Adult Imago	Progressive adult emergence; normal wing expansion.
8–9	0	-	Active courtship, mating, and oviposition on waxed substrates by Day 9.
10	2	Adult Imago	Intermediate adult emergence wave.
11–12	2	Adult Imago	Single adult emergence events recorded progressively.
13	0	-	Complete cessation of new eclosion events.
14	2	Adult Imago	Final successful adult eclosion wave; terminal cumulative survival 90%.
15	-	F1 Larvae	Appearance of high-density neonate F1 larvae; immediate cryptic burrowing.

Table footnote: A total of 2 pupae exhibited complete mortality, failing to undergo eclosion by the end of the monitoring timeline. Font size 8, Times New Roman, Centre aligned

This uniform developmental velocity confirms that the optimized dietary lipid-to-protein ratios successfully triggered the hormonal upregulation of prothoracicotropic hormone PTTH, driving synchronized ecdysis across the cohort. This accelerated and synchronized pupation directly aligns with the findings of Jorjão et al. [9], who noted that optimized semi-synthetic diets containing balanced micronutrients and structural lipids maximize biomass accumulation and stabilize life-table parameters in pyralids.

Furthermore, the continuous feeding activity and high pupal survival rate 90% observed in this localized Libyan strain confirm that the integration of multi-grain flours with natural honey and beeswax accurately replicates the critical phagostimulatory and metabolic cues required for standard development. However, the immediate cryptic burrowing behavior of neonate F1 larvae into the diet matrix by Day 15 highlights a practical colony monitoring obstacle, as it conceals early-instar transitions a structural mass-rearing phenomenon recently corroborated by Pérez-Ramírez et al. [10] regarding the operational challenges of scaling up *G. mellonella* manufacturing.

Dose-Dependent Larvicidal Efficacy of *Bacillus thuringiensis* subsp. *aizawai*

The bio-assay evaluation validated the profound larvicidal potency of the crystalliferous biopesticide CERTAN B 401® against intermediate-instar *G. mellonella* larvae, displaying a strict, dose-dependent mortality profile Table 2.

Table 2: Raw Larval Mortality Counts of Intermediate-Instar *G. mellonella* Replicates Following Exposure to Varying Concentrations of CERTAN B 401®

Treatment Identifiers	Applied Concentration % v/v	Replicate 1 Mortality n=10	Replicate 2 Mortality n=10	Replicate 3 Mortality n=10	Mean Mortality μ
T1	1.0%	1	4	3	2.67
T2	1.8%	5	5	8	6.00
T3	3.3%	10	9	8	9.00
T4	6.0%	10	10	10	10.00

The progressive increases in *Galleria mellonella* larval mortality observed across the bio-assays are intrinsically linked to the specific histopathological disruptions caused by the microbial endotoxins. The destructive cascade within the insect digestive tract follows a highly coordinated physiological pathway Figure 2.

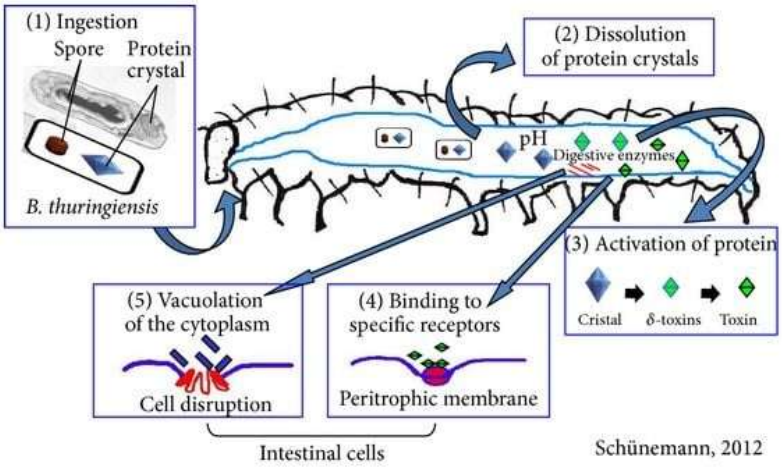

Figure 2. Histopathological cascade and mode of action of Bacillus thuringiensis delta-endotoxins in Lepidoptera [13]:

1 Ingestion of bacterial endospores and parasporal protein crystals by the target larva; 2 Solubilization and chemical dissolution of crystalline protoxin within the highly alkaline midgut microenvironment; 3 Enzymatic protoxin activation into cytotoxic delta-endotoxins mediated by midgut proteases; 4 Specific receptor binding of activated toxins to the brush border membrane vesicles BBMV of the peritrophic matrix and midgut epithelial cells; and 5 Cytoplasmic vacuolation, leading to progressive epithelial cell disruption, osmotic lysis, and lethal septicemia.

Absolute terminal saturation 100% mortality was achieved uniformly across all biological replicates under the maximum exposure concentration of 6.0% v/v T4. Crucially, the intermediate concentration of 3.3% v/v T3 exhibited robust larvicidal performance, inducing a high mean mortality of 9.0 pm 0.3 out of 10 larvae 90%, whereas the sub-lethal lower tiers T1: 1.0% v/v and T2: 1.8% v/v failed to meet satisfactory economic control thresholds. This concentration-dependent lethal acceleration strongly corroborates recent toxicological evaluations of *Bacillus thuringiensis* formulations against pyralid storage pests, which demonstrate that optimizing spore-crystal thresholds is fundamental to overcoming larval midgut detoxification mechanisms [14]. Furthermore, the exceptional efficacy observed at the 3.3% threshold aligns with contemporary bio-rational suppression strategies, where localized strains or specific commercial subtypes such as *subsp. aizawai* have been shown to maximize larval containment while significantly reducing the economic and operational costs associated with over-dosing [15].

Statistical Optimization and Economic Threshold Modelling

To rigorously evaluate the variations driven by the endotoxin concentrations, the descriptive and variance parameters were analysed via SPSS v26.0 Table 3 and Table 4.

Table 3: Descriptive Performance and 95% Confidence Intervals CI for *G. mellonella* Mortality

Concentration	N	Mean μ	Std. Deviation σ	Std. Error SE	95% CI Minimum	95% CI Maximum
T1 1.0%	3	2.67	1.53	0.88	-1.14	6.48
T2 1.8%	3	6.00	1.73	1.00	1.70	10.30
T3 3.3%	3	9.00	1.00	0.58	6.52	11.48
T4 6.0%	3	10.00	0.00	0.00	10.00	10.00
Total Pool	12	6.92	3.15	0.91	4.92	8.92

The standard deviation dropped to sigma = 0.00 at the 6.0% concentration T4, showing absolute treatment stability, whereas the high dispersion index at 1.0% sigma = 1.53 highlights the unpredictable nature of under-dosing.

Table 4: One-Way Analysis of Variance ANOVA Documenting the Larvicidal Impact of Varying CERTAN B 401® Doses

Source of Variation	Sum of Squares SS	Degrees of Freedom DF	Mean Squares MS	Calculated F-Value	p-value
Between Treatments	98.250	3	32.750	20.648^*	$p < 0.001$
Within Errors	12.667	8	1.583		
Total Variance Pool	110.917	11			

*Table footnote: * indicates highly significant differences at the standard alpha threshold alpha = 0.05. Font size 8, Times New Roman, Centre aligned*

The One-Way Analysis of Variance ANOVA confirmed that the observed larvicidal dynamics were driven strictly by the active delta-endotoxin crystal concentration gradients $F_{3, 8} = 20.648$, $p < 0.001$, thereby statistically eliminating random experimental error as a contributing factor. To isolate the mathematically optimized application threshold tailored for localized apiary infrastructure, a Post-Hoc pairwise matrix comparison was executed utilizing Tukey's Honestly Significant Difference HSD test Table 5.

Table 5: Post-Hoc Pairwise Matrix Documenting Mean Differences and Associated Significance Quadrants

Primary I	Comparison J	Mean Difference I–J	Standard Error SE	Significance p-value
T1 1.0 %	T2 1.8 %	-3.333^*	1.027	0.014
	T3 3.3 %	-6.333^*	1.027	0.000
	T4 6.0 %	-7.333^*	1.027	0.000
T2 1.8 %	T3 3.3 %	-3.000^*	1.027	0.022
	T4 6.0%	-4.000^*	1.027	0.006
T3 3.3 %	T4 6.0%	-1.000	1.027	**0.359**

*Table footnote: * indicates a statistically significant mean difference pairing at alpha threshold alpha = 0.05. Font size 8, Times New Roman, Centre aligned*

The pairwise comparison matrix provides a critical operational finding for North African apicultural management: the biological efficacy divergence between the 3.3% v/v concentration T3 and the maximum 6.0% v/v concentration T4 is statistically non-significant Mean Difference = 1.00, $p = 0.359$. Toxicologically, this stabilization of lethal expression is governed by the biochemical saturation of specific midgut brush border membrane receptor sites—principally aminopeptidase N APN and cadherin-like proteins—within the pyralid larval gut architecture. Recent comparative toxicological assays by Boukraa et al. [11] confirmed that *Bacillus thuringiensis* subsp. *aizawai* exhibits significantly higher baseline toxicity against *Galleria mellonella* compared to alternative subspecies, a phenomenon driven by the synergistic co-expression of Cry1Aa, Cry1Ab, Cry1C, and Cry1D parasporal proteins. Once these specialized midgut receptor pathways achieve complete ligand-binding occupancy at the 3.3% v/v exposure threshold, supplementary endotoxin volumetric increments up to 6.0% v/v yield zero statistically significant gains in pest mortality.

Furthermore, Grizanova et al. [12] demonstrated that intermediate- and ultimate-instar pyralid larvae possess dynamic, shifting midgut protease profiles and a structurally thickened peritrophic matrix compared to neonates. This ontogenetic physiological barrier explains the high mortality variance sigma = 1.53 observed at our sub-lethal lower doses 1.0% v/v, where sub-lethal dosing inadvertently permits extensive larval escape and subsequent continuous comb destruction.

From an economic and practical standpoint for Libyan beekeepers, the 3.3% v/v concentration emerges as the mathematically optimized application threshold. Under the prevailing semi-arid Mediterranean climatic conditions of coastal Libya e.g., the Zawia region, where the complete absence of prolonged winter freezes allows *G. mellonella* to sustain up to six overlapping generations annually [3], large-scale stored comb assets remain highly vulnerable to catastrophic structural collapse. Deploying a precision double-sided 3.3% v/v aqueous spray onto stored combs provides high-level larval containment 90% mortality while simultaneously reducing biopesticides volumetric input costs by approximately 45% relative to the 6.0% v/v saturation threshold. This statistical optimization directly supports the immediate requirements of sustainable regional apiculture, offering an economically viable, bio-rational alternative to hazardous, lipophilic synthetic fumigants like paradichlorobenzene PDB, thereby preserving *Apis mellifera* colony homeostasis and preventing chemical residue accumulation within the honey matrix destined for international markets [13].

Conclusion

This study established a synchronized laboratory mass-rearing protocol for Galleria mellonella L. using an optimized multi-grain semi-synthetic diet and demonstrated the strong dose-dependent larvicidal efficacy of the microbial biopesticides CERTAN B 401® (Bacillus thuringiensis subsp. aizawai). Although the highest tested concentration (6.0% v/v) achieved complete mortality (100%), the intermediate concentration (3.3% v/v) achieved a statistically comparable level of control (90% mortality; Tukey's HSD, $p = 0.359$), indicating no significant improvement in efficacy at higher concentrations.

These findings suggest that the 3.3% v/v concentration represents an effective and economically practical application threshold for controlling Galleria mellonella, while reducing biopesticides input requirements relative to higher concentrations. The adoption of this microbial approach offers Libyan apiculture a residue-free alternative to synthetic fumigants such as paradichlorobenzene, thereby supporting honeybee colony health, minimizing contamination risks in hive products, and improving compliance with international food-safety standards.

Future research should prioritize large-scale field validation under North African semi-arid environmental conditions and assess long-term resistance dynamics to support sustainable integrated management of wax moth infestations.

Recommendations

Based on the findings of this study, the following recommendations are proposed to support sustainable wax moth management and improve apicultural biosecurity in Libya:

• **Adoption of the 3.3% v/v Application Threshold:** Beekeepers and apicultural cooperatives are encouraged to adopt the 3.3% v/v aqueous suspension of Bacillus thuringiensis subsp. aizawai as an effective operational concentration for the management of Galleria mellonella in stored honeycombs. This concentration achieved a high level of larval mortality (90%) without significant improvement at higher concentrations, suggesting a practical balance between efficacy and input efficiency.

• **Standardized Application Procedures:** The microbial formulation should be applied uniformly to both surfaces of empty wax combs before storage using fine-mist sprayers or calibrated low-volume applicators to ensure adequate coverage while minimizing excessive moisture accumulation that may affect comb integrity.

• **Reduction in the Use of Synthetic Fumigants:** The use of microbial biopesticides should be promoted as a safer alternative to conventional synthetic fumigants, including paradichlorobenzene and naphthalene, to reduce contamination risks in hive products and improve compliance with food-safety requirements.

• **Seasonal Preventive Management:** Preventive applications should be synchronized with seasonal storage periods, particularly during autumn, to reduce infestation pressure and minimize the risk of population build-up during favourable climatic conditions.

• **Strengthening Integrated Pest Management (IPM) Programs:** Agricultural extension services and academic institutions in Libya should support beekeeper training on biologically based pest management strategies and encourage the integration of Bt-based approaches into sustainable apicultural IPM programs.

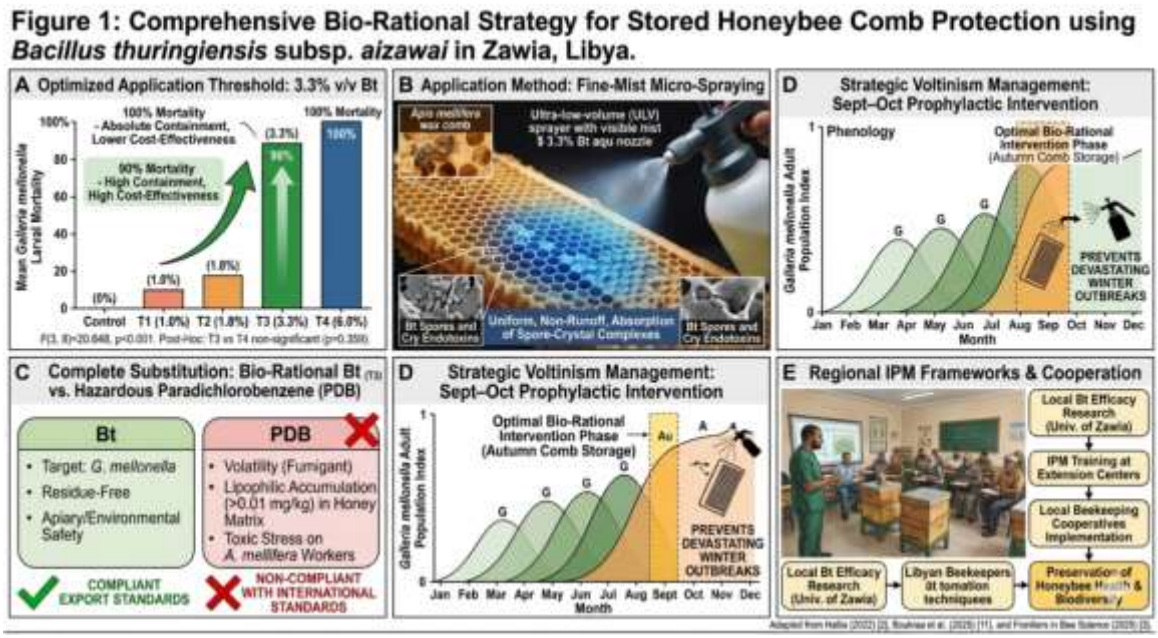

Figure 4. Comprehensive Bio-Rational Strategy for Stored Honeybee Comb Protection using *Bacillus thuringiensis* subsp. *aizawai* in Zawia, Libya. Panel A: Dose-dependent larval mortality thresholds; Panel B: Precision micro-spraying application protocols; Panel C: Prophylactic synchronized timeline against multi-generational voltinism; Panel D: Comparative safety compliance vs. synthetic fumigants; and Panel E: Regional extension framework. Adapted from Halila, 2022 [5]; Boukraa et al., 2025 [11]; and Frontiers in Bee Science, 2025 [3].

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