PLOS ONE

Entomopathogenic potential of bacteria associated with soil-borne nematodes and insect immune responses to their infection

Ameni Loulou[1], Maristella Mastore[2], Sara Caramella[2], Aashaq Hussain Bhat[3], Maurizio Francesco Brivio[2]*, Ricardo A. R. Machado[3]*, Sadreddine Kallel[1]*

1 Department of Plant Health and Environment, Laboratory of Bio-Aggressor and Integrated Protection in Agriculture, National Agronomic Institute of Tunisia, University of Carthage, Tunis, Tunisia, 2 Department of Theoretical and Applied Sciences, Laboratory of Comparative Immunology and Parasitology, University of Insubria, Varese, Italy, 3 Faculty of Sciences, Experimental Biology Research Group, Institute of Biology, University of Neuchâtel, Neuchâtel, Switzerland

* kallel.sadreddine@gmail.com (SK); maurizio.brivio@uninsubria.it (MFB); ricardo.machado@unine.ch (RARM)

Abstract

Soil-borne nematodes establish close associations with several bacterial species. Whether they confer benefits to their hosts has been investigated in only a few nematode-bacteria systems. Their ecological function, therefore, remains poorly understood. In this study, we isolated several bacterial species from rhabditid nematodes, molecularly identified them, evaluated their entomopathogenic potential on *Galleria mellonella* larvae, and measured immune responses of *G. mellonella* larvae to their infection. Bacteria were isolated from *Acrobeloides* sp., *A. bodenheimeri*, *Heterorhabditis bacteriophora*, *Oscheius tipulae*, and *Pristionchus maupasi* nematodes. They were identified as *Acinetobacter* sp., *Alcaligenes* sp., *Bacillus cereus*, *Enterobacter* sp., *Kaistia* sp., *Lysinibacillus fusiformis*, *Morganella morganii* subsp. *morganii*, *Klebsiella quasipneumoniae* subsp. *quasipneumoniae*, and *Pseudomonas aeruginosa*. All bacterial strains were found to be highly entomopathogenic as they killed at least 53.33% *G. mellonella* larvae within 72h post-infection, at a dose of 10^6 CFU/larvae. Among them, *Lysinibacillus fusiformis*, *Enterobacter* sp., *Acinetobacter* sp., and *K. quasipneumoniae* subsp. *quasipneumoniae* were the most entomopathogenic bacteria. Insects strongly responded to bacterial infection. However, their responses were apparently little effective to counteract bacterial infection. Our study, therefore, shows that bacteria associated with soil-borne nematodes have entomopathogenic capacities. From an applied perspective, our study motivates more research to determine the potential of these bacterial strains as biocontrol agents in environmentally friendly and sustainable agriculture.

Introduction

Over the past decades, entomopathogenic bacteria (EPBs) and their toxins have been successfully commercialized as microbial insecticides and used in biological control programs as eco-

friendly and sustainable alternatives to synthetic pesticides [1,2]. Several entomopathogenic bacteria species from different families such as *Bacillaceae*, *Pseudomonadaceae*, *Enterobacteriaceae*, *Streptococcaceae*, *Micrococcaceae*, *Paenibacillaceae*, *Neisseriaceae*, *Rickettsiaceae* and *Coxiellaceae* are commonly used [3–5]. In this context, Bacillus thuringiensis, *Lysinibacillus sphaericus*, Paenibacillus popilliae, *Yersinia entomophaga*, *Chromobacterium subtsugae*, and *Burkholderia rinojensis* have received the most attention as microbial control agents [6–9]. Furthermore, species of *Xenorhabdus* and *Photorhabdus* stand out for their entomopathogenic potential [10,11]. They are mutualistically associated with entomopathogenic nematodes (EPNs) of the genera *Steinernema* and *Heterorhabditis*, respectively [12–14]. Both genera bacteria colonize the intestines of the dauer larvae of EPNs [15–17]. Their insecticidal potential is mostly based on the secretion of toxic compounds into the hemocoel that suppress the insect immune responses, causing host death [18,19]. Also some species of *Serratia* show a highly lethal effect on insects [20–22]. For example, *Serratia* sp. isolated from *Caenorhabditis briggsae* (Rhabditida: Rhabditidae) is extremely pathogenic to *Galleria mellonella* [23]. In addition, bacteria of the genus *Serratia* is frequently found in association with other nematode species such as *Oscheius carolinensis*, *O. chongmingensis*, and *O. rugaoensis*, and have been isolated from the gut of the infective juvenile (IJ) and from the cuticle of these nematodes [24–28]. These bacteria have also been shown to be entomopathogenic [26]. *Oscheius* nematodes associate also with other entomopathogenic bacteria such as *B. cereus*, *Ochrobactrum tritici*, *Flavobacterium* sp., *Providencia vermicola*, and *S. marcescens* [29–32]. Clearly, nematodes may be a suitable source of bacteria with entomopathogenic potential. The efficacy of EPBs can be assessed by studying the immunological responses of host insects which are composed by two closely interconnected reactions: cellular and humoral defenses [33,34]. Insect hemocytes mediate cellular immune responses, including processes such as, phagocytosis, nodulation, encapsulation, and production of antimicrobial compounds [33,35–37]. Immunocompetent cells circulate freely in the insect hemolymph and can also be found in the digestive tract and fat bodies [36,38,39]. Different specialized cells prohemocytes, plasmatocytes, granulocytes, spherulocytes and oenotocytes are the most common types of hemocytes [40,41]. Their number in the hemolymph can vary during bacterial infection. They can for instance increase due to migration from different insect organs and tissues to the hemolymph. They can also decrease due to phagocytosis, nodulation and/or encapsulation processes [36]. Different type of hemocytes act in concert in the process of nodulation, which eliminates a large number of pathogens by forming layers of cells around them. This response is preceded by the activation of phenoloxidases, which in turn, leads to the melanization of nodules [33,40]. In the encapsulation process, granular cells recognize large foreign bodies in the hemolymph, and promote plasmatocyte binding. When capsule formation begins, the number of hemocytes circulating in the hemolymph decreases and increases after capsule formation [42]. Besides, pathogens could be destroyed by enzymes released by hemocytes through degranulation, in the process of cytotoxicity [36,43]. Stimulation of insect humoral responses after infection leads to the synthesis of a pool of antimicrobial peptides (AMPs) such as moricin-like peptides, cecropins, gloverin, Gm proline-rich peptides, Gm anionic peptides, galiomycin, gallerimycin, inducible serine protease inhibitors, defensins, cobatoxins, and heliocin-like peptides [44–47]. Certain immune-relevant proteins and peptides, including lysozyme, apolipophorins, and anionic peptides, are normally present in the hemolymph of healthy larvae and their concentration could be altered after pathogen infection [45,48]. Lysozyme acts on the peptidoglycan layer present in fungi and bacteria [36,49,50]. These enzymes participate in the cellular and humoral immune responses and affect host–parasite interactions [51,52]. During a microbial infection, prophenoloxidase cascade (proPO system), released from various hemocytes, is activated by serine proteases [53]. In its activated form, PO converts tyrosine to dihydroxyphenylalanine (DOPA) and oxidizes phenolic

substances to quinones and melanin, in a process that often induces the darkening of infected larvae [54–56]. Moreover, proPO system activation can lead to synthesis of antimicrobial peptides and activation of some cellular immune responses [57,58].

In this study, we isolated several bacterial strains from different soil-borne nematode species, tested their entomopathogenic potential, and evaluated the immune responses of insects to their infection. Our study highlights a promising potential of several bacterial strains to be used as bioinsecticides for the biological control of agricultural pests.

Materials and methods

Bacteria isolation

Bacterial strains were obtained from ten soil-borne nematode strains: *Oscheius tipulae* OC2 and TC2, *Acrobeloides* sp.TC9, TG13A, and HW0, *Acrobeloides bodenheimeri* TC7 and KG18, *Pristionchus maupasi* IN18 and IN7, *Heterorhabditis bacteriophora* EN01, and from an unidentified soil-borne nematode, IN1 (Table 1). The nematode isolation procedure is described in Loulou *et al.*, (2022) [59]. To isolate the bacteria from all but IN1 nematodes, approximately 200 specimens of each nematode strain were washed several times with sterile phosphate-buffered saline (PBS, 1mM KH$_2$PO$_4$, 1mM K$_2$HPO$_4$, 5M NaCl, pH 7.2). Then, the nematodes were incubated in a 1% v/v sodium hypochlorite/PBS solution for 5 min under gentle orbital agitation. After this incubation period, the nematodes were recovered by decanting, washed with sterile PBS, and incubated again in a 1% v/v sodium hypochlorite/PBS solution. After this incubation period, the nematodes were again recovered by decanting, washed several times with PBS, and sonicated in sterile PBS at 4˚C for 30 sec and at 100W burst using an ultrasonic processor (Labsonic-L, B-Braun Biotech Inc, Allentown, PA, USA). The nematodes body fragments were pelleted by centrifugation at 650g (10 min, 4˚C). Then, 100 μL of supernatants were cultured in LB medium overnight at 30˚C under constant shaking (180 rpm). An aliquot of the resulting cultures was plated on LB solid agar and incubated at 30˚C for 24–48 h. Single colonies were sub-cultured and used for further experiments. Bacteria associated with IN1 nematodes were isolated by plating drops of hemolymph from *Galleria mellonella* larvae on LB agar plates. For this, larvae were dipped first in 70% ethanol, and then punctured with a sterile

Table 1. Characteristics of the bacterial strains isolated in this study.

Code	Bacterial ID	Associated nematode	Place of nematode	Insect host
Lf_OC2	*Lysinibacillus fusiformis*	*Oscheius tipulae* OC2	Ouzra	*C. capitata* pupae
K_TC2	*Kaistia* sp.	*Oscheius tipulae* TC2	Takilsa	*C. capitata* pupae
A_TC9	*Alcaligenes* sp.	*Acrobeloides tricornis* TC9	Takilsa	*C. capitata* pupae
E_TC7	*Enterobacter* sp.	*Acrobeloides bodenheimeri* TC7	Takilsa	*C. capitata* pupae
Kqq_KG18	*Klebsiella quasipneumoniaes* ubsp. *quasipneumoniae*	*Acrobeloides bodenheimeri* KG18	Kobba	*G. mellonella* larvae
Bc_IN18	*Bacillus cereus*	*Pristionchus maupasi* IN18	INAT-Tunis	*G. mellonella* larvae
Bc_IN7	*Bacillus cereus*	*Pristionchus maupasi* IN7	INAT-Tunis	*G. mellonella* larvae
A_IN1	*Acinetobacter* sp.	Unidentified sp. IN1	INAT-Tunis	*G. mellonella* larvae
Bc_TG3A	*Bacillus cereus*	*Acrobeloide ssp.*TG3A	Takilsa	*G. mellonella* larvae
Pa_HWO	*Pseudomonas aeruginosa*	*Acrobeloides tricornis* HWO	Chott-Meriem	Locust egg
Mmm_EN01	*Morganella morganii* subsp. *morganii*	*Heterorhabditis bactriophora* EN01	E-nema Germany	*G. mellonella* larvae

https://doi.org/10.1371/journal.pone.0280675.t001

needle in the ventral part. The released hemolymph was cultured in LB broth and incubated overnight at 30˚C for 24h under constant shaking (180 rpm). An aliquot of the resulting cultures was plated on LB agar and incubated at 30˚C for 24–48 h. Single colonies were sub-cultured and used for further experiments. Bacterial stocks were stored at -80˚C in LB supplemented with 20% (v /v) glycerol. The obtained bacterial strains were named using the initial letters of their genus and species/subspecies as a prefix and then using the strain name of their nematode host (Table 1). Hence, the strain designations are Lf_OC2, K_TC2, A_TC9, E_TC7, Kqq_KG18, Bc_IN18, Bc_IN7, A_IN1, Bc_TG3A, Pa_HWO, and Mmm_EN01 (Table 1).

Bacteria identification

Bacterial strains were identified based on 16S rRNA gene sequences and/or whole genome sequences using the EzBioCloud's identification service and The Type (Strain) Genome Server (TYGS), two free bioinformatics platforms available under https://www.ezbiocloud.net and https://tygs.dsmz.de, respectively [60,61]. Whole genome sequences were obtained as described [62]. Briefly, genomic DNA was extracted and purified using the GenElute Bacterial Genomic DNA Kit (Sigma–Aldrich, Switzerland) following the manufacturer's instructions. The resulting DNA was used for library preparation using the TruSeq DNA PCR–Free LT Library Prep (FC–121–3003) kit. Indexed libraries were then pooled at equimolar concentrations and sequenced (2 × 150 bp) on an Illumina HiSeq 3000 instrument. Genomes were assembled using the Bactopia pipeline [63]. To this end, the raw Illumina reads were quality trimmed using Trimmomatic 0.39 [64]. The resulting reads were assembled with SPAdes 3.14.1 (k–mer sizes of 31, 51, 71, 91, and 111 bp) [65]. Scaffolds with a mean read–depth smaller than 20% of the median read–depth of the longer scaffolds ($\geq$ 5,000 bp) as well as scaffolds that were shorter than 200 bp were removed. The final assemblies were polished using Pilon 1.22 [66].

To obtain 16S rRNA gene sequences, the 16S rRNA gene was amplified by polymerase chain reaction (PCR) using the following universal primers: 27F (5'-AGAGTTT–GATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') and the following cycling conditions: 1 cycle at 94˚C for 10 min followed by 40 cycles at 94˚C for 60 s, 55˚C for 60 s, 72˚C for 60 s and a final extension at 72˚C for 5 min. PCR products were separated by electrophoresis in a1% TAE-agarose gel stained with GelRed nucleic acid gelstain (Biotium), gel-purified (QIAquick gel purification kit Qiagen) and sequenced by Sanger sequencing (Microsynth). Obtained16S rRNA raw sequences were manually curated using Bioedit 7.2.5 [67].

Bacterial pathogenicity against *G. mellonella* larvae

To evaluate the entomopathogenic potential of the isolated bacteria, *G. mellonella* larvae were injected with the different bacterial strains at different bacterial concentrations. To this end, overnight cultures were centrifuged at 1500g for 20 min. Bacterial pellets were then washed several times with sterile PBS. For larvae infection, three bacterial concentrations, 10^2, 10^4, and 10^6 CFU/larva, obtained by serial dilutions, were used. Five µl of bacterial solution were injected in the abdominal spiracle of the larvae using a sterile gas-tight syringe with a 30 gauge hypodermic needle (Hamilton Co, Reno, NE, USA). Ten surface-sterilized last instar larvae per bacterial strain and concentration were injected. Controls consisted of larvae injected with PBS buffer. The larvae were placed in sterile plastic boxes and kept in the dark at 25˚C. Insect mortality was determined at 12, 24, 48 and 72h post-injection. All assays were carried out in triplicate.

Immune response of *G. mellonella* larvae to bacterial infection

Hemolymph collection. To collect hemolymph for hemocyte number determination, and to measure phenoloxidase and lysozyme activity, last-instar *G. mellonella* larvae were injected with the different bacterial strains at a concentration of 10^2 CFU/larva as described above. Then, the larvae were incubated in sterile plastic boxes and kept in the dark at 25°C. Larvae were collected 4, 24, and 48h after injections, anesthetized on ice, surface-sterilized with 70% ethanol, and punctured with a sterile needle in the ventral region. Released hemolymph was collected in siliconized Eppendorf microcentrifuge tubes, and centrifuged at 250g for 5 min at 4°C to separate humoral fraction from hemocytes. In all samples few crystals of phenylthiourea (PTU) were added to prevent melanization, except those samples used for phenoloxidase activity assays. Samples of cell-free hemolymph and hemocytes were used for phenoloxidase and lysozyme activity, and total hemocyte counts. All assays were carried out at 4, 24, and 48h post-infection with three replicates. Controls included hemolymph from non-injected and PBS-injected larvae [68].

Total hemocyte counts (THC). To determine the total number of hemocytes present in hemolymph samples, 10 μL of hemolymph were disposed on a Neubauer hemocytometer, counting the hemocytes a Corning Cell counter (Corning Inc., NY, USA) at a magnification of 5X. The data obtained were processed by CytoSmart® [68].

Phenoloxidase activity. Relative phenoloxidase activity was determined by measuring the absorbance spectrophotometrically (Jasco V-560 spectrophotometer, Easton, MD, USA) at 490 nm, every 5 min, for 30 min, using L-Dopa (L-3,4-Dihydroxyphenylalanine) as substrate. Phenoloxidase activity was recorded as the formation of dopachrome from L-Dopa substrate and expressed in absorbance units per μl of hemolymph (ΔAbs_{490}/30min/10μl). All the assays were carried out with 5μl of hemolymph in 995μl of L-Dopa buffer (8 mM L-Dopa in 10 mM Tris-HCL pH 7.2) [69].

Lysozyme activity. Hemolymph lysozyme activity was evaluated by their lytic action on *Micrococcus luteus* [70]. Assays were carried out by turbidimetry changes in samples measuring the decrease of absorbance induced by lysozyme cell lysis. Absorbance was recorded by a Bio Rad iMark™ Microplate Absorbance reader, at 450 nm, for 10 min, in hemolymph collected from larvae at 4, 24 and 48h after bacterial injections. One unit of lysozyme activity is defined as the change of 0.001 units/min of a suspension of *M. luteus*. For each sample, three replicates were carried out, and *M. luteus* suspension, hemolymph from PBS-injected and non-injected larvae were used as control [69].

Chemicals and instruments

All reagents were supplied by Sigma Chemicals (St. Louis, MO, USA), Merck Millipore Ltd. (Tullagreen, Cork, Ireland). Instruments were provided by Bio-Rad Laboratories (Detroit, MI, USA) and Euroclone S.p.A. (Milan, Italy, EU). Centrifugations were carried out with a SIGMA 1–14 (SciQuip Ltd., Newtown, Wem, Shropshire, UK) microcentrifuge and Eppendorf 5804 (Eppendorf, AG, Hamburg, Germany) centrifuge. Spectrophotometric analysis was performed with a Jasco V-560 spectrophotometer (Easton, MD, USA). All materials, buffers, and solutions were autoclaved or filtered with 0.22 μm Minisart filters (Sartorius, Goettingen, Germany).

Statistical analysis

Differences in the bacterial pathogenicity against *G. mellonella* larvae and in insect immunological responses to the different bacterial strains were assessed by one-way ANOVA. Normality and equality of variance were verified using Shapiro–Wilk and Levene's tests at P = 0.05. In

some cases, square transformation was performed. Duncan post-hoc test was used for multiple comparisons. Dunnett tests were carried out to compare treatment data to control data with a probability of 0.05. Kruskall and Wallis tests were carried out to compare data across all treatments with P = 0.05. All statistical analyses were conducted using SPSS software 16.0® [71,72].

Results

Bacteria identification

Based on the analysis of 16S rRNA gene sequences and/or whole genome sequences the bacteria isolated from the different nematode isolates were identified as *Lysinibacillus fusiformis*, *Kaistia* sp., *Alcaligenes* sp., *Enterobacter* sp., *Klebsiella quasipneumoniae* subsp. *quasipneumoniae*, *Bacillus cereus* (three different strains), *Acinetobacter* sp., *Pseudomonas aeruginosa*, and *Morganella morganii* subsp. *morganii* (Table 1, S1 Fig). The bacterial strains identified as *Kaistia* sp., *Alcaligenes* sp., *Enterobacter* sp., and *Acinetobacter* sp., represent new species, which will be formally described elsewhere (Table 1).

Entomopathogenic potential

When injected into the hemocoel of *G. mellonella* larvae, all bacteria effectively killed these insects in a bacterial strains, time, and concentration dependent manner (Table 2). When the bacteria were injected at a concentration of 10^6 CFU/insect, most of them killed more than 80% of the insects within 48h. Some bacteria were more pathogenic and killed more than 80% of the insects even within 24h post injection. This was the case of: *B. cereus* Bc_IN18, *B. cereus* Bc_TG3A, *Enterobacter* sp. E_TC7, *K. quasipneumoniae* Kqq_KG18, *L. fusiformis* Lf_OC2, and *M. morganii* Mmm_EN01 (Fig 1, Table 2). Some other bacteria, such as *B. cereus* Bc_IN7 and *Kaistia* sp. K_TC2, were much less pathogenic and killed no more than 50–80% of the insects within 72h at the same bacterial concentration. When the bacteria were injected at concentrations of 10^4 CFU/larva, lower mortalities were caused by most of the bacterial strains (Fig 1). Notably, *L. fusiformis* Lf_OC2 still killed 90% of the insects within 24h, while *Acinetobacter* sp. A_IN1, *Enterobacter* sp. E_TC7, *B. cereus* Bc_TG3, and *K. quasipneumoniae* Kqq_KG18 killed between 50-70% of the insects within 72h (Fig 1, Table 2). When bacteria were injected at a concentration of 10^2 CFU/larva, only *L. fusiformis* Lf_OC2 killed up to 90% of the insects within 72h. At the same concentration, *Acinetobacter* sp. A_IN1 killed about 50% of the insects, while the other bacterial strains were ineffective and killed less than 30% of the insect within 72h post-treatment (Fig 1, Table 2).

The immune response of larvae to infection

Total hemocytes counts (THC). The number of hemocytes in the hemolymph of bacteria-injected larvae increased 4h post-infection (Fig 2). In some cases, however, the number of hemocytes was only slightly higher in bacteria-injected larvae compared to controls, i.e. with insects injected with *B. cereus* Bc_IN18 and *Enterobacter* sp. E_TC7. The highest number of hemocytes was found in the hemolymph of insects injected with *Acinetobacter* sp. A_IN1. The number of hemocytes decreased sharply 24h post-infection, and only minor differences in the number of hemocytes were observed. 48h post-infection, the number of hemocytes increased again and was slightly higher in insects injected with most of the bacterial strains, apart from those injected with *L. fusiformis* Lf_OC2 and *M. morganii* subsp. *morganii* Mmm_EN01.

Lysozyme activity. Lysozyme activity in the hemolymph of bacteria-injected larvae increased 4h post-infection (Fig 3). There were no major differences in enzymatic activity in the hemolymph of insects injected with the different bacteria 4h post-infection. The highest

Table 2. Virulence of bacteria isolates against *Galleria mellonella* larvae at 72h post-treatment and time (h) required to obtain 50% (TL50) and 90% (TL90).

		Dose CFU/larva	
	10^2	10^4	10^6
Lysinibacillus fusiformis	%M 96.66 a*	100 a*	100 a*
	TL50 33.2	17.5	5.8
	TL90 44.5	22	10
Kaistia sp.	%M 26.66 c*	30.00 de	53.33 c*
	TL50 -	-	45
	TL90 -	-	-
Alcaligenes sp.	%M 3.33 e	43.33 cd*	100 a*
	TL50 -	-	24
	TL90 -	-	42,5
Enterobacter sp.	%M 16.66 cde	56.66bc*	100 a*
	TL50 -	45	6
	TL90 -	-	15
Klebsiella quasipneumoniae subsp. *quasipneumoniae*	%M 10.00 de	56.66 bc*	100 a*
	TL50 -	44	12
	TL90 -	-	20
Bacillus cereus Bc_IN18	%M 10.00 de	36.66 cd*	96.66 a*
	TL50 -	-	16,2
	TL90 -	-	27
Bacillus cereus Bc_TG13 A	%M 10.00 de	56.66 bc*	100 a*
	TL50 -	46.8	17.2
	TL90 -	-	17
Acinetobacter sp.	%M 53.33 b*	70.00 b*	93.33 a*
	TL50 52,4	44,8	18,2
	TL90 -	-	48
Bacillus cereus Bc_IN7	%M 6.66 de	13.33 ef*	76.66 b*
	TL50-	-	15
	TL90 -	-	-
Pseudomonas aeruginosa	%M 13.33 cde	43.33 cd*	100 a*
	TL50 -	-	7.2
	TL90 -	-	30
Morganella morganii subsp. *morganii*	%M 20.00 cd	33.33 de*	100 a*
	TL50 -	-	17
	TL90 -	-	21
Control	%M 8.18de	8.18 f	8.18 d
	TL50 -	-	-
	TL90 -	-	-

The means by bacteria followed by the same letters (a, b) are not significantly different according to the test of Duncan and Kruskall and Wallis with P < 0.05.The means by bacteria followed by (*) are not significantly different according to the test of Dunnett(P = 0.050).

enzymatic activity was observed in the hemolymph of insects injected with *L. fusiformis* Lf_OC2, the lowest activity was observed in the hemolymph of insects injected with *P. aeruginosa* Pa_HWO. 24h post-infection, lysozyme activity decreased in most cases, but remained at similar levels as those observed 4h post-infection in the hemolymph of insects injected with *Enterobacter* sp. E_TC7, *Acinetobacter* sp. A_IN1, and *P. aeruginosa* Pa_HWO. The highest enzymatic activity was observed in the hemolymph of insects injected with *Acinetobacter* sp.
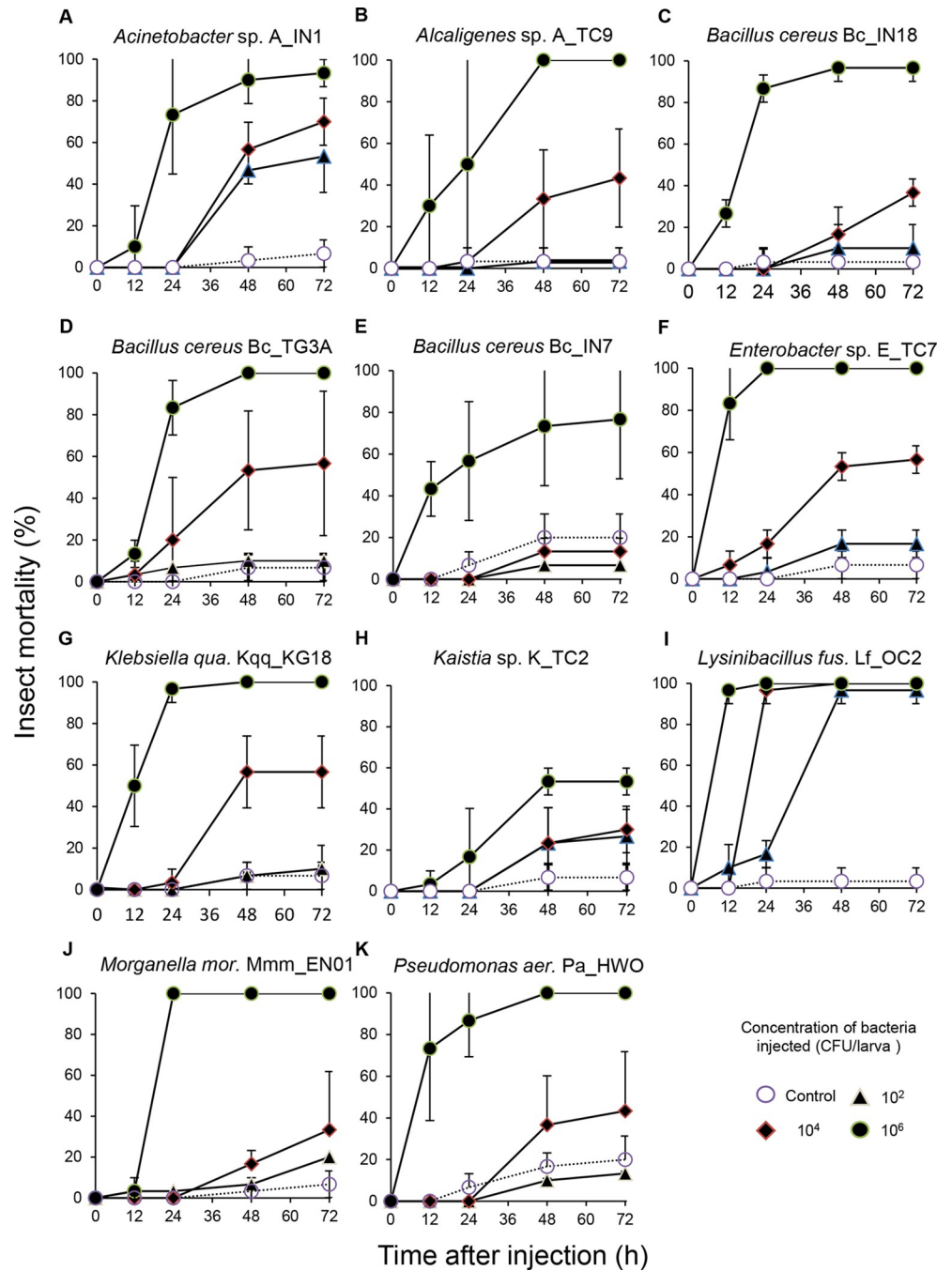
Fig 1. Entomopathogenic potential of different bacterial strains against G. mellonella larvae. Insects were injected with three bacterial concentrations 10^2, 10^4, or 10^6 CFU/larva of the following bacterial strains. A) *Acinetobacter* sp. A_IN1. B) *Alcaligenes* sp. A_TC9. C) *Bacillus. cereus* Bc_IN18. D) *Bacillus cereus* Bc_TG3A. E) *Bacillus cereus* Bc_IN7. F) *Enterobacter* sp. E_TC7. G) *Klebsiella quasipneumoniae* subsp. *quasipneumoniae* Kqq_KG18. H) *Kaistia* sp. K_TC2. I) *Lysinibacillus fusiformis* Lf_OC2. J) *Morganella morganii* subsp. *morganii*. Mmm_EN01. K) *Pseudomonas aeruginosa* Pa_HWO. Bars correspond to confidence intervals (P = 0.05).

https://doi.org/10.1371/journal.pone.0280675.g001

A_IN1 and *P. aeruginosa* Pa_HWO, the lowest was observed in the hemolymph of insects injected with *L. fusiformis* Lf_OC2. 48h post-infection, the enzymatic activity remained at similar levels as those observed 24h post-infection, although it decreased in the hemolymph of
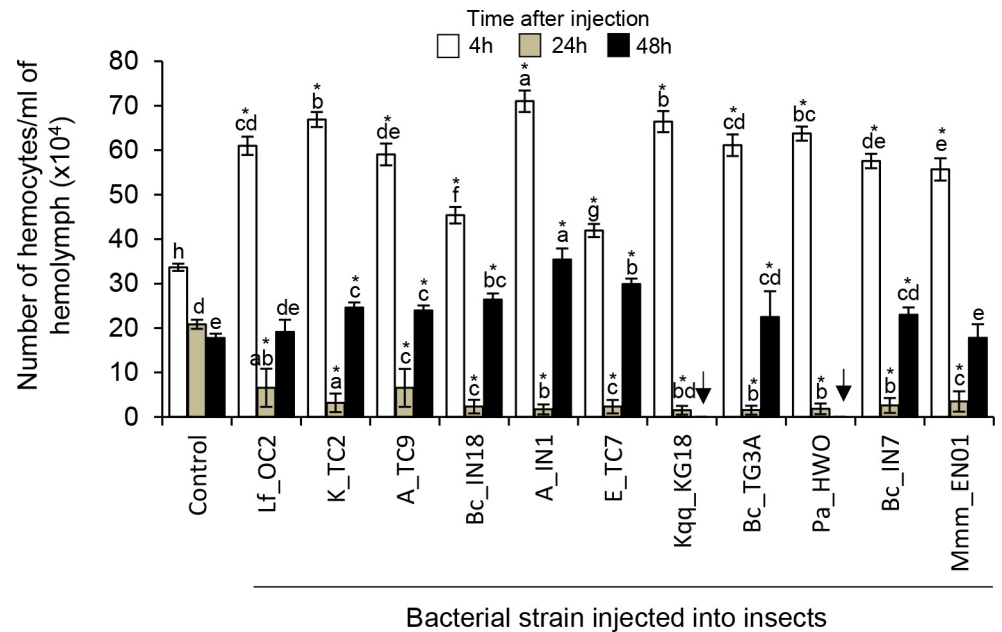
Fig 2. Total Hemocyte Counts (THC) in bacteria-infected insects. Mean hemocyte number per ml of hemolymph of *G. mellonella* larvae injected with different bacteria or with buffer as controls at 4h, 24h and 48h post-treatment. Bars correspond to confidence intervals (P = <0.05). Different letters indicate significant differences between the number of hemocytes across all treatments within each time point (Duncan test, Kruskall, and Wallis test, P<0.05). Asterisks indicate significant differences in the number of hemocytes of control larvae and bacteria-injected larvae within each time point (Dunnet test, P<0.05). Arrows indicate that the insects died.

insects injected with *L. fusiformis* Lf_OC2 and *M. morganii* subsp. *morganii* Mmm_EN01, and increased in the hemolymph of insects injected with *K. quasipneumoniae* subsp. *quasipneumoniae* Kqq_KG18. The highest enzymatic activity was observed in the hemolymph of insects injected with *K. quasipneumoniae* subsp. *quasipneumoniae* Kqq_KG18, the lowest was observed in the hemolymph of insects injected with *L. fusiformis* Lf_OC2 and *M. morganii* subsp. *morganii* Mmm_EN01 (Fig 3).

    Phenoloxidase activity. Phenoloxidase activity in the hemolymph of bacteria-injected larvae increased 4h post-infection and remained higher in the hemolymph of bacteria-injected larvae than in the hemolymph of control, non-injected insects for 48h post-infection (Fig 4). There were no major differences in enzymatic activity in the hemolymph of insects injected with the different bacteria between 4-48h post-infection. The highest enzymatic activity was observed in the hemolymph of insects injected with *L. fusiformis* Lf_OC2 (Fig 4).

Relationships between insect immune responses and bacteria pathogenesis

No statistically significant correlations were observed between bacteria pathogenesis, measured as the insect mortality caused by bacterial infection 72h post-infection, and insect immune responses, measured as the number of hemocytes, lysozyme activity or phenoloxidase activity in the hemolymph of *G. mellonella* larvae injected with different bacteria 4h post-infection or 24h post-infection (Fig 5).

Discussion

In this study, we isolated several bacterial strains from soil-borne nematodes and tested their pathogenesis against *G. mellonella* larvae and the immune responses of these insects to
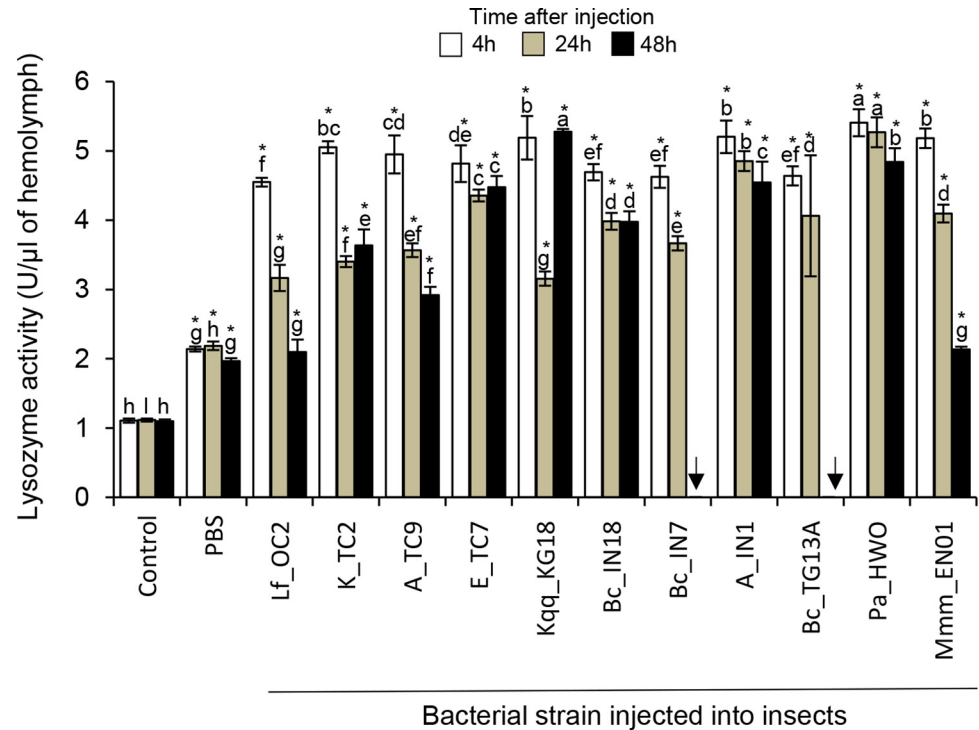
Fig 3. Lysozyme activity in the hemolymph of bacteria-infected insects. Mean lysozyme activity in the hemolymph of *G. mellonella* larvae injected with different bacteria or with buffer as controls at 4h, 24h and 48h post-treatment. Bars show confidence intervals (P = 0.05). Different letters indicate significant differences in lysozyme activity across all treatments within each time point (Duncan test, Kruskall and Wallis test, P<0.05). Asterisks indicate significant differences in lysozyme activity between control larvae and bacteria-injected larvae within each time point (Dunnet test, P<0.05). Arrows indicate that the insects died.

bacterial infection. The bacterial strains isolated were identified as *L. fusiformis*, *Kaistia* sp., *Alcaligenes* sp., *Enterobacter* sp., *K. quasipneumoniae* subsp. *quasipneumoniae*, *B. cereus*, *Acinetobacter* sp., *P. aeruginosa*, and *M. morganii* subsp. *morganii*.

Several of the bacterial species isolated in this study have been previously observed in association with soil-borne nematodes [73]. For instance, *Alcaligenes* sp., *Klebsiella* sp., *Acinetobacter* sp., *Pseudochrobactrum* sp., *Comamonas* sp., and *Leucobacter* sp. were isolated from specimens of *Rhabditis regina* [74]. Furthermore, numerous species of *Oscheius* are vectors of bacteria [75–77]. For instance, several species of *Acinetobacter*, *Bacillus*, *Klebsiella*, *Enterobacter*, *Pseudomonas*, *Lysinibacillus* and *Alcaligenes* have been isolated from different *Oscheius* species [30,78]. Recently, nematodes from two *O. tipulae* strains, TC2 and OC2, were found to efficiently kill *G. mellonella* larvae and *C. capitata* at different developmental stages [59]. The nematode-killing abilities could potentially be achieved by the pathogenic effects of the bacteria they carry.

In support of the above hypothesis, we observed that all the bacterial strains tested in this study, including some that were isolated from *O. tipulae* TC2 and OC2, were pathogenic and rapidly killed *G. mellonella* larvae. Among them, *L. fusiformis* Lf_OC2 isolated from *O. tipulae* (OC2) showed the highest effectiveness. *Lysinibacillus* species are ubiquitous [79]. In addition, *L. fusiformis* acts as a biocontrol agent against a wide range of plant diseases, due to its capacity to produce different antimicrobial compounds such as salicylic acid, chitinase, protease, and β-endoglucanase [80–83]. In contrast to our observations, other *L. fusiformis* strains have a low entomopathogenic effect against *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae)
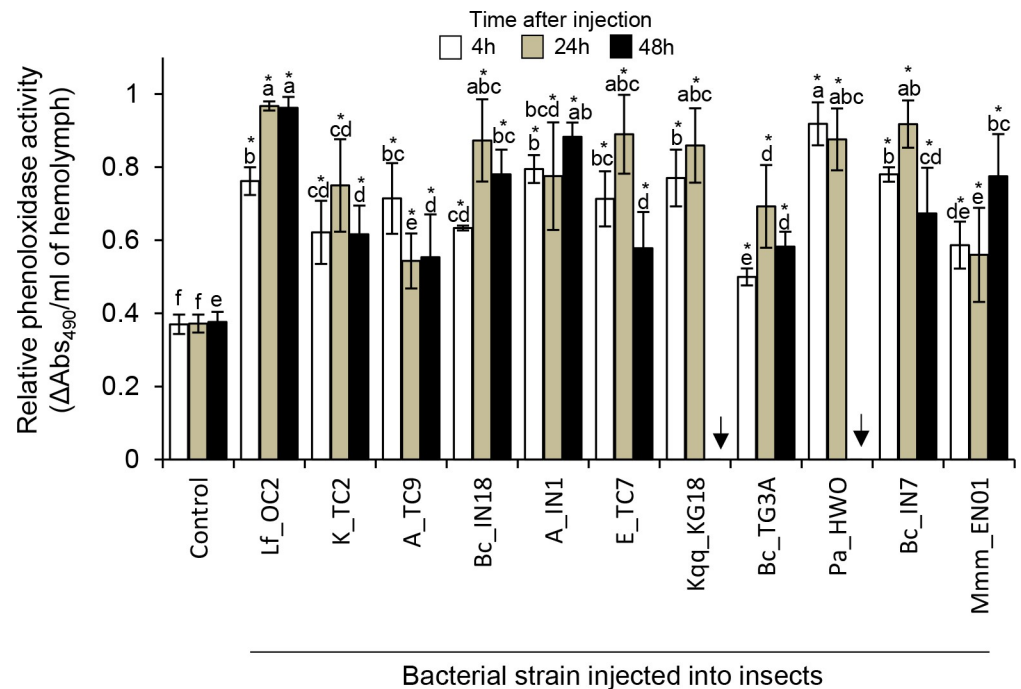
Fig 4. Relative phenoloxidase activity in the hemolymph of bacteria-infected insects. Mean relative phenoloxidase activity per ml of hemolymph of *G. mellonella* larvae injected with different bacteria or with buffer controls at 4h, 24h, and 48h post-treatment. Absorbance measurements were taken 30 min after hemolymph incubation. Bars correspond to the confidence intervals at P = 0.05. Different letters indicate significant differences in phenoloxidase activity across all treatments within each time point (Duncan test, Kruskall and Wallis test, P<0.05). Asterisks indicate significant differences in phenoloxidase activity between control larvae and bacteria-injected larvae within each time point (Dunnet test, P<0.05). Arrows indicate that the insects died.

https://doi.org/10.1371/journal.pone.0280675.g004

[84]. However, other strains of this species are highly lethal to *Drosophila melanogaster* and show miticidal effect against *Varroa destructor* [85,86]. These previous literature reports together with our results indicate a great entomopathogenic potential of *L. fusiformis* against certain arthropods.

The entomopathogenic abilities of several other species isolated in this study have been documented in previous studies. This is the case of *P. aeruginosa* and *B. cereus*. *Pseudomonas aeruginosa* occupies diverse ecological niches [87]. It is an opportunistic pathogen of vertebrates, humans, insects and nematodes [88–93]. This bacterium is associated with nematodes such as *H. bacteriophora* and has been shown to kill other insects such as *Trichoplusia ni* [94]. On the other hand, *B. cereus* is widely distributed in many environments, and is used as a biocontrol agent against different plant diseases and insect pests [95–101].

The pathogenicity of several other bacteria isolated in this study has not been previously reported. This is the case of *K. quasipneumoniae* subsp. *quasipneumoniae* Kqq_KG18 isolated from *A. bodenheimeri* nematodes. This bacterial species is an ubiquitous opportunistic pathogen of humans and animals that is often found in soil, groundwater, animals, and plants [102]. However, closely related species such as *K. pneumoniae* and *K. oxytoca* kill *Trichoplusia ni* and *Culex pipiens*, respectively [94,103].

An important aspect of our study is regarding the type of association established between the bacterial strains isolated in this study and the nematodes. Soil-borne nematodes are frequently vectors of bacteria. *Oscheius* nematodes for instance carry different *Gammaproteobacteria* (*Enterobacter, Proteus, Providencia, Pseudomonas, Stenotrophomonas*), *Betaproteobacteria* (*Alcaligenes*), and *Bacilli* (*Bacillus, Enterococcus, Lysinibacillus*) [75,78]. Our experimental
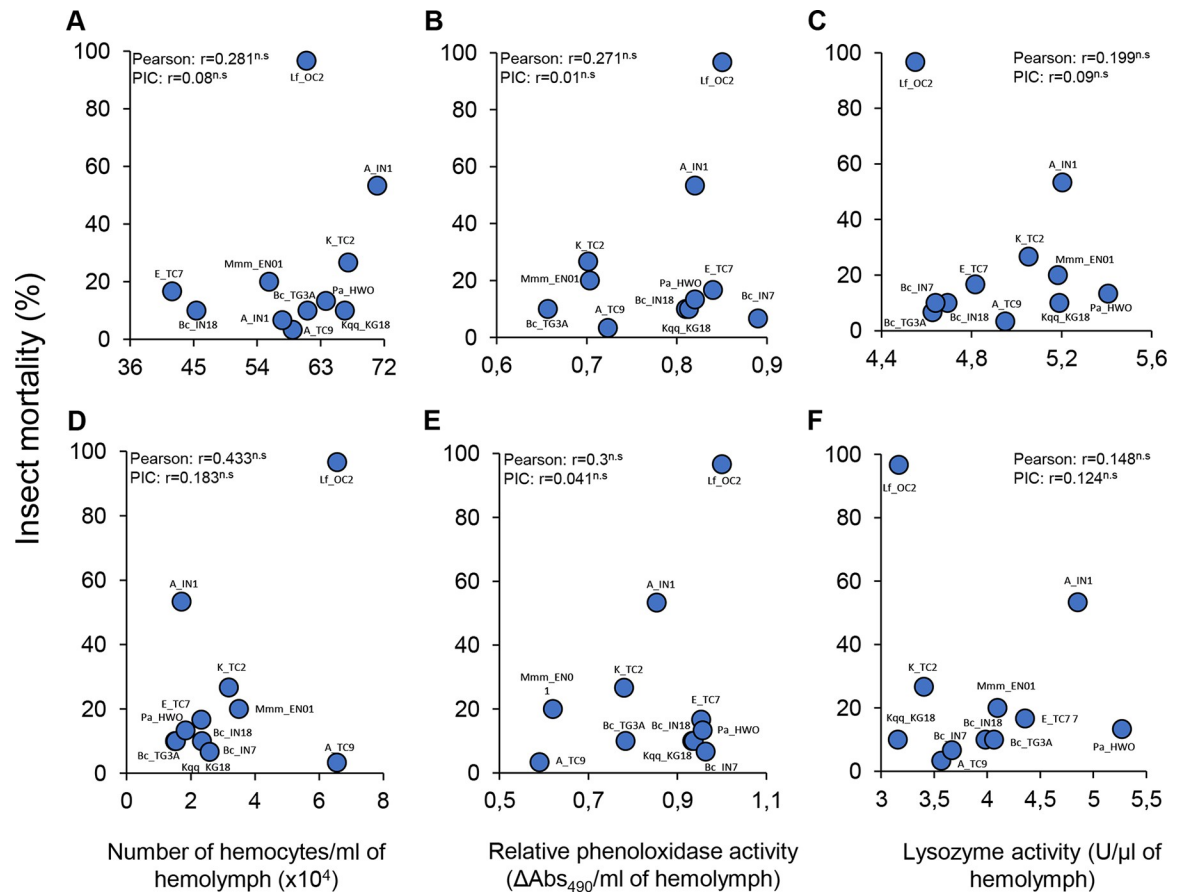
Fig 5. Relationship between insect immune responses to bacterial infection and bacteria pathogenesis. Correlation between insect mortality caused by bacterial infection 72h post-infection and: A) Mean hemocyte number per ml of hemolymph of *G. mellonella* larvae injected with different bacteria 4h post-infection; B) Mean lysozyme activity in the hemolymph of *G. mellonella* larvae injected with different bacteria 4h post-infection; C) Mean relative phenoloxidase activity per ml of hemolymph of *G. mellonella* larvae injected with different bacteria 4h post-treatment; D) Mean hemocyte number per ml of hemolymph of *G. mellonella* larvae injected with different bacteria 24h post-infection; E) Mean lysozyme activity in the hemolymph of *G. mellonella* larvae injected with different bacteria 24h post-infection; and F) Mean relative phenoloxidase activity per ml of hemolymph of *G. mellonella* larvae injected with different bacteria 24h post-treatment. Correlations were statistically assessed by person test and by independent phylogenetic contrast (PIC). n.s.: Statistically not significant.

approach does not allow to determine whether the bacteria and the nematodes establish an obligate mutualistic symbiosis similar to that established by *Photorhabdus* and *Heterorhabditis* or by *Xenorhabdus* and *Steinernema* [10,11,104]. As several of the nematodes we studied here are soil-borne, bacteriophagus organisms, it is likely that they passible acquire the different bacteria while foraging and that the bacteria are carried on their cuticles or in their digestive tracts. Interestingly, we observed that some nematodes were associated with bacterial strains known to produce highly toxic metabolites, such as *M. morganii* subsp. *morganii* [105–109]. This observation opens up the question on whether these bacteria can also be detrimental to the nematodes. Our interpretation is that it is likely so, but it depends on bacterial densities. Under low bacterial densities, certain toxin-producing bacteria can associate with and be carried by the nematodes on their cuticle, and aid them killing soil-borne insects, while under high bacterial densities, nematodes might be killed by these bacteria. It is, therefore, possible that the nematodes have evolved mechanisms to avoid soil areas where toxic bacteria occur at high densities to avoid their toxic effects.

Insects strongly responded to the infection of all the bacteria tested in this study by increasing the number of hemocytes in the hemolymph, and also by activating key immune enzymes such as phenoloxidases and lysozymes. Interestingly, and contrary to our expectations, we observed no correlation between bacteria pathogenesis, measured as the insect mortality caused by bacterial infection, and insect immune responses, measured as the number of hemocytes, lysozyme activity, and phenoloxidase activity in the hemolymph of *G. mellonella* larvae injected with different bacterial strains. A potential explanation of these results is that insect immune responses might not be effective at suppressing the infection of certain bacterial species [110–113]. Additional experiments are required however to test this hypothesis and to understand the relative contribution of the different immune responses to limit bacterial infection.

Conclusions

Bacteria associated with soil-borne nematodes have entomopathogenic capacities. From an applied perspective, our study motivates more research to determine their potential as biocontrol agents in environmentally friendly and sustainable agriculture.

Supporting information

S1 Fig. Phylogenetic tree based on ribosomal gene sequences of the bacteria isolated in this study and several related species. Phylogenetic relationships based on 16S rRNA gene sequences were inferred by using the Maximum Likelihood method based on the Kimura 2-parameter model. The tree with the highest log likelihood (-6121.67) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. There were a total of 932 positions in the final dataset. NCBI accession numbers of the sequences used for the analyses are shown.
(PDF)

S1 File.
(DOCX)

Acknowledgments

Author Contributions

Conceptualization: Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Data curation: Ameni Loulou, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Formal analysis: Ameni Loulou, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Funding acquisition: Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Investigation: Ameni Loulou, Maristella Mastore, Sara Caramella, Aashaq Hussain Bhat, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Methodology: Ameni Loulou, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Project administration: Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Resources: Ameni Loulou, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Supervision: Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Validation: Ameni Loulou, Ricardo A. R. Machado, Sadreddine Kallel.

Visualization: Ameni Loulou, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

Writing – original draft: Ameni Loulou, Ricardo A. R. Machado, Sadreddine Kallel.

Writing – review & editing: Ameni Loulou, Maristella Mastore, Sara Caramella, Aashaq Hussain Bhat, Maurizio Francesco Brivio, Ricardo A. R. Machado, Sadreddine Kallel.

References

1. Glare TR, Jurat-Fuentes J-L, O'Callaghan M. Basic and Applied, Research: Entomopathogenic Bacteria. Microbial Control of Insect and Mite Pests. 2017; 47–67.

2. Da Silva WJ, Pilz Junior HL, Heermann R, Da Silva OS. The great potential of entomopathogenic bacteria *Xenorhabdus* and *Photorhabdus* for mosquito control: a review. Parasites & Vectors. 2020; 13: 1–14.

3. Kalha CS, Singh PP, Kang SS, Hunjan MS, Gupta V, Sharma R. Integrated pest management: Current concepts and ecological perspective. Entomopathogenic viruses and bacteria for insect-pest control. Academic Press. 2014; 225–244.

4. Sakr HH. Insecticidal activity of *Brevibacillus brevis* (Paenibacillaceae) bacterial culture filtrate on *Spodoptera littoralis* (Lepidoptera: Noctuidae) larvae. The Egyptian Society of Experimental Biol. 2017; 13: 331–342.

5. Gouli VV, Marcelino JAP, Gouli SY. Microbial Pesticides: Biological Resources, Production and Application. Chapter 1 The basic biological resources for the production of microbial pesticides. Elsevier Inc. 2021; 1–134. https://doi.org/10.1016/C2020-0-00269-0.

6. Jenson I. Bacillus-Introduction. Encyclopedia of Food Microbiology, (Second Edition). 2014; 111–117.

7. Cordova-Kreylos AL, Fernandez LE, Koivunen M, Yang A, Flor-Weiler L, Marrone PG. Isolation and Characterization of *Burkholderia rinojensis* sp. nov., a Non-*Burkholderia cepacia* complex soil bacterium with insecticidal and miticidal activities. Applied and Environmental Microbiology. 2013; 79: 7669–7678.

8. Blackburn MB, Sparks ME, Gundersen-Rindal DE. The genome of the insecticidal *Chromobacterium subtsugae* PRAA4-1 and its comparison with that of *Chromobacterium violaceum* ATCC 12472. Genomics Data. 2016; 10:1–3.

9. Hurst MRH, Jones SA, Binglin T, Harper LA, Jackson TA, Glare TR. The main virulence determinant of *Yersinia entomophaga* MH96 is a broad-host-range toxin complex active against insects. Journal of Bacteriology. 2011; 193: 1966–1980.

10. Koppenhöfer AM, Fuzy EM. *Steinernema scarabaei* for the control of white grubs. Biological Control. 2003; 28: 47–59.

11. Machado RAR, Thönen L, Arce CC, Theepan V, Prada F, Wüthrich D, et al. Engineering bacterial symbionts of nematodes improves their biocontrol potential to counter the western corn rootworm. Nature Biotechnology. 2020; 38: 600–608. https://doi.org/10.1038/s41587-020-0419-1 PMID: 32066956

12. Bhat AH, Chaubey AK, Shokoohi E, Machado RAR. Molecular and phenotypic characterization of *Heterorhabditis indica* (Nematoda: Rhabditida) nematodes isolated during a survey of agricultural soils in Western Uttar Pradesh, India. Acta Parasitologica. 2021; 66: 236–252.

13. Hill V, Kuhnert P, Erb M, Machado RAR. Identification of *Photorhabdus* symbionts by MALDI-TOF MS. Microbiology. 2020; 166: 522–530.

14. Machado RAR, Muller A, Ghazal SM, Thanwisai A, Pagès S, Bode HB, et al. *Photorhabdus heterorhabditis* subsp. *aluminescens* subsp. nov., *Photorhabdus heterorhabditis* subsp. *heterorhabditis* subsp. nov., *Photorhabdus australis* subsp. *thailandensis* subsp. nov., *Photorhabdus australis* subsp. *australis* subsp. nov., and *Photorhabdus aegyptia* sp. nov. isolated from *Heterorhabditis* entomopathogenic nematodes. International Journal of Systematic and Evolutionary Microbiology. 2021; 71: 004610.

15. Wang JX, Bedding RA. Population development of *Heterorhabditis bacteriophora* and *Steinernema carpocapsae* in the larvae of *Galleria mellonella*. Fund Appl Nematol. 1996; 19: 363–367.

16. Goodrich-Blair H, Clarke DJ. Mutualism and pathogenesis in *Xenorhabdus* and *Photorhabdus*: two roads to the same destination. Molecular microbiology. 2007; 64(2): 260–268.

17. Hinchliffe SJ, Hares MC, Dowling AJ. Insecticidal toxins from the *Photorhabdus* and *Xenorhabdus* bacteria. The Open Toxinology Journal. 2010; 3(1): 83–100.

18. Shrestha S, Kim Y. An entomopathogenic bacterium, *Xenorhabdus nematophila*, inhibits hemocyte phagocytosis of *Spodoptera exigua* by inhibiting phospholipase A2. J Invertebr Pathol. 2007; 96: 64–70.

19. Ahmed S, Kim Y. Differential immunosuppression by inhibiting PLA2 affects virulence of *Xenorhabdus hominickii* and *Photorhabdus temperate temperata*. J Invertebr Pathol. 2018; 157: 136–46.

20. Lysyk TJ, Kalischuk-Tymensen LD, Selinger LB. Comparsion of selected growth media for culturing *Serratia marcescens*, *Aeromona* ssp., and *Pseudomonas aeruginosa* as pathogens of adult *Stomoxys calcitrans* (Diptera: Muscidae). J Med Entomol. 2002; 39: 89–98.

21. Nunez-Valdez ME, Calderon MA, Aranda E, Hernandez L, Ramirez-Gama RM, Lina L, et al. Identification of a putative mexican strain of *Serratia entomophila* pathogenic against root-damaging larvae of Scarabaeidae (Coleoptera). Appl. Environ. Microbiol. 2008; 74: 802–810.

22. Patil CD, Patil SV, Salunke BK, Salunkhe RB. Prodigiosin produced by *Serratia marcescens* NMCC46 as a mosquito larvicidal agent against *Aedes aegypti* and *Anopheles stephensi*. Parasitol Res. 2011; 109: 1179–1187.

23. Abebe E, Akele FA, Morrison J, Cooper V, Thomas WK. An insect pathogenic symbiosis between a *Caenorhabditis* and *Serratia*. Virulence. 2011; 2: 158–161.

24. Liu Q, Mracek Z, Zhang L, Puza V, Dong L. Re-description of *Oscheius-chongmingensis* (Zhang *et al.*, 2008) (Nematoda: Rhabditidae) and its entomopathogenicity. Nematology. 2012; 14: 139–149.

25. Ye W, Torres-Barragan A, Cardoza Y. *Oscheius carolinensis* n.sp. (Nematoda: Rhabditidae), a potential entomopathogenic nematode from vermicompost. Nematology. 2010; 12: 121–135.

26. Torres-Barragan A, Suazo A, Buhler WG, Cardoza YJ. Studies on the entomopathogenicity and bacterial associates of the nematode *Oscheius carolinensis*. Biol Contr. 2011; 59: 123–129.

27. Zhang KY, Liu XH, Tan J, Wang Y, Qiao L, Yedid G, et al. *Heterorhabditidoides rugaoensis* n.sp. (Rhabditida: Rhabditidae), a novel Highly Pathogenic entomopathogenic Nematode Member of Rhabditidae. J Nematol. 2012; 44: 348–360.

28. Lephoto TE, Featherston J, Gray VM. Draft whole-genome sequence of Serratia sp. strain TEL, associated with *Oscheius* sp. TEL-2014 (Nematoda: Rhabditidae) isolated from a grassland in South Africa. Genome Announcements. 2015; 3(4): e00747–15.

29. Mohandas C, Sheeba M, Firoza AJ, Rajamma P. Bacteria associated with Rhabditis (*Oscheius*) spp. (Rhabditidae: Nematoda) for the biocontrol of insect pests. Proceeding of the National Seminar on Achievements and Opportunities in Post harvest Management and Value Addition in Root and Tuber-Crops (NSRTC–2) (Trivandrum). 2007; 195–198.

30. Deepa I, Mohandas C, Siji JV. Molecular characterization of novel symbiotic bacteria from entomopathogenic nematodes. Nature Seminar on Climate Change and Food Security: challenges and opportunities. Tuber Crops. 2011; 24: 399–404.

31. Tambong JT. Phylogeny of bacteria isolated from Rhabditis sp. (Nematoda) and identification of novel entomopathogenic *Serratia marcescens* strains. Current Microbiology. 2013; 66: 138–144.

32. Fu JR, Liu QZ. Evaluation and entomopathogenicity of gut bacteria associated with dauer juveniles of *Oscheius chongmingensis* (Nematoda: Rhabditidae). Microbiologyopen. 2019; 8 (9): e00823.

33. Browne N, Heelan M, Kavanagh K. An analysis of the structural and functional similarities of insect hemocytes and mammalian phagocytes. Virulence. 2013; 4: 597–603. https://doi.org/10.4161/viru.25906 PMID: 23921374

34. Satyavathi VV, Minz A, Nagaraju J. Nodulation: An unexplored cellular defense mechanism in insects. Cell Signal. 2014; 26: 1753–1763. https://doi.org/10.1016/j.cellsig.2014.02.024 PMID: 24704121

35. Jiang M, Chen Y, Wang Y, Loor JJ, Ye Y, Wen Y, et al. Yak (Bos grunniens) stomach lysozyme: Molecular cloning, expression and its antibacterial activities. Anim. Biotechnol. 2010; 21: 25–35. https://doi.org/10.1080/10495390903339899 PMID: 20024784

36. Kavanagh K, Reeves EP. Exploiting the potential of insects for *in vivo* pathogenicity testing of microbial pathogens. FEMS Microbiol Rev. 2004; 28: 101–112.

37. Strand MR. The insect cellular immune response. Insect Sci. 2008; 15: 1–14.

38. Sheehan G, Kavanagh K. Analysis of the early cellular and humoral responses of *Galleria mellonella* larvae to infection by *Candida albicans*. Virulence. 2018; 9: 163–172. https://doi.org/10.1080/21505594.2017.1370174

39. Holz A, Bossinger B, Strasser T, Janning W, Klapper R. The two origins of hemocytes in *Drosophila*. Development. 2003; 130: 4955–4962.

40. Lavine MD, Strand MR. Insect hemocytes and their role in immunity. Insect Biochem Mol Biol. 2002; 32: 1295–1309. https://doi.org/10.1016/s0965-1748(02)00092-9 PMID: 12225920

41. Boman HG, Hultmark D. Cell-free immunity in insects. Annu Rev Microbiol. 1987; 41: 103–126. https://doi.org/10.1146/annurev.mi.41.100187.000535 PMID: 3318666

42. Grizanova EV, Semenova AD, Komarov DA, Chertkova EA, Slepneva IA, Dubovskiy IM. Maintenance of redox balance by antioxidants in hemolymph of the greater wax moth *Galleria mellonella* larvae during encapsulation response. Arch Insect Biochem Physiol. 2018; 98, e21460.

43. Baggiolini M, Wymann MP. Turning on the respiratory burst. Trends Biochem Sci. 1990; 15: 69–72. https://doi.org/10.1016/0968-0004(90)90179-f PMID: 2186518

44. Tsai CJ, Loh JM, Proft T. *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. Virulence. 2016; 7: 214–229.

45. Brown SE, Howard A, Kasprzak AB, Gordon KH, East PD. A peptidomics study reveals the impressive antimicrobial peptide arsenal of the wax moth *Galleria mellonella*. Insect Biochem Mol Biol. 2009; 39: 792–800.

46. Vilcinskas A. Anti-infective therapeutics from the Lepidopter an model host *Galleria mellonella*. Curr Pharm Des. 2011; 17: 1240–1245.

47. Taszłow P, Vertyporokh L, Wojda I. Humoral immune response of *Galleria mellonella* after repeated infection with *Bacillus thuringiensis*. J Invertebr Pathol. 2017; 149: 87–96.

48. Mak P, Zdybicka-Barabas A, Cytry´ nska M. A different repertoire of *Galleria mellonella* antimicrobial peptides in larvae challenged with bacteria and fungi. Dev Comp Immunol. 2010; 34: 1129–1136.

49. Zdybicka-Barabas A, Staçzek S, Mak P, Skrzypiec K, Mendyk E, Cytryn´ ska M. Synergistic action of *Galleria mellonella* apolipophorin III and lysozyme against Gram-negative bacteria. Biochim Biophys Acta. 2013; 1828: 1449–1456.

50. Zdybicka-Barabas A, Mak P, Klys A. Skrzypiec K, Mendyk E, Fiołka MJ, Cytry´nska M. Synergistic action of *Galleria mellonella* anionic peptide 2 and lysozyme against Gram-negative bacteria. Biochim Biophys Acta. 2012; 1818: 2623–2635.

51. Büyükgüzel E, Tunaz H, Stanley D, Büyükgüzel K. Eicosanoids mediate *Galleria mellonella* cellular immune response to viral infection. J Insect Physiol. 2007; 53: 99–105.

52. Stanley D, Haas E, Miller J. Eicosanoids: Exploiting Insect Immunity to Improve Biological Control Programs. Insects. 2012; 3: 492–510. https://doi.org/10.3390/insects3020492 PMID: 26466540

53. Ling E, Shirai K, Kanehatsu R, Kiguchi K. Reexamination of phenoloxidase in larval circulating hemocytes of the silkworm, *Bombyx mori*. Tissue and Cell. 2005; 37: 101–107. https://doi.org/10.1016/j.tice.2004.10.007

54. Ashida M. The prophenoloxidase cascade in insect immunity. Res Immunol. 1990; 141: 908–910. https://doi.org/10.1016/0923-2494(90)90191-z PMID: 2129210

55. Cerenius L, Lee BL, Soderhall K. The proPO-system: Pros and cons for its role in invertebrate immunity. Trends Immunol. 2008; 29: 263–271. https://doi.org/10.1016/j.it.2008.02.009 PMID: 18457993

56. Lu A, Zhang Q, Zhang J, Yang B, Wu K, Xie W, Luan YX, Ling E. Insect prophenoloxidase: The view beyond immunity. Front Physiol. 2014; 5: 252. https://doi.org/10.3389/fphys.2014.00252 PMID: 25071597

57. Hoffmann JA, Reichhart JM, Hetru C. Innate immunity in higher insects. Curr. Opin. Immunol. 1996; 8: 8–13. https://doi.org/10.1016/s0952-7915(96)80098-7 PMID: 8729440

58. Park SY, Kim CH, Jeong WH, Lee JH, Seo SJ, Han YS, Lee IH. Effects of two hemolymph proteins on humoral defense reactions in the wax moth, *Galleria mellonella*. Dev Comp Immunol. 2005; 29: 43–51.

59. Loulou A, M'saad Guerfali M, Muller A, Bhat AH, Abolafia J, Machado RA, Kallel S. Potential of *Oscheius tipulae* nematodes as biological control agents against *Ceratitis capitata*. PloS one. 2022; 17: e0269106.

60. Meier-Kolthoff JP, Göker M. TYGS is an automated high-throughput platform for state-of-the-art genome-based taxonomy. Nature Communications. 2019; 10: 1–10.

61. Yoon SH, Ha SM, Kwon S, Lim J, Kim Y, Seo H, Chun J. Introducing EzBioCloud: A taxonomically united data base of 16S rRNA and whole genome assemblies. Int J Syst Evol Microbiol. 2017; 67: 1613–1617.

62. Machado RAR, Bhat AH, Abolafia J, Muller A, Bruno P, Fallet P, Toepfer S. Multi-locus phylogenetic analyses uncover species boundaries and reveal the occurrence of two new entomopathogenic nematode species, *Heterorhabditis ruandica* n.sp. and *Heterorhabditis zacatecana* n.sp. Journal of Nematology. 2021; 53: 1–42.

63. Petit RA, Read TD. Bactopia: a flexible pipeline for complete analysis of bacterial genomes. Msystems. 2020; 5: e00190–20. https://doi.org/10.1128/mSystems.00190-20 PMID: 32753501

64. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics (Oxford, England). 2014; 30: 2114–2120. https://doi.org/10.1093/bioinformatics/btu170 PMID: 24695404

65. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. Journal of Computational Biol. 2012; 19: 455–77. https://doi.org/10.1089/cmb.2012.0021 PMID: 22506599

66. Walker BJ, Abeel T, Shea T, Priest M, Abouelliel A, Sakthikumar S, et al, Pilon: an integrated tool for comprehensive microbial variant detection and genome assembly improvement. PloS one. 2014; 9: e112963. https://doi.org/10.1371/journal.pone.0112963 PMID: 25409509

67. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl Acids Symp Ser. 1999; 41: 95–98.

68. Mastore M, Caramella S, Quadroni S, Brivio MF. *Drosophila suzukii* susceptibility to the oral administration of *Bacillus thuringiensis*, *Xenorhabdus nematophila* and its secondary metabolites. Insects. 2021; 12: 635.

69. Mastore M, Quadroni S, Toscano A, Mottadelli N, Brivio MF. Susceptibility to entomopathogens and modulation of basal immunity in two insect models at different temperatures. Journal of thermal biology. 2019; 79: 15–23. https://doi.org/10.1016/j.jtherbio.2018.11.006 PMID: 30612676

70. Mastore M, Brivio MF. Basic methods to evaluate humora immunity processes in Lepidoptera larvae. *Immunity in Insects*, Humana, New York, NY. 2020; 127–144. https://doi.org/10.1007/978-1-0716-0259-1_8

71. Paradis E, Claude J, Strimmer K. APE: analysis of phylogenetics and evolution in R language. Bioinformatics. 2004; 20: 289–290.

72. Harmon LJ, Weir JT, Brock CD, Glor RE, Challenger W. GEIGER: investigating evolutionary radiations. Bioinformatics. 2008; 24: 129–131. https://doi.org/10.1093/bioinformatics/btm538 PMID: 18006550

73. Shokoohi E, Mashela PW, Machado RA. Bacterial communities associated with *Zeldia punctata*, a bacterivorous soil-borne nematode. International Microbiology. 2022; 25: 207–216.

74. Jiménez-Cortés JG, Canales-Lazcano J, Lara-Reyes N, Rosenblueth M, Martínez-Romero E, Contreras-Garduño J. Microbiota from *Rhabditis Regina* may alter nematode entomopathogenicity. Parasitology Research. 2016; 115: 4153–4165.

75. Park HW, Kim YO, Ha JS, Youn SH, Kim HH, Bilgrami AL, Shin CS. Effects of associated bacteria on the pathogenicity and reproduction of the insect-parasitic nematode *Rhabditisblumi* (Nematoda: Rhabditida). Can J Microbiol. 2011; 57:750–758. https://doi.org/10.1139/w11-067

76. Rana A, Bhat AH, Chaubey AK, Půža V, Abolafia J. Redescription and synonymization of *Oscheius citri* Tabassum, Shahina, Nasira and Erum, 2016 (Rhabditida, Rhabditidae) from India and its taxonomical consequences. Journal of Helminthology. 2021; 95: E24. https://doi.org/10.1017/S0022149X21000134

77. Bhat AH, Gautum S, Rana A, Chaubey AK, Abolafia J, Půža V. Morphological, morphometrical and molecular characterization of *Oscheius siddiqii* Tabassum and Shahina, 2010 (Rhabditida, Rhabditidae) from India with its taxonomic consequences for the subgenus *Oscheius* Andrássy,1976. Biology. 2021; 10: 1239. https://doi.org/10.3390/biology10121239

78. Sangeetha BG, Jayaprakas CA, Siji JV, Rajitha M, Shyni B, Mohandas C. Molecular characterization and amplified ribosomal DNA restriction analysis of entomopathogenic bacteria associated with *Rhabditis* (*Oscheius*) spp. Biotechnology. 2016; 6: 1–13. https://doi.org/10.1007/s13205-015-0326-1

79. Fekete T. *Bacillus* species and related genera other than *Bacillus anthracis*, in Principles and Practice of Infectious Diseases, Seventh Ed, Mandell G.L., Bennett J. E., and Dolin R. Churchill Livingstone Elsevier, Philadelphia, PA. 2010; 209: 2727–2731.

80. Ahmed I, Yokota A, Yamazoe A, Fujiwara T. Proposal of *Lysinibacillus boronitolerans* gen. nov. sp. nov., and transfer of *Bacillus fusiformis* to *Lysinibacillus fusiformis* comb. nov. and *Bacillus sphaericus* to *Lysinibacillus sphaericus* comb. Nov. Int J Syst Evol Microbiol. 2007; 57: 1117–1125.

81. Passera A, Rossato M, Oliver JS, Battelli G, Cosentino E, Sage JM, Toffolatti SL, Lopatriello G, Davis JR, Kaiser MD, Delledonne M. Characterization of *Lysinibacillus fusiformis* strain S4C11: *in vitro*, in planta, and in silico analyses reveal a plant-beneficial microbe. Microbiol Res. 2020; 244: 126665.

82. Trivedi P, Spann T, Wang N. Isolation and characterization of beneficial bacteria associated with citrus roots in Florida. Microb Ecol. 2011; 62: 324–336. https://doi.org/10.1007/s00248-011-9822-y PMID: 21360139

83. Singh RK, Kumar DP, Solanki MK, Singh P, Srivastva AK, Kumar S, et al. Optimization of media components for chitinase production by chickpea rhizosphere associated *Lysinibacillus fusiformis* B-CM18. J Basic Microbiol. 2013; 53: 451–460.

84. Eski A, Demir I, Güllü M, Demirbağ Z. Biodiversity and pathogenicity of bacteria associated with the gut microbiota of beet armyworm, *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae). Microbial Pathogenesis. 2018; 121: 350–358.

85. Smith BR, Unckless RL. Draft Genome Sequence of *Lysinibacillus fusiformis* Strain Juneja, a Laboratory-Derived Pathogen of *Drosophila melanogaster*. Genome Announcements. 2018; 6: e01571–17. https://doi.org/10.1128/genomeA.01571-17 PMID: 29437109

86. Usta M. Isolation and determination of bacterial microbiota of *Varroa destructor* and isolation of *Lysinibacillus* sp. from it. Egyptian Journal of Biological Pest Control. 2021; 31: 1–8. https://doi.org/10.1186/s41938-021-00482-7

87. Silby MW, Winstanley C, Godfrey SA, Levy SB, Jackson RW. *Pseudomonas genomes*: diverse and adaptable. FEMS Microbiol Rev. 2011; 35: 652–680.

88. Yorgey P, Rahme LG, Tan MW, Ausubel FM. The roles of mucD and alginate in the virulence of *Pseudomonas aeruginosa* in plants, nematodes and mice. Mol Microbiol. 2001; 41: 1063–1076.

89. Maciel-Vergara G, Jensen AB, Eilenberg J. Cannibalism as a possible entry route for opportunistic pathogenic bacteria to insect hosts, exemplified by *Pseudomonas aeruginosa*, a pathogen of the giant mealworm *Zophobasmorio*. Insects. 2018; 9: 88. https://doi.org/10.3390/insects9030088

90. Jander G, Rahme LG, Ausubel FM. Positive correlation between virulence of *Pseudomonas aeruginosa* mutants in mice and insects. J. Bacteriol. 2000; 182: 3843–3845.

91. Chieda Y, Iiyama K, Lee JM, Kusakabe T, Yasunaga-Aoki C, Shimizu S. Virulence of an exotoxin A-deficient strain of *Pseudomonas aeruginosa* toward the silkworm, *Bombyx mori*. Microb Pathog. 2011; 51: 407–414.

92. Tan MW, Mahajan Miklos S, Ausubel FM. Killing of *Caenorhabditis elegans* by *Pseudomonas aeruginosa* used to model mammalian bacterial pathogenesis. Proc Natl Acad Sci, USA. 1999; 96: 715–720.

93. Papaioannou E, Wahjudi M, Nadal-Jimenez P, Koch G, Setroikromo R, Quax WJ. Quorum-quenching acylasereduces the virulence of *Pseudomonas aeruginosa* in a *Caenorhabditis elegans* infection model. Antimicrob Agents Chemother. 2009; 53: 4891–4897.

94. He Y, Qin Q, DiLegge MJ, Vivanco JM. Isolation of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* from entomopathogenic nematode-insect host relationship to examine bacterial pathogenicity on *Trichoplusiani*. Microbial Pathogenesis. 2019; 135: 103606.

95. Ehling-Schulz M, Frenzel E, Gohar M. Food–bacteria interplay: pathometabolism of emetic *Bacillus cereus*. Front Microbiol. 2015; 6: 704. https://doi.org/10.3389/fmicb.2015.00704

96. Sezen K, Demir I, Kati H, Demirbag Z. Investigations on bacteria as a potential biological control agent of summer chafer, *Amphimallon solstitiale* L. (Coleoptera: Scarabaeidae). J. Microbiol. 2005; 43: 463–468.

97. Selvakumar G, Mohan M, Sushil SN, Kundu S, Bhatt JC, Gupta HS. Characterization and phylogenetic analysis of an entomopathogenic *Bacillus cereus* strain WGPSB-2 (MTCC 7182) isolated from white grub *Anomala dimidiata* (Coleoptera: Scarabaeidae). Biocontrol Sci Technol. 2007; 17: 525–534.

98. Sushil SN, Mohan M, Selvakumar G, Bhatt JC, Gupta HS. Isolation and toxicity evaluation of bacterial entomopathogene against phytophagous white grubs (Coleoptera: Scarabaeidae) in Indian Himalayan hills. Int J Pest Manag. 2008; 54: 301–307.

99. Abdallah RA, Nefzi A, Jabnoun-Khiareddine H, Messaoud C, Stedel C, Papadopoulou KK, et al. A putative endophytic *Bacillus cereus* str. S42 from *Nicotiana glauca* for biocontrol of *Fusarium* wilt

disease in tomato and gas chromatography-mass spectrometry analysis of its chloroform extract. Archives of Phytopathology and Plant Protection. 2016; 49: 343–361.

100. Ehling-Schulz M, Lereclus D, Koehler TM. The *Bacillus cereus* group: *Bacillus* species with pathogenic potential. Microbiol Spectrum. 2019; 7:1–60.

101. Kumar SN, Nath VS, Chandran RP, Nambisan B. Cyclic dipeptides from rhabditid entomopathogenic nematode associated *Bacillus cereus* have antimicrobial activities. World J Microbiol Biotechnol. 2014; 30: 439–449.

102. Bagley ST. Habitat Association of *Klebsiella* Species. Cambridge University Press. 2015. https://doi.org/10.1017/S0195941700062603 PMID: 3882590

103. Fouda A, Awad MA, Kabadaia MM. The insecticidal activity of entomopathogenic bacteria isolated from *Musca domestica*, *Chrysomya albiceps* and *Lucilia sericata* against the Mosquito larvae of *Culex pipiens*. Egyptian Academic J of Biological Sciences E Medical Entom and Parasitology. 2021; 13: 23–28.

104. Boemare N, Akhurst R, Mourand RG. DNA relatedness between *Xenorhabdus* spp. (Entero-bacteria-cae), symbiotic bacteria of entomopathogenic nematodes, and a proposal to *Xenorhabdus luminescens* to a new genus, *Photorhabdus* gen. nov. International. Journal of Systematic bacteriology. 1993; 43: 249–255.

105. Chattopadhyay A, Bhatnagar NB, Bhatnagar R. Bacterial insecticidal toxins. Critical reviews in microbiology. 2004; 30: 33–54. https://doi.org/10.1080/10408410490270712 PMID: 15116762

106. Janda JM, Abbott SL, Khashe S, Robin T. Biochemical investigations of biogroups and subspecies of *Morganella morganii*. Journal of clinical microbiology. 1996; 34: 108–113.

107. Lalitha K, Kalaimurgan D, Nithya K, Venkatesan S, Shivakumar MS. Antibacterial, antifungal and mosquitocidal efficacy of copper nanoparticles synthesized from entomopathogenic nematode: Insect–host relationship of bacteria in secondary metabolites of *Morganella morganii* sp. (PMA1). Arabian Journal for Science and Engineering. 2020; 45: 4489–4501.

108. Kumar SN, Siji JV, Rajasekharan KN, Nambisan B, Mohandas C. Bioactive stilbenes from a *Bacillus* sp. N strain associated with a novel rhabditid entomopathogenic nematode. Lett Appl Microbiol. 2012; 54: 410–417.

109. Kumar SN, Mohandas C, Nambisan B, Sreerag RS, Jayaprakas CA. Cyclo(l-Pro-d-Arg): a new anti-bacterial and antitumour diketopiperazine from *Bacillus cereus* associated with a rhabditid entomo-pathogenic. Folia Microbiol. 2014; 59: 197–202.

110. Freitak D, Ots I, Vanatoa A, Hörak P. Immune response is energetically costly in white cabbage butter-fly pupae. Proceedings of the Royal Society of London. Series B: Biological Sciences. 2003; 270: S220–S222. https://doi.org/10.1098/rsbl.2003.0069 PMID: 14667388

111. Schmid-Hempel P. Variation in immune defence as a question of evolutionary ecology. Proceedings of the Royal Society of London. Series B: Biological Sciences. 2003; 270: 357–366. https://doi.org/10.1098/rspb.2002.2265 PMID: 12639314

112. Siva-Jothy MT, Moret Y, Rolff J. Insect immunity: an evolutionary ecology perspective. Advances in insect physiology. 2005; 32: 1–48.

113. Ardia DR, Gantz JE, Strebel S. Costs of immunity in insects: an induced immune response increases metabolic rate and decreases antimicrobial activity. Functional ecology. 2012; 26: 732–739.