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BACILLUS MYCOIDES: A NOVEL INDIGENOUS ISOLATE FOR PROTECTING HONEYBEES FROM PESTICIDE INDUCED TOXICITY

Berjin Viju M G[1], Muthamilselvan Muthukumar[2*]

[1]Department of Entomology, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Maduranthagam Taluk, Chengalpattu District, Tamil Nadu, 603201, India Email ID: bm5736@srmist.edu.in
[2*]Department of Entomology, SRM College of Agricultural Sciences, SRM Institute of Science and Technology, Baburayanpettai, Maduranthagam Taluk, Chengalpattu District, Tamil Nadu, 603201, India Email ID: muthukumar.tnau@gmail.com
*Corresponding Author: Muthamilselvan Muthukumar

ABSTRACT
The extensive use of neonicotinoid insecticides, particularly imidacloprid in agricultural pest management has raised significant concerns regarding their negative impacts on honeybee health and pollination services. The study evaluated the protective potential of the beneficial gut-associated bacterium *Bacillus mycoides* against imidacloprid-induced toxicity in the Indian honeybee, *Apis cerana*. A preliminary toxicity assay was performed using five imidacloprid concentrations (0.5, 1, 2, 5 and 10 ppm) to determine the 72-h median lethal concentration (LC_{50}), which was estimated to be 0.896 ppm. Newly emerged worker bees were assigned to four treatment groups: control, *B. mycoides* treated, imidacloprid exposed and imidacloprid + *B. mycoides*. Bee survival was assessed using Kaplan–Meier survival analysis. Significant variation in survival rates was observed among treatments (log-rank test, $P < 0.0001$). Imidacloprid exposure alone resulted in the lowest survival (2.00 ± 0.82 bees), whereas supplementation with *B. mycoides* significantly enhanced survival (6.00 ± 0.82 bees; Tukey's HSD, $P < 0.001$). The survival of bees treated with *B. mycoides* alone was comparable to that of the untreated control group (9.25 ± 0.50 and 8.75 ± 0.50 bees, respectively). These findings indicate that *B. mycoides* mitigates imidacloprid-associated toxicity and improves the resilience of *A. cerana* under pesticide stress.

KEYWORDS: *Apis cerana*; *Bacillus mycoides*; Imidacloprid; Bioassay; Kaplan–Meier survival; LC_{50}.

INTRODUCTION

Honeybees play an indispensable role as pollinators in both agricultural and natural ecosystems, contributing to the reproduction of nearly one-third of global food crops. Beyond their ecological role honeybees produce economically valuable hive products including propolis, honey, pollen, royal jelly and beeswax each with diverse application in the food, cosmetic and pharmaceutical industries (Hristov *et al*., 2020). Among managed pollinator species, the Indian honeybee (*Apis cerana* Fabricius, 1793) is widely distributed across the diverse agroclimatic regions of India and plays a pivotal role as a key pollinator for numerous agricultural crops, making it an essential component of sustainable apiculture (Ghosh *et al*., 2025). Over the past few decades, honeybee population has reported serious decline worldwide, raising concerns about pollinator conservation (Tang *et al*., 2023). The decline of honey bee populations is associated with multiple stressors, including predators, parasites and pathogens (Maqbool *et al*., 2025). Additionally, exposure to pesticides widely used in agriculture for controlling insect pests, mites, plant pathogens and weeds poses a serious threat to honey bee populations (Almasri *et al*., 2021). Although these pesticides are mainly used to improve crop productivity their widespread applications inadvertently expose non-target organisms particularly bees and other pollinators, leading to adverse toxic effects (Sánchez-Bayo, 2012). During foraging, honeybees are frequently exposed to pesticides through contaminated nectar, pollen, water and direct contact with treated plants, making pesticide exposure an unavoidable environmental stressor (Démares *et al*., 2022). Extensive use of neonicotinoid insecticides in agriculture has become an important environmental issue because these compounds remain in the environment for prolonged periods and can adversely affect non target organisms particularly bees, even at sublethal concentration (van der Sluijs *et al*., 2013). Imidacloprid is one of the most extensively applied neonicotinoid insecticides and has remained an important component of pest management programs since its introduction (Simon-Delso *et al*., 2015). Microbial biodegradation is an efficient and environmentally sustainable approach for reducing pesticide residues. Microorganisms utilize pesticides as source of carbon, nitrogen and phosphorus transforming complex toxic compounds into simpler less harmful products while supporting their growth and metabolism (Bhatt *et al*., 2021). Among various microorganisms including bacteria, fungi and algae, bacteria are the most widely used due to their metabolic diversity and adaptability (Huang *et al*., 2020). Oral supplementation with beneficial gut bacteria has emerged as a promising strategy to enhance honeybee tolerance to pesticide stress. In *A. mellifera*, gut bacterial supplementation improved survival acetamiprid and deltamethrin exposure by enhancing detoxification, antioxidant and immune responses (Al-Humayd *et al*., 2024). Despite these advances, no published study has isolated imidacloprid-degrading gut bacteria and evaluated their protective potential through

supplementation feeding in *A. cerana*. Therefore, the present study aimed to evaluate the effects of the previously isolated imidacloprid-degrading honeybee gut bacterium on the survival and physiological responses of *A. cerana* following oral exposure to imidacloprid. We hypothesized that bacterial supplementation would mitigate pesticide induced toxicity by enhancing honey bee survival and modulating the expression of detoxification, antioxidant and immune related genes. Collectively, this study highlighting its potential as a probiotic strategy against neonicotinoid toxicity.

MATERIALS AND METHODS

Collection of Bees

Newly emerged worker bees were collected from healthy colonies maintained at the apiary of SRM College of Agricultural Sciences, Chengalpattu, Tamil Nadu, India (12.38575° N latitude and 79.73683° E longitude). Bees were collected directly from brood frames of a randomly selected healthy colony and transported to the laboratory in ventilated cages. For safe handling, bees were immobilized by cooling at -20 °C for 3–5 min, following the protocol described by Tutun *et al*. (2020). Subsequently, healthy, active and undamaged bees were randomly allocated to well-ventilated experimental cages at a density of 15 bees per cage for the oral feeding bioassay.

Bacterial Isolates and Culture Preparation

The bacterial isolates used in this study *Bacillus mycoides* (GenBank accession no. PZ234880), was obtained from our previously characterized bacterial collection. Isolation and molecular identification of the isolates were performed in our earlier study. A pure culture of *B. mycoides* was streaked onto Nutrient Agar (NA). A single colony from the pure culture was then inoculated into sterile nutrient broth and incubated at 32 ± 2 °C for 24 hours. Following incubation, the bacterial culture was stored at 4°C until further use in the experimental procedure.

Experimental Cages

Each treatment consisted of 15 bees maintained in a separate transparent square experimental cage made of polystyrene (HiMedia Laboratories Insect Breeding Dish, TCP014) measuring 72 × 72 × 100 mm. Each cage was fitted with a 40 mm ventilation opening at the bottom covered with a 0.053 mm mesh to provide adequate airflow while preventing bee escape. The treatment solution was supplied using absorbent cotton saturated with the respective solution and placed about the mess allowing the bees to feed freely.

Experimental Insecticide

Imidacloprid, a systemic insecticide belonging to the neonicotinoid group with an active ingredient concentration of 70% water dispersible granules (WG) marketed as Admire® (Bayer CropScience) was selected for the present study. The molecular formula and chemical structure of imidacloprid are presented in **(Table 1)** according to the National Centre for Biotechnology Information (NCBI).

Preliminary Toxicity Assessment of Imidacloprid

A preliminary toxicity bioassay was conducted to determine the appropriate test concentration of imidacloprid (70% WG) for the subsequent supplementary feeding experiment. Bees were orally exposed to five concentrations of imidacloprid (0.5, 1, 2, 5 and 10 ppm), each prepared in a 50% (w/v) sucrose solution. Each treatment consisted of 15 adult bees maintained in separate experimental cages. A control group was provided with a 50% (w/v) sucrose solution without insecticide. Bees that did not respond to gentle tactile stimulation were considered dead. Mortality was recorded 72 h after exposure and the resulting data were used to estimate the median lethal concentration (LC_{50}) of imidacloprid, which was used to select the test concentration for the subsequent supplementary feeding experiment.

Treatment Details and Preparation of Supplementary Feeding Solutions

The study was conducted to evaluate the effect of the gut bacterium *B. mycoides* on the toxicity of imidacloprid in newly emerged worker honeybees. Each treatment was administered to bees (n=15) maintained in separate well-ventilated experimental cages, as described in the preliminary bioassay, with four replications under laboratory conditions **(Table 2)**. The bacterial suspension was prepared by inoculating *B. mycoides* into nutrient broth (NB) and incubating the culture for 24 hours at 37°C under orbital shaking at 150 rpm. The grown culture was standardized to approximately 1×10^8 CFU mL^{-1} and mixed with 50% (w/v) sucrose solution immediately before feeding. The imidacloprid solution was prepared at the previously determined LC_{50} test concentration by dissolving the insecticide in a 50% (w/v) sucrose solution. Fresh treatment solutions were prepared daily and supplied throughout the experimental period (Al-Humayd *et al*., 2024). The bees were maintained under laboratory conditions and mortality was recorded at every 24 hours. Bees that failed to respond to gentle tactile stimulation were considered dead.

Statistical Analysis

All experimental data were analyzed using R software (CRAN R version 4.6.1). Probit analysis was performed to estimate the median lethal concentration (LC_{50}) of imidacloprid using the MASS package (Venables & Ripley, 2026). A probit regression model was fitted to the concentration-mortality data to generate the dose–response curve and used to estimate the median lethal concentration LC_{50} value. Differences among treatment groups were evaluated using one-way analysis of variance (ANOVA). Statistical significance was determined at $P < 0.05$. Survival data were analyzed using the Kaplan–Meier method using the survival package (Therneau, 2026) and the survminer package (Kassambara *et al*., 2026).

Differences in survival distributions among treatments were assessed using the log-rank test, with statistical significance set at $P < 0.05$.

RESULT

Determination of Lc_{50} Of Imidacloprid

A preliminary bioassay was conducted to determine the median lethal concentration (LC_{50}) of imidacloprid for use in the subsequent oral feeding experiment. Newly emerged worker honeybees were orally exposed to five concentrations of imidacloprid (0.5, 1, 2, 5 and 10 ppm). The observed mortality increased progressively with increasing imidacloprid concentration, demonstrating a clear dose-dependent response. A nonlinear dose–response curve was fitted to the mortality data, and the LC_{50} value was estimated as 0.896 ppm, indicating the concentration required to cause 50% mortality after 72 h of oral exposure **(Figure 1)**. Mortality increased in a concentration dependent manner ranging from 26.7% at 0.5 ppm to 53.3% at 1 ppm, 80.0% at 2 ppm and reached 100% at both 5 and 10 ppm. Based on the estimated LC_{50} value, 0.896 ppm of imidacloprid was selected as the test concentration for the subsequent oral feeding experiment to evaluate the protective effect of *B. mycoides* against pesticide induced toxicity.

Effect of *Bacillus mycoides* Supplementation on Survival of *A. cerana*

The present study evaluated the protective effect of the gut bacterium *B. mycoides* on the survival of newly emerged worker honeybees following oral exposure to imidacloprid. Kaplan–Meier survival analysis revealed significant differences in survival among the four treatments (log-rank test, $P < 0.0001$) **(Figure 2)**. Honeybees supplemented with *B. mycoides* (T_2) exhibited the highest survival throughout the experimental period with a mean number of surviving bees was 9.25 ± 0.50, which was comparable to the control treatment (T_1) 8.75 ± 0.50 bees. No significant difference was observed between T_1 and T_2 (Tukey's HSD, $P > 0.05$), indicating that supplementation with *B. mycoides* alone did not adversely affect honeybee survival.

When honeybees exposed to imidacloprid alone (T_3) showed the lowest survival, with a final mean survival of 2.00 ± 0.82 bees, demonstrating the toxic effect of imidacloprid on worker honeybees. However, supplementation with *B. mycoides* in combination with imidacloprid (T_4) significantly improved survival, resulting in a final mean survival of 6.00 ± 0.82 bees, which was significantly higher than that of the imidacloprid only treatment (T_3) (Tukey's HSD, $P < 0.001$) **(Table 3)**. One-way ANOVA of the final survival data also demonstrated a significant treatment effect ($F = 96.36$, $P < 0.0001$). These findings suggest that oral supplementation with *B. mycoides* reduced the harmful effects of imidacloprid and enhanced the survival of newly emerged worker honeybees, indicating its potential protective role against imidacloprid induced toxicity.

DISCUSSION

Honey bees are among the most economically important pollinators worldwide, yet their health is increasingly threatened by neonicotinoid insecticides such as imidacloprid (Yang *et al*., 2023). Consistent with this concern, the present study demonstrated that dietary supplementation with the imidacloprid degrading bacterium *B. mycoides* partially protected *A. cerana* against imidacloprid toxicity by improving survival, suggesting its potential role in mitigating pesticide induced stress.

Li *et al*. (2017) reported that *A. cerana* is more sensitive to imidacloprid than *A. mellifera*, exhibiting markedly lower LD_{50} values in comparative toxicity studies. Consistent with these findings, the LC_{50} estimated for *A. cerana* in the present study further confirms the high susceptibility of this species to imidacloprid exposure. Henriques Martins *et al*. (2024) also identified *A. cerana* as one of the most imidacloprid sensitive pollinator species, with an LD_{50} lower than that of *A. mellifera*. This supports the use of a species-specific LC_{50} in the present study and highlights the greater vulnerability of *A. cerana* to neonicotinoid exposure. Carneiro *et al*. (2022) demonstrated that imidacloprid induces midgut epithelial damage, oxidative stress and apoptosis in honey bees. Consistent with these findings, the high mortality observed in the imidacloprid-treated group in the present study likely reflects such pesticide-induced physiological damage and supports the use of the estimated LC_{50} as an appropriate concentration for evaluating the protective effect of *B. mycoides* supplementation.

Hussain *et al*. (2023) reported that honey bees with a more diverse gut microbiota exhibited greater resilience to insecticide exposure. Consistent with this finding, *A. cerana* supplemented with *B. mycoides* showed improved survival following imidacloprid exposure in the present study, supporting the protective role of gut bacteria against pesticide-induced stress. Al-Ghamdi *et al*. (2020) confirmed that gut-derived Bacillus isolates were not themselves harmful to honeybees and could be safely incorporated as beneficial symbionts. Notably, *B. mycoides* supplementation alone did not differ from the untreated control in the present study, confirming its safety and specificity as a pesticide mitigating symbiont rather than a general growth-promoting agent. El Khoury *et al*. (2022) showed that endogenous honeybee gut bacteria are capable of directly degrading neonicotinoid compounds such as clothianidin in vitro. Similarly, the improved survival of imidacloprid-exposed bees supplemented with *B. mycoides* in the present study suggests that this strain may exert a comparable direct or indirect detoxifying effect on imidacloprid. Leska *et al*. (2022) demonstrated that probiotic bacteria isolated from *A. mellifera* reduced the cytotoxic effects of imidacloprid. Similarly, *A. cerana* supplemented with *B. mycoides* showed significantly higher survival than bees exposed to imidacloprid alone, supporting the protective role of beneficial gut bacteria against insecticide toxicity. Al-Humayd *et al*. (2024) reported that gut bacteria, including Bacillus spp., enhanced the survival of *A. mellifera jemenitica* exposed to deltamethrin and acetamiprid by reducing pesticide toxicity, oxidative stress and improving immune responses. Similarly, the increased survival of *A. cerana* supplemented with *B. mycoides* in the present study suggests a comparable protective role against imidacloprid toxicity. Leska *et al*.

(2022) concluded that microbiological interventions represent a promising avenue for mitigating insecticide-related harm to honeybee populations. Collectively, the findings of the present study support this view, indicating that probiotic supplementation with *B. mycoides* is a sustainable strategy for pesticide risk mitigation in managed *A. cerana* colonies.

CONCLUSION

The findings of this study demonstrate that the gut bacterium *B. mycoides* significantly improved the survival of *A. cerana* exposed to the neonicotinoid insecticide imidacloprid. Bees treated with *B. mycoides* alone showed survival similar to the untreated control, indicating that the bacterium is safe and does not negatively affect bee health. These results suggest that *B. mycoides* can help reduce the harmful effects of imidacloprid and may serve as a beneficial probiotic for protecting honeybee colonies from pesticide stress. The use of pesticide-degrading gut bacteria as a dietary supplement could be a sustainable and eco-friendly approach to improving honeybee health. However, further studies are needed to determine the most effective dose and method of administering *B. mycoides* under field conditions. Future studies should investigate the mechanisms involved in protection, including pesticide degradation, antioxidant activity and the regulation of immune and detoxification related genes during long-term exposure to sublethal pesticide levels.

Author Contributions

Berjin Viju: investigation, data curation, writing - original draft, Muthukumar: conceptualization, supervision, resources, writing - review and editing.

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Conflicts of Interest

The authors declare no conflicts of interest

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TABLES

Table 1. Molecular formula and chemical structure of Imidacloprid.

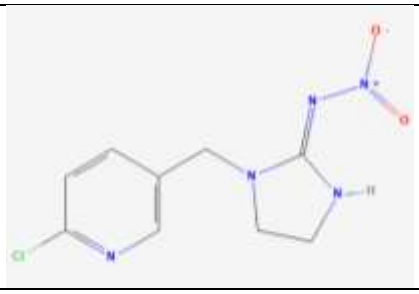
Insecticide	Molecular Formula	Chemical Structure
Imidacloprid	$C_9H_{10}ClN_5O_2$	

Table 2. Treatment details for the oral feeding experiment

Treatment	Description
T_1	50% sucrose solution (Control)
T_2	50% sucrose solution + *B. mycoides*
T_3	50% sucrose solution + Imidacloprid
T_4	50% sucrose solution + *B. mycoides* + Imidacloprid

Table 3. Final survival of honeybees under different treatments expressed as mean ± SD with Tukey's HSD grouping.

Treatment	Mean ± SD	Group
T_1	8.75 ± 0.50	a
T_2	9.25 ± 0.50	a
T_3	2.00 ± 0.82	c
T_4	6.00 ± 0.82	b

Treatments sharing the same letter are not significantly different ($P > 0.05$), whereas treatments with different letters differ significantly ($P < 0.05$).

FIGURES

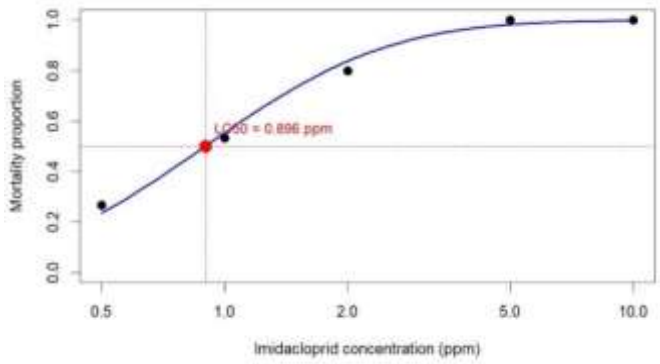


Figure 1. Nonlinear dose–response curve illustrating the concentration-dependent mortality of *A. cerana* workers following 72 h of oral exposure to Imidacloprid. The estimated LC_{50} is indicated by the red point at 50% mortality.

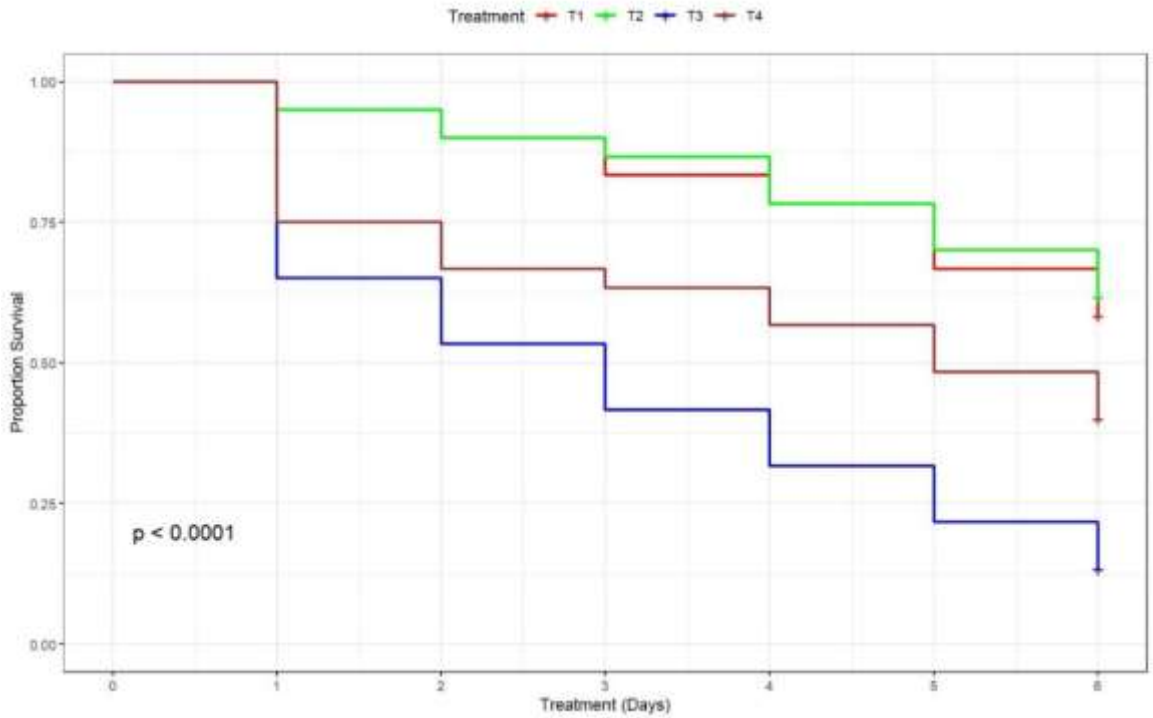


Figure 2. Kaplan–Meier survival curves of *A. cerana* workers during the 6-day laboratory experiment. The x-axis represents the experimental duration (days) and the y-axis represents the Kaplan–Meier estimates of survival probability.