



Larvicidal activity of the entomopathogenic fungi and nematode against the cattle tick *Rhipicephalus microplus* Canestrini (Ixodida: Ixodidae)

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The livestock industry is a critical component of global agriculture, supporting the nutrition and livelihoods of over one billion people (Adesogan *et al.* 2026). In the Philippines, cattle and water buffalo contribute substantially to rural livelihoods through meat, milk, and draft power production (Villanueva *et al.* 2025). Bukidnon is widely recognized as a major cattle-producing province in the Philippines and recorded the highest provincial value of agricultural and fisheries production nationwide in 2024 (Philippine Statistics Authority 2025). However, infestations by the cattle tick *Rhipicephalus microplus* Canestrini (Ixodida: Ixodidae) reduce livestock productivity and facilitate the transmission of pathogens, including *Rickettsia* spp. (Rickettsiales: Rickettsiaceae) and *Coxiella burnetii* (Legionellales: Coxiellaceae) (Galay *et al.* 2020; Githaka *et al.* 2022). Although chemical acaricides remain the primary control strategy, widespread resistance threatens their long-term efficacy (Heylen *et al.* 2024). Consequently, alternative control approaches, including biological control agents, are increasingly being explored for sustainable tick management (FAO 2004; Alota *et al.* 2021).

Entomopathogenic fungi (EPF), particularly *Metarhizium* spp. (Hypocreales: Clavicipitaceae) and *Beauveria* spp. (Hypocreales: Cordycipitaceae), have demonstrated considerable pathogenicity against cattle ticks and are regarded as promising alternatives to conventional acaricides (Alonso-Díaz and Fernández-Salas 2021). Several studies have reported the efficacy of both EPN and EPF against *Rhipicephalus microplus*, highlighting their potential role in sustainable tick management programs (Alonso-Díaz and Fernández-Salas 2021; Filgueiras *et al.* 2025). EPN and EPF occupy similar soil habitats but utilize different infection mechanisms. Fungal infection weakens the host cuticle and facilitates nematode invasion, while nematode-associated symbiotic bacteria accelerate host mortality following infection. Their combined application may enhance tick mortality through additive or complementary effects (Wakil *et al.* 2017). However, studies evaluating EPF–EPN interactions against *R. microplus* remain limited,



particularly in the Philippines. To address this gap, this study assessed the larvicidal activity of *Steinernema siamkayai* Stock, Somsook & Reid, and *Metarhizium pingshaense* Q.T. Chen & H.L. Guo applied individually and in combination against *R. microplus* larvae to determine the effects of co-inoculation on tick mortality.

Metarhizium pingshaense strain MT6025 (GenBank accession no. PZ165967) was isolated from soil collected in Malaybalay City, Bukidnon, Philippines (8° 09' N, 125° 08' E) using the *Tenebrio molitor* baiting method (Sharma *et al.* 2018). *Steinernema siamkayai* Thai strain SST1 was obtained from the National Biological Control Research Center (NBCRC), Khon Kaen University, Thailand. Engorged female *R. microplus* ticks were collected from cattle in Malaybalay, Bukidnon (Katalbas *et al.* 2020) and morphologically verified (Walker *et al.* 2003). Engorged females were incubated individually in 5 mL vials at room temperature and 85% $\pm$ 5% relative humidity for oviposition. The first-instar larvae were harvested 10–20 days post-hatching for immediate bioassays at the NBCRC.

Preliminary bioassays were conducted to optimize the working concentrations of the local entomopathogens. Based on the mortality responses, 1.0×10^7 conidia mL⁻¹ for *M. pingshaense* and 250 infective juveniles (IJs) for *S. siamkayai* were selected for the main bioassays. Bioassays followed a Completely Randomized Design (CRD) using a modified Larval Packet Test (LPT) adapted from the FAO (2004) framework, with five treatments, four replicates, and 20 larvae per replicate. In Treatment 1 (Combined Biocontrol), larvae inside Whatman No. 1 filter paper packets were exposed to 1 mL of local *M. pingshaense* 1.0×10^7 conidial suspension for 24 hours before *S. siamkayai* 250 infective juvenile (IJ) inoculation. Treatments 2 and 3 served as positive (2 mL L⁻¹ Amitraz) and negative (sterile water + 0.1% Tween 80) controls, respectively. Packets were held in sterile Petri dishes and incubated at room temperature (28 $\pm$ 2 °C) under dark, 80% relative humidity conditions; mortality was recorded 72 hours post-exposure.

Data analysis was conducted via RStudio (v2023.12.1). Baseline concentrations were optimized using preliminary bioassay trends. Mortality data was evaluated for normality using the Shapiro–Wilk test. Because parametric assumptions were violated, treatment differences were determined using the non-parametric Kruskal–Wallis test, followed by Dunn's post-hoc multiple comparison test with a Bonferroni adjustment ($\alpha = 0.05$).

Table 1. Experimental treatment groups used in the larval packet test (LPT) bioassay against *Rhipicephalus microplus*.

Treatment Group	Description	Application Method
T1: Combined Biocontrol	<i>M. pingshaense</i> (1.0×10^7 conidia mL ⁻¹) + <i>S. siamkayai</i> thai strain SST1 (250 IJs)	Sequential: 1 mL conidial suspension for 24 hours, followed by EPN IJ inoculation
T2: Positive Control	Tickamit 12.5 EC(Amitraz)	Standard field-dose acaricide solution applied to the packet
T3: Negative Control	Vehicle Control	Sterile distilled water + 0.1% Tween 80

The bioassays demonstrated significant differences in mortality among treatments (Kruskal–Wallis test: $\chi^2 = 9.80$, df = 2, $P = 0.0074$). The combined application of local EPF + EPN (T1) and the positive chemical control (T4) produced the highest mortality, with mean values of $98.75 \pm 1.25\%$ SE (79/80 larvae) and $100.00 \pm 0.00\%$ SE (100/100 larvae), respectively (Fig. 1). No mortality was observed in the negative control. Post hoc comparisons indicated that the combined biological treatment was statistically comparable to Amitraz (adj.p = 1.000) and differed significantly from the negative control (adj.p = 0.0110). Collectively, these findings demonstrate the potential of entomopathogenic fungi, *Metarhizium pingshaense* and nematodes, *Steinernema siamkayai*, produced high mortality against *R. microplus* larvae under the present experimental conditions. Moreover, the results suggest application of these entomopathogens can be a biological alternative for cattle tick management.

The combined EPF + EPN treatment produced high larval mortality and was statistically comparable to Amitraz under laboratory conditions. However, because the individual local agents were not evaluated separately for the main bioassay, the results do not provide definitive evidence of synergism. The observed response may be influenced by treatment concentration, exposure duration, or complementary modes of infection between the fungal and nematode agents (Mathulwe *et al.* 2025).

During sequential exposure, *M. pingshaense* initiates cuticular infection by synthesizing mechanical and enzymatic cascades that compromise the tick's outer integument, producing microscopic perforations (Rajput *et al.* 2024). These localized structural failures likely facilitate easier penetration pathways for *S. siamkayai* IJs, which enter through natural spiracles, orifices, or fungal lesions. Once inside the hemocoel, the EPN releases its mutualistic bacteria, inducing septicemia and rapid mortality (Hummadi *et al.* 2021). Although these mechanisms may explain the high mortality observed in the combined treatment, they were not directly investigated in the study. Nevertheless, the findings suggest that sequential application of locally isolated entomopathogens has potential as a biological control strategy against *R. microplus* larvae. Similar integrated biological approaches have been proposed to overcome some of the limitations associated with single-agent applications while reducing reliance on conventional chemical acaricides (Koller *et al.* 2023; Mathulwe *et al.* 2025).

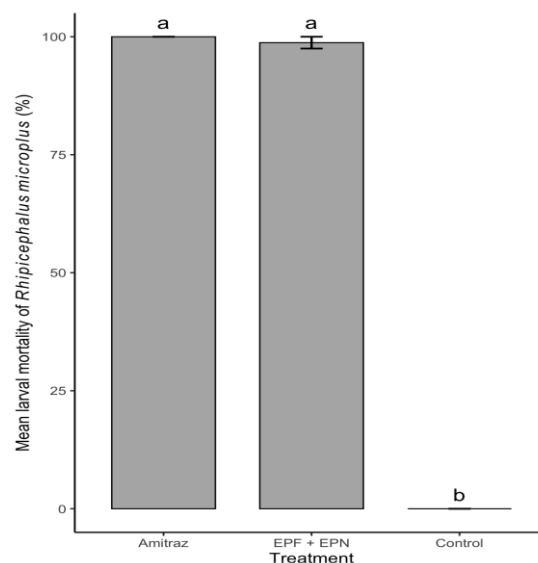


Figure 1. Mean larval mortality (%) of first-instar *Rhipicephalus microplus* after 72h exposure to Amitraz, local EPF + EPN combination, and negative control under laboratory conditions ($28 \pm 2^\circ\text{C}$; $80 \pm 10\%$ relative humidity). Bars represent mean mortality $\pm$ SE. Different letters above bars indicate groups based on Dunn's post hoc test with Bonferroni adjustment following the Kruskal–Wallis test ($P < 0.05$).

The combined application of *Metarhizium pingshaense* and *Steinernema siamkayai* showed potential as a biological control strategy against the first-instar *Rhipicephalus microplus* larvae with mortality statistically comparable to Amitraz under laboratory conditions. Further studies are needed to evaluate the individual effects of the two agents and determine whether their interaction is synergistic, additive, or antagonistic under laboratory and field conditions.

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