scientific reports

Check for updates

OPEN

Temperature dependent immunological responses of *Spoladea recurvalis* exposed to entomopathogenic fungi

Alex Byonanebye[1,2], Fathiya M. Khamis[1,3]✉, Maina Mwangi[2], Everlyne Samita Namikoye[2], Inusa J. Ajene[1] & Komivi Senyo Akutse[1,4]✉

Spoladea recurvalis **is a major pest of amaranth, capable of causing up to 100% yield loss if not controlled. Current control measures rely on synthetic chemicals; however, these chemicals are unsafe due to the crop's short maturity that make pre-harvest intervals observation difficult. A sustainable alternative to synthetic chemicals would be entomopathogenic fungi (EPF) though, a few bottlenecks still exist in the effective use of these biological solutions. Studies show low susceptibility of *S. recurvalis* to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18, with larval mortalities of 58.3 and 6.7%, respectively. Temperature can affect the efficacy of EPF and how the insect's immunity responds to fungal infections, but the effect of temperature on *S. recurvalis* and their interactions with the EPF is unknown. Therefore, we evaluated immune responses and gut microbiome of *S. recurvalis* when exposed to EPF at 15, 20, 25, 30 and 35 °C. From the results, *S. recurvalis* susceptibility increased at 30 °C, with ICIPE 30 causing the strongest effect. Total hemocyte counts in ICIPE 30-treated larvae reduced from 30.25×10^4/ml on day 3 to 10.75×10^4/ml on day 7. Granulocytes and plasmatocytes also decreased significantly. The dominant bacterial genera were *Enterobacter*, *Enterococcus*, and *Klebsiella*, with microbial diversity highest in ICIPE 30-treated larvae on day 1 and ICIPE 18-treated larvae on day 7, and lowest in ICIPE 30-treated larvae on day 7. The Findings of this study demonstrate that temperature influences this EPF's efficacy and provide important information on the optimal conditions for this biopesticide in the pest's management.**

Keywords *Spoladea recurvalis*, Amaranth, *Metarhizium anisopliae*, Hemocytes, Gut microbiome

Amaranth is a common name for *Amaranthus* genus of the Amaranthaceae family[1]. It is inexpensive to grow, incredibly nutritious, and adapts to different environments[2]. Being highly nutritious and cheap, amaranth is being promoted to the vulnerable people in society, especially pregnant women, children[3] and immunocompromised individuals, including people with HIV, to strengthen their immune systems[4]. Amaranth provides farmers with up to 7,117 USD/ha income[5]. Despite the importance of this crop, its production is affected by insect pests majorly lepidopteran leaf webber *Spoladea recurvalis* (Fabricius) (Lepidoptera: Crambidae). *Spoladea recurvalis* has been reported to be the major amaranth pest in America, Asia and Africa[6]. The larval stages feed on leaf lamina, leaving only the veins to make a skeleton and also webs the remaining parts to form a shelter[7]. In Kenya, *S. recurvalis* has been reported to cause up to 100% loss to farmers if not managed[8]. To manage this pest, farmers rely on synthetic pesticides use[9] and no adequate biological measures are used in the management of amaranth pests[10]. However, the use of synthetic pesticides on such vegetables, which are quick-maturing and have a short pre-harvest interval, may leave residues that are hazardous to consumers, including humans and animals, and with negative repercussions to the environment and biodiversity[11]. In this instance, biological control agents like entomopathogens are promising alternatives that are safe to humans and eco-friendly[12–14]. Though this is a feasible alternative, a previous study by Opisa et al.[12] reported moderate mortality of *S. recurvalis* by a

[1]International Centre of Insect Physiology and Ecology (icipe), P.O. Box 30772-00100, Nairobi, Kenya. [2]Department of Agricultural Science and Technology, Kenyatta University, P.O. Box 43844-00100, Nairobi, Kenya. [3]Department of Zoology and Entomology, University of Pretoria, Private Bag X20, Hatfield 0028, South Africa. [4]Unit of Environmental Sciences and Management, North-West University, Private Bag X6001, Potchefstroom 2520, South Africa. ✉email: fkhamis@icipe.org; kakutse@icipe.org

Metarhizium anisopliae isolate ICIPE 30 of 58.3% while *M. anisopliae* ICIPE 18 could only induce 6.7% mortality to second instar larvae.

Efficacy of entomopathogens and host response to biological control agents (BCA) is affected by temperature variations[15–17], which can limit the use of these entomopathogens for effective pest control[18]. Temperature affects the ecological suitability of insects by adjusting their physiology[19], hence studies on the effects of temperature on BCA and host response are relevant for successful biological control programs. Reaction to cold and pathogen invasion/infection leads to variation in immune responses[20,21]. Wang et al.[22] conducted immunological studies in *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) exposed to *M. rileyi* and realized that there was impairment of cellular immunity, while Maingi et al.[17] also got similar results with *Phthorimaea absoluta* (Meyrick) (Lepidoptera: *Gelechiidae*) after exposure to *M. anisopliae* ICIPE 18 and ICIPE 20 isolates. However, this has not been explored in *S. recurvalis*. Cooler temperatures may slow the growth of entomopathogens and increase insects' ability to fight infections[23]. Insect immune systems and pathogens may behave differently as the temperature varies significantly, impacting biological control programs[24]. Insects use hemocytes to carry out mechanisms such as encapsulation and nodulation to fight pathogen invasion[25]; encapsulation involves hemocytes that activate and form multilayered capsules around the pathogens, while nodulation involves a number of cells that aggregate around a pathogen. Granulocytes and plasmatocytes are actively involved in defense against pathogen infection[26,27], and these granulocytes are the first hemocytes that initiate the encapsulation process[28,29]. The gut microbiome also aids the insects in digestion, detoxification, stress tolerance, and immune defense by producing metabolites and signals that inhibit pathogens, activate systemic immunity, and create conditions unfavorable for fungal growth[30–32]. Therefore, this study was carried out to determine temperature-dependent immunological responses and gut bacterial community diversity in *S. recurvalis* following infection with *M. anisopliae* fungal isolates (ICIPE 30 and ICIPE 18) to provide information on the optimal conditions for integration of the biopesticide in the pest's management system.

Results

Mortality of *Spoladea recurvalis* larvae

Cross-analysis of treatments and temperatures in relation to mortality revealed significant differences for both temperature ($\chi2 = 167.58$, $df = 2$, $p < 0.001$) and treatment ($\chi2 = 871.27$, $df = 2$, $p < 0.001$). The mortality rates significantly differed in EPF-treated larvae across days at 20 °C ($\chi2 = 106.227$, $df = 2$, $p < 0.001$), 25 °C ($\chi2 = 340.19$, $df = 2$, $p < 0.001$), and 30 °C ($\chi2 = 340.19$, $df = 2$, $p < 0.001$) (Fig. 1). At 20 °C, *M. anisopliae* ICIPE 30 caused 21.5% mortality, while *M. anisopliae* ICIPE 18 caused only 6.5% (Fig. 1). As the temperature increased to 25 °C, mortality rates also rose, with *M. anisopliae* ICIPE 30 causing 57.5% and *M. anisopliae* ICIPE 18 causing 8.5%. At 30 °C, the highest mortality rates were observed, with *M. anisopliae* ICIPE 30 achieving 82.5% compared to 17.5% for *M. anisopliae* ICIPE 18 (Fig. 1). All mycosis tests conducted on the *S. recurvalis* larvae that died at 20 °C, 25 °C, and 30 °C showed over 95% positivity.

Hemocytes of *Spoladea recurvalis*

Total hemocyte counts (THC)

This study evaluated the effect of *M. anisopliae* ICIPE 30 and ICIPE 18 isolates on total hemocytes in the hemolymph of *S. recurvalis* at different temperature regimes. Cross analysis of treatments, temperatures and days in relation to hemocyte density was run and significant differences were observed between temperature

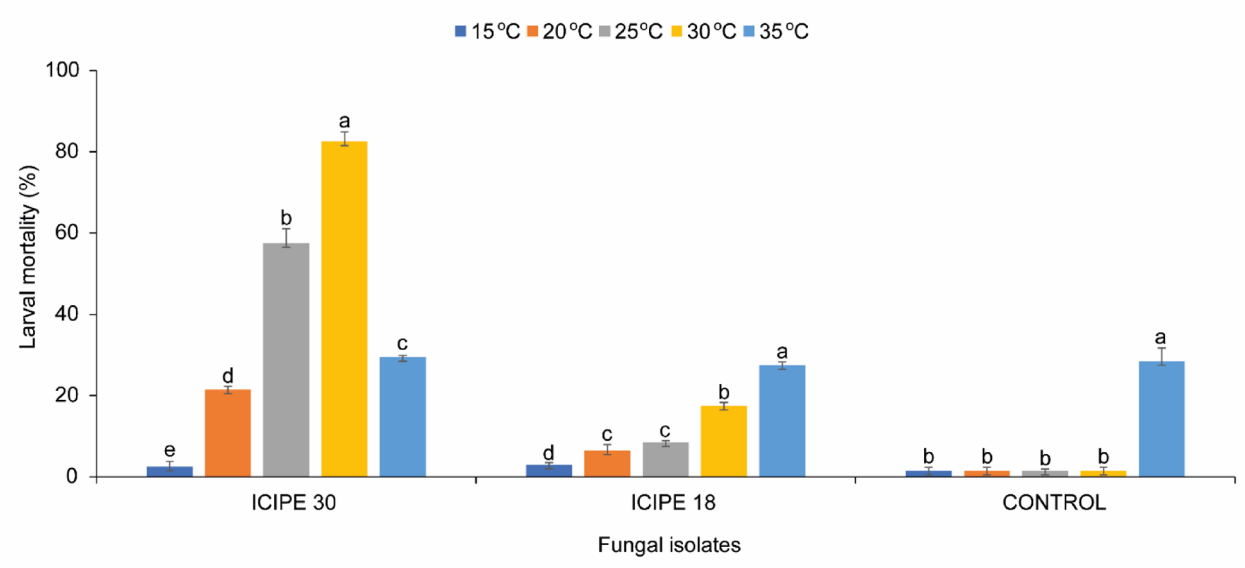

Fig. 1. Mean mortality (%) of *S. recurvalis* larvae post exposure to *M. anisopliae* isolates ICIPE 30 and ICIPE 18 at 20, 25 and 30 °C.

regimes ($\chi2 = 460.49$, $df = 2$, $p < 0.001$) and there was a strong interaction between temperature, treatments and days of the experiment ($\chi2 = 29.62$, $df = 4$, $p < 0.001$). Further analysis was done at each regime and at 20 °C, a significant difference between treatments ($\chi2 = 208.382$, $df = 2$, $p < 0.001$) and days ($\chi2 = 73.196$, $df = 3$, $p < 0.001$) was exhibited as well as an interaction between days and treatments ($\chi2 = 16.061$, $df = 6$, $p < 0.05$) (Fig. 2A). The application of *M. anisopliae* ICIPE 30 resulted in a significant rise in *S. recurvalis*' total hemocytes, from 15.5×10^4/ml on day 1 to 41.25×10^4/ml on day 3, peaking at 48.25×10^4/ml on day 5, before experiencing a slight decline to 37×10^4/ml on day 7. Similarly, larvae treated with *M. anisopliae* ICIPE 18 exhibited a significant increase in total hemocyte counts, reaching 19×10^4/ml by day 3. However, by days 5 and 7, the mean hemocyte counts in the *M. anisopliae* ICIPE 18-treated larvae were not significantly different from those in the control group (Fig. 2A).

For bioassays conducted at 25 °C (Fig. 2B), significant differences were observed in larval total hemocytes per treatment ($\chi^2 = 422.41$, df = 2, $p < 0.001$), across the days ($\chi^2 = 201.63$, df = 3, $p < 0.001$), and there was an interaction between days and treatments ($\chi^2 = 92.37$, df = 6, $p < 0.001$). A rapid increase in total hemocytes up to day 3 (96.5×10^4/ml) was noted in larvae treated with *M. anisopliae* ICIPE 30, followed by a drastic decline on day 5 (62×10^4/ml) and day 7 (40.5×10^4/ml). In larvae treated with *M. anisopliae* ICIPE 18, there was a statistically significant increase in the total hemocyte count on day 3 (42.75×10^4/ml), day 5 (49.25×10^4/ml), and day 7 (52.25×10^4/ml). However, in control larvae, THC showed an insignificant increase on day 3 (13.5×10^4/ml), day 5 (14.75×10^4/ml), and day 7 (16.75×10^4/ml) (Fig. 2B).

The analysis did not show significant differences at 30 °C (Fig. 2C) among the treatments ($\chi2 = 4.480$, df = 2, $p = 0.1065$); but there were significant variations across the days ($\chi2 = 57.458$, df = 3, $p < 0.001$) and in the interaction between days and treatments ($\chi2 = 35.336$, df = 6, $p < 0.001$). *Metarhizium anisopliae* ICIPE 30 induced the highest number of hemocyte counts on day 3 (30.25×10^4/ml), but these counts declined drastically to 13.75×10^4/ml on day 5 and 10.75×10^4/ml on day 7. Similarly, an increase in total hemocytes was also observed in larvae treated with *M. anisopliae* ICIPE 18 on day 3 (19.5×10^4/ml), although the counts were lower compared to *M. anisopliae* ICIPE 30. The THC remained constant (19.25×10^4/ml) on day 5, but with a slight decline observed at day 7 (18×10^4/ml). However, in control larvae, THC gradually increased from day 3 (13.75×10^4/ml) to day 5 (16.5×10^4/ml) and then to day 7 (17×10^4/ml) (Fig. 2C).

Differential hemocyte count (DHC)

Microscopic observations revealed five types of cells, viz. granulocytes, prohemocytes, plasmatocytes, spherulocytes and oenocytes in the hemolymph of *S. recurvalis* (Fig. 3). We observed that at 20 °C, total number of granulocytes in *S. recurvalis* larvae did not show any significant differences among treatments ($\chi2 = 4.1530$, df = 2, $p = 0.1254$) and days ($\chi2 = 2.1921$, df = 3, $p = 0.5335$). However, the number of granulocytes in larvae treated with *M. anisopliae* ICIPE 30 insignificantly increased from day 1 to day 7, with percentages of 59.65%, 59.93%, 64.13%, and 67.42%, respectively (Fig. 4A1). Larvae treated with *M. anisopliae* ICIPE 18 showed an increased number of granulocytes compared to the control on day 5 (60.75%) and day 7 (61.25%). Granulocytes in the control groups ranged between 57.63% on day 1 to 58.25% on day 7 (Fig. 4A1). The percentage of plasmatocytes also did not differ significantly between treatments ($\chi2 = 3.3230$, df = 2, $p = 0.18985$), although they varied across the days of the experiment ($\chi2 = 8.9290$, df = 3, $p = 0.03025$). Plasmatocytes in both larvae treated with *M. anisopliae* ICIPE 30 and ICIPE 18, and controls, did not show significant changes except on day 7 (15.78%) in ICIPE 30-treated larvae (Fig. 4B1–B3).

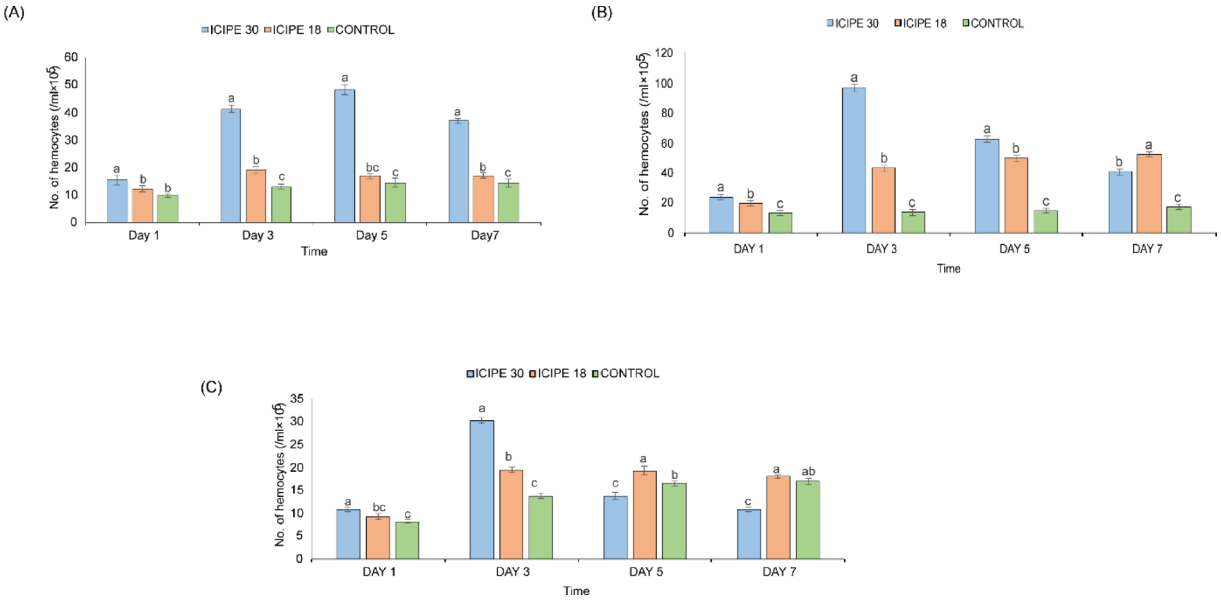

Fig. 2. Mean Total Hemocyte Counts in hemolymph of *Spoladea recurvalis* after exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18 at 20 (**A**), 25 (**B**) and 30 °C (**C**).

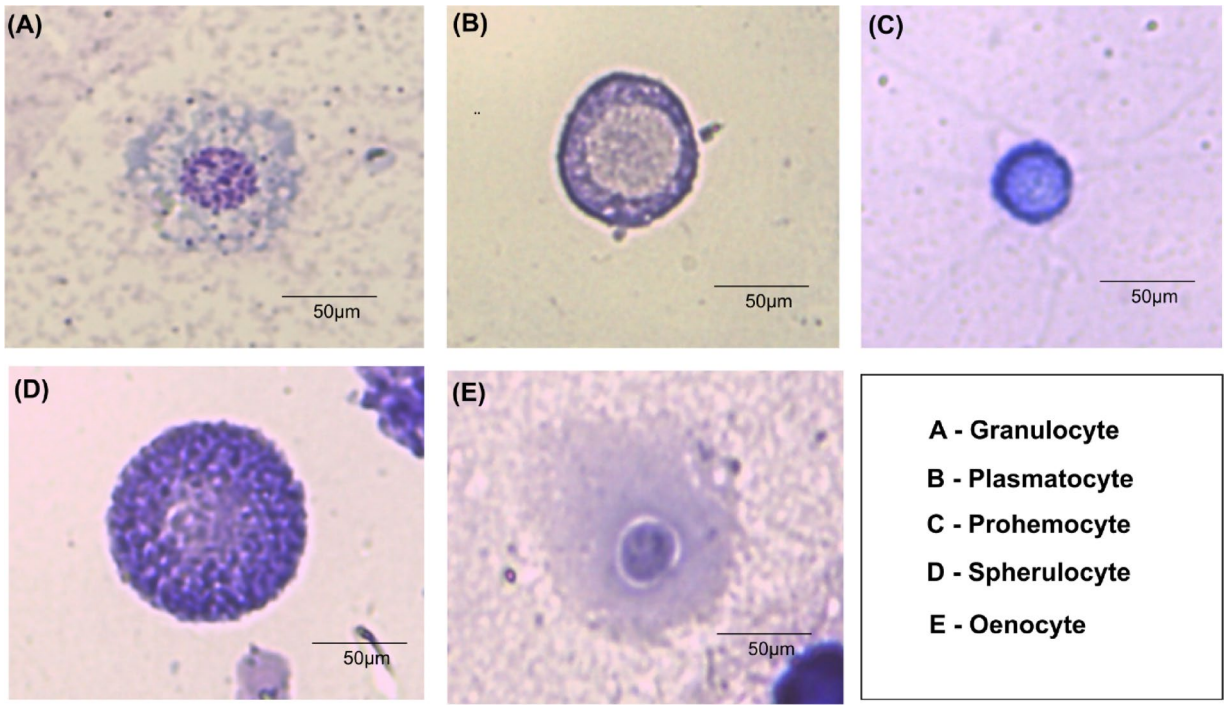

Fig. 3. Images of hemocyte types, granulocytes (**A**), plasmatocytes (**B**), prohemocytes (**C**), spherulocytes (**D**) and oenocytes (**E**), observed in hemolymph of *Spoladea recurvalis* larvae post exposed to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18.

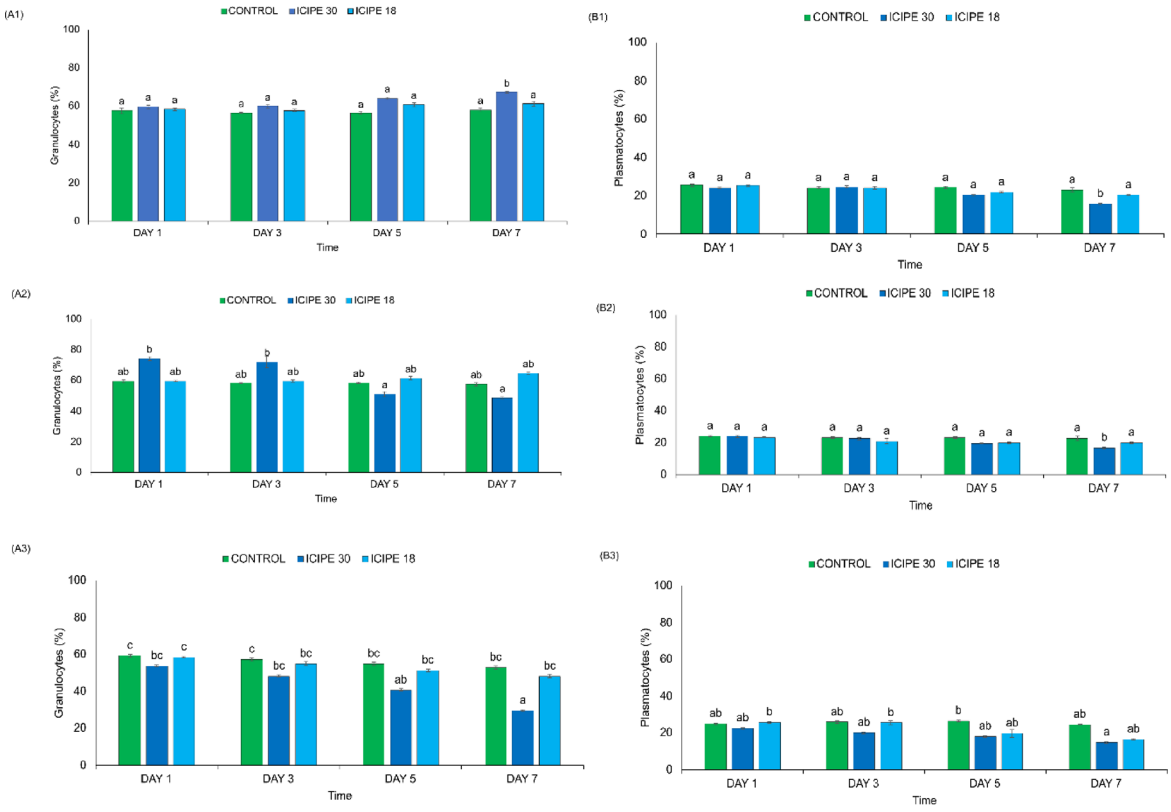

Fig. 4. Mean percent granulocytes at 20 (**A1**), 25 (**A2**) and 30 °C (**A3**) and plasmatocytes at 20 (**B1**), 25 (**B2**) and 30 °C (**B3**) observed in hemolymph of *Spoladea recurvalis* after exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18.

For *S. recurvalis* larvae which were treated at 25 °C, there was notable changes in total number of granulocytes per treatment ($\chi2 = 10.0390$, df = 2, $p < 0.006$) and day ($\chi2 = 9.7400$, df = 3, $p < 0.02$), with a strong interaction between days and treatments ($\chi2 = 27.7256$, df = 6, $p < 0.001$). Total granulocytes (Fig. 4A1) in larvae exposed to *M. anisopliae* ICIPE 30 increased sharply from day 1 (74.21%) up to day 5 and 7, where a decline was observed (50.90% and 48.69%, respectively). In *M. anisopliae* ICIPE 18-treated larvae, granulocytes (Fig. 4A1) increased gradually, with higher values obtained on days 5 (61.48%) and 7 (64.82%). However, in the control groups, granulocytes declined slightly from 59.59% on day 1 to 57.57% on day 7 (Fig. 4A1). Plasmatocytes did not differ for both treatments ($\chi2 = 3.0554$, df = 2, $p = 0.2170$) and days ($\chi2 = 5.0748$, df = 3, $p = 0.1664$). The percent number of plasmatocytes did not show any significant changes across treatments and days, except for *M. anisopliae* ICIPE 30-treated larvae on day 7 (16.55%) (Fig. 4B2).

At 30 °C, the total number of granulocytes (Fig. 4A3) differed between treatments ($\chi2 = 29.743$, df = 2, $p < 0.001$), days ($\chi2 = 25.003$, df = 3, $p < 0.001$), and there was an interaction between days and treatments ($\chi2 = 13.438$, df = 6, $p = 0.03659$). Total granulocytes in larvae exposed to *M. anisopliae* ICIPE 30 decreased in number from day 1 (53.89%) and continued to decline on days 3, 5, and 7 (48.33%, 40.83%, and 29.47%, respectively). *Metarhizium anisopliae* ICIPE 18-treated larvae had a gradual reduction in the number of granulocytes, with 58.50% on day 1, 55% on day 3, 51.34% on day 5, and 48.33% on day 7. However, the percent granulocytes in control larvae was also slightly reduced, from 59.24%, 57.27%, 54.79%, and 52.76% on days 1, 3, 5, and 7, respectively, without any significant variations.

The number of plasmatocytes also was significantly different across treatments ($\chi2 = 16.3538$, df = 2, $p < 0.001$) and days ($\chi2 = 11.4586$, df = 3, $p < 0.01$) but did not show any interaction between days and treatments ($\chi2 = 7.4801$, df = 6, $p = 0.28$). Total number of plasmatocytes in *M. anisopliae* ICIPE 30-treated larvae declined from day 1 (22.5%) to day 7 (14.74%) (Fig. 4B3). The same trend was observed in *M. anisopliae* ICIPE 18-treated larvae, but the percentages were higher than in *M. anisopliae* ICIPE 30-treated larvae, with 25.94% on day 1, 25.59% on day 3, 18.06% on day 5, and 16.67% on day 7. Control groups changed in the proportion of plasmatocytes from day 1 (24.95%) to day 5 (26.53%) and a slight decrease on day 7 (24.69%), but without any significant variations.

Microbiome of *Spoladea recurvalis*

The 16 S rRNA sequencing produced a total of 2,330,328 reads and the analysis revealed 1,119 bacteria genera in *S. recurvalis* gut. Their abundance per sample depended on the treatment, temperature and days post-infection. The most abundant bacterial genera were *Enterobacter*, *Enterococcus*, and *Klebsiella*. All bacterial genera with abundance below 0.5% were merged into others (Fig. 5).

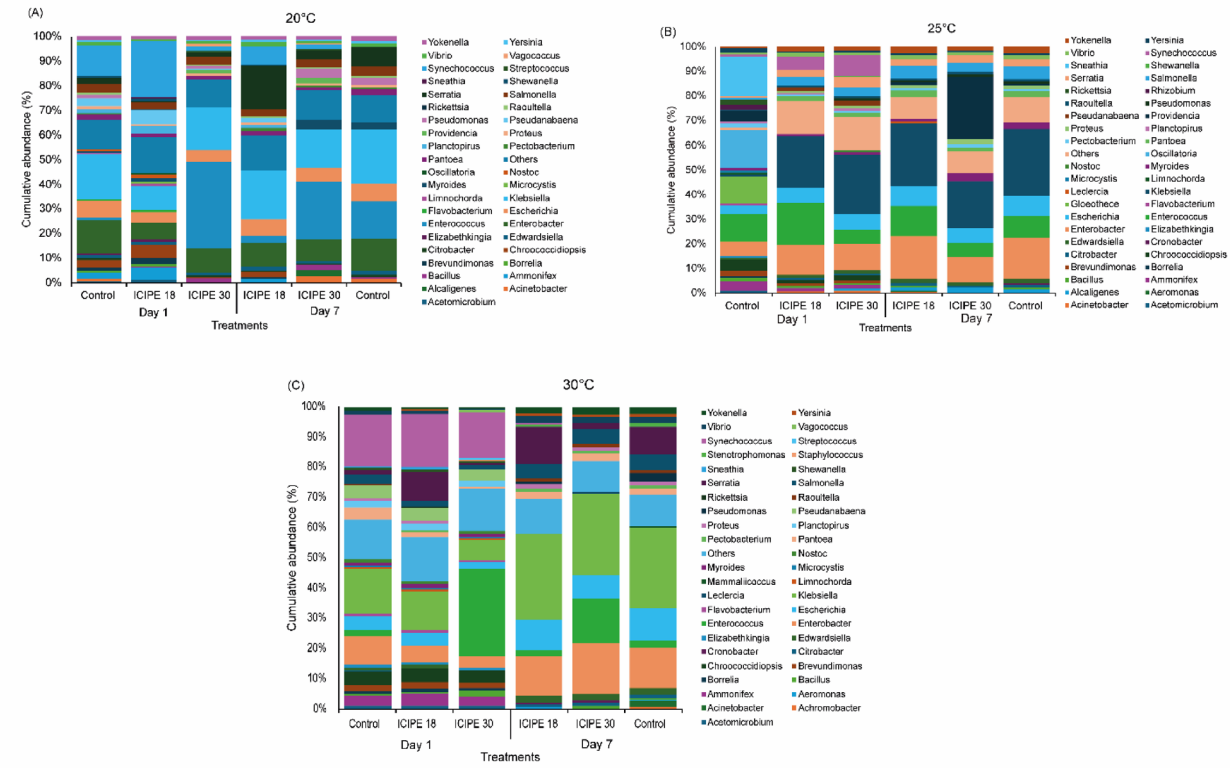

Fig. 5. Relative abundance of bacteria genera in *Spoladea recurvalis* gut after exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18 at 20 °C (**A**), 25 °C (**B**) and 30 °C (**C**). All bacteria genera below 0.5% abundance were grouped into others.

Enterococcus was abundant in larvae treated with *M. anisopliae* isolates ICIPE 30 and ICIPE 18 compared to controls. However, the abundance of *Enterococcus* was observed to decrease over time in fungal-isolate-exposed larvae. On the other hand, the abundance of *Enterococcus* in control larvae increased over time for all days and at various temperatures. The highest abundance of *Enterococcus* spp. (34.85%) was observed in larvae treated with *M. anisopliae* ICIPE 30 at 20 °C and 30 °C on day 1 (Fig. 5A and C).

Klebsiella abundance increased with time in the control larvae for all temperatures. The percentage abundance was observed rising from day 1 to 7 at 20 °C (18.30% to 21.99%), 25 °C (11.02% to 27.09%) and 30 °C (14.79% to 25.59%) (Fig. 5A–C). Larvae treated with *M. anisopliae* isolates ICIPE 30 and ICIPE 18 also showed an increase in *Klebsiella* abundance though their trend irregularly varied; for example, larvae treated with *M. anisopliae* ICIPE 30 showed a significant decline in *Klebsiella* abundance at 20 °C (17.22% on day 1 to 15.45 on day 7) and 25 °C (24.07% on day 1 to 19.29% on day 7). However, there was a rapid rise of *Klebsiella* spp. abundance in larvae treated with *M. anisopliae* ICIPE 30 at 30 °C from 6.59% on day 1 to 26.85% on day 7 (Fig. 5C).

Enterobacter spp. was abundant with time throughout all temperatures, regardless of the treatments, but there was a rapid rise in its abundance in larvae treated with *M. anisopliae* ICIPE 30 at 30 °C from 3.88% on day 1 to 16.93% by day 7. The same trend was observed in *M. anisopliae* ICIPE 18-treated larvae at 30 °C, where *Enterobacter* spp. abundance increased from 5.70% on day 1 to 13.16% by day 7 (Fig. 5C). The highest and rapid change of *Enterobacter* spp. abundance was observed in the larvae treated with *M. anisopliae* ICIPE 30 at 30 °C from 3.88% on day 1 to 16.93% on day 7 (Fig. 5C).

We also observed that *Serratia* spp. population/community had significant changes where its abundance increased with time, though this increase was slight but significant in larvae treated with *M. anisopliae* ICIPE 30 at 30 °C from 0.69% on day 1 to 2.16 on day 7 (Fig. 5C). The highest abundance was observed in larvae treated with *M. anisopliae* ICIPE 18 at 20 °C on day 7 (17.49%) (Fig. 5C), while the larvae that were exposed to *M. anisopliae* ICIPE 30 at 30 °C had their *Serratia* spp. abundance reduced to as low as 2.16% by day 7.

Alpha diversity of the bacteria varied depending on treatments, temperatures and days post-infection. It was observed that bacterial diversity reduced with time and temperature, influencing the alpha diversity. True Shannon (Fig. 6) ranged between 1.4 and 3.7 and the highest diversity (Index 3.6) was observed in the control larvae at 25 °C on day 1 (Fig. 6B), while the lowest (Index 2.7) was observed in *M. anisopliae* ICIPE 30 at 30 °C on day 7 (Fig. 6C). *Metarhizium anisopliae* ICIPE 30 at 30 °C led to the reduction in bacterial diversity from Index 3.2 on day 1 to 2.7 by day 7 (Fig. 6C).

The bacterial richness (Fig. 7A–C) ranged between 420 and 645 species across all treatments, temperatures and days post-infection. Larvae treated with *M. anisopliae* ICIPE 18 at 30 °C on day 7 showed the highest average species richness (442.3), while the lowest species richness was observed in the control larvae at 25 °C on day 7

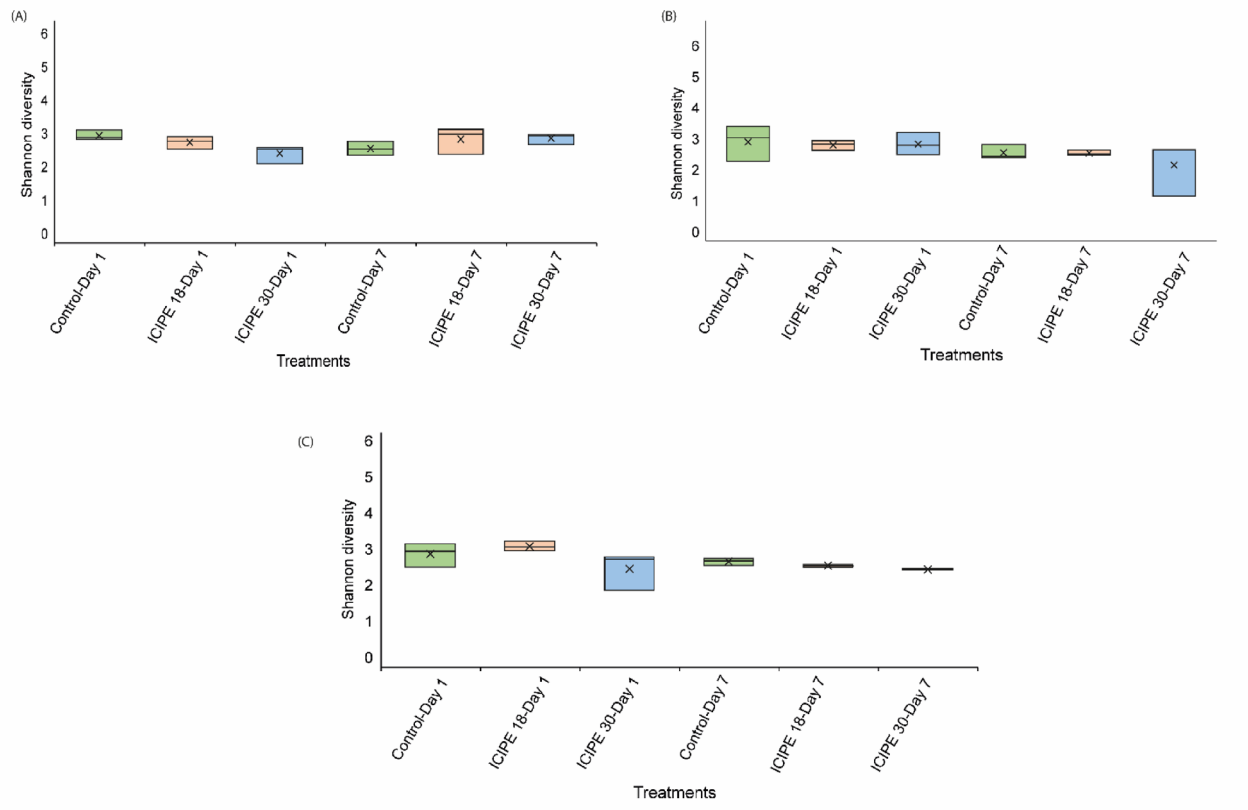

Fig. 6. Alpha diversity of bacteria (Shannon-Wiener Index) in *Spoladea recurvalis* gut after exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18 at 20 °C (**A**), 25 °C (**B**) and 30 °C (**C**).

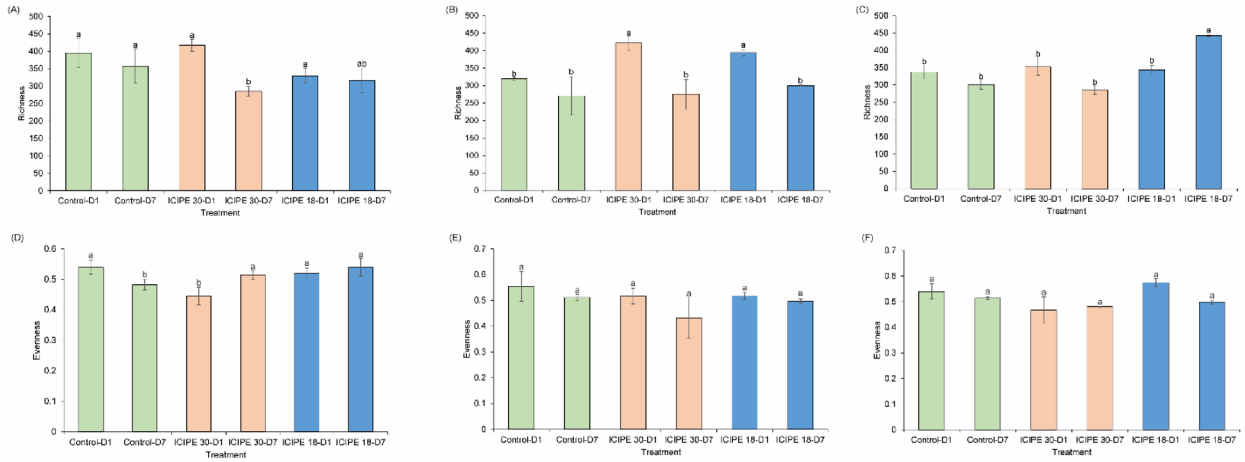

Fig. 7. Bacterial richness at 20 °C (**A**), 25 °C (**B**), 30 °C (**C**) and evenness at 20 °C (**D**), 25 °C (**E**), 30 °C (**F**) in gut of *Spoladea recurvalis* larvae post exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18.

(271). Bacterial evenness index (Fig. 7A–F) ranged between 0.4 and 0.6 across all samples. Bacteria distribution was even more (0.57) in control larvae at 25 °C on day 1. The lowest distribution was observed in larvae treated with *M. anisopliae* ICIPE 30 at 25 °C on day 7 (0.43).

Beta diversity (Fig. 8) was analyzed using the Jaccard dissimilarity index and the PCoA clustered samples differently per temperature. At 20 °C (Fig. 8A), the highest microbial diversity was observed between *M. anisopliae* ICIPE 30-treated larvae on day 1 and those treated with *M. anisopliae* ICIPE 18 on day 1 (68.43%), while the lowest was obtained between *M. anisopliae* ICIPE 30-treated larvae on day 7 and those in the control on day 1 (21.57%). For 25 °C (Fig. 8B), the highest diversity was recorded between *M. anisopliae* ICIPE 30-treated larvae on day 1 and day 7 (50.00%), while the lowest was observed between *M. anisopliae* ICIPE 30-treated larvae on day 7 and the control larvae on day 7 (16.30%). Finally, for larvae treated at 30 °C (Fig. 8C), the highest microbial diversity was observed between *M. anisopliae* ICIPE 30-treated larvae on day 1 and those treated with *M. anisopliae* ICIPE 18 on day 7 (68.44%), while the lowest was recorded between *M. anisopliae* ICIPE 30-treated larvae on day 7 and those in the control on day 7 (13.60%).

Discussion

Regardless of the number of studies conducted on the efficacy of entomopathogens on various insect pests, a number of avenues have remained unexplored[18,33,34]. In our study, we demonstrate the impact of entomopathogenic fungal isolates *M. anisopliae* ICIPE 30 and ICIPE 18 on both cellular and gut microbiome immunity of *S. recurvalis* at varying temperatures.

Cellular-wise, we noted differences in hemocyte counts for each treatment, with the highest hemocyte numbers recorded in larvae treated with *M. anisopliae* ICIPE 30 at 25 °C. It is likely that larvae were stable at the optimum temperature and initiated strong defense mechanisms upon infection, thus an increase in hemocyte counts[12,17]. While both isolates triggered an increase in hemocyte numbers across all temperatures and days, a decline was observed starting from day 5 going forward, with the highest decline observed in *M. anisopliae* ICIPE 30-treated larvae. These differences are relatable to the germination and mycelial growth rates of these isolates since effective germination and mycelial growth are important factors in terms of the pathogenicity and virulence of the entomopathogen after host pest infection[35,36]. The two fungal isolates have been studied, and it was established that *M. anisopliae* ICIPE 30 has higher germination and mycelial growth rates than *M. anisopliae* ICIPE 18 at 20, 25 and 30 °C[37,38].

Overall, across all fungal-treated larvae, a rapid rise in hemocyte numbers was recorded between day 1 and day 3 before a decline that continued up to day 7. It is possible that in the first days, larvae imposed a quick response to fight the fungal infection as it has been a defense mechanism of most lepidopteran insects, as reported by Gillespie et al.[39]. According to this study, circulating hemocytes in hemolymph can change rapidly in response to infection, and this translates to an initial increase in THC followed by a decline after the pathogen gains momentum[39]. This kind of decline has also been reported by Hung and Boucias[40] in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) after exposure to *Beauveria bassiana*. Furthermore, although we observed variations in hemocyte counts among the fungal-treated larvae, the control groups also exhibited slight, though statistically insignificant, changes under the different temperature regimes. However, hemocyte numbers in all control groups consistently remained lower than those in the treatment groups. This phenomenon could be attributed to the normal hemocyte changes that can happen during larval growth and instar transformations, as well as life changes[41,42]. Our results demonstrated that *S. recurvalis* immune responses differed depending on the incubation temperatures and the fungal virulence, which is in line with previous studies[17,43]. *Metarhizium anisopliae* fungal isolates have been reported to be more effective at 30 °C[37,38] and this may also explain the highest decline of hemocytes at 30 °C in this study.

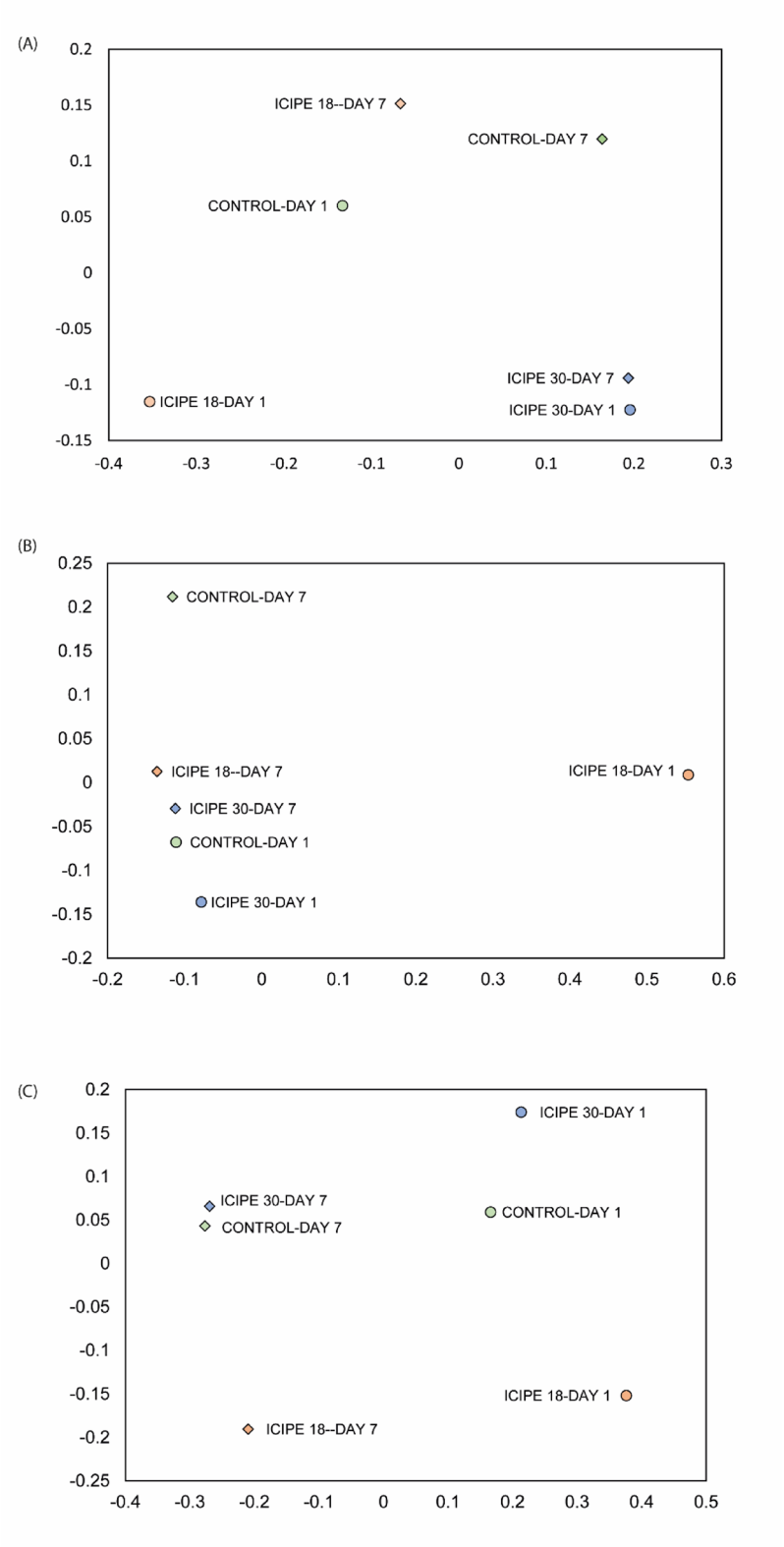

Fig. 8. Beta diversity of bacteria in the gut of *Spoladea recurvalis* larvae post exposure to *Metarhizium anisopliae* isolates ICIPE 30 and ICIPE 18 at 20 °C (**A**), 25 °C (**B**) and 30 °C (**C**).

In this study, we considered the identification, classification and counting of different cell types to ascertain the effect of the tested fungal isolates on cell types in the hemolymph of *S. recurvalis*. Notably, granulocytes and plasmatocytes were the most observed cell types across all the treatments though their total counts varied depending on the fungal isolate, temperature and days after infection. However, we noted that both hemocytes in fungal isolate-treated larvae at 20 °C showed a gradual increase up to day 7. This trend differs from that seen at other temperatures, where hemocyte levels drastically rose but later declined. We attribute this difference to the relatively unfavorable conditions at 20 °C, which may have hindered the fungal isolates from fully establishing early on. Nonetheless, they still triggered an immune response in the larvae, leading to a sustained increase in hemocyte levels[38].

On the other hand, the number of granulocytes in *M. anisopliae* ICIPE 30-treated larvae at 25 °C and 30 °C increased significantly right from day 1 and later started declining from day 3 however, the highest decline was noted at 30 °C by day 7. Insects use hemocytes to carry out mechanisms such as encapsulation and nodulation to fight pathogen invasion[25]; encapsulation involves hemocytes that activate and form multilayered capsules around the pathogens, while nodulation involves a number of cells that aggregate around a pathogen. Granulocytes and plasmatocytes are actively involved in defense against pathogen infection[26,27], and these granulocytes are the first hemocytes that initiate the encapsulation process[28,29]. It is possible that the increase in percentage granulocytes and plasmatocytes in the larvae was a response to infection with fungal isolates, while a decline might have been due to other processes, such as apoptosis, where cells can be damaged in the process of defense and hemolymph completely colonized[44].

The observed changes in total and differential hemocytes prompted us to explore the effect of *M. anisopliae* isolates ICIPE 18 and ICIPE 30 on the gut microbiome of *S. recurvalis*, where we identified over 1,000 bacterial genera, indicating a rich and diverse microbial community; consistent with previous findings that lepidopteran guts can harbor a vast number of bacteria, both pathogenic and non-pathogenic[45]. Overall, we observed low susceptibility to the tested fungal isolates in *S. recurvalis* larvae that exhibited higher bacterial abundance and diversity. Notably, larvae treated with fungal isolates at 20 °C and 25 °C on days 1 and 7, as well as at 30 °C on day 1, showed both higher bacterial abundance and diversity, which coincided with reduced susceptibility to the fungal isolates. Meanwhile, *M. anisopliae* ICIPE 30-treated larvae had the lowest bacterial abundance and diversity by day 7 and that might be why we observed the highest mortality. Several studies have reported similar results that are in line with ours, where gut bacteria are implicated to offer strong immunological responses, protecting insects from infections and biological control agents[46,47]. A study by Dillon and Charnley[48] reported a strong correlation between gut bacteria of the desert locust *Schistocerca gregaria* Forsskål (Orthoptera: Acrididae) and its defense against pathogens. Additionally[49], reported that lepidopteran insects use their gut microbiome to protect themselves from infection. Furthermore, for insect survival, it is critical for them to mount immunological responses against fungal infections, as also observed in our current study[33].

Some bacterial genera have been reported to have effects on the insect immune responses[50]; for example, *Enterococcus* can reduce entomopathogenic fungal spore germination, making the host insects resistant to infection[51]. In addition, *Enterococcus mundtii* has been reported to protect *Spodoptera littoralis* Boisduval (Lepidoptera: Noctuidae) from pathogens through resisting colonization of the gut by these external microbes[52,53]. Xia et al.[54] also reported that some species of *Enterobacter* can reduce larval mortality in high abundance of these bacteria. Furthermore, most gut bacteria can boost the immunity of their host; for example, Snyman et al.[55] noted that *Klebsiella* could produce specific enzymes that aid in digestion and facilitate good nutrition in *Busseola fusca* Fuller (Lepidoptera: Noctuidae), and it is known that insects can resist infections if their nutritional state is stable[56,57]. All these highlighted mechanisms could have contributed to larval resistance to fungal isolates before the gut bacterial community was disrupted by the tested fungal isolates.

Bacteria of the genus *Serratia* have also been reported to induce the immunity of lepidopteran insects such as *Hyphantria cunea* Drury (Lepidoptera: Erebidae)[58]. However, when the insect gut is destroyed, *Serratia* may shift and develop outside the gut[59]. This may explain why we observed a reduction in the abundance of *Serratia* in *M. anisopliae* ICIPE 30-treated larvae on day 7, indicating the most significant shift of microbiome structures and the highest larval mortality. Our results showed the lowest bacterial richness and evenness in *S. recurvalis* larvae treated with *M. anisopliae* ICIPE 30 at 30 °C by day 7. This finding presents evidence of dysbiosis compared to Maingi et al.[17], who observed similar results in *P. absoluta* when challenged with entomopathogenic fungal isolates.

Beta diversity, clustered differently the larvae treated with *M. anisopliae* ICIPE 30, ICIPE 18 and those in the controls. This result shows that treatments had a significant effect on the gut microbiome structure. The lowest microbial diversity was observed in larvae treated with *M. anisopliae* ICIPE 30 at 30 °C on day 7, and this suggests that *M. anisopliae* ICIPE 30 caused the highest shift in *S. recurvalis* gut microbiome structure. Generally, the gut microbiome was impacted by the entomopathogenic fungal isolates according to our cumulative abundance, beta and alpha diversity. Our results are in line with Maingi et al.[17], who also observed gut community disruption in *P. absoluta* post-infection with entomopathogenic fungi *M. anisopliae* ICIPE 20 and ICIPE 18.

Conclusion

Our study demonstrated that, at 30 °C, *M. anisopliae* ICIPE 30 could weaken the microbiome of *S. recurvalis* and lower cellular immunity. We also noticed that temperature influenced the immune response of the insect as well as the activity of the tested fungal isolates. Furthermore, *S. recurvalis* has a robust bacterial community that provides immunity to it when it gets infected but this could be degraded at favorable temperatures. An abundant and diverse gut microbiome was responsible for immunity in the *S. recurvalis* larvae, while dysbiosis did the opposite at 30 °C. Since *M. anisopliae* ICIPE 30 worked well at higher temperatures, it might be a good biocontrol option in the field and therefore, more research studies are warranted for its development as a biopesticide for sustainable management of *S. recurvalis* in vegetable cropping systems.

Materials and methods

Spoladea recurvalis colony

Spoladea recurvalis adults and larvae were obtained from amaranth fields in Transmara, Narok county (0°35′32.892″N 3°0′49.14″E) and Yatta, Machakos County (01°08.295′S 037°25.892′E) in May and June 2014 and a colony was established. At the start of this experiment, field collections were frequently done to infuse the colony to avoid inbreeding and here, the moths were maintained in ventilated sleeved Perspex cages (40 × 40 × 45 cm) and fed on 10% honey solution. Potted amaranth plants were placed in the cages for oviposition. After 24 h, plants were removed and relocated to a table and transferred into wooden cages (50 × 50 × 60 cm) until the eggs hatched. Three days later, leaves with larvae were removed from the plants and placed in clear plastic dishes (20 × 15 × 7 cm) lined with paper towel to absorb excess moisture and fine netting infused lid for ventilation. The larvae were daily supplied with fresh amaranth leaves to obtain the second instar larvae required for use in the bioassays. To maintain the colony, the remaining larvae were fed on new amaranth leaves every day until pupation. Using a fine camel hair-brush, the pupae were removed from the clear plastic dishes (20 × 15 × 7 cm) and placed into other clean clear plastic dishes (20 × 15 × 7 cm) and put into Perspex cages (40 × 40 × 45 cm) for adult emergence. The colony was kept at the temperature of 25 ± 2 °C, 50–70% Relative Humidity and 12:12 L: D photoperiod.

Fungal isolates

The entomopathogenic fungi (EPF) isolates, *Metarhizium anisopliae* ICIPE 30 and ICIPE 18, were obtained from *icipe*'s Arthropod Germplasm Centre. The isolates were sub cultured on SDA media using streaking method and maintained at 25 ± 2 °C in complete darkness. The method involves use of a fine streaking loop to evenly spread fungal spores on media to make a four quadrant streak lines. To obtain fungal suspensions, conidia were harvested from two-to-three-week-old fully mature plates using a sterile spatula and suspended in 10 ml of distilled water containing 0.05% Triton X- 100 (MERCK KGaA, Darmstadt, Germany) solution. The suspension was vortexed for five minutes at about 700 rpm to obtain a uniform/homogenous spore suspension. The conidial concentration was determined using a new improved Neubauer chamber and a light microscope where a serial dilution was conducted to adjust the suspension to 1×10^8 conidia/ml. Prior to the bioassays, a viability test was conducted where fungal suspensions were adjusted to a concentration of 3×10^6 conidia/ml and 0.1 ml of the suspension was equally spread over SDA plates. Three sterile microscope cover slips were placed on the surface of each plate and incubated at 25 ± 2 °C. After 18 h, conidia germination was examined by counting 100 randomly selected germinated conidia beneath each coverslip under a light microscope. Conidia were considered to have germinated when the length of the germ tube was at least twice the diameter of the conidium[60,61]. Four replicate plates were used per isolate, and viability of each isolate was determined, where more than 95% of the conidia germinated.

Laboratory experiment

Second instar larvae were exposed to *M. anisopliae* ICIPE 30 and ICIPE 18 at the temperatures of 15 20, 25 30 and 35 °C. Petri dishes were lined with two pieces of filter paper and 50 s instar larvae per EPF treatment, per temperature regime, were placed on the filter paper using a soft camel brush. These larvae were sprayed with 10 ml at the concentration of 1×10^8 conidia/ml of each of the fungal isolates ICIPE 30 and ICIPE 18 at room temperature using potter precision laboratory spray tower (Burkard Manufacturing Co Ltd, UK). Control groups were sprayed with 0.05% Triton X-100 (MERCK KGaA, Darmstadt, Germany) solution.

Sprayed larvae were transferred into clear plastic dishes (10 cm diameter), well-ventilated by a fine net on the lid lined with sterile paper towel and containing pre-sterilized fresh amaranth leaves. Larvae were kept under the various defined temperatures in MIR-554 cooled incubator (PHC Europe B.V, Netherlands), monitored daily and fed on fresh surface-sterilised amaranth leaves until the end of the experiment. Each set up was replicated four times. Surviving larvae were randomly selected at days 1, 3, 5 and 7 for the studies on immunological responses and the effects of the EPF on the gut microbiota of *S. recurvalis*. In addition, dead larvae were also recorded at days 1, 3, 5 and 7 to determine the mortality rates at the different temperature regimes. For all mortality observed, mycosis tests were conducted to confirm whether death was due to infection by the fungus used in the bioassays. The insect cadavers were surface sterilized with 70% ethanol, rinsed three times in distilled water, and then kept separately in 9 cm Petri dishes lined with sterile moistened filter paper to allow for fungal outgrowth and verify if mortality could be attributed to the respective fungal isolates they were treated with. Mortality due to fungal infection was confirmed by the presence of hyphae and conidia on the surface of the cadavers.

Hemocyte study

Hemocyte studies included total hemocyte count (THC) and differential hemocyte count (DHC). For THC, selected surviving larvae were put in sterile falcon tubes and placed in ice bucket for 10 min to inactivate them. Inactive larvae were then surface sterilized with 70% ethanol, 1.5% sodium hypochlorite followed by three washes of distilled water. Larvae were placed on sterile paper tower at room temperature for 10 min to allow draining of excess distilled water and returning to normal physiological processes. Larvae were carefully pierced using blood lancet and hemolymph collected using sterile micro-capillary glass tubes. Hemolymph was quantified to have 1 µl for THC and another 1 µl for DHC[17]. For THC, 1 µl of hemolymph was added in Eppendorf tube containing 9 µl of ice cold sterile anticoagulant buffer (186 mM NaCl, 41 mM citric acid, 98 mM NaOH and 17 mM EDTA), mixed well and placed in ice. The 10 µl mixture was loaded on hemocytometer and total cells counted under light microscope. THC was reported as "Number of cells/ml×10^4".

To identify and count different cell types, hemolymph was spread evenly on sterile glass slides and allowed to air dry at room temperature. The cells were fixed by dipping the slide in a fixative made by mixing methanol

and glacial acetic acid in 3:1 ratio for about 5 s followed by air drying at room temperature. Staining was done by dipping the dried slides in staining glasses containing 5% Giemsa/PBS (v/v) for 35–45 min. Slides were then gently rinsed with distilled water to remove excess stain and air dried at room temperature. Dry slides were viewed under light microscope (Leica DM 2500 LED, Leica Microsystems Wetzlar Germany) to identify and count the different hemocytes present. For each slide, 100 cells were counted and grouped into different cell types according to[62]. Images were captured using the Leica LASZ Software, Leica Microsystems Wetzlar Germany.

Gut microbiome study

To establish the effect of the tested fungal isolates on gut microbiota, the insect larvae were aseptically surface sterilized as described above and dissected to remove the gut. DNA was extracted from the gut using ISOLATE II Genomic DNA Kit, (Bioline, London, UK) according to the manufacturer's procedure. DNA quantity and purity was assessed using Nanodrop 2000 Spectrophotometer (Thermo Fischer Scientific, Wilmington, US), from where samples within A_{260nm}/A_{280nm} ratio of 1.8-2 were barcoded. Library preparation was done using the 16 S Barcoding Kit 1–24 (SQK-16S024, Oxford Nanopore Technologies, UK) following the manufacturer's protocol. The library was prepared using a 50 µl mix containing; PCR Water (20.7 µl) (New England Biolabs, US), MyTaq buffer (8 µl) (Meridian Biosciences, UK), MyTaq enzyme (0.5 µl) (Meridian Biosciences, UK), 25mM.$MgCl_2$ (0.8 µl) (New England Biolabs, US), 16 S Barcode (10 µl) (Oxford Nanopore Technologies) and DNA template (10 µl).

Amplification was done using Master Cycler Nexus gradient thermal cycler (Eppendorf, Germany) by running 1 cycle for 2 min at 95 °C for initial denaturation, 40 cycles for 30 s at 95 °C for denaturation, 40 cycles for 45 s at 55 °C for annealing, 40 cycles for 1 min at 72 °C for extension, 1 cycle for 10 min at 72 °C for final extension and then was held at 10 °C. Amplicons were then purified using AMPure beads following the ONT protocol and checked for quality and quantity using Nanodrop 2000 Spectrophotometer, sampled and pooled to a 1.5 ml Eppendorf DNA LoBind tube and 1 µl of RAP adapters (Oxford Nanopore Technologies, UK) added. This was mixed gently by flicking and down spinning, incubated at room temperature for 20 min before loading for sequencing.

The prepared library was loaded into a FLO-MIN106 flow cell (Oxford Nanopore Technologies, UK) and sequenced on an ONT MK1C device (Oxford Nanopore Technologies, UK) with live base-calling for 4 h.

Data analysis

Data from THC and larval mortality were subjected to a normality test using the Shapiro-Wilk test[63] and then analyzed through the Generalized Linear model with negative binomial regression in the MASS package[64]. DHC data were analyzed using beta regression implemented in the Betareg package[65]. GLM served as a unifying framework that generated a structured model object, which was then used in the subsequent ANOVA step to break down the variance and test the significance of specific factors. Post hoc was performed using the estimated marginal means with a Tukey adjustment using the R packages multcomp[66] and emmeans[67] to compare treatment means at $p \leq 0.05$. The analysis examined the effects of explanatory variables: temperature, fungal isolates, and the interaction with time on response variables, including total hemocyte density, hemocyte types, and larval mortality percentage. Model significance was assessed through analysis of deviance using Chi-square tests.

Sequencing data/reads were uploaded to the EPI2ME Desktop agent software (3.7.3)[68] to identify classified bacteria. Genera with cumulative abundance below 0.5% were classified as others. Alpha diversity[69] statistics, such as the Shannon-Wiener index, richness, abundance, and evenness, were used to evaluate bacterial diversity in each sample. The Jaccard[70] dissimilarity index was used to calculate beta diversity among bacterial genera in samples. Finally, using the "vegan"[71] package, the inter-sample microbiome relationship was calculated using principal coordinate analysis (PCoA). All the analyses were run in R. Software (4.3.1)[72].

Data availability

Sequences generated from this study were deposited in the GenBank database ([www.ncbi.nlm.nih.gov/genbank](http:/www.ncbi.nlm.nih.gov/genbank)) under the BioProject: PRJNA1328078. All other relevant data are within the paper.

Received: 31 October 2025; Accepted: 19 March 2026
Published online: 27 March 2026

References

1. Muriuki, E. N., Sila, D. N. & Onyango, A. Nutritional diversity of leafy amaranth species grown in Kenya. *J. Appl. Biosci.* **79**, 6818 (2014).
2. Wambugu, P. W. The State of Plant Genetic Resources for Food and Submitted To Fao Commission on Plant Genetic. (2009).
3. Achigan-Dako, E. G., Sogbohossou, O. E. D. & Maundu, P. Current knowledge on *Amaranthus* spp.: Research avenues for improved nutritional value and yield in leafy amaranths in sub-Saharan Africa. *Euphytica* **197**, 303–317 (2014).
4. Zipporah, N. W., Elizabeth, K., Nicholas, G. & Dorcus, M. Contribution of Amaranth Grain (A. Cruentus) on dietary intake and Nutritional Status of Adults Living with HIV in Mweiga, Nyeri, Kenya. *J. Basic. Appl. Sci. Res.* **4**, 196–203 (2014).
5. Kooten, O., Van Harbinson, J., Mose, L. O., Imungi, J. K. & Onyango, C. M. Feasibility of commercial production of amaranthus leaf vegetable by small-scale farmers In Kenya. In *9th African Crop Science Conference Proceedings, Cape Town, South Africa* 767–772, 14 (2009).
6. Aderolu, I. A. & Occurrence, O. A. Abundance and control of the major insect pests associated with Amaranths in Ibadan, Nigeria. *Entomol. Ornithol. Herpetol. Curr. Res.* **02**, (2013).
7. James, B. et al. Integrated pest management in vegetable production: A guide for extension workers in West Africa. *Trop. Agric.* (2010).
8. Kahuthia-Gathu, R. *Invasion of amaranth and spinach by the spotted beet webworm Hymenia species in Eastern Province of Kenya*. (2011).

9. Clarke-Harris, D., Fleischer, S., Fuller, C. & Bolton, J. Evaluation of the efficacy of new chemistries for controlling major lepidoptera pests on vegetable amaranth in Jamaica. *CARDI Rev.* 12–19 (2004).
10. Nampeera, E. L. et al. Farmers' knowledge and practices in the management of insect pests of leafy amaranth in Kenya. *J. Integr. Pest Manag.* **10**, (2019).
11. Carvalho, F. P. Pesticides, environment, and food safety. *Food Energy Secur.* **6**, 48–60 (2017).
12. Opisa, S., du Plessis, H., Akutse, K. S., Fiaboe, K. K. M. & Ekesi, S. Effects of Entomopathogenic fungi and Bacillus thuringiensis-based biopesticides on *Spoladea recurvalis* (Lepidoptera: Crambidae). *J. Appl. Entomol.* **142**, 617–626 (2018).
13. Miller, S. O. *Management of Spoladea recurvalis (Lepidoptera: Crambidae) on amaranths using biopesticides* (North- West University, 2019).
14. Imam, M., Hassan, N., Khatun, S. & Rahman, M. *Bacillus thuringiensis* increases the efficacy of bio-pesticides against eggplant shoot and fruit borer, *Leucinodes orbonalis* Guenee. *J. Nat. Pestic Res.* **6**, 100055 (2023).
15. Kryukov, V. Y. et al. Fungal infection dynamics in response to temperature in the lepidopteran insect *Galleria mellonella*. *Insect Sci.* **25**, 454–466 (2018).
16. Agbessenou, A., Akutse, K. S., Yusuf, A. A., Wekesa, S. W. & Khamis, F. M. Temperature-dependent modelling and spatial prediction reveal suitable geographical areas for deployment of two *Metarhizium anisopliae* isolates for *Tuta absoluta* management. *Sci. Rep.* **11**, 1–16 (2021).
17. Maingi, F., Akutse, K., Ajene, I., Omolo, K. & Khamis, F. Immunological responses and gut microbial shifts in *Phthorimaea absoluta* exposed to Metarhizium anisopliae isolates under different temperature regimes. *Front. Microbiol.* **14**, 1–16 (2023).
18. Maina, U. M., Um, M., Zakaria, D., Galadima, I. B. & Gambo, F. M. A review on the use of entomopathogenic fungi in the management of insect pests of field crops. *J. Entomol. Zool. Stud.* **6**, 27–32 (2018).
19. Shamakhi, L., Zibaee, A., Karimi-Malati, A. & Hoda, H. Effect of thermal stress on the immune responses of *Chilo suppressalis* walker (Lepidoptera: Crambidae) to Beauveria bassiana. *J. Therm. Biol.* **84**, 136–145 (2019).
20. Ferguson, L. V. & Sinclair, B. J. Insect Immunity Varies Idiosyncratically During Overwintering. *J. Exp. Zool. Part. Ecol. Integr. Physiol.* **327**, 222–234 (2017).
21. Zhang, J. et al. Variation in gut microbial communities of *Chilo suppressalis* in the typical bivoltine areas of northern China. *J. Appl. Entomol.* **146**, 860–874 (2022).
22. Wang, J. L. et al. Infection of the entomopathogenic fungus *Metarhizium rileyi* suppresses cellular immunity and activates humoral antibacterial immunity of the host *Spodoptera frugiperda*. *Pest Manag Sci.* **78**, 2828–2837 (2022).
23. Linder, J. E., Owers, K. A. & Promislow, D. E. L. The effects of temperature on host-pathogen interactions in *D. melanogaster*: Who benefits? *J. Insect Physiol.* **54**, 297–308 (2008).
24. Laughton, A. M., O'Connor, C. O. & Knell, R. J. Responses to a warming world: Integrating life history, immune investment, and pathogen resistance in a model insect species. *Ecol. Evol.* **7**, 9699–9710 (2017).
25. Dubovskiy, I. M., Kryukova, N. A., Glupov, V. V. & Ratcliffe, N. A. Encapsulation and nodulation in insects. *Invertebr Surviv J.* **13**, 229–246 (2016).
26. Pourali, Z. & Ajam, M. The effect of thermal stresses on the immune system of the potato tuber moth, *Phthorimaea operculella* (Lepidoptera: Gelechiidae). *J. Entomol. Soc. Iran.* **37**, 515–525 (2018).
27. Strand, M. R. & Lavine, M. D. Insect Hemocytes and Their Role in Immunity. *Insect Biochem. Mol. Biol.* **32**, 25–47 (2002).
28. Lavine, M. D. & Strand, M. R. Surface characteristics of foreign targets that elicit an encapsulation response by the moth *Pseudoplusia includens*. *J. Insect Physiol.* **47**, 965–974 (2001).
29. Pech, L. L. & Strand, M. R. Granular cells are required for encapsulation of foreign targets by insect haemocytes. *J. Cell. Sci.* **109**, 2053–2060 (1996).
30. Kryukov, V. Y. et al. Fungus *Metarhizium robertsii* and neurotoxic insecticide affect gut immunity and microbiota in Colorado potato beetles. *Sci. Rep.* **11**, 1–13 (2021).
31. Wei, G. et al. Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality. https://doi.org/10.1073/pnas.1703546114 (2017).
32. Fan Zhang, X. X. et al. W. The interactions between gut microbiota and entomopathogenic fungi: a potential approach for biological control of *Blattella germanica* (L). *Pest Manag Sci.* **74**, 438–447 (2017).
33. Zibaee, A. & Malagoli, D. Immune response of *Chilo suppressalis* Walker (Lepidoptera: Crambidae) larvae to different entomopathogenic fungi. *Bull. Entomol. Res.* **104**, 155–163 (2014).
34. Shen, D., Li, M., Chu, Y., Lang, M. & An, C. Cellular immune response of the Asian corn borer, *Ostrinia furnacalis* (Lepidoptera: Pyralidae), to infection by the entomopathogenic fungus, Beauveria bassiana. *Eur. J. Entomol.* **113**, 415–422 (2016).
35. Altinok, H. H., Altinok, M. A. & Koca, A. S. Modes of action of entomopathogenic fungi. *Curr. Trends Nat. Sci.* **8**, 117–124 (2019).
36. Mora, M. A. E., Castilho, A. M. C. & Fraga, M. E. Classification and infection mechanism of entomopathogenic fungi. *Arq. Inst. Biol. (Sao Paulo)*. **84**, 1–10 (2018).
37. Dimbi, S., Maniania, N. K., Lux, S. A. & Mueke, J. M. Effect of constant temperatures on germination, radial growth and virulence of *Metarhizium anisopliae* to three species of African tephritid fruit flies. *BioControl* **49**, 83–94 (2004).
38. Ekesi, S., Maniania, N. K. & Ampong-Nyarko, K. Effect of temperature on germination, radial growth and virulence of *Metarhizium anisopliae* and *Beauveria bassiana* on *Megalurothrips sjostedti*. *Biocontrol Sci. Technol.* **9**, 177–185 (1999).
39. Gillespie, J. P., Burnett, C. & Charnley, A. K. The immune response of the desert locust *Schistocerca gregaria* during mycosis of the entomopathogenic fungus, *Metarhizium anisopliae* var acridum. *J. Insect Physiol.* **46**, 429–437 (2000).
40. Hung, S. Y. & Boucias, D. G. Influence of *Beauveria bassiana* on the cellular defense response of the beet armyworm, *Spodoptera exigua*. *J. Invertebr Pathol.* **60**, 152–158 (1992).
41. Gardiner, E. M. M. & Strand, M. R. Hematopoiesis in larval *Pseudoplusia includens* and *Spodoptera frugiperda*. *Arch. Insect Biochem. Physiol.* **43**, 147–164 (2000).
42. Siddiqui, M. I. & Al-Khalifa, M. S. Review of haemocyte count, response to chemicals, phagocytosis, encapsulation and metamorphosis in insects. *Ital. J. Zool.* **81**, 2–15 (2014).
43. Mowlds, P. & Kavanagh, K. Effect of pre-incubation temperature on susceptibility of *Galleria mellonella* larvae to infection by *Candida albicans*. *Mycopathologia* **165**, 5–12 (2008).
44. Altunta, H., Kili, A. Y., Ukan, F. & Ergin, E. Effects of gibberellic acid on hemocytes of *Galleria mellonella* L. (lepidoptera: Pyralidae). *Environ. Entomol.* **41**, 688–696 (2012).
45. Douglas, A. E. Multiorganismal insects: Diversity and function of resident microorganisms. *Annu. Rev. Entomol.* **60**, 17–34 (2015).
46. Ruokolainen, L., Ikonen, S., Makkonen, H. & Hanski, I. Larval growth rate is associated with the composition of the gut microbiota in the *Glanville fritillary* butterfly. *Oecologia* **181**, 895–903 (2016).
47. Yang, H. T. et al. Pathogen invasion changes the intestinal microbiota composition and induces innate immune responses in the zebrafish intestine. *Fish and Shellfish Immunology* **71** (Elsevier Ltd, 2017).
48. Dillon, R. & Charnley, K. Mutualism between the desert locust *Schistocerca gregaria* and its gut microbiota. *Res. Microbiol.* **153**, 503–509 (2002).
49. Wu, K. et al. Gut immunity in Lepidopteran insects. *Dev. Comp. Immunol.* **64**, 65–74 (2016).
50. Mollah, M. I., Choi, H. W., Yeam, I. & Lee, J. M. Salicylic acid, a plant hormone, suppresses phytophagous insect immune response by interrupting. **12**, (2021).
51. Zhang, X. et al. The gut commensal bacterium *Enterococcus faecalis* LX10 contributes to defending against Nosema bombycis infection in Bombyx mori. *Pest Manag Sci.* **78**, 2215–2227 (2022).

52. Shao, Y. et al. Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota. *Cell. Chem. Biol.* **24**, 66–75 (2017).
53. Shao, Y., Arias-Cordero, E., Guo, H., Bartram, S. & Boland, W. In vivo Pyro-SIP assessing active gut microbiota of the cotton leafworm, *Spodoptera littoralis*. *PLoS One*. **9**, 1–13 (2014).
54. Xia, X. et al. DNA sequencing reveals the midgut microbiota of diamondback moth, *Plutella xylostella* (L.) and a possible relationship with insecticide resistance. *PLoS ONE* **8**, (2013).
55. Snyman, M., Gupta, A. K., Bezuidenhout, C. C., Claassens, S. & van den Berg J. Gut microbiota of *Busseola fusca* (Lepidoptera: Noctuidae). *World J. Microbiol. Biotechnol.* **32**, (2016).
56. Chandra, R. K. Nutrition, immunity and infection: From basic knowledge of dietary manipulation of immune responses to practical application of ameliorating suffering and improving survival. *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14304–14307 (1996).
57. Lochmiller, R. L. & Deerenberg, C. Trade-offs in evolutionary immunology: just what is the cost of immunity? *OIKOS* **88**, 87–98 (2000).
58. Wang, Z., Feng, K., Tang, F. & Xu, M. Activation of the host immune response in *Hyphantria cunea* (Drury) (lepidoptera: Noctuidae) induced by serratia marcescens bizio. *Insects* **12**, 1–17 (2021).
59. Peng, Y. et al. Reconstruction of Gut Bacteria in *Spodoptera frugiperda* Infected by Beauveria bassiana Affects the Survival of Host Pest. *J. Fungi*. **9**, 906 (2023).
60. Horner, W. E., Lehrer, S. B. & Salvaggio, J. E. Fungi. *Immunol. Allergy Clini. North Am.* **14** (1994).
61. Inglis, G. D., Enkerli, J. U. E. R. G. & Goettel, M. S. *Laboratory techniques used for entomopathogenic fungi: Hypocreales. Manual of techniques in invertebrate pathology*. **2** (2012).
62. Qyli, M., Aliko, V. & Faggio, C. Physiological and biochemical responses of Mediterranean green crab, *Carcinus aestuarii*, to different environmental stressors: Evaluation of hemocyte toxicity and its possible effects on immune response. *Comp. Biochem. Physiol. Part. - C Toxicol. Pharmacol.* **231**, 108739 (2020).
63. Shapiro, S. S. & Wilk, M. B. An analysis of variance test for normality (complete samples). *Biometrika* **52**, 591–611 (1965).
64. Venables, W. N. & Ripley, B. D. MASS: Support Functions and Datasets for Venables and Ripley's MASS. https://cran.r-project.org/package=MASS (2022).
65. Cribari-neto, F. & Gruen, B. Package 'Betareg". Beta regression in R'. *J. Stat. Softw.* **34**, 1–24 (2022).
66. Bretz, F., Westfall, P., Heiberger, R. M., Schuetzenmeister, A. & Scheibe, S. Package 'Multcomp': simultaneous inference in general parametric models. (2022).
67. Lenth, R. V. Emmeans estimated marginal means, aka least-squares means. (2022).
68. Oxford Nanopore Technologies. EPI2ME Desktop Agent software. https://nanoporetech.com/software/other/epi2me-desktop-application?utm_source=chatgpt.com (2025).
69. Magurran, A. E. *Measuring Biological Diversity* (Blackwell Publishing, 2004).
70. Jaccard, P. Étude comparative de la distribution florale dans une portion des Alpes et des Jura. *Bull. la. Société Vaudoise des. Sci. Nat.* **37**, 547–579 (1901).
71. Oksanen, J. et al. The vegan package. *J. Veg. Sci.* **14**, 927–930 (2007).
72. Team, R. C. R: A language and environment for statistical computing. https://www.r-project.org/ (2024).

Acknowledgements

The authors gratefully acknowledge the *icipe* core funding provided by the Swedish International Development Cooperation Agency (Sida); the Swiss Agency for Development and Cooperation (SDC); the Australian Centre for International Agricultural Research (ACIAR); the Government of Norway; the German Federal Ministry for Economic Cooperation and Development (BMZ); and the Government of the Republic of Kenya. The first author was also supported through the Inter-University Council for East Africa (IUCEA) of the East African Community (EAC) Scholarship Programme. We are thankful to Jane Kimemia, Sospeter Wafula, Felix Muthama, and Levi Ombura for their technical assistance. The views expressed herein do not necessarily reflect the official opinion of the donors.

Author contributions

Conceptualization: KSA and FMK, Funding acquisition: KSA, Experimental design: AB, KSA, IJA and FMK, Software: AB, IJA and KSA, Investigation: AB, Visualization: AB, KSA, IJA and FMK, Resources: KSA and FMK; Supervision: MM, ESN, KSA and FMK, Project administration: KSA, Writing—original draft preparation: AB, Writing—review and editing: AB, FMK, KSA, IJA, MM, ESN. All authors have read and approved the manuscript.

Funding

This research was funded by Biovision Foundation and Liechtensteinischer Entwicklungs Dienst (LED) through AGROVEG project "Intensified agroecological-based cropping systems to enhance food security, environmental safety, and income of smallholder producers of crucifers and African traditional vegetables in East Africa" (DPP-020/2022–2024).

Declarations

Competing interests

The authors declare no competing interests.

Additional information

Correspondence and requests for materials should be addressed to F.M.K. or K.S.A.