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ORIGINAL PAPER

Bacillus subtilis and *B. licheniformis* Isolated from *Heterorhabditis indica* Infected Apple Root Borer (*Dorysthenes huegelii*) Suppresses Nematode Production in *Galleria mellonella*

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Abstract

Purpose *Heterorhabdits indica* successfully controlled apple root borer *Dorysthenes huegelii* in the orchards, but nematode-infected cadavers revealed the presence of non-symbiotic bacterial *B. subtilis* and *B. licheniformis,* and no subsequent generations of *H. indica* were produced (hampered recycling phenomenon). Intrigued, we tested the effect of the two *Bacillus* species on symbiotic association of *H. indica—Photorhabdus luminescens.*

Methods One-to-one competitive parallel line *in vitro* assays were carried out between *P. luminescens* and the two *Bacillus* spp., while *in vivo H. indica* development was studied on the test insect *Galleria mellonella* which were fed with *Bacillus* mixed diet, followed by nematode exposure.

Results Where *P. luminescens* was flanked by either of the two *Bacillus* species, only *B. subtilis* significantly suppressed its growth, while in reversed assays both the *Bacillus* growth was unaffected. *Heterorhabditis indica* was able to kill *Galleria* larvae pre-fed with the two *Bacillus spp.*; these cadavers did not develop the characteristic evenly distributed brick red coloration. Besides *P. luminesecns*, both *Bacillus* spp. were found to coexist in these cadavers. Development of hermaphrodites was not affected, but second-generation females, and final nematode progeny was reduced significantly. Monozenic lawns of *B. subtilis* and *B. licheniformis* did not support *H. indica* development.

Conclusion These results show the reduced development of *H. indica* by the presence of the non-symbiotic bacteria in *G. mellonella* is likely to affect their ability to recycle in other insect larvae. Reduced recycling caused by non-symbiotic bacteria will reduce the overall long-term pest control benefits and have implications in the development of application strategies using entomopathogenic nematodes (EPNs) as insect control agents.

Keywords *Bacillus. subtilis* · *B. licheniformis* · *P. luminescens* · *H. indica* · *G. mellonella* · Symbiosis · Re-cycling

Introduction

The entomopathogenic nematode belonging to genera *Heterorhabditis* with the aid of its symbiotic gram negative entero-bacterium *Photorhabdus* confer efficient mortality to insect pests and widely used in inundative biological pest control programmes [1]. True mutualism exists between the two—(i) the bacteria needs the nematode to be vectored inside the insect host; and (ii) the nematode relies upon the bacterial symbiont to kill the host, preserve the resulting cadaver, and create a nutrient-rich environment for its development [2, 3].

The commercial success of *Heterorhabditis* spp. is well documented in the fields especially against the coleopteran pests [4, 5]. In recent years the apple root borer, *Dorysthenes huegelii* Redtenbacher 1848 (Coleoptera: Cerambycidae) is devastating the orchards in the Indian state of Himachal Pradesh located in the Himalayan region at 2150 m altitude. Due to strict regulations on pesticide usage, the management of *D. huegelii* by non-chemical methods is given a serious priority to secure the livelihood of more than two hundred-thousand farmers cultivating apple in over a

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hundred-thousand-hectare area [6]. Soil application of *H. indica* for over two seasons (2015–2016 and 2016–2017) provided effective mortality to *D. huegelii*, but the nematode failed to develop beyond the hermaphrodite stage in eighty per cent of the cadavers, collected and observed, post-application from the treated orchards (Mohan, unpublished). Reports suggest that the susceptible target and non–target host insects can contribute significantly to increase and conserve their numbers to prolong pest suppression and reduce the need for subsequent applications [7–11], however, recycling of EPN under field conditions could be hampered by various abiotic (temperature, humidity) [12] and biotic factors (microbial antagonists) [13–16]. Presence of asymptomatic contaminant *Sphingomonas koreensis* suppressed the growth and reproduction on *H. indica in vivo* [17].

We observed that nematode infected *D. huegelii* cadavers did not exhibit the characteristic uniform brick red coloration typical of *H. indica* infection. Besides *Photorhabdus luminescens*, the symbiont of *H. indica*, we could isolate *Bacillus subtilis* and *B. licheniformis* predominantly present in these cadavers [18]. We hypothesized that the two *Bacillus* species might be one of the limiting factors towards a successful *H. indica—P. luminescens* symbiosis and nematode development. Intrigued by the observations in the orchard we investigated the individual roles of the two *B. subtilis* and *B. licheniformis* on the growth and development of *H. indica* and *P. luminescens* in the greater-wax moth larvae, *Galleria mellonella* as the test insect. The study could not be carried out on *D. huegelii* because of their cryptic habitat (tunnel inside the roots) they cannot be collected in required numbers.

Materials and Methods

Bacterial Isolates

B. subtilis (KU894788) and *B. licheniformis* (KU894782) originally isolated from field population of *H. indica* infected *D. huegelii* cadavers collected from nematode treated apple orchard were selected for the studies [17]. Fresh cultures were prepared in Nutrient Broth (Hi Media Cat No. M002) incubated at 30 °C overnight to obtain approximately 1.5×10^8 cfu/ml. *Photorhabdus luminescens* was isolated and purified from the infective juveniles (IJ) of *H. indica* (IARI strain) [19] following the routine procedure described by Akhurst [20]

Competitive Bioassays of *B. subtilis* and *B. licheniformis* with *P. luminescens*

Reciprocal parallel line bioassays were carried out to evaluate the growth response of *B. subtilis* and *B. licheniformis* on *P. luminescens*, and vice versa [17]. In the first experiment the two non-symbiotic bacteria were individually streaked in the centre between two parallel streaks of *P. luminescens* at 1 cm eqi-distance on 90 mm Nutrient Agar plates (Hi Media, Cat No.012). In the reverse bioassay, a *P. luminescens* streak was flanked individually by each of the two *Bacillus* spp. Control streaks for each bacterium (i.e. no flanking bacteria) were maintained on separate plates. All the tests were replicated five times. Each streak was marked with five random points before incubating the plates at 30 °C for 48 h. The width of the streak was measured at the pre-determined points which were averaged to obtain the growth of individual bacterium.

Effect of *B. subtilis* and *B. licheniformis* on the Tripartite Interaction Involving *H. indica*, *P. luminescens* and *G. mellonella*

Axenic *Galleria* larvae were obtained as per Han and Ehlers [21]. The experiment consisted of two treatments in which the larvae were orally fed with 30 g of artificial *Galleria* diet (Wheat flour 200 g, Maize flour 200 g, Milk powder 75 g, Yeast extract 25 g, Glycerol 100 ml, Honey 100 ml) mixed with freshly prepared overnight broth culture of either *B. subtilis* or *B. licheniformis* (1.5×10^6 cfuml^{-1}). The control larvae were fed with Nutrient Broth mixed diet and artificial diet alone. Twenty larvae each for the two treatments and two controls were maintained at 28 °C for 15 days. The initial and final weights of the larvae were recorded. Preliminary screenings had indicated mortality in larvae fed with *B. subtilis* mixed diet; therefore, taking this into account, a separate set of 60 larvae was maintained as reserve and those larvae which died after 15 days were replaced with the live ones to complete the experimental set-up. Subsequently, each larva was individually infected with 30 *H. indica* IJs in 35 mm plastic Petri dishes lined with filter paper discs.

Observations on the development of hermaphrodites was recorded on the 3rd day and amphimictic females on the 6th day by dissecting 5 *Galleria* cadavers for each treatment. Simultaneously, the symbiotic (*P. luminescens*) and non-symbiotic (*B. subtilis* and *B. licheniformis*) were re-isolated from the hemolymph of these cadavers. Five cadavers were placed on the White's Trap on the 8th day for counting the final IJ emergence and the remaining 5 were dissected to observe the three bacterial populations coinciding with IJ emergence.

Before dissecting, the cadavers were surface sterilized by dipping in 70% ethanol, passed over a flame for 3 s and plunged in sterile distilled water. They were carefully cut open longitudinally from ventral side. Using sterile loop 5 aliquots (0.005 ml each) of haemolymph were taken from 5 different points and pooled in separate eppendorph for each

Galleria. The colony forming unit (cfu) counts were made by spreading 100 µl of 10^{-7} dilutions of each replicate.

In Vitro Development of Axenic *H. indica* on *B. subtilis* and *B. licheniformis*

Lawns of *B. subtilis* and *B. licheniformis* were prepared overnight on Lipid Agar media (NB 16 g + Agar 12 g + Sunflower Oil 5 ml + Distilled water 1 L). Hermaphrodites were dissected from pre-infected *Galleria* cadavers and rinsed in Ringer's Solution (×2) to clean any adhering debris. To surface sterilize they were suspended in 0.1% Merthiolate for 2 h with intermittent agitation to release the eggs. The deposited eggs were separated and re-suspended in fresh Merthiolate for another 2 h [22]. After rinsing with sterile distilled water, approximately 100 eggs were transferred on the bacterial lawns, with three replications, and incubated at 28 °C. Plates with *P. luminescens* lawns served as positive control. The hatching of the eggs and subsequent nematode development was observed at 24 h interval till 96 h.

Statistical Analysis

The experiments were performed twice in time with similar results and the data from the latest experiments are presented. The data for 2.2 was subjected to *t* Test. Based on the Mean (*M*) and Standard Error (SE) values of $P < 0.01$ were considered statistically significant. The data for 2.3 was subjected to Analysis of Variance using SAS 9.3 (Statistical Analysis Software). Significant and non-significant differences between the treatments were tested using Least Significant Difference (LSD). The values of $P < 0.05$ were considered statistically significant. In the figures, the letters "a, b, c" are used to denote significant difference between the treatments.

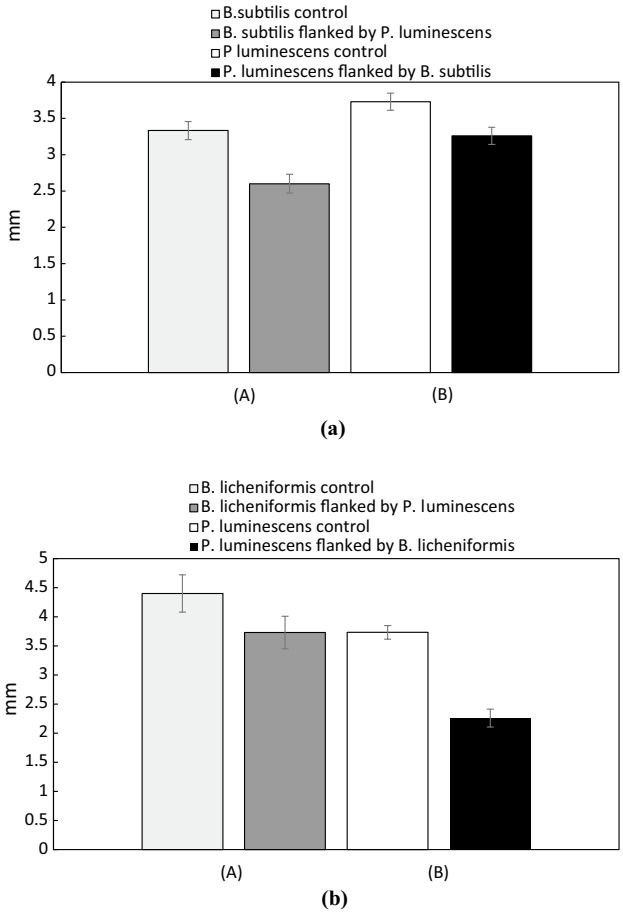

Fig. 1 **a** Bacterial growth in parallel line assays (A) Growth of *B. subtilis* when flanked between *P. luminescens* ($t = 1.147$; df (14); $P < 0.01$); (B) Growth of *P. luminescens* when flanked between *B. subtilis* ($t = 0.0067$; df (14); $P < 0.01$). Error bars represent the mean SE. **b** Bacterial growth in parallel line assays (A) Growth of *B. licheniformis* when flanked between *P. luminescens* ($t = 0.077$; df (14); $P < 0.01$); (B) Growth of on *P. luminescens* when flanked between *B. licheniformis* ($t = 0.000001160$; df (14); $P < 0.01$) Error bars represent the mean SE

Results

Competitive Parallel Line Bioassay

In parallel line bioassays *P. luminescens* ($M = 3.33$; SE = 0.125) suppressed the growth of *B. subtilis* ($M = 2.6$; SE = 0.130) non-significantly ($t = 1.147$; df (14); $P < 0.01$) whereas *B. subtilis* ($M = 3.73$; SE = 0.118) significantly suppressed ($t = 0.0067$; df (14); $P < 0.01$) the growth of *P. luminescens* ($M = 3.26$; SE = 0.118) (Fig. 1a). Similarly, *P. luminescens* ($M = 4.4$; SE = 0.320) did not suppress ($t = 0.077$; df (14); $P < 0.01$) the growth of *B. licheniformis* ($M = 3.73$; SE = 0.28), but *B. licheniformis* ($M = 3.73$; SE = 0.118) significantly suppressed ($t = 0.000001160$; df (14); $P < 0.01$) the growth of *P. luminescens* ($M = 2.26$; SE = 0.153) (Fig. 1b).

Effect of *B. subtilis* and *B. licheniformis* on the Tripartite Interaction Involving *H. indica, P. luminescens* and *G. mellonella*

After 15 days of feeding, there was a significant increase (LSD = 0.04; $P < 0.05$; SD = 0.07) in the weight of *Galleria* larvae from initial 0.15–0.26 g and 0.28 g having fed with diet alone and NB mixed diet, respectively, when compared to 0.17 g for those fed with *B. subtilis* and 0.16 g for *B. licheniformis* mixed diets (Fig. 2). Seventy percent of the larvae fed with *B. subtilis* died by the 15th day and were replaced with the ones which were live in the reserve set. Subsequently, the larvae treated with the two *Bacillus* species and the controls were infected with *H. indica* which resulted in 100% mortality within 24 h. Initially all

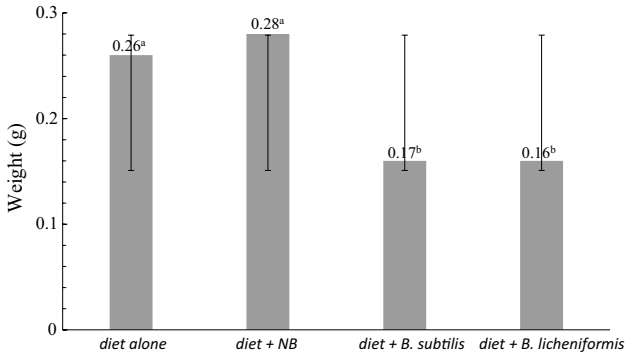

Fig. 2 Weight of *Galleria* larvae after 15 days of feeding with different diets (LSD = 0.04; $P < 0.05$; SD = 0.07). NB: Nutrient Broth; Bars having same alphabets in superscript are not significantly different

the larvae took characteristic pink coloration, but by day 3 the *Bacillus* treated larvae exhibited uneven coloration with patches of black and grey; unlike in control larvae which appeared dark brick red in color.

There was no significant difference in the production of hermaphrodites in *B. subtilis* (LSD = 12.35; $P < 0.05$) and *B. licheniformis* (LSD = 11.55; $P < 0.05$) fed cadavers on day 3 (Fig. 3a, b). On day 6, the development of females significantly declined in the two treatments (LSD = 155.25; $P < 0.05$) and (LSD = 154.65; $P < 0.05$), respectively (Fig. 4a, b); while the final nematode emergence significantly declined in *B. subtilis* (LSD = 20890.50; $P < 0.05$) and *B. licheniformis* (LSD = 23174.42; $P < 0.05$) (Fig. 5a, b) fed cadavers on day 8. The bacterial propagation on day 3, 6 and 8 coinciding with the nematode development was recorded and the log transformed values of the colony forming units (cfu) are presented in Fig. 6 a, b. The bacterial load of the two *Bacillus* spp. was negligible in the hemolymph on day 3; but interestingly, both *B. subtilis* (4.4×10^8) and *B. licheniformis* (2.5×10^9) species over-grew *P. luminescens* (8.4×10^2 and 5.6×10^2) in their respective treatments on day 8; however, the growth of *P. luminesens* remained similar to that of control (1.4×10^3).

We had also fed a set of 15 *Galleria* larvae on diet mixed with both the *Bacillus* spp. After 15 days only 2

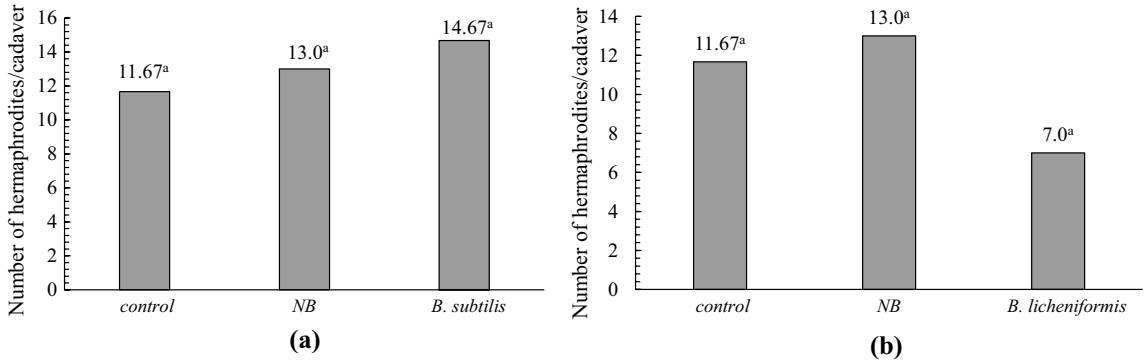

Fig. 3 Development of hemaphrodites in *Galleria* larvae fed with (A) *Bacillus subtilis* (LSD = 12.35; $P < 0.05$) and (B) *B. licheniformis* (LSD = 11.55; $P < 0.05$); Bars having same alphabets in superscript are not significantly different

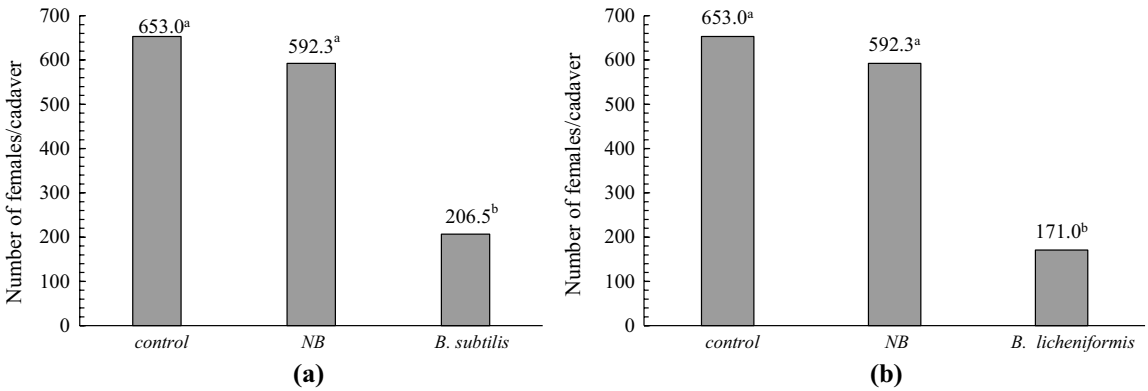

Fig. 4 Development of females in *Galleria* larvae fed with (A) *Bacillus subtilis* (LSD = 155.25; $P < 0.05$) and (B) *B. licheniformis* (LSD = 154.65; $P < 0.05$); Bars having same alphabets in superscript are not significantly different

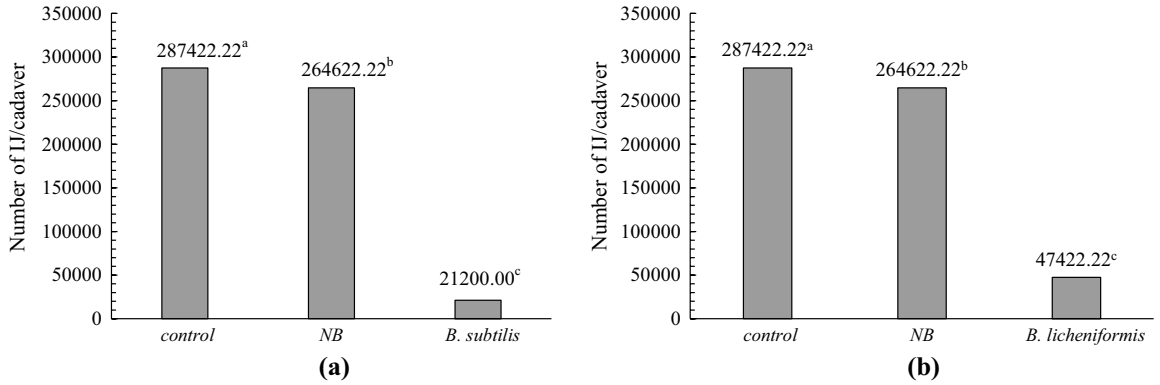

Fig. 5 Development of infective juveniles in *Galleria* larvae fed with (A) *Bacillus subtilis* (LSD = 20890.50; $P < 0.05$) and (B) *B. licheniformis* (LSD = 23174.42; $P < 0.05$); Bars having same alphabets in superscript are not significantly different

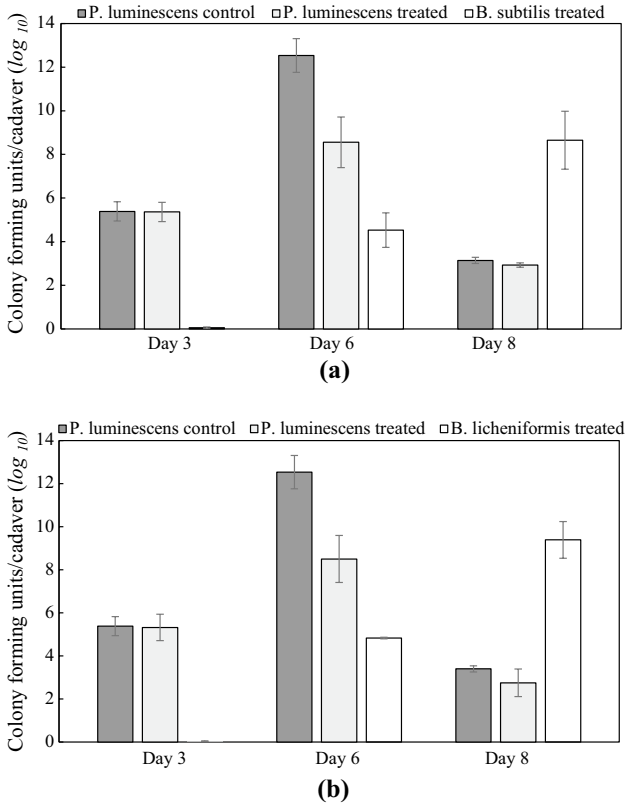

Fig. 6 **a** Comparative growth of *P. luminescens* in *Galleria* fed with artificial diet and *B. subtilis* mixed diet (bars indicate standard deviation on log transformed means of colony forming units). **b** Comparative growth of *P. luminescens* in *Galleria* fed with artificial diet and *B. licheniformis* mixed diet (bars indicate standard deviation on log transformed means of colony forming units)

larvae survived which when infected with *H. indica* died within 24 h. Upon dissecting these cadavers on the 3rd day to observe the hermaphrodites, we found dead *H. indica* IJs without any evidence of development (data not presented).

In Vitro Development of Axenic *H. indica* on *B. subtilis* and *B. licheniformis*

The axenic eggs hatched and nematode development was evident within 48 h on *Photorhabdus* lawns (control). However, only 80% of the eggs hatched within 24 h on the lawns of *B. subtilis* and *B. licheniformis,* and the juveniles died within 48 h. Embryological development was not detected in the unhatched eggs under the compound microscope till 96 h which clearly indicated that they were dead. Therefore, the experiment was terminated (data not included).

Discussion

Our results indicate that the presence of non-symbiotic bacteria *B. subtilis* and *B. licheniformis* can have an overall suppressive effect on the *H. indica—P. luminescens* symbiotic expression. *Photorhabdus* is known to secrete antibiotics, antibacterial and antifungal compounds to significantly reduce or eliminate populations of microorganisms mainly emerging from the insect intestinal microflora that are likely to compete with them for food [23, 24]. The bacterium creates a monoxenic environment conducive for its symbiont nematode to complete the life cycle and emerge in large numbers [2]; to make them available for the subsequent insect control in the fields. The *B. subtilis* group, which includes *B. licheniformis*, produce numerous antimicrobial compounds displaying a broad range of biological functions favouring their ubiquitous distribution in soil, aquatic environments, food and gut microbiota of arthropods and mammals [25–28]. Therefore, they possibly negatively interacted with *P. luminescens* to affect nematode development and reproduction. In the *in vitro* line assays the growth of *P. luminescens* declined by 15.78% and 28.94% when sandwiched between *B. subtilis* and *B. licheniformis*, respectively, but interestingly when *P. luminescens* was vectored

by its symbiont *H. indica*, it was able to impart mortality to *Galleria* larvae pre-fed with the two *Bacillus* species.

B. licheniformis was non-pathogenic to *Galleria* larvae when fed orally, but 70% of them fed with *B. subtilis* mixed diet died within 15 days. Insecticidal effect of *B. subtilis* has been widely reported against larval and pupal stages of mosquitoes [29, 30], *S. littoralis* [31, 32], *Ephestia kuehniell* [33], *S. litura* [34] and *Ectomyelois ceratoniae* [35], *Drosophilla melanogaster* [36]. Ramachandran et al. [26] reported an antimicrobial substance RLID 12.1 in *B. subtilis* having biocontrol potential against drug-resistant pathogens, while *B. licheniformis* is reported to possess antifungal properties [37, 38]. Pre-exposure of host insect to non-pathogenic bacteria or low dose of pathogenic microorganisms can provide some degree of protection against a subsequent pathogenic infection which has been documented in *Manduca sexta* [39], *Trichoplusia ni* [40], *Apis mellifera* [41], *Bombyx mori*, [42] *Bombus terrestris* [43] Patrnogic et al. [44] did not observe any prolongation in the life span of *D. melanogaster* pre-exposed to non-pathogenic strain of *Escherichia coli* alone or in combination with *Micrococcus luteus*, followed by introducing *P. luminescens* and *P. asymbiotica*, although there was an upregulation in the antibacterial peptide immune response in the young adult flies. In our studies *Galleria* pre-fed either with *B. licheniformis* and *B. subtilis* succumbed to the combined pathogenic effect of *H. indica—P. luminescens* symbionts.

Further, the initial development of hermaphrodite from the penetrated IJs was not affected significantly in the haemolymph. Possibly, at the time of *H. indica* infection, *B. subtilis* and *B. licheniformis* were predominantly present inside the *Galleria* gut having fed orally and did not contaminate the hemolymph to compete with *P. luminescens*. Examination of the hemolymph resulted in their negligible re-isolation. *Heterorhabditis indica* IJs that entered via spiracles or cuticle, directly accessed the haemolymph to release *P. luminescens* which killed the insect. By the time *P. luminescens* could dissolve the insect gut, to spread and allow the two *Bacillus* to propagate in the hemolymph, the *H. indica* IJs had already developed into hermaphrodites.

From this stage onwards, there was a significant decline observed in the nematode development. Propagation of *B. subtilis* and *B. licheniformis* in the haemolymph was observed by day 6 and they possibly started competing with *P. luminescens* and interfered with the bioconversion of insect tissues, which is vital for providing nourishment for nematode development. Nematode reproduction and recycling are optimal only when *Photorhabdus* dominates the microbiota of the insect cadaver by producing an array of antimicrobial compounds that suppress the growth of any competing microbe [45]. We observed that the female production declined by 68.37% and 73.81% in cadavers contaminated with *B. subtilis* and *B. licheniformis*, respectively. This further led to significantly low second-generation IJ production which decline hugely by 92.92% and 83.50%, respectively, with simultaneous increase in the cfu counts of the two *Bacillus* spp. Presence of non-symbiont bacteria can reduce *Heterorhabditis* yields *in vitro* [46] and *in vivo* [47] while certain contaminant species may also induce morphological and behavioural abnormalities in *Heterorhabditis* [48]. Blackburn et al. [49, 50] reported mortality in *Leptinitarsa decemlineata* by *H. marelatus*, but the nematode could not reproduce within it because of *Lactococcus* interfering with the growth of *P. temperata,* while Kamra and Mohan [51] reported antagonistic effect of *Pseudomonas fluorescens* on the development of *H. indica* inside the *Galleria* larvae.

H. indica completely failed to develop in 80% *D. huegelii* cadavers collected from the nematode treated apple orchards from which *B. subtilis* and *B. licheniformis* were initially isolated [18]. Similarly, the co-infection of both the *Bacillus* spp. in *Galleria* larvae also led to the complete failure of nematode multiplication. Soil rhizosphere is a heterogeneous environment, where multiple infections are obvious, leading to complex interactions in turn resulting in EPN reproduction failure.

Finally, we were interested to know, if apart from *P. luminescens*, whether or not *B. subtilis* and *B. licheniformis* could support axenic *H. indica* development to any degree under *in vitro* conditions. Within 24 h none of them survived on the lawns of the two *Bacillus* species. The mutualistic interaction between the entomopathogenic nematodes and their symbiotic bacteria is highly advance and specific [52, 53]. Axenic *H. bacteriophora* H06 could not develop when exposed to *S. litura* insect cell cultures, and the cell-free filtrates or cells of different non-symbiotic cultures, including *B. subtilis, B. thuringiensis, P. fluorescens, P. aeruginosa, Micromonospora purpurea, Rhizopus delemar, Streptomyces venezuelae, S. antibioticus, Penicillium citrnum, Ganoderma lucidum, Agaricusbisporus, Pleurotusostreatus, Rhizobium legumiunosarum*, and *Photobacterium phosphoreum* [54].

Non-symbiotic bacteria as described here interrupted the symbiotic relationship between *H. indica* and *P. luminescens* which is imperative for successful recycling of the nematode. A thorough understanding of how other microbes may affect EPN symbiosis and subsequent insect control, is essential for successful exploitation of EPNs as effective biocontrol agents.

Conclusion

In our studies we found that the non-symbiotic *B. subtilis* and *B. licheniformis*, were not eliminated by *Photorhabdus* and therefore the next generation of nematodes was greatly

reduced. Our results are based on one-to-one competition studies between individual non-symbiotic bacteria within the context of the *H. indica – P. luminescens* – insect interaction that provided new insights. This work suggests that the application of EPNs in one season may not necessarily be maintained (or perhaps enhance) in subsequent seasons due to the restricted nematode production caused by microbial contaminants which negatively impact their recycling ability.Therefore the efficiency of EPN as a bio-pesticide and their sustained effect for pest management strategies can be challenged.

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Author contribution SM: conceptualization, design, data curation, formal analysis, supervision, validation, visualization, original draft preparation, review and editing. AU: design, execution of experiments, data compilation, formal analysis, draft preparation.

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Declarations

Conflict of interest This is an original research work based on our observations in the ongoing field trials using *H. indica* for insect pest management. To best of our knowledge there is no duplication and conflict of interest with any other research group.

Human and animal rights No human participants/animals were involved.

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