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XXXV CYCLE

STUDIES ON THE POTENTIAL AND SAFETY OF EMERGING ENTOMOPATHOGENIC BACTERIA

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ABSTRACT

The present thesis had the objective of studying some emerging entomopathogenic bacteria determining their potential against invertebrate pests (i.e., insects and nematodes) and evaluating their safety profile for non-targets insects. Accordingly, experiments involved different strains of the spore former *Brevibacillus laterosporus* and of the multitalented plant growth promoting species *Pseudomonas protegens*. More in detail, spores of an insecticidal strain of *B. laterosporus* (UNISS 18) were assayed on a range of water-dwelling invertebrates including mosquito larvae and some non-target species, revealing a high susceptibility of mosquito larvae, and different degrees of susceptibility of other species, which suggests a careful use of this bacterium in the aquatic environment. A honeybee-borne strain of *B. laterosporus* (F5) was instead assayed on soil-dwelling nematodes including the pest species *Meloidogyne incognita*. A protein fraction containing different virulence factors extracted from the culture supernatant of this bacterial strains proved to be effective in causing a significant nematode mortality. The biocidal potential against *Culex pipiens* and *Aedes albopictus* of a recently isolated strain of *P. protegens* (COI) was determined by laboratory bioassays in which this bacterium was found to be active against both species. In addition to biocidal effects, when larvae were exposed to sub-lethal concentrations, also immature development was significantly affected. Comparative experiments including reference strain CHA0 were also conducted, which confirmed for the first time the potential of *P. protegens* against mosquitoes. To investigate the safety of this bacterial species that has primary importance for the agroecosystem management, investigations were conducted on the lacewing *Chrysoperla carnea*. This study confirmed the safety of this entomopathogenic bacterium to green lacewing immatures and adults, with no significant effects on development and adult reproductive potential. According to the results of this thesis, both entomopathogenic bacterial species appears promising in the prospect of their future exploitation for the management of invertebrate pests of agricultural and medical-veterinary importance.

CHAPTER I

1. INTRODUCTION

1.1 Microbial control

The term microbial control was first used by Steinhaus to indicate the management of pest populations employing microorganisms as biological control agents (BCAs). Protections of crops, farm animals and humans from insect pests have historically relied on the intense use of insecticidal substances, mostly chemicals, that have fed an industry that developed highly specific formulations, including co-formulants and coadjuvant, ensuring an improved efficacy of commercially available products (Green, 2000). Because of the concerns to human health and the environment associated with the use of chemicals, such as the risks of environmental pollution, contamination of food and water, intoxication of operators, health effects on non-target organisms, including insect predators and pollinators, the search for alternatives with lower ecological impact are fostered. As an additional risk, repeated chemical applications have been the cause of insect population selection pressure, especially when high doses were used, which have often resulted in rapid insect adaptation and resistance development. Accordingly, more than 500 species of insects and mites were reported to have developed resistance to insecticides and miticides (Georghiou and Lagunes-Tejeda, 1991). For all these reasons, the use of low-impact insecticidal products is strongly encouraged. Such eco-sustainable approach has led to the implementation of integrated pest management (IPM) programs involving the combined use of modern chemical products and biocontrol strategies (Ruiu, 2018). This critical need for new, selective and safe pest control system is stimulating considerable interest in using microbes as a means of controlling pests in a sustainable manner. In 2018, the global market for microbial pesticides was evaluated at USD1.94 billion and is expected to reach USD 4.75 billion in 2024 (MordorIntelligence Report 2021). The microbial agents used as bioinsecticides usually include microorganims (viruses, bacteria, fungi, protozoa, and entomopathogenic nematodes) as well as toxic substances they produce (Tanada and Kaya, 2012). Depending on their formulation,

microbials can often be applied in a similar fashion as traditional insecticidal sprays, dusts, liquid drenches, liquid concentrates, wettable powders, or granules. Although frequently bioinsecticides require specific precautions in application in order to maximize their effectiveness and persistence. The greatest strength of these products is their safety, as they are generally regarded as safe to non-target organisms, including humans.

Despite their benefits and their safety profile, the efficacy of microbial insecticides is often limited, and they can be rapidly inactivated in the environment. Accordingly, research activities are in progress to face the limitations of bioinsecticides, like their narrow spectrum of targets often related to a specific mechanism of action. In line with these research efforts, new generation microbial insecticides are expected to face most of these limitations playing an increasingly important role in pest management in the near future.

1.2 Entomopathogenic bacteria

Among the diversity of bacteria that evolved ways of interactions with insects, entomopathogenic species are those that developed in the direction of overcome insect's immune system initiating a pathogenic process leveraging ad hoc evolved mechanisms. Among all microbial entomopathogens, bacteria include largely employed and studied agents, especially sporogenic species, even though several non-spore forming species are emerging (Ruiu, 2015). Today, several plant protection products and biocides used in agriculture for crop pests' biocontainment and in the animal husbandry context for the management of parasites and vectors, like flies and mosquitoes, are based on entomopathogenic bacteria and their bioactive compounds. A major component of entomopathogenic bacteria efficacy in the field is represented by the physico-chemical characteristics of the commercial formulations and the way they are applied, which should ensure appropriate coverage of the target crops. A specialized industry for bacterial processing and their product formulation has grown in the last decades, with major goals focused on maximizing shelf life, enhance dispersion and adhesion, lessen spray drift, and most importantly, boost effectiveness (Lacey et al., 2001).

The primary category of entomopathogenic bacteria and the most significant for application purposes, has historically been represented by the Bacillaceae family, with the two genera *Clostridium* and *Bacillus*. These Gram-positive bacteria, typically reproduce by sporulation. The species that have had the largest use in the biological control of insects belong to the genus *Bacillus*. The larvicidal potential of *Bacillus thuringiensis* (Bt) against Lepidoptera, Diptera, and Coleoptera (Palma et al., 2014), is mostly related with the production of crystal proteins (Cry) acting at the midgut level after ingestion, determining the disruption of the intestinal barriers and paving the way to the haemocoel. After ingested, protoxins are solubilized in the intestine and activated by specific enzymes, they bind to specific epithelial receptors in which eventually led to the formation of pores altering cell membrane permeability up to cell lysis and disruption. This is normally followed by insect paralysis and death (Ruiu, 2015). Distinct strains of Bt produce different Cry toxins, but they may also release other insecticidal toxins including Insecticide Vegetative Proteins (VIP), thermolabile α-hexotoxin, thermostable β-exotoxin or Turingiensin. Among the variety of Bt strains, the subspecies *israelensis* (Bti) was discovered in 1977 by Goldberg and Margalit as a mosquito larval pathogen. This discovery prompted extensive investigation into its potential application in the management of mosquitoes. Another *Bacillus* species, *B. sphaericus*, was produced to complement Bti in the control of certain mosquito species, less susceptible to Bti.

Earlier Bt-based foliar insecticides and some of the current ones are based on wild type strains, meaning that they were discovered in nature in the same form in which they are used as active substance of commercial products. On the other hand, research work demonstrated that some artificial Cry protein combinations may produce synergistic effects. According to a more complex mechanism of action, certain bacterial enzymes, including chitinase and protease, may take part in the natural insect pathogenic process leading to the degradation of larval tissues favoring the germination of spores and the eventual bacterial septicaemia (Grady et al., 2016). It is widely acknowledged that Bt has been an essential tool in biological and integrated pest management in line with an environmentally friendly pest control strategy. However,

because of its mode of action and the resulting narrow action spectrum, its employment is successful on in certain niche market segments (Glare et al., 2012). For these reasons, industry and academia are working continuously to find novel Bt strains, alternative bacterial species, and insecticidal genomic traits that have a wider target range and sufficient potency. On the other hand, the marketability of new microbial products depends also on their environmental and non-target organism safety. Accordingly, risk-assessment tests using beneficial insects like pollinators (eg. honeybees), predators and parasites (eg. Chrysopids, coccinellids, hymenopteran wasps), are needed for pre-market registration procedures (Ruiu, 2018). The side-effects that could result from the use of a bioinsecticide are not limited to the acute toxicity that they might have against some non-targets, but important effects could be brought on by ingesting sub-lethal doses that may affect immature development, longevity and reproductive efficiency (Cloyd, 2012). These factors have led to an increase in the significance of studies looking at the potential impact of entomopathogenic bacteria and their metabolites on these non-target species. In this contest, in addition to Bt, other entomopathogenic bacterial species are attracting attention because of their potential toward different target pests. Among them, *Brevibacillus laterosporus* and *Pseudomonas protegens*, that are object of the present thesis.

1.2.1 Brevibacillus laterosporus

The aerobic spore former *Brevibacillus laterosporus* (Laubach), another entomopathogenic bacterium, is distinguished by the development of a distinctive canoe-shaped lamellar body connected to one side of the spore-coat. Although this species was initially isolated from water (Laubach, 1916), it may also be collected from soil where it engages in a range of interactions with other living organisms and participates in the biogeochemical cycles (Ruiu, 2013). As a member of the rhizosphere's microbial community, it supports plant growth by competing with phytopathogens for nutrients and space (Chen et al., 2017). The synthesis of several substances, including peptides and antibiotics, gives *B. laterosporus* a significant antibacterial potential in addition to its

indirect protective function (Jiang et a., 2015; Zhao et al., 2012). At the beginning of the 20th century, this bacterial species was isolated from diseased honeybees, suggesting that it could be an insect pathogen, even though its presence in the hive was then understood to be secondary (McCray, 1917). More recently, the insecticidal properties of *B. laterosporus* have been demonstrated against mosquito larvae of various species (*Aedes aegypti* L., *Aedes albopictus* (Skuse), *Anopheles stephensi* Liston, *Culex pipiens* L., and *Culex quinquefasciatus* Say) and black flies [*Simulium vittatum* (Zetterstedt)] (Favret and Yousten, 1985; Rivers et al., 1991, Ruiu et al., 2007; Zubasheva et al, 2010), against coleopteran and lepidopteran larvae (Oliveira et al., 2004), and against nematodes and molluscs (Singer, 1996). There have also been reports of some *B. laterosporus* strains producing parasporal inclusions resembling those produced by *B. thuringiensis* (Smirnova et al., 1996). The toxicity of some of these *B. laterosporus* strains against mosquitoes was associated to these inclusions, although their composition was not examined in depth. Additionally, entomopathogenic activity has also been reported in the absence of these inclusions (Orlova et al., 1998). Previous research has shown that the muscoid fly parasitoid wasp *Muscidifurax raptor* Girault and Sanders (Hymenoptera: Pteromalidae) is slightly susceptible when exposed to higher doses than those that are effective against the host flies (Ruiu et al., 2007). Such large spectrum of action is the result of an arsenal of protein toxins, virulence factors, enzymes (eg. proteases, chitinases) and various polyketides and nonribosomal peptides it produces (Djukic et al., 2011, Marche et al., 2018; Glare et al., 2020). Spores or cells of this bacterial entomopathogen act in the gut of the host causing a disruption of the intestinal barriers (Ruiu et al., 2012).

Brevibacilus laterosporus represents an interesting example to define the specificity of bacterial pathogenicity, given that some strains are toxic to certain insect species while are not active against others, with special regard to non-targets.

1.2.2 Pseudomonas protegens

The soil-dwelling bacterium *Pseudomonas protegens* is well-known among the variety of bacteria that live in the soil where it competes in the rhizosphere- an ecological hotspot that attracts many kinds of organisms- establishing several interactions with the plant and other microbials (Lugtenberg and Kamilova, 2009, Hol et al., 2013). Due to its capacity to play important roles in the agroecosystem by filling specific ecological niches, the interest in this pseudomonad species is increasing (Agaras et al., 2017). Such beneficial properties include the interaction with the plant root system, leading to increased access to nutrients in the soil through support in nitrogen fixation, siderophore secretion, phosphorus solubilization, phytohormone synthesis, and improvement in water uptake (Sivasakthi et al., 2014). Additionally, the bacterium's capacity to colonize the root leads to the development of protective biofilms and the development of a closer bond with the plant, up to endophytism (Morales-Cedeño et al., 2021). Because of the increased competition for nutrients and available space, as well as the production of antibiotics and other bioactive compounds, this results in greater plant resistance to phytopathogens (Ramamoorthy et al., 2001; Ramette et al., 2011). Studies on *P. protegens* reference strain CHA0 provided a wider view on the variety of bioactive compounds this species can produce, such as chitinase and phospholipase C (Flury et al., 2016), hydrogen cyanide, the cyclic lipopeptide orfamide (Flury et al., 2017, Jang et al., 2013) the toxin rhizoxin (Loper et al., 2016), and certain lipopolysaccharide O-antigens (Kupferschmied et al., 2016). Different strains of this bacterium have consistently been reported to be effective, either orally or through intra-haemocoelic injection, against a range of insect pests (Vesga et al., 2020). The insecticidal action by ingestion implies colonizing the insect gut after oral intake and transmigrating into the hemolymph, where systemic infections take place up to the eventual death of several Lepidopteran, Dipteran, Coleopteran, and Hemipteran insect species (Ruffner et al., 2013, Flury et al., 2019). This action is supported by enzymes like chitinases, possibly involved in the degradation of peritrophic matrix (Ruiu and Mura, 2021), and by toxins interacting with the epithelial cell membranes, such as the fluorescent insecticidal toxin (Fit) secretion system that include the high molecular weight protein, FitD (McCarthy et al., 2012). This toxin was also found to harbor a pore-forming domain (Ruffner et al.,

2015). According to the production of several insect virulence factors and a significant pathogenic potential, *P. protegens* appears also to be a promising candidate for pest management (Kupferschmied et al., 2013; Keel, 2016).

1.3 Target pests and non-target species

In this thesis, different insect and nematode species were used according to the aim of each study, assaying the biopesticidal potential and/or the safety of the abovementioned entomopathogenic bacteria.

1.3.1 Mosquitoes

Two distinct species of mosquitoes widely distributed and noxious in the Mediterranean area, were involved in laboratory bioassays: *Aedes albopictus* (Skuse) and *Culex pipiens* L.. Eggs of *Ae. albopictus* was collected in the urban center of Sassari (Sardinia, Italy) to produce colonies, while *C. pipiens* form *pipiens* was established in the laboratory after collecting larvae in Oristano province (Sardinia, Italy). The insectary of the Laboratory of Entomology and Vector Control of the Istituto Zooprofilattico Sperimentale della Sardegna in Sassari, Italy, has housed both species in stable colonies.

At the time of the stable colony's establishment, a molecular characterization of the *pipiens* form was first carried out according to the protocol of Bahnck and Fonseca (2006). Insect rearing was conducted in an insectary with specific environmental parameters, where mosquito populations were raised in cages. Adults were fed sucrose solutions (10%) and given a blood meal once a week. Larvae and pupae were reared in distilled water and fed with food cat powder.

1.3.2 Physella acuta

Invasive snail adult of *P. acuta* collected from rearing field at the Department of Agriculture, Food and Environment were then moved to laboratory conditions, where they

were molecularly characterized and identified (Benelli et al., 2015). *P. acuta* snails were kept in polypropylene aquaria. Three times per week, dead snails and faeces were removed from the aquarium during cleaning. The leaves of lettuce were consumed. For bioassays, only adult snails were employed.

1.3.3 Cloeon dipterum

The mayfly *C. dipterum* nymphs, non-target water-dwelling species, were collected from rearing field by the Department of Agriculture, Food and Environment, and identified using the keys provided by Grandi (1960). Mayflies were reared under laboratory conditions in polyethylene aquaria containing tap water and fed leaf litter. For the bioassays, late instar nymphs were utilized.

1.3.4 Chironomus riparius

The harlequin flies *Chironomus riparius*, a non-biting species, was reared in cages with a water bowl filled with sediment at the bottom for oviposition. The swarming behavior, however, is extremely adaptable and varies between individuals (Caspary et al. 1971).

1.3.5 Chrysoperla carnea

Several chrysopid species, predators of several plant pests (aphids, lepidopterans, mites, thrips, and whiteflies) (Principi and Canard, 1984; Senior and McEwen, 2001), have been successfully used in pest management programs to ensure the conservation and growth of their populations in the agricultural ecosystem (Cordeiro et al., 2010).

The effects of various chemically active compounds on these helpful insects have been the subject of several research, but the potential toxicity or pathogenicity of entomopathogenic microbials on non-target organisms is still not well known (Michaud and Grant, 2003).

1.3.6 Panagrellus redivivus

The free-living nematode *Panagrellus redivivus*is frequently used in studies related to the water and soil toxicity, highlighting the interest in its reproduction, movement and feeding influenced by pollutants and toxins (Boyd and Williams 2003, Niu et al. 2010).

This model species was employed in preliminary bioassays testing the efficacy of entomopathogenic bacteria in the laboratory.

1.3.7 Meloidogyne incognita

Meloidogyne incognita, root-knot nematode (RKN), known for its severe effect on vegetables leading to crop production losses (Ibrahim et al., 2010).

To manage serious plant pathogens, including plant parasitic nematodes, the use of organic amendments or biofumigants deriving from plant residues and agro-industrial wastes has been a point of interest to conduct different research by numerous authors (Ploeg and Stapleton. 2001; Mashela, 2002; Bailey and Lazarovits, 2003; López- Pérez et al., 2005; López-Pérez et al., 2010; Karavina and Mandumbu, 2012; Kruger et al., 2013).

1.4 Thesis objectives and achievements

The general thesis objective was to contribute to increase the knowledge on the bioinsecticidal potential of the emerging entomopathogenic bacteria *Brevibacillus laterosporus* and *Pseudomonas protegens*, against specific targets pests, including insects and nematodes, and to evaluate their safety to some non-target insects.

The specific objectives are listed below:

- Study and characterize the potential of a honeybee-borne strain of *B. laterosporus* against soil-dwelling nematodes.
- Evaluate the lethal and sublethal activity of *Brevibacillus laterosporus* on the mosquito *Aedes albopictus* and the possible side effects on non-target water-dwelling invertebrates.
- Test and characterize the lethal and sub-lethal effects of a recently isolated *P. protegens* strain against mosquitoes with medical and veterinary importance
- Asses the safety of this *P. protegens* strain to the beneficial predatory insect *Chrysoperla carnea*.

These studies led to the production of the following three co-authored manuscripts already published in peer-reviewed international scientific journals, and one manuscript under submission:

1. Stefano Bedini, Barbara Conti, Rim Hamze, Elen Regozino Muniz, Éverton K.K. Fernandes, Luca Ruiu, 2021**.** Lethal and sublethal activity of *Brevibacillus laterosporus* on the mosquito *Aedes albopictus* and side effects on non-target water-dwelling invertebrates. [Published on Journal of Invertebrate Pathology (2021), Vol 184, 107645]
2. Rim Hamze, Maria Tiziana Nuvoli, Carolina Pirino, Luca Ruiu, 2022. Compatibility of the bacterial entomopathogen *Pseudomonas protegens* with the

natural predator *Chrysoperla carnea* (Neuroptera: Chrysopidae). [Published on Journal of Invertebrate Pathology (2022), Vol 194, 107828]

3. Rim Hamze and Luca Ruiu, 2022. *Brevibacillus laterosporus* as a natural biological control agent of soil-dwelling nematodes [Published on Agronomy (2022) 12, 2686].

4. Rim Hamze, Cipriano Foxi, Salvatore Ledda, Giuseppe Satta, Luca Ruiu, 2022. Mosquitocidal activity of *Pseudomonas protegens* [under submission]

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CHAPTER II

Lethal and sub-lethal activity of *Brevibacillus laterosporus* on the mosquito *Aedes albopictus* and side effects on non-target water-dwelling invertebrates

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2.1. Abstract

The biocidal potential of *Brevibacillus laterosporus* against mosquitoes of major medical importance has been widely documented, but its effects on non-target invertebrates are still poorly known. In this study, we determined the lethal and sub-lethal effects of *B. laterosporus* strain UNISS 18, an entomopathogenic bacterium known for its effectiveness against synanthropic Diptera, on the larvae of the Asian tiger mosquito *Aedes albopictus*, a vector of several pathogens to humans. Moreover, we compared the larvicidal activity with the lethal action on the invasive snail *Physella acuta* and on two non-target water-dwelling species: the mayfly *Cloeon dipterum,* and the harlequin fly *Chironomus riparius*. *B. laterosporus* exhibited significant lethal effects on all the tested species with a concentration-dependent activity. However, the susceptibility varied among species, with a higher susceptibility of *Ae. albopictus* (LC_{50} = 0.16 × 10^7 spores mL^{-1}) than the other species (LC_{50} = 0.31, 0.33, and 0.30 × 10^7 spores mL^{-1} for *C. dipterum*, *C. riparius*, and *P. acuta*, respectively). While 1st instar mosquito larvae were very susceptible to the bacterial infection, no effects on preimaginal development stages and adult emergence were observed at sub-lethal spores' concentrations. Even if the efficacy of *B. laterosporus* against *Ae. albopictus* and the invasive freshwater snail *P. acuta* is promising for their control, the susceptibility of non-target beneficial aquatic insects, highlights the need of accurate evaluations before applying *B. laterosporus* for pest management in water environments.

Key words: biocide; pest management; mosquitoes, bacteria, non-target

2.2. Introduction

Brevibacillus laterosporus is a spore-forming bacterium found in various matrices, including soil, water, invertebrates, plants, and food, and known for its biopesticidal properties against a wide range of invertebrate pests including insects in different orders (Coleoptera, Lepidoptera, Diptera), nematodes and mollusks (Ruiu, 2013). Such large spectrum of action is the result of an arsenal of toxins and virulence factors it produces (Marche et al., 2018; Glare et al., 2020). Among Diptera, *B. laterosporus* is active against mosquito larvae of different species including *Aedes aegypti* L., *Aedes albopictus* (Skuse), *Anopheles stephensi* Liston, *Culex pipiens* L., and *Culex quinquefasciatus* Say (Favret and Yousten, 1985; Ruiu et al., 2007a; Zubasheva et al, 2010). The insecticidal activity of *B. laterosporus* normally involves the ingestion of spores or cells that act in the gut of the host causing a disruption of the intestinal barriers, as a consequence of a toxin-mediated process (Ruiu et al., 2012). Although active against various dipteran pests, *B. laterosporus* has been shown to be safe for several terrestrial non-target insects such as green lacewings (Ruiu et al., 2020) and muscoid fly pupal parasitoids (Ruiu et al., 2007b). According to these results, a coevolutionary process in which *B. laterosporus* has developed potential pathogenicity specific to certain insect groups could be assumed. A similar coevolutionary process has not been documented for aquatic environments where the mosquito larvae live. Therefore, specific studies are needed to increase knowledge on the interactions that this microorganism can establish with other water-associated invertebrates.

Accordingly, the aim of this study was to evaluate the insecticidal and the sublethal effects of *B. laterosporus* against *Ae. albopictus* and to compare them with the bacterial activity against the invasive snail *P. acuta* and the two non-target water-dwelling insect species, *C. dipterum*, and *C. riparius*.

2.3. Materials and methods

2.3.1. Bacterial preparations

Spores of *Brevibacillus laterosporus* strain UNISS 18 (= NCIMB 41419), an entomopathogenic bacterial strain known for its insecticidal activity against Diptera (Bedini et al., 2020) were produced in LB broth in a shaking incubator at 180 rpm and 30

°C, according to the methods described by Marche et al. (2017). The spores were harvested from sporulated cultures by centrifugation at 15,000 × *g* at 4 °C for 15 min and resuspended in water to obtain a stock concentration of 1×10^9 spores $mL^{-1}$and stored at -20 °C until use.

2.3.2. Invertebrate samples

Aedes albopictus eggs were maintained at the Department of Agriculture Sciences (University of Sassari, Italy) in tap water to allow hatching of larvae that were fed with cat food powder. Samples of the mayfly *Cloeon dipterum*, the harlequin fly *Chironomus riparius* as well as the samples of bladder snail *Physella acuta* were collected from a field water tank at the Department of Agriculture, Food and Environment (Pisa, Italy); they were reared in the laboratory in polyethylene aquaria (40 × 30 × 30 cm) containing about 10 L of tap water. *C. dipterum* and *C. riparius*, were fed with leaf litter. Adult snails of *P. acuta* were collected and identified at the species level by molecular characterization and fed with lettuce leaves (Benelli et al., 2015).

2.3.3. Bioassays

2.3.3.1 Lethal bioassays on Aedes albopictus

Ae. albopictus larvae of each developing instar (1st, 2nd, and a mixture of 3rd/4th instar) were put in 50 mL plastic cups exposed to *B. laterosporus* tap water suspension (Lacey, 1997). The larvae were fed with cat food powder and inspected every day. Median lethal concentration (LC_{50}) was determined on 1st instar larvae assaying the following range of concentrations: 1.0×10^5; 5.0×10^5; 8.0×10^5; 1.0×10^6; 2.0×10^6; 2.5×10^6; 4.0×10^6; 5.0×10^6; 7.5×10^6; 1.0×10^7; 1.0×10^8 spores mL^{-1} for 48 h. At least three replicates' of 10/20 larvae for each developing stage were used for each concentration assayed, and the whole experiment was repeated three times. The experiments were conducted in an incubator at 25 °C.

2.3.3.2 Sub-lethal bioassays on Aedes albopictus

Ae. albopictus larvae (1st instar) were maintained individually at 25 °C until pupation and adult emergence in plastic wells (2 cm diameter each) filled with tap water, an equal amount of cat food powder, and an appropriate sub-lethal concentration of *B. laterosporus* spores. The experimental design involved three replicates of 24-well plates each. The sub-lethal concentrations 0.25 and 0.1 × 10^6 spores mL^{-1} were assayed in comparison with untreated control containing only water and the same amount of cat food powder.

Treated and control insects were inspected daily to record the dates of larval moult, pupation, adult emergence, and death during the bioassay.

2.3.3.3 Concentration-response bioassays on non-target water-dwelling invertebrates

Groups of 10 individuals of *C. dipterum* late instars nymphs (length 3.9 mm ± 0.2 mm), *C. riparius* third instars larvae, and *P. acuta* adult (length 6.1 ± 0.2 mm) were put in 100 mL beakers and exposed to *B. laterosporus* suspension at the doses of 3 × 10^6, 6 × 10^6, 1.2 × 10^7, 2.5 × 10^7, and 5.0 × 10^7 spores mL^{-1} in tap water. In the control group, 10 individuals were put into tap water only. Mortality was recorded after 48 and 96 h. During the bioassays, *C. dipterum* and *C. riparius* were fed with leaf litter, *P. acuta* with small pieces of lettuce leaves. The bioassays were performed under laboratory conditions (25 °C, 65% R.H., natural photoperiod). Three replicates for each treatment were performed.

2.4 Statistical analysis

Data of the *Ae. albopictus* larval and pupal development time, and percentage of adult emergence were processed with R software (R Development Core Team, 2016) and analysed by ANOVA followed by Least Significant Difference (LSD) tests for post-hoc comparison of means when necessary. Two-ways ANOVA was used to analyse data of experiments involving two independent variables (larval instar and bacterial spore concentration).

The median lethal concentration (LC_{50}) of the *B. laterosporus* spores against the mosquito *Ae. albopictus* and the non-target invertebrate species *C. dipterum*, *C. riparius*, and *P. acuta* were calculated by Log-probit regression (Finney, 1971) by SPSS 22.0 software (IBM SPSS Statistics, Armonk, North Castle, New York, USA).

2.5 Results

2.5.1 Lethal and sub-lethal effects on Aedes albopictus

Mortality percentages of *Ae. albopictus* 1st and 2nd instar larvae exposed for 48 h to progressive concentrations of *B. laterosporus* spores are shown in Fig. 1. In these comparative experiments, mortality was significantly affected by both the larval instar ($F_{1,64}$ = 25.16, $P < 0.0001$) and the spores' concentration ($F_{2,64}$ = 3.09, $P < 0.0001$), with a concentration-dependent effect and higher susceptibility of younger larvae. Concentration dependence was also observed in a separate experiment with a cluster of 3rd/4th instar larvae ($F_{2,12}$ = 3.11, $P < 0.0001$), that appeared to be less susceptible, requiring at least 10^7 spores mL^{-1} to achieve a mortality percentage (35 %) significantly different from untreated control, and a concentration of 10^8 spores mL^{-1} to reach 100 % mortality in 48 h.

The duration of the preimaginal development and the percentage of adults' emergence from individuals that survived to the exposure to sub-lethal doses of *B. laterosporus* are shown in Table 1. No significant differences were detected among control and treatment groups regarding the larval development time ($F_{2,89}$ = 1.69, P = 0.191), the pupal development time ($F_{2,78}$ = 2.36, P = 0.102), and the percentage of adults' emergence ($F_{2,8}$ = 0.09, P = 0.919) calculated based on the number of pupae.

2.5.2 Concentration-response effects on aquatic invertebrates

B. laterosporus spore suspensions showed significant toxicity towards all the species tested in this study, with a concentration-dependent effect (Fig. 2). According to the Probit analysis, the different insects tested showed varied susceptibility to *B. laterosporus*. The highest effectiveness of the bacterial spores was registered against *Ae.*

albopictus larvae, with a LC_{50} value of 0.157×10^7 spores mL^{-1}, whereas the weakest effect was demonstrated against *C. riparius*, whose LC_{50} value was 0.330×10^7 spores mL^{-1} (Table 2).

2.6 Discussion

The ecological relationships that *B. laterosporus* has evolved with insects is still controversial; it is a pathogen for some species (Ruiu, 2013), including the mosquitoes of major medical importance (Bedini et al., 2020), but it is also a common resident in the body of other insect species (Marche et al., 2016), with beneficial effects to the health of honey bees (Brady et al., 2018, Marche et al., 2019), and other terrestrial (Porubcan, 2003) and aquatic (Atmomarsono and Susianingsih, 2020) organisms.

In this study, the survival of both target (*Ae. albopictus*, and *P. acuta*) and non-target invertebrate species (*C. dipterum*, and *C. riparius*) was significantly affected by *B. laterosporus* spores, although with different degrees of susceptibility. The high effectiveness of *B. laterosporus* documented in this study against the target mosquito *Ae. albopictus* is in line with previous studies (Ruiu, 2013; Barbieri et al., 2021) and confirms its high potential as a biological control agent (Glare et al., 2020). Besides mosquitoes, the bioinsecticidal properties of *B. laterosporus* against dipteran pests of medical and veterinary importance are well documented and include the house fly *Musca domestica* L. (Ruiu et al., 2006; Zimmer et al., 2013) and blowflies, such as *Lucilia caesar* L., *Calliphora vomitoria* L., and *Chrysomya* spp. (Bedini e al., 2020; Carramaschi et al., 2017). The pathogenicity against these pests is mediated by several virulence factors including enzymes (i.e., proteases and chitinases) and a variety of toxins including homologous of Cry proteins (Marche et al., 2018; Bowen et al., 2021). The mechanism of action takes place at the insect midgut where activation of the local innate immune response is observed (Mura & Ruiu, 2017), followed by damages to the epithelium and consequent paralysis and death (Ruiu et al., 2012). Similar effects were observed on Nematocera like mosquitoes after being exposed to sporulated cultures of *B. laterosporus* strains producing (Zubasheva et al, 2010) or lacking (Ruiu et al., 2007a) parasporal crystals.

As expected, in our study, the activity of *B. laterosporus* against the Asian tiger mosquito was more significant on younger larvae and a significant insecticidal activity (LC_{95}) was found at spore concentrations above 10^6 spores mL^{-1}, below this limit no sub-lethal effects on the development of surviving larvae were detected. The LC_{50} values observed in this experiment for *Ae. albopictus* were comparable with those previously detected for *Ae. aegypti* and *Culex pipiens* for which LC_{50} values were slightly above 10^5 spores mL^{-1} (Bedini et al., 2020). A higher susceptibility was observed in the laboratory for larvae of *Culicoides* biting midges exposed to the same bacterial strain (UNISS18) on which an LC_{50} level of $2.4x10^4$ spores mL^{-1} was observed (Foxi et al., 2019). Median lethal concentration levels always above 10^8 spores mL^{-1} were instead reported for larvae of Diptera Brachycera, including muscoids and fruit flies (Ruiu et al., 2011; Bedini et al., 2020). Similar effects were shown by Zimmer et al. (2013) on *Musca domestica* (Diptera, Muscidae). The authors observed sub-lethal effects on the development of the flies, reducing both adult size, and impairing the reproductive performance of the species after exposing newly emerged larvae to concentration of 10^7 to 10^9 spores mL^{-1} of the entomopathogenic bacteria *B. laterosporus*, *Bacillus thuringiensis* var. *israelensis* (*Bti*), *B. thuringiensis* var. *kurstaki*, and a commercial formulation of *Bacillus sphaericus* (Zimmer et al., 2013).

In this study, we assessed the effect of *B. laterosporus* also on the invasive freshwater snail *P. acuta*; the observed lethal effect was expected, and it is in line with the toxicity of *B. laterosporus* towards slugs previously reported (Singer et al., 1997). From this perspective, *B. laterosporus,* together with the use of active botanicals (Bedini et al., 2016), might represent a promising control agent for regulating the population dynamics of this invasive snail.

Although the insecticidal and molluscicidal activity of *B. laterosporus* could be useful for the biocontainment of human and agricultural pests, the possible risk it may pose to non-target species if applied to the environment should be also evaluated. The side effects of biocidal treatments to non-target species have substantial implications for a proper use of biocides and for the implementation of the necessary precautions to avoid adverse impact on beneficial aquatic organisms. For this reason, here we extended the risk-

assessments of using *B. laterosporus* by documenting its bioactivity also against two non-target species, the mayfly *C. dipterum*, and the harlequin fly *C. riparius*.

The toxicity tests performed in this study showed that the susceptibility of *C. dipterum* and *C. riparius* to *B. laterosporus* is slightly higher than *Ae. albopictus*. In a recent study, Bordalo et al. (2021) reported a clear lethal and sub-lethal toxicity of *Bti* and *Beauveria bassiana*-based bioinsecticides to *C. riparius* at concentrations below the ones recommended for field application, with potential population-level effects. Our tests indicated that, on average, susceptibility of the non-target species *C. dipterum* and *C. riparius* to *B. laterosporus* is about the half of the susceptibility of *Ae. albopictus* (0.31, 0.33, and 0.16 × 10^7 spores mL^{-1} for *C. dipterum, C. riparius*, and *Ae. albopictus*, respectively). Such difference in the susceptibility might not be sufficient to support an actually higher specificity of *B. laterosporus* than *Bti* and *B. bassiana* to *C. dipterum* and *C. riparius.* Such information could be relevant for the selection of bioinsecticides with reduced adverse effects on non-target aquatic organisms.

Overall, the present study contributes to increase knowledge on the interactions that *B. laterosporus* can establish with invertebrate species in aquatic habitats. Leveraging the large arsenal of toxins and virulence factors it produces (Marche et al., 2018; Glare et al., 2020), *B. laterosporus* is a promising candidate for the development of new bacterial-based bioinsecticides. However, this study showing a range of biocidal activity of *B. laterosporus* against non-target water-dwelling invertebrates, including harmful and non-harmful species indicate that careful consideration must be given to the potential side-effects to the beneficial fauna in aquatic environments. Further studies, including a wider target range, possibly performed under field conditions, will better clarify the actual role of this bacterium within the freshwater community.

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2.8 Figures and tables

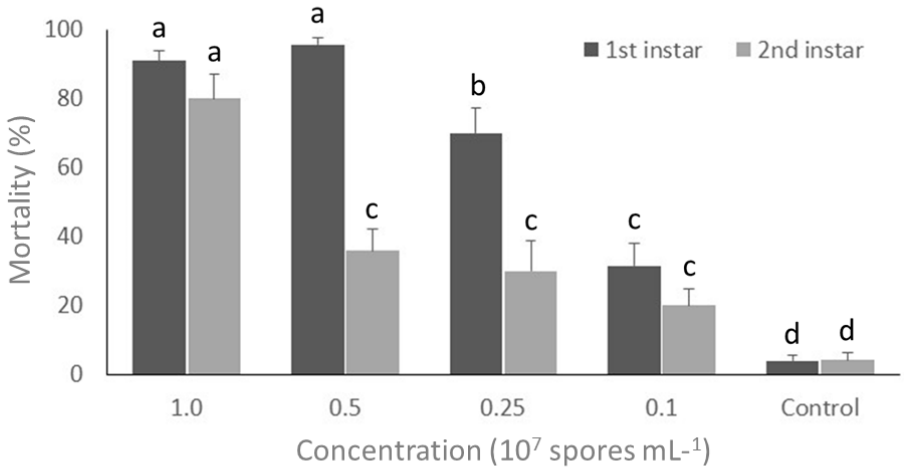

Fig. 1. Percentage mortality (mean ± SE) of first and second instar larvae of *Ae. albopictus* exposed for 48 h to progressive concentrations of *B. laterosporus* spores. Different letters above bars indicate significantly different means (two-way ANOVA followed by LSD test, $P < 0.05$).

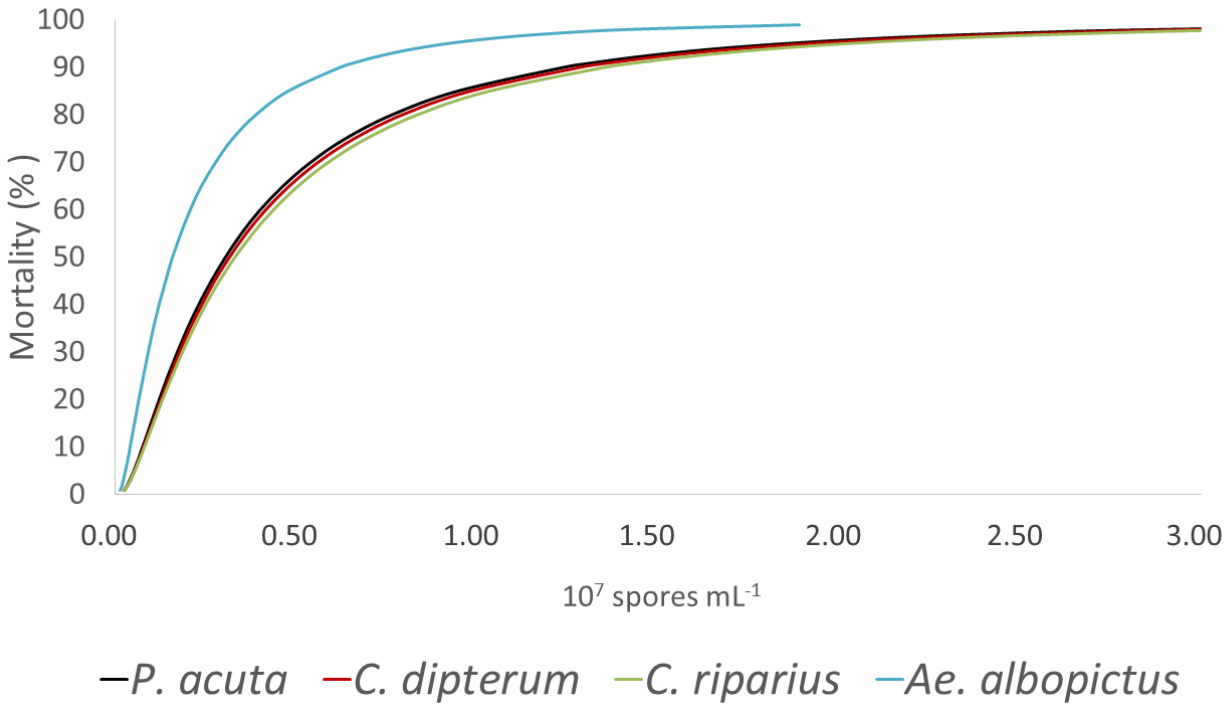

Fig. 2. Concentration-effect relationship of the mortality (%) of the 1st instar mosquito *Aedes albopictus,* adults of *Physella acuta* and the non-target aquatic invertebrates *Cloeon dipterum* and *Chironomus riparius,* exposed to *Brevibacillus laterosporus* spores as predicted by the Probit model.

Table 1: Means (±SE) of larval development time, pupal development time, and percentage of adult emergence of *Aedes albopictus* surviving exposure to sub-lethal concentrations of *Brevibacillus laterosporus* spores.

Bacterial concentration (spores/mL)	n[a]	Larval development time[b] (days)	n[c]	Pupal development time[d] (days)	Adult emergence[e] (%)
2.5×10^6	27	7.92 ± 0.40*	23	2.65 ± 0.13	89.68 ± 5.20
1.0×10^6	37	7.16 ± 0.22	33	2.33 ± 0.10	86.31 ± 8.27
Control	26	7.65 ± 0.31	23	2.34 ± 0.10	89.81 ± 6.48

[a] number of pupated *Aedes albopictus* larvae

[b] days from egg hatching to pupation

[c] number of emerged *Aedes albopictus* adults

[d] days from pupation to adult emergence

[e] calculated on the number of pupated insects.

*No significant differences among means were detected ($P > 0.05$)

Table 2. Biocidal effect of *Brevibacillus laterosporus* spores to the mosquito *Aedes albopictus* and other aquatic invertebrates: *Cloeon dipterum*, *Chironomus riparius*, and *Physella acuta.*

Species	**LC_{50}[a]**	**95% CI[b]**	**LC_{95}[c]**	**95% CI[b]**	**Intercept ± SE**	***P***
Ae. albopictus	0.16	0.11-0.23	0.91	0.55-2.00	1.73 ± 0.13	< 0.001
C. dipterum	0.31	0.18-0.54	1.82	1.00-4.29	1.08 ± 0.13	< 0.001
C. riparius	0.33	0.19-0.59	1.91	1.01-4.78	1.03 ± 0.14	< 0.001
P. acuta	0.30	0.18-0.49	1.76	1.02-3.87	1.11 ± 0.12	< 0.001

[a] concentration of bacterial spores, given as 10^7 spores mL^{-1}, that kills 50% of the insects

[b], confidence interval

[c], concentration of bacterial spores, given as 10^7 spores mL^{-1}, that kills 95% of the insects.

Data are calculated by Probit regression analysis Model slope = 2.10 ± 0.13.

Pearson Goodness-of-Fit Test, $\chi^2 = 82.537$, df = 19, $P < 0.001$ (since $P < 0.150$, a heterogeneity factor was used in the calculation of confidence limits).

Parallelism Test, $\chi^2 = 61.192$, df = 4, $P < 0.001$.

CHAPTER III

Compatibility of the bacterial entomopathogen *Pseudomonas protegens* with the natural predator *Chrysoperla carnea* (Neuroptera: Chrysopidae)

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3.1 Abstract

The susceptibility of the green lacewing *Chrysoperla carnea* to the soil-dwelling bacterial entomopathogen *Pseudomonas protegens* CHA0 was investigated in this study. Laboratory bioassays were conducted on larval instars exposed to different bacterial concentrations by both direct feeding and indirectly by offering a pre-treated insect prey. Potential toxicity was assessed through dose-response bioassays, while possible sublethal effects were evaluated on immature development time and the reproductive performance (fecundity) of adults emerging from treated juveniles. As a result, no significant effects were observed on larval survival and development in a comparison between treated and untreated (control) groups. No significant impact on adult emergence and no detrimental effects on female fecundity were detected. Everything considered, the use of *P. protegens* in the agroecosystem appears to be compatible with chrysopids.

Key words: biocontrol; non-target; safety; pest management; biopesticide.

3.2 Introduction

Among the diversity of the bacterial community living in the soil, the interest in *Pseudomonas protegens* is growing due to its ability to perform significant activities in favor of cultivated plants by occupying different ecological niches in the agroecosystem (Agaras et al., 2017). Such beneficial properties include the interaction with the plant root system, leading to increased access to nutrients in the soil through support in nitrogen fixation, siderophore secretion, phosphorus solubilization, phytohormone synthesis, and improvement in water uptake (Sivasakthi et al., 2014; Trivedi et al., 2020). Added to this is the bacterium's ability to colonize the root, which results in both the formation of protective biofilms and the establishment of a more intimate relationship with the plant, up to endophytism (Morales-Cedeño et al., 2021). This leads to improved resistance to phytopathogens as a result of increased competitiveness for nutrients and space, release of antibiotics and various bioactive molecules (Ramamoorthy et al., 2001; Ramette et al., 2011).

According to the production of several insect virulence factors and a significant pathogenic potential, *P. protegens* appears also to be a promising candidate for pest management (Kupferschmied et al., 2013; Keel, 2016; Pronk et al., 2022). Consistently, different strains of this bacterial species were reported to be active against a variety of insect pests, either orally and by intra-haemocoelic injection (Flury et al., 2016; Vesga et al., 2021). The insecticidal action by ingestion implies overcoming gut barriers, which can be supported by enzymes like chitinases possibly involved in the degradation of peritrophic matrix (Flury et al., 2016; Ruiu and Mura, 2021) and by toxins interacting with the epithelial cell membranes like the fluorescent insecticidal toxin FitD that was also found to harbor a pore-forming domain (Ruffner et al., 2015).

While the expected multiple beneficial action of *P. protegens* in the agro-ecosystem is promising for the deriving application potential, still limited information on the possible side effects on non-target organisms, including natural pest parasites and predators, is available. It has in some cases been observed that *P. protegens* could use less susceptible

insects as vectors for its spread in the soil, which further complicate the diversity of ecosystem relationships that this bacterial species would have evolved (Flury et al., 2019).

A prominent biocontainment role against several insect pests such as aphids, thrips, whiteflies, and some lepidopterans is played by diverse lacewing predatory species within the *Chrysoperla carnea* Stephens group, in different agricultural systems (Principi and Canard, 1984, Senior and McEwen, 2001). In addition to their natural action as biological control agents (BCAs), these species are commercially produced in biofactories and employed in augmentative (inundative) biocontrol programs in different parts of the world (Pappas et al., 2011). The susceptibility of chrysopids to some entomopathogenic bacteria has occasionally been reported even though most of the entomopathogens usually employed for pest management are normally considered selective in respect to these predators (Romeis et al., 2004).

This study aimed at investigating the possible lethal and sub-lethal effects that *P. protegens* may cause to the lacewing *C. carnea*. For this purpose, the effects on survival of predatory larvae were studied by both directly exposing the larvae to the bacterium through feeding and indirectly by offering larvae a previously treated prey (tritrophic interaction). The potential effect on several parameters such as immature developments, adult emergence and fecundity was examined.

3.3 Materials and methods

3.3.1 Bacterial strain and preparations

The entomopathogenic bacterium *P. protegens* strain CHA0 (CFBP 6595^{T}), whose entomopathogenic properties are well known, was employed in this study (Stutz et al., 1986). Bacterial cultures were routinely conducted in flasks at 30 °C in LB broth, shaken at 180 rpm, from which bacterial cells were harvested by centrifugation at 15,000 x g at 4 °C for 10 minutes after 48-72 h, before being resuspended in water to make suspensions used in bioassays. Bacterial concentration was assessed by plating serial dilutions on LB agar and determining the number of colony forming units (CFUs).

3.3.2 Insect bioassays

Different sets of experiments were conducted to evaluate the possible direct effect of *P. protegens* by feeding larvae on a treated liquid suspension, or the indirect effect potentially resulting from feeding larvae on prey previously treated with the bacterium. Following these approaches, several parameters related to survival and sublethal effects, especially in terms of preimaginal development and adult reproductive capacity, were studied comparing treated and control specimens.

All experiments were conducted in a bioassay room at 25 °C under a photoperiod of L14:D10.

3.3.2.1 Insect rearing

First instar larvae of *C. carnea* were provided by the insect rearing facility of Bioplanet Srl (Cesena, Italy) and immediately employed in bioassays.

Larvae of the mealworm beetle *Tenebrio molitor* L. (Coleoptera*:* Tenebrionidae) used in bioassay as a lacewing prey, were provided by the insect rearing facility of the Department of Agricultural Sciences of the University of Sassari (Italy) (Ruiu et al., 2020).

3.3.2.2 Direct feeding bioassays

The possible direct toxicity of *P. protegens* to lacewing larvae was evaluated by feeding young larvae with a suspension containing bacterial cells. For this purpose, 1st instar larvae were maintained individually inside plastic jars (2 cm diameter x 3 cm high) into which a drop (4 µl) of a 20% fructose suspension containing a variable bacterial cell concentration (treatments) or lacking bacterial cells (control), was placed. To mitigate possible effects of fructose on bacterial cell viability/pathogenicity, this mixture was prepared fresh just before being administered to the insects. On the other hand, no reduction in pathogenicity of *P. protegens* against susceptible targets was previously observed when bacterial cells were mixed with sugar (Ruiu et al., 2017). Accordingly, a fresh drop was

provided daily to each larva for a 5-day period. From the sixth day on, the lacewing larvae were instead offered one larva per day of the artificial prey *T. molitor* which ensured that they were well fed until the end of their life cycle. The experimental design involved groups of ten individually reared larvae for each bacterial concentration assayed (10^9, 10^8, 10^7, 10^6, 10^5 CFU/ml) and for the control. Each treatment involved three replicates (10 individuals/replicate). Insects were examined daily to assess mortality, larval moults, pupation, and emergence of adults. Emerging adults were moved to new cages maintaining them within the same treatment group (respecting a male to female ratio of 1 to 1), where they could mate. These cages had a removable sheet of paper on their inner surface which allowed oviposition and egg counting during the subsequent 3-week period. Adult feeding in this period was based on the *ad libitum* administration of honeybee pollen and water. Insect death in the cage was also recorded.

3.3.2.3 Tritrophic bioassays

According to the natural predatory behaviour of *C. carnea*, the possible effect of feeding on a prey that consumed *P. protegens*, was evaluated offering chrysopid larvae pre-treated mealworms. For this purpose, *T. molitor* larvae were starved for a week before being exposed for 3 days to wheat bran moistened with the bacterial suspension at a concentration of 10^9 CFU/ml or just water (control), as the sole liquid food source. After this period, mealworm larvae were alive with none or slight symptoms of infection (movement; response to external stimuli) and were immediately offered for predation to chrysopid larvae. The presence of the bacterium in the body of treated mealworm samples was confirmed by plate culture of the homogenized gut. Groups of ten 1st instar *C. carnea* larvae, were individually kept in plastic jars (2 cm diameter x 3 cm high) where a newly treated or untreated (control) mealworm was daily offered for predation over the next 7 days. In the following days, however, lacewing larvae were all fed with untreated mealworms. Mortality, date of moults and of pupation, were recorded. The experimental design involved three replicates (10 individuals / replicate).

Emerging adults within a group were moved to reproduction cages, fed on honeybee pollen and water, and allowed to mate and oviposit eggs on a removable paper sheet as described for the previous experiment. The number of eggs laid per female were counted on the following 3-week period. Mortality when detected in a cage was also recorded.

3.4 Statistical analysis

Data processing and statistics were conducted using R software version 4.0.4 (R Core Team, 2020). Data on immature (larval and pupal) development time were analysed by 2-ways ANOVA (factors: treatment, insect stage), while data on adult emergence rate and oviposition were analyzed by 1-way ANOVA. No post-hoc comparison was conducted as no significant difference between means of treated and control groups was found.

Direct comparisons between treated and control group means were based on *t*-tests.

3.5 Results

3.5.1 Direct feeding

Treatment of newly hatched *C. carnea* larvae with a fructose solution incorporating *P. protegens* CHA0 at a concentration ranging between 10^9 and 10^5 CFU/ml did not cause detectable changes in their survival rate in different developmental stages (larvae, pupae, and adults) compared with the control ($F_{5,71} = 1.51$; $P = 0.2057$) (Fig.1).

The average immature development time of treated 1st instar larvae is reported in Table 1. These treatments caused a slight though not significant reduction in the development time of larvae treated with concentrations in a range between 10^8 and 10^6 CFU/ml ($F_{5,159} = 2.25$; $P = 0.0518$), and non-significant effects on the duration of the pupal stage ($F_{5,153} = 0.26$; $P = 0.9337$), compared with the control.

No significant differences were observed between adults emerging form larvae treated with different bacterial concentrations and untreated, in terms of both emergence

rate ($F_{5,17}$ = 0.89; P = 0.5181) and average oviposition per female ($F_{5,27}$ = 0.67; P = 0.6535) (Table 2).

3.5.2 Tritrophic interaction

The lacewing survival rate after feeding larvae with mealworms pre-treated with *P. protegens* CHA0, is shown in Fig. 2. A non-significant reduction in the survival rate was observed in the transition from preimaginal to adult stage of treated insects ($F_{3,23}$ = 2.25; P = 0. 1219). However, no differences in the overall survival were observed between treated and control groups ($F_{1,23}$ = 3.13; P = 0. 0962).

Exposition to treated preys did not affect the development time of larvae (t = -0.010; df = 46; P = 0.4960) and pupae (t = -0.1557; df = 48; P = 0.4385) in respect to a control feeding on untreated mealworms (Table 3). A slight reduction in adult emergence (18%) observed in treated insects compared with control was not statistically significant (t = 1.508; df = 4; P = 0.1031).

The average oviposition rate per lacewing female was not affected by exposing them at the larval stage to treated preys (Table 4) and no significant differences between treated and control groups were detected (t = -0-025; df = 6; P = 0.4904).

3.6 Discussion

Studies on the insect pathogenesis potential of *Pseudomonas protegens* highlight its ability to rapidly reproduce and develop in the insect haemocoel, once inside, which would therefore represent a suitable and nutrient-rich environment (Ruffner et al., 2013). On the other hand, this bacterium harbors an arsenal of virulence factors allowing its penetration inside the insect body through the intestinal barriers (Péchy-Tarr et al., 2008; Loper et al., 2016; Vesga et al., 2020). Such behavior has been observed on diverse target species in different orders, including Lepidoptera, Coleoptera, and Diptera (Flury et al., 2016; Flury et al., 2019; Ruiu and Mura 2021). These results support an increasing interest

in this bacterium, also in relation to its multitalented properties including the ability to interact with the plant root system triggering its immune response, and to act as a phytopathogen antagonist (Keel, 2016). While such wide range of beneficial activities is very promising for a biological control agent candidate, no information is available on its selectivity to non-target beneficial organisms in the agroecosystem, such as pest predators and parasites. In fact, these organisms, while performing their ecosystem services, may come into contact with biological control agents administered through bio-insecticidal applications. This includes both direct feeding on droplets of microbial based products and possible indirect effects resulting from the predatory or parasitic action on their hosts, which are the actual targets of insecticidal treatments. When young larvae of *C. carnea* were exposed to sweet drops incorporated even with high concentrations of *P. protegens* strain CHA0, up to one billion CFU/ml, no detectable effects on either survival, preimaginal development or reproductive performance of emerging adults were observed. These observations would support the lack of an effective capacity of the bacterium to go through the intestinal barriers of chrysopid larvae. While the mechanism of action is not completely understood, according to a recently proposed model, *P. protegens* pathogenic process after bacterial cell ingestion by a susceptible host leverages several toxins and virulence factors (Vesga et al., 2020). Among these, the lipopeptide orfamide A and chitinases are supposed to be involved in adhesion and disruption of the peritrophic matrix, respectively (Flury et al., 2017). The successive disruption of the epithelial cell layer is believed to be the result of a combined action of phospholipase, exopolysaccharides, and two-partner secretion proteins (TPS) (Job et al., 2022). In addition, the FitD toxin containing a pore-forming domain, was observed to be expressed at the gut level (Flury et al., 2017; Vesga et al., 2020; Ruiu and Mura, 2021), which may give further support for breaking the intestinal barrier and entering the hemocoel. At the present state of the art, no information on the level of specificity against a given target is available. However, a different degree of susceptibility of diverse insect targets to *P. protegens* CHA0 has been reported (Vesga et al., 2021). In experiments with the cabbage root fly, *Delia radicum*, it was demonstrated that larvae of this dipteron fed with *P. protegens,* were not affected and even behaved as vectors of the bacterium that could spread to the roots of other plants

exerting its beneficial action (Flury et al., 2019). This therefore implies a complex evolutionary process that would explain variable susceptibility of different hosts. In the case of other entomopathogenic bacteria, such as the most known *B. thuringiensis*, insect toxicity is commonly related to a highly specific binding of Cry proteins with epithelial cell membrane receptors of susceptible hosts (Jurat-Fuentes and Crickmore, 2017). Accordingly, biopesticidal strains of *B. thuringiensis* were found to be generally nontoxic to *C. carnea* and no specific interaction of Cry toxins with midgut receptors was observed (Romeis et al., 2004; Rodrigo-Simón et al., 2006). On the other hands, some deleterious effects were occasionally observed under specific laboratory conditions (Hilbeck et al., 1998), which, however, is not necessarily representative of the natural environmental conditions under which chrysops could come into contact with bacteria (Bourguet et al., 2002; Lövei et al., 2009). The absence of susceptibility to *P. protegens* CHA0 by ingestion we observed in laboratory experiments would therefore support the lack of a specific action toward this non-target insect. However, in addition to the possibility of direct contact with the bacterium, the health of a predator could also be undermined in the ecosystem by feeding on an entomopathogen-affected host, which may lead to either infection or the availability of a prey with poorer nutritional characteristics (Ruiu et al., 2007; Salama et al., 1991; Eubanks and Denno, 2000). No such detrimental effects on survival, development, or reproductive potential of *C. carnea* feeding on pre-treated mealworms were observed, thus supporting the compatibility of *P. protegens* CHA0 within a tritrophic interaction system involving predator, prey, and pathogen.

According to the results of this study, neither contraindications nor indications of risk to the use of *P. protegens* strain CHA0 as a biological control agent in the agroecosystem emerged. Moreover, the conditions of artificial exposure of chrysopid larvae to the bacterium in the laboratory should be considered extreme, considering that increased dilution in the environment is expected and that additional behavioural factors (i.e., repellency, avoidance) may result in a further reduction in the chances of contact with significant doses of the pathogen (Cordeiro et al., 2010). On the other hand, the environmental conditions under which an insect pathogen could act in the ecosystem are

more complex than a laboratory, and other factors, such as various stressors could increase the susceptibility of the host *(*Donegan and Lighthart, 2003).

The development of a high plasticity and adaptability to interact with different components of the ecosystem's biological community (i.e, plants, phytopathogens, insect pests) and the safety profile for chrysopids that emerged from this study, make this soil-dwelling bacterium a valuable resource in the agricultural context. However, further studies are needed to screen a wider range of non-target species, before concluding that its use in pest management is safe for beneficial entomofauna.

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3.9 Figures and tables

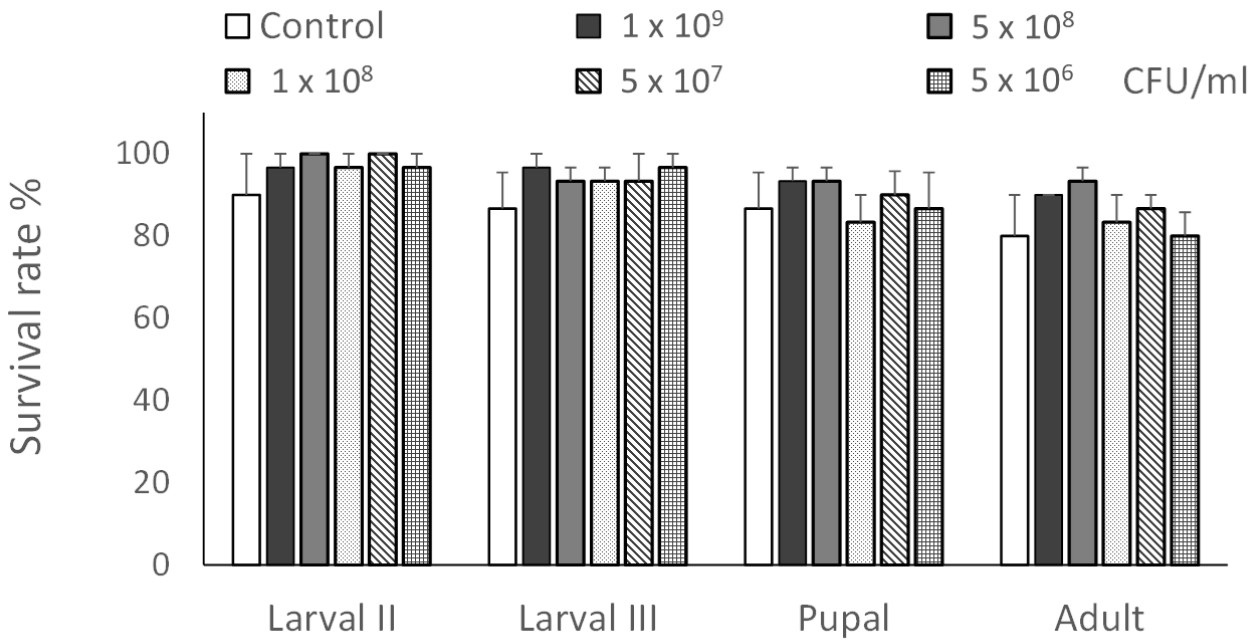

Fig. 1. Survival percentage (mean ± SE) of different *Chrysoperla carnea* stages from 1st instar larvae fed with different concentrations (CFU/ml) of *Pseudomonas protegens* CHA0 cell suspensions. Means were not significantly different (ANOVA, $P > 0.05$).

Table 1. Means (± SE) of immature (larval and pupal) development time and adult emergence rate of *Chrysoperla carnea* feeding on different concentrations of *Pseudomonas protegens* CHA0 cell suspensions.

Bacterial concentration (CFU/ml)[a]	Development time (days)		Adult emergence[d] %
	Larvae[b]	Pupae[c]	
10^9	21.4 ± 0.84[e]	11.9 ± 0.10	90.0 ± 0.00
10^8	18.6 ± 0.48	11.8 ± 0.15	93.3 ± 3.33
10^7	19.0 ± 0.64	12.1 ± 0.28	83.3 ± 6.67
10^6	18.9 ± 0.68	11.7 ± 0.15	86.7 ± 3.33
10^5	20.3 ± 0.97	12.3 ± 1.31	80.0 ± 5.77
Control	20.9 ± 0.98	11.5 ± 0.20	80.0 ± 10.00

[a] larvae were exposed to a cell suspension containing 20% fructose

[b] time from egg hatching to pupation

[c] time from pupation to adult emergence

[d] based on the initial number of larvae

[e] means in each column were not significantly different (2-ways ANOVA, $P > 0.05$).

Table 2. Fecundity (mean ± SE) in *Chrysoperla carnea* females from larvae exposed to different concentrations of *Pseudomonas protegens* CHA0 cell suspensions.

Bacterial concentration (CFU/ml)[a]	Eggs/female[a]
10^9	309.8 ± 88.51[b]
10^8	249.8 ± 44.01
10^7	270.8 ± 39.31
10^6	253.1 ± 63.43
10^5	298.1 ± 3.33
Control	153.6 ± 49.14

[a] Total number of eggs laid over a 3-week period.

[b] means in the column were not significantly different (ANOVA, $P > 0.05$).

Table 3. Means (± SE) of immature (larval and pupal) development time and adult emergence rate of *Chrysoperla carnea* feeding on insect preys treated with *Pseudomonas protegens* CHA0.

Treatment[a]	Development time (days)		Adult Emrgence[d] %
	Larvae[b]	Pupae[c]	
Treated	18.5 ± 0.95[e]	11.7 ± 0.17	76.7 ± 8.82
Control	18.4 ± 0.89	11.6 ± 0.19	93.3 ± 6.67

[a] Treated indicates *C. carnea* fed *T. molitor* larvae pre-treated with *P. protegens*

[b] time from egg hatching to pupation

[c] time from pupation to adult emergence

[d] based on the initial number of larvae

[e] means in each column were not significantly different (*t*-test, P > 0.05).

Table 4. Fecundity (mean ± SE) in *Chrysoperla carnea* females from larvae fed on insect preys treated with *Pseudomonas protegens* CHA0.

Treatment[a]	Eggs/female[b]
Treated	263.7 ± 70.47[c]
Control	261.7 ± 39.18

[a] Treated indicates *C. carnea* fed *T. molitor* larvae pre-treated with *P. protegens*.

[b] Total number of eggs laid over a 3-week period.

[c] means in the column were not significantly different (*t*-test, P > 0.05).

CHAPTER IV

Brevibacillus laterosporus **as a natural biological control agent of soil-dwelling nematodes**

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4.1 Abstract

The nematocidal potential of an entomopathogenic strain of *B. laterosporus* previously known for its antimicrobial properties was assessed on the free-living nematode microworm *Panagrellus redivivus* and the root-knot nematode *Meloidogyne incognita.* Laboratory bioassays showed significant nematocidal properties of the culture supernatant of the bacterium, achieving over 90 % mortality of both microworms and *M. incognita* juveniles when supernatant was collected at the sporulation phase, which related to the progressive production and release of virulence factors and toxins in the culture medium at this stage of bacterial growth. A protein fraction obtained by precipitation from the bacterial culture supernatant was found to be very active against nematodes with a concentration-dependent effect and an LC_{50} value of 0.4 µg/µl on *M. incognita*. Bacterial preparations either based on spores or culture supernatant proved to be effective in reducing *M. incognita* density in treated compared to untreated soil, which makes the use of *B. laterosporus* as a biological control agent of soil-dwelling nematode pests particularly promising.

Keywords: Biocontrol; BCA; pest management; toxins; virulence factors; biopesticide

4.2 Introduction

Brevibacillus laterosporus Laubach is a ubiquitous bacterial species, whose spores, typically associated with a canoe-shaped parasporal body, are frequently isolated from the soil, where it interacts with a wide variety of living organisms, taking part in the biogeochemical cycles (Ruiu, 2013). As a component of the rhizosphere microbial community, it promotes plant growth competing for space and nutrients with phytopathogens (Chen et al., 2017**)**. In addition to this indirect protective action, *B. laterosporus* shows high antimicrobial potential due to the production of several compounds, including peptides and antibiotics (Jiang et al., 2015, Zhao et al., 2012). The biological control properties of this bacterial species are also expressed against invertebrates, such as insects, nematodes, and mollusks (Ruiu et al., 2013). The recent studies on its genome revealed the presence of several traits associated with such potential, with the involvement of protein toxins, enzymes (eg. proteases, chitinases), and various polyketides and nonribosomal peptides (Djukic et al., 2011). The nematocidal properties have preliminarily emerged in some studies highlighting a likely prominent role of cuticle degrading proteases, which, however, would not be a prerogative of *B. laterosporus*, being these gene traits shared with several *Bacillus* species (Lian et al., 2007). The latter include the most studied bacterial entomopathogen *Bacillus thuringiensis* Berliner, whose nematocidal action appears instead to be mainly due to the Cry proteins it produces (Wei et al., 2003). The pore-forming mechanism of action of these toxins involves highly specific binding to receptors of the intestinal epithelial cell membrane, which would support a common evolution of this bacterium and nematodes. According to different routes of interaction of *B. thuringiensis* with nematodes, even if its ecology is still not well understood, the nematode was proposed to be an alternative dominant host (Ruan et al., 2015). Similarly, other bacterial species were found to show pathogenic behaviour against soil nematodes, which points to their potential exploitation in pest management (Tian et al., 2007). The nematocidal action of specific *B. laterosporus* strains have been reported emphasizing the role of extracellular proteases, although a more complex mechanism of action with the involvement of several yet unidentified virulence factors has been proposed (Huang et al., 2005). On the other

hands, most work have been conducted on free-living nematode species, while to determine the actual potential against plant parasitic nematodes would be very important for the purposes of their employment as biological control agents (Soliman et al., 2019). Soil-borne nematode pests affecting agricultural crops are responsible for significant economic losses and their management, historically based on the heavy use of chemicals, is of vital importance to protect agricultural production (Abd-Elgawad, 2014). In this scenario, the development of eco-friendly biocontrol strategies, such as the application of effective entomopathogenic bacteria derived products, is a priority (Tian et al., 2007).

The aim of this study was to assess the nematocidal potential of an entomopathogenic strain of *B. laterosporus*. For this purpose, preliminary bioassays were conducted on the free-living nematode microworm *Panagrellus redivivus* (Panagrolaimidae, Tylenchida) (Linnaeus) Goodey, followed by experiments with the root-knot nematode *Meloidogyne incognita* (Heteroderidae, Tylenchida) (Kofold & White).

4.3 Materials and Methods

4.3.1 Bacterial preparations and analyses

The bioinsecticidal *Brevibacillus laterosporus* strain F5 maintained in the collection of the University of Sassari and originally isolated from the honeybee body was used in this study (Marche et al., 2016). Bacterial cultures were routinely conducted in Luria Bertani (LB) broth at 30 °C shaking at 180 rpm, and harvested by centrifugation at 15,000x*g* at 4 °C for 15 min. The sporulation medium T3 was used to obtain synchronised bacterial growth (Marche et al., 2017), in order to collect the culture supernatant at different stages of growth (exponential, stationary, sporulation). The stage of growth was routinely checked under a phase-contrast microscope. For soil experiments, pure spore suspensions were dehydrated under a laminar flow hood, made into powder using a grinder, and quantified by CFU counts. Fresh culture supernatants, after being sterilized by Minisart® filters (pore size = 0.2 μm), were directly used in bioassays or subjected to protein extraction by ammonium sulfate precipitation, followed by dialyzes against phosphate-buffered saline (PBS) as described elsewhere (Marche et

al., 2019). The protein profile of this fraction extracted from sporulated culture supernatant, was determined by Liquid chromatography–mass spectrometry (LC–MS/MS), in a previous work in which its antimicrobial properties were investigated (Figure S1) (Marche et al., 2019).

Protein concentration of the preparations used in nematode bioassays was determined through the Bradford dye-binding method employing the Bio-Rad Protein Assay.

4.3.2 In-vitro bioassays with Panagrellus redivivus

These bioassays had the purpose of evaluating in vitro the bionematocidal activity of different bacterial preparations against *P. redivivus*, in order to identify the most active bacterial fractions. The nematocidal activity of the whole bacterial culture was determined in a preliminary experiment, which was followed by more focused bioassays employing the culture supernatant collected at different stages of growth. The pure protein fraction extracted from the culture supernatant at the sporulation phase was also assayed at the following concentrations: 0.1 µg, 0.5 µg, and 1 µg/µl.

Microworms (*P. redivivus*) were provided by the rearing facility of the Department of Agricultural Sciences of the University of Sassari (Italy) where they were maintained on a rearing substrate containing wheat bran, water and brewer's yeast.

Lethal effects were determined by dose-response bioassays using 96-well polystyrene microplates filled with different bacterial preparations (200 µl/well) or just sterile water (control). The bioassay design included 4 replicates per treatment represented by 4 wells, each containing 20 nematodes. Plates were covered to avoid liquid evaporation and maintained in an incubator at 27 °C. Nematode mortality was checked daily for 48 h under a stereomicroscope, considering dead the nematodes that did not move even after being touched with a needle. Each experiment was repeated three times.

4.3.3 Experiments with M. incognita

These experiments had the purpose of assessing the efficacy against root-knot nematodes of the bacterial fractions and the protein extracts selected in previous bioassays with microworms.

Second-stage juveniles (J2s) of *M. incognita* were obtained from egg masses collected from the roots (galls) of tomato plants (*Solanum lycopersicum L.*) used to maintain nematode rearing under controlled conditions.

A first experiment was conducted in vitro, using 96-well microplates according to the same experimental design and procedures previously described for *P. redivivus*. These bioassays assessed the nematocidal potential of both the culture supernatant collected at different stages of growth and its protein precipitate at the sporulation phase at progressive concentrations in the range between 0.1 and 1 µg/µl to determine the median lethal concentration (LC_{50}). This experiment was repeated three times.

A second experiment was carried out to evaluate the potential of *B. laterosporus* to act in the natural nematode environment, i.e., the soil. For this purpose, bacterial spore powder was mixed with sterile medium texture soil to a final concentration of 10^9 spores/g soil. Similarly, filter-sterilized culture supernatant of sporulated *B. laterosporus* (5 ml) was mixed with soil (10 g). Controls that received no treatment or treated with sterile water at the same dose as the culture supernatant were included. The experimental unit was represented by a 25 ml vial containing 10 g soil in which 200 nematode juveniles were placed. Nematode recovery number from treated and control samples was determined after 7 days by collecting active specimens through a 38 µm sieve placed on a Baermann funnel (Viglierchio and Schmitt, 1983). Each treatment had 5 replicates and the whole experiment was repeated twice.

4.4. Statistical analyses

Data for statistics were processed using R software version 4.2.0 (R Core Team, 2022). Direct comparison between treated and control group in the preliminary experiment with *P. redivivus* and the whole bacterial culture was based on t-test.

Data on nematode percentage mortality and recovery number in different experiments with *P. redivivus* and *M. incognita* were analyzed by 1-way ANOVA (factor: treatment) followed by LSD test for post-hoc comparison of means.

The relationship between nematode mortality and protein extract concentration was analyzed by linear regression analyses, while probit regression was used to calculate the median lethal concentration (LC_{50}).

4.5 Results

4.5.1. Bioassays with Panagrellus redivivus

Treatment of *P. redivivus* with the whole *B. laterosporus* sporulated culture in preliminary experiments determined a highly significant mortality level (92.9%) compared with the control (1.2 %) ($t = 5.6937$; $df = 13$; $p < 0.001$).

Significant lethal effects were associated with the culture supernatant of *B. laterosporus*, albeit with different levels of effectiveness depending on the stage of bacterial growth at which it was collected (Table 1). The highest mortality percentage after 48 h exposure was caused by the culture supernatant harvested at the sporulation stage (> 90%), followed by the stationary (58%) and the exponential (28%) phases ($F_{3,44} = 218.47$; $p < 0.001$).

The protein extract obtained from the culture supernatant of the bacterium collected at the sporulation phase showed significant mortality of microworms ($F_{3,44} = 363.08$, $p < 0.001$). The lethal effects were concentration-dependent and the average percentage mortality after 48 hours exceeded 90 % at a concentration of 1 µg/µl (Figure 1).

4.5.2 Experiments with Meloidogyne incognita

Second-stage juveniles of the root-knot nematode appeared to be highly susceptible to the culture supernatant of *B. laterosporus*, especially when it was collected at the sporulation phase of bacterial cultures ($F_{3,44} = 633.35$; $p < 0.0001$). In the latter phase, the supernatant determined more than 90% mortality, which remained around 50 % when treatments were made with the culture supernatant collected at the stationary phase (Figure 2).

This nematode species survival was also affected by the protein extract from the supernatant of the bacterial culture at sporulation phase with a concentration-dependent effect (Figure 3). Based on linear regression analyses, a significant correlation between concentration and mortality was observed (adjusted $R^2 = 0.84$, $F = 510.60$, $p < 0.0001$). According to Probit analysis, LC_{50} (CI) values for the protein extract were 0.42 (0.32-0.49) µg/µl (Slope = 1.70 ± 0.28; $\chi 2 = 13.78$; df = 94).

In the second experiment with *M. incognita*, nematode recovery 7 days after inoculation from soil treated with *B. laterosporus* spores (10^9/g) or culture supernatant (0.5 ml/ g) was significantly affected by treatments compared to water and untreated controls ($F_{3,36} = 79.26$, $p < 0.0001$). In more detail, spores determined a decrease in the percentage recovery of around 50% in respect to the untreated control, while a reduction of around 80% was caused by the culture supernatant treatment compared to the water control (Figure 4).

4.6 Discussion

Brevibacillus laterosporus is a ubiquitous bacterial species frequently isolated from different types of soil (Ruiu, 2013). While the ecological role of this species has yet to be fully understood, there is significant evidence of its ability to contribute to fertility and to support plant health (Javed et al., 2020; Ruiu, 2020; Wang et al., 2022). In particular, the potential of different strains of this species as antagonists of phytopathogens or as bioinsecticides was explored (Chen et al., 2017; Bedini et al., 2020). Some studies using specific bacterial strains have also shown a promising

nematocidal effect (Zheng et al., 2016). Accordingly, our experiments with the free-living nematode *P. redivivus* and the crop pest *M. incognita*, proved a high susceptibility of these species to the bacterium. The nematocidal effect was associated with the culture supernatant, and a higher mortality was achieved when the latter was collected at the sporulation phase. This finding is in line with previous knowledge on *B. laterosporus* which is known to produce and release in the culture medium, especially during this growth phase, several compounds including proteins, small peptides, and antibiotics (Marche et al., 2017; Glare et al., 2020). The insecticidal and antimicrobial activities of the bacterial strain employed in our study, originally isolated from the honeybee body (Marche et al., 2016; Marche et al., 2019), were previously reported. These bioactivities were observed to be associated with different bacterial fractions. In particular, high insecticidal properties were associated with the live spores (Marche et al., 2017), while high antimicrobial power was attributed to the same protein fraction that we tested *in vitro* on nematodes (Marche et al., 2019). According to these studies, the action of bacterial bioactive compounds was concentration-dependent, which corroborates our observations on nematodes. Previous investigations reported the potential of *B. laterosporus* culture supernatant against some nematode species including *Panagrellus* and *Meloidogyne* species (Tian et al., 2007; Ann, 2013). Among proteins that were found to be implicated in the nematocidal action, extracellular proteases were found to be major virulence factors allowing the enzymatic degradation of the nematode cuticle (Huang et al., 2005). Consistently, the protein extract that caused a high nematocidal activity in our experiments contained proteases and chitinases, even though they were not the main component. On the other hand, specific production of these enzymes by the bacterial cell is expected to be stimulated in the presence of the host, which may support their greater implication when living cells/spores would come into contact with nematodes in the soil. According to a more complex mechanism of action, our study highlights other *B. laterosporus* virulence factors possibly involved in the nematocidal effects. The most abundant component of the bioactive protein mixture was the 5.7 kDa antimicrobial peptide laterosporulin, whose toxicity potential, although previously highlighted, was not reported for nematodes (Singh et al., 2012). Our study for the first time highlights the

importance that this small peptide might also have toward these targets. However, it is more likely that the nematocidal action is the result of a combined effect of multiple virulence factors that, in addition to the above proteolytic enzymes, would include other bioactive extracellular components that we identified in the nematocidal extract. Among these, a lectin domain protein and a 60kDa chaperonin, and several putative uncharacterized proteins whose functional properties still need to be specifically evaluated (Marche et al., 2019).

Interestingly, the culture supernatant of *B. laterosporus* was active against *M. incognita* juveniles in our soil experiments, which looks very promising in the prospect of applying it directly against this nematode species in its natural environment. Additionally, significant nematode control potential in the soil was also found to be associated with living bacterial spores. The spores of *B. laterosporus* are characterized by a typical spore coat-canoe-shaped parasporal body (SC-CSPB) complex, associated with insecticidal activity due to the presence of certain protein virulence factors (Marche et al., 2017). In addition, spore germination would trigger a pathogenic process that brings into play numerous other virulence factors potentially leading the nematode to death, as observed against other invertebrate pests (Marche et al., 2016, Marche et al., 2018, Mura and Ruiu 2017). From an ecological point of view, the spore represents the most frequent form in which this bacterial species is found in soil (Ruiu, 2013). Therefore, it is not unlikely that this species has co-evolved in the same environment as soil nematodes, corroborating the same hypothesis already made for *B. thuringiensis*, i.e., that in nature the nematode may represent an alternative host (Ruan et al., 2015). According to the results of our study, from a practical point of view, the exploitation of *B. laterosporus* bioactive strains as a resource for nematode pest management in the soil appears to be very promising. Field trials involving appropriately developed *B. laterosporus*-based formulations are needed to a full evaluation of the actual pest biocontainment potential under the complex conditions characterizing the soil ecosystem.

4.7 Author Contributions

Conceptualization, methodology and investigation, R.H. and L.R.; resources, L.R.; data curation, writing—review and editing, R.H. and L.R.; supervision, L.R.; funding acquisition, L.R. All authors have read and agreed to the published version of the manuscript.

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4.10 Figures and tables

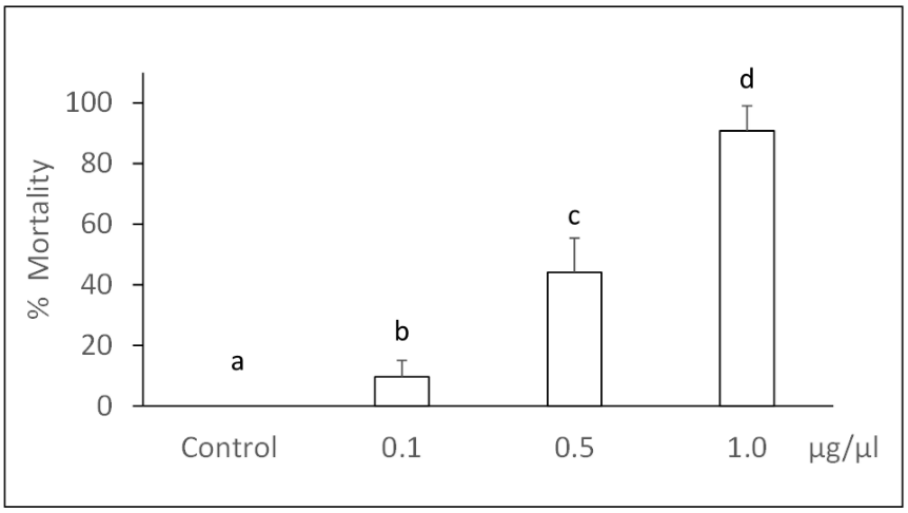

Fig. 1. Mortality percentage (mean ± sd) of *Panagrellus redivivus* exposed for 48 h to different concentrations of the protein extract from the culture supernatant of *Brevibacillus laterosporus*. Different letters above bars indicate significantly different means (1-way ANOVA followed by LSD test, $p < 0.001$)

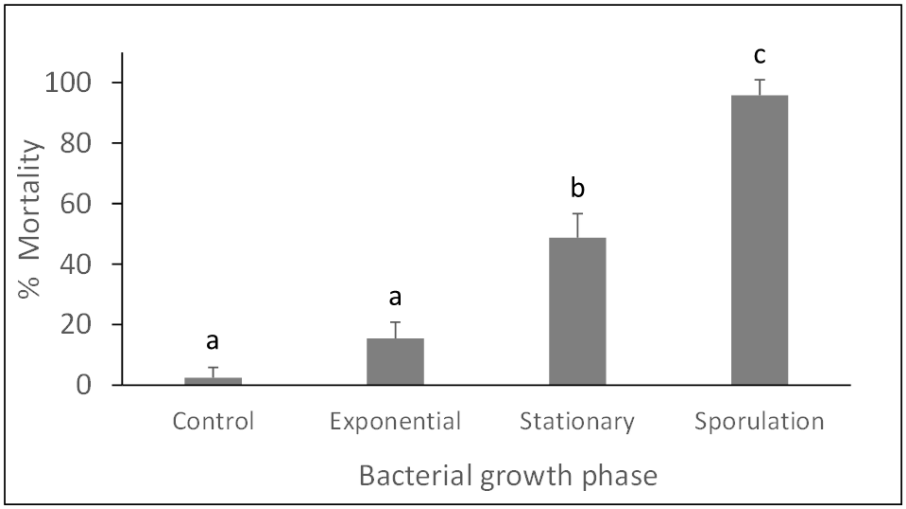

Fig. 2. Mortality percentage (mean ± sd) of *Meloidogyne incognita* exposed for 48 h to culture supernatant of *Brevibacillus laterosporus* at different growth phases. Different letters above bars indicate significantly different means (1-way ANOVA followed by LSD test, $p < 0.001$)

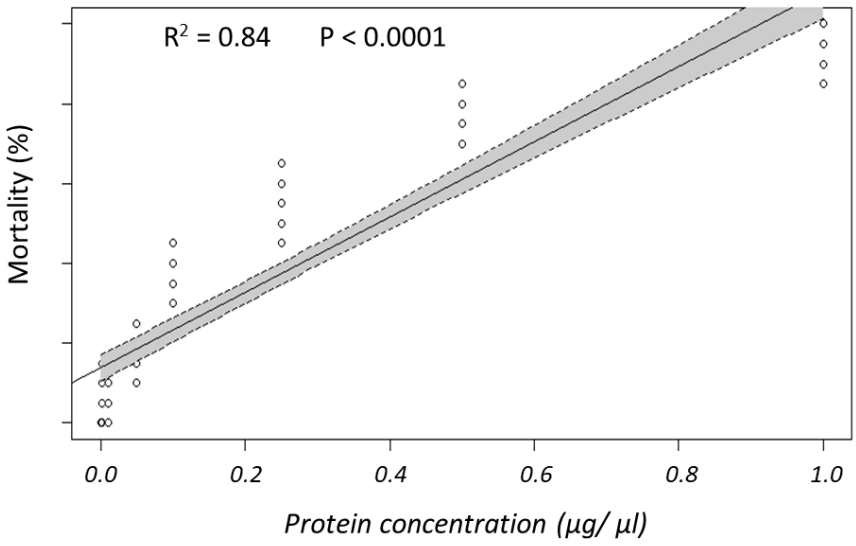

Fig. 3. Relationship between *Meloidogyne incognita* mortality and concentration of the protein extract from *Brevibacillus laterosporus* culture supernatant at the sporulation phase. Shaded areas in the linear regression plot represent 95% confidence intervals.

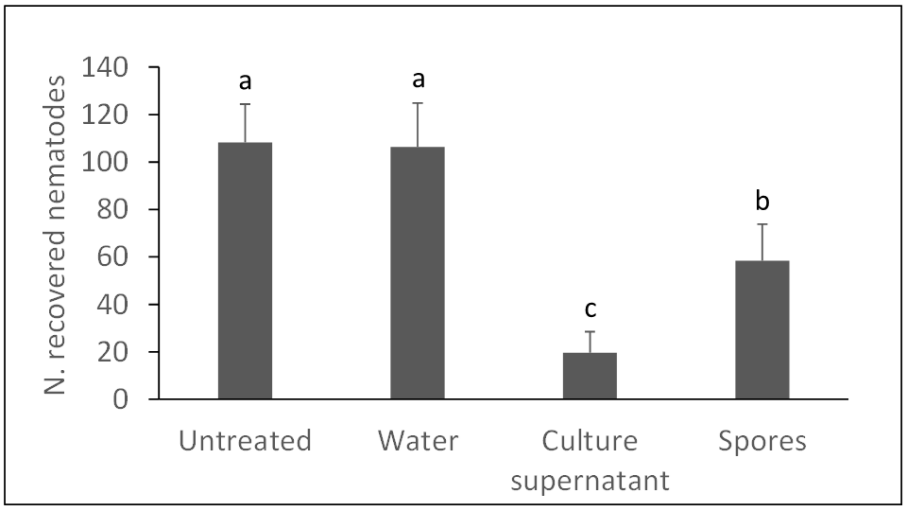

Fig. 4. Number (mean ± sd) of *Meloidogyne incognita* individuals recovered from soil samples treated with different *Brevibacillus laterosporus* preparations and inoculated with 200 nematode juveniles. Different letters above bars indicate significantly different means (1-way ANOVA followed by LSD test, $p < 0.001$).

Protein description	UniprotKB Acc. No.
Putative laterosporulin	A0A075R4X6
Jacalin-like lectin domain protein OS	A0A075R7C5
60 kDa chaperonin	A0A075QZ29
Chitinase A1 (ChiA1)	A0A075R595
ATP-dependent protease	H0U930
Protease 3	A0A075R6T9
Zinc metalloprotease FtsH	A0A075QY12
Serine protease	A0A0F6XZ09
Mosquitocidal toxin	A0A075R1T8

Fig. S1. Selection of proteins identified in the *Brevibacillus laterosporus* culture supernatant collected at the sporulation phase and related to potential nematocidal activity (Marche et al., 2019)

Table 1. Mortality (mean ± sd) of *Panagrellus redivivus* after exposure for 48 h to *Brevibacillus laterosporus* culture supernatant collected at different bacterial growth stages.

Bacterial growth phase	**Mortality[1] %**
Control	2.1 ± 3.3 a
Exponential	28.3 ± 15.7 b
Stationary	58.3 ± 7.5 c
Sporulation	94.6 ± 5.8 d

[1] Means followed by different letters are significantly different (1-way ANOVA followed by LSD test, $p < 0.001$)

CHAPTER V

Mosquitocidal activity of *Pseudomonas protegens*

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5.1 Abstract

This study investigated the biocidal potential of a novel *Pseudomonas protegens* strain, isolated from diseased insects, on mosquito larvae of the two species *Culex pipiens* and *Aedes albopictus*, representing major threats for disease transmission in the Mediterranean area and worldwide. Lethal effects were observed on mosquito larvae after ingestion, with over 90% mortality achieved 72 h after exposition to a bacterial concentration of 100 million CFU/ml. Lethal effects were concentration dependent and a significantly higher susceptibility was associated with younger larvae of both mosquito species. Significant slowdown of immature (larval and pupal) development and decrease in adult emergence rate after treatment with sub-lethal doses of the bacterium were also detected.

Keywords: bioinsecticide; biopesticide; biocontrol; *Aedes*; *Culex*; pest management.

5.2 Introduction

Pseudomonas protegens is a soil-dwelling bacterium belonging to the *P. fluorescens* group, whose interest for application in plant protection is raising (Ruiu, 2020). This is primarily due to its ability to stimulate root growth through biofilm formation and by improving the availability of environmental resources to the plant (Ramette et al., 2011). On the other hands, this species can protect the plant against pathogens and parasites by indirect mechanisms of competition for space and nutrients, by triggering plant immune-response, and by the direct action of several bioactive compounds it may produce (Ramamoorthy et al., 2001). The latter include antifungal metabolites like orfamide A, hydrogen cyanide (HCN), 2,4-diacetylphloroglucinol (DAPG), and some extracellular enzymes like proteases and chitinases (Philmus et al., 2015). In addition to this antimicrobial action, *P. protegens* may exert inhibitory or toxic effects on invertebrates (Flury et al., 2016). Accordingly, several gene traits harboured by this bacterial species have been associated with the insecticidal action after ingestion or injection into the haemocoel of living cells. Among these are included genes encoding for chitinases, phospholipase C, two-partner secretion (TPS) proteins, and the fluorescent insecticidal toxin (FitD) (Vesga et al., 2020). The insecticidal potential has been demonstrated against a still limited range of targets including lepidopteran and dipteran species (Ruiu et al., 2022). Most knowledge regards insects related to the agroecosystem, representing the natural living environment for this bacterium. Little is known on the potential of *P. protegens* against insect species of medical and veterinary importance, apart from some recent studies on the house fly *Musca domestica* L. (Diptera: Muscidae) which demonstrated a significant pathogenic potential against this target (Ruiu and Mura, 2021). However, the relationship of *P. protegens* with dipterans appears to be puzzling. In contrast with an expected pathogenic behaviour, it was reported that larvae of the cabbage root fly, *Delia radicum* L. (Diptera: Anthomyiidae) were not affected by the bacterium and even behaved as carrier, spreading it to the plant root system where it could work as a beneficial microbial (Flury et al., 2019). No information is available on the pathogenic potential against mosquitoes, that are known to be susceptible to other entomopathogenic bacteria, including the well-studied *Bacillus thuringiensis israelensis*

(Goldberg and Margalit, 1977) and *Lysinbacillus sphaericus* (Singer, 1973), whose mosquitocidal action leverages specific crystal proteins and other toxins acting at the midgut level and causing the disruption of the intestinal barrier (Feldmann et al., 1995; Thomas and Ellar, 1983; Darboux et al., 2001). While these biological control agents have proved to be effective in field conditions, the emergence of resistant insects has occasionally been observed (Paul et al., 2005; Su et al., 2018). Another concern associated with these microbials is their sensitivity to UV radiations, which reduce their persistence, and consequently their effectiveness, in field conditions (Myasnik et al., 2001). For these reasons the search for alternative biological solutions to contain mosquito populations is fostered, which includes the screening of other microbial control agents.

This study aimed to determine the susceptibility of major mosquito species larvae to *P. protegens*. For this purpose, the lethal effects on the common house mosquito and Asian tiger mosquito larvae of different instars exposed to diverse bacterial doses were assayed. The potential effects of sub-lethal doses to mosquito immature development and survival were also evaluated.

5.3 Materials and methods

5.3.1 Bacterial strain and preparations

The *Pseudomonas protegens* strain COI, originally found in association with entomopathogenic nematode infective juveniles and maintained in the collection of the University of Sassari (Ruiu et al., 2022), was employed in this study. As a reference strain CHA0 (CFBP 6595^{T}) was also used (Stutz et al., 1986).

Bacteria cultured in Luria Bertani (LB) broth at 30 °C in flasks, shaken at 180 rpm for 48-72 h, were harvested by centrifugation at 15,000 x g at 4 °C for 10 minutes. Collected cells were resuspended in water to adjust concentrations as needed for bioassays. Bacterial concentration was routinely determined counting the number of colony forming units (CFUs) on LB agar plates.

5.3.2 Insect bioassays

The effects of *P. protegens* were studied in parallel on the two mosquito species, *Culex pipiens pipiens* L. (Diptera: Culicidae) and *Aedes* (*Stegomyia*) *albopictus* Skuse (Diptera: Culicidae) with different experiments. Both the lethal effects resulting from exposing insects to different concentrations of the bacterium and the sub-lethal impact on immature development were studied.

All laboratory bioassays were conducted in an incubator at 25 °C.

5.3.2.1 Insect rearing

Insect rearing was carried out in the insectary of the Laboratory of Entomology and Vector control of the Istituto Zooprofilattico Sperimentale della Sardegna (Sassari, Italy) where maintenance of *Culex pipiens* form *pipiens* and *Aedes albopictus* colonies were routinely conducted (Ledda et al., 2022). Adults were maintained in transparent plastic cages (30 × 30 × 30 cm) in an insect room at 25 ± 2°C, 60% ± 5% relative humidity, under a photoperiod 14:10 h (LD). Adults were provided *ad libitum* with a saccharose solution (10%) and with a blood meal administered weekly. Larvae and pupae were reared in trays containing distilled water and powdered cat food.

5.3.2.2 Dose-response bioassays

Larvae of both mosquito species employed in bioassays were grown form fresh eggs provided by the rearing laboratory. Experiments were carried out in plastic cups (3.5 cm diameter) filled with appropriate dilutions of dechlorinated tap water and each containing 10 coetaneous larvae transferred by Pasteur pipette from a rearing tray (Bedini et al., 2020). Larvae were inspected daily, and mortality was recorded for 72 h. The experimental design involved four replicates for each treatment and the whole experiment was repeated 3 times.

A preliminary experiment was conducted to assess *P. protegens* lethal potential against both *C. pipiens* and *Ae. albopictus* 2nd instar larvae exposed to a concentration of

10^8 CFU/ml. This experiment included a comparison between bacterial strains COI and CHA0, while all the following experiments used only strain COI.

A second experiment had the purpose of assessing larval susceptibility to different bacterial concentrations to determine a possible concentration-dependent effect. Thus, the following range of concentrations was assessed against 2nd instar larvae of both mosquito species: $1x10^9$, $2x10^8$, $1x10^8$, $7.5x10^7$, $6x10^7$, $5x10^7$, $2.5x10^7$, 1x10 7, $1x10^6$ cells/ml.

According to the same experimental design, a third experiment was conducted to compare the susceptibility of 1st and 2nd instar larvae of the two insect species to *P. protegens* COI at a concentration of 1, 0.5, and 0.25 x 10^8 CFU/ml.

5.3.3 Sub-lethal bioassays

Just hatched 1st instar larvae of *C. pipiens* and of *Ae. albopictus* were individually transferred to multiwell plates (1 larva/well) and maintained on tap water (500 µl/well) appropriately mixed with the bacterial cell suspension to a final concentration of 2.5 and 5.0 x 10^7 cells/ml, which allowed survival of a significant number of insects to assay development parameters.

Groups of 24 larvae were used for each treatment and for the control. An equal amount of powdered cat food was added to each well at the beginning of the bioassay to ensure adequate nourishment for immature development. Insects were inspected daily to record mortality, moulting, pupation, and adult emergence. Pupae were moved individually to water containing plastic cups (2 cm diameter) for adult emergence observation. The experimental design was based on three replicates (24 individuals / replicate) per treatment. The whole experiment was repeated twice.

5.4 Statistical analysis

Statistical analyses were conducted using R software version 4.2.0 (R Core Team, 2020).

Data on the percentage of mosquito mortality in different experiments involving only one factor (treatment) were analysed by 1-way ANOVA, followed by Tukey test for post-hoc comparison of means. Data on larval mortality in experiments comparing 1st and 2nd instars and different bacterial strains, were analysed by 2-ways ANOVA (factors: treatment, larval instar/strain), followed by Tukey test for means comparison.

The relationship between larval mortality and bacterial concentration was subjected to linear regression analyses.

Over time insect survival rate was analysed by repeated measures ANOVA (PROC MIXED), and means were separated by LSMEANS comparison (adjust=Tukey).

5.5. Results

5.5.1 Dose-response bioassays

Exposing *C. pipiens* and *Ae. albopictus* to *P. protegens* at a concentration of 10^8 CFU/ml determined a significant larval mortality exceeding 80 % after 72 h compared to control ($F_{2,66} = 563.85$, $p < 0.001$), with no significant differences between bacterial strains COI and CHA0, and between the two mosquito species (Figure 1).

These lethal effects were concentration-dependent and the correlation between mortality and concentration was significant for both *C. pipiens* (adjusted $R^2 = 0.89$, $F = 115.1$, $p < 0.0001$) (Figure 2) and *Ae. albopictus* (adjusted $R^2 = 0.87$, $F = 147.2$, $p < 0.0001$) (Figure 3).

A higher susceptibility of younger larvae was detected in experiments comparing mortality of first and second instars of both species exposed to different bacterial concentrations. In these experiments lethal effects were significantly affected by the larval instar (*C. pipiens*: $F_{1,88} = 705.75$, $p < 0.0001$; *Ae. albopictus*: $F_{1,88} = 91.55$, $p < 0.0001$), concentration (*C. pipiens*: $F_{3,88} = 70.98$, $p < 0.0001$; *Ae. albopictus*: $F_{3,88} = 429.99$, $p < 0.0001$), and the interaction between these factors (*C. pipiens*: $F_{3,88} = 16.11$, $p < 0.0001$; *Ae. albopictus*: $F_{3,88} = 15.26$, $p < 0.0001$). Accordingly, over 90 % mortality after 72 h was observed on both *C. pipiens* and *Ae. albopictus* 1st instar exposed to 1 x

10^8 CFU/ml, while mortality of 2nd instar larvae was around 70% and just over 60 %, respectively (Figures 4 and 5). A mortality of around 40 % and 20 % for *C. pipiens* and of 60 % and 40 % for *Ae. albopictus* was achieved at a concentration of 5 x 10^7 CFU/ml for 1st and 2nd instars, respectively.

5.5.2 Sub-lethal bioassays

Over time survival rate of *C. pipiens* and *Ae. albopictus* larvae exposed to *P. protegens* is shown in Figures 6 and 7, respectively. A significant decrease in the survival rate was detected in both *C. pipiens* ($F_{6,105} = 39.61$; $p < 0.0001$) and *Ae. albopictus* ($F_{6.105} = 83.44$; $p < 0.0001$), for both concentrations assayed, in comparison with the control. While the survival rate in the control was around 90% after 7 days, it was reduced to around 40 % and 70 % and to 30 % and 70 % in *C. pipiens* and *Ae. albopictus* exposed to a lower (2.5 x 10^7 CFU/ml) or a higher (5 x 10^7 CFU/ml) bacterial concentration, respectively.

Exposition to the bacterium affected significantly the larval (*C. pipiens*: $F_{2,15} = 12.56$, $p < 0.001$; *Ae. albopictus*: $F_{2,15} = 2.12$, $p < 0.001$) and pupal (*C. pipiens*: $F_{2,15} = 4,96$, $p = 0.0221$; *Ae. albopictus*: $F_{2,15} = 3.91$, $p = 0.0429$) development time of surviving mosquitoes in respect to the control. A slight though statistically significant slowdown in larval (19%) and pupal (20%) development time of *C. pipiens* exposed to the higher concentration was observed (Table 1). Similarly, a small but significant increase in larval (16%) and pupal (15%) development time was detected in *Ae. albopictus* (Table 2). A significant reduction in adult emergence calculated on the number of pupated insects (*C. pipiens*: $F_{2,15} = 3.73$, $p = 0.0486$; *Ae. albopictus*: $F_{2,15} = 11.93$, $p < 0.001$) was assessed in both *C. pipiens* (- 16%) and *Ae. albopictus* (-30%) exposed to the higher concentration compared with the control

5.6 Discussion

The interest in the bacterium *Pseudomonas protegens* is raising as a result of recent studies highlighting its potential as a plant protection product enhancing plant health and acting against some phytopathogens and pests (Keel., 2016; Vesga et al., 2020). Significant entomopathogenic properties have been observed against a still limited range of pests, mostly including Lepidoptera and Diptera (Kupferschmied et al., 2013; Flury et al., 2016; Ruffner et al., 2015; Ruiu et al., 2017).

Beyond the agroecosystem context, in which this bacterial species is attracting most interest, no specific information is available on its potential against animals and humans' parasites and disease vectors such as mosquitoes. This study investigated the pathogenicity of the recently isolated *P. protegens* strain COI on the two major mosquito species *C. pipiens* and *Ae. albopictus*, reporting significant lethal effects. Although *Ae. albopictus* appeared slightly more susceptible to the bacterium, no substantial differences in the biocidal efficacy were observed between the two dipteran targets. This entomopathogenic properties corroborates previous studies employing this strain against larvae of the wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae), the gypsy moth *Lymantria dispar* L. (Lepidoptera: Erebidae), the corn earworm *Helicoverpa armigera* (Hübner) (Leptidoptera: Noctuidae), the Mediterranean fruit fly *Ceratitis capitata* Wied. (Diptera: Tephritidae), and the house fly *Musca domestica* L. (Diptera: Muscidae) (Ruiu and Mura, 2021; Ruiu et al., 2022;). Mortality of mosquito larvae was concentration-dependent and higher mortality was achieved against younger larvae and with a longer exposure time, which is in line with what is generally expected for a bacterial entomopathogen (Jurat-Fuentes and Jackson, 2012). Similarly, a significant correlation between mortality and concentration was observed in experiments with *P. protegens* COI and muscoid fly larvae (Ruiu and Mura, 2021). The level of oral toxicity observed in these experiments on *M. domestica* larvae was similar to what we observed on mosquito larvae with over 90% mortality achieved exposing larvae to 100 millions CFU/ml. While the mechanism of action against fly and mosquito larvae might be different, it was observed that the oral administration to fly larvae of living *P. protegens* cells was

associated with the expression of bacterial genes encoding for the fluorescent insecticidal toxin FitD and the chitin degrading enzyme chitinase D that are known to be involved in overcoming intestinal barriers (Keel, 2016). Equivalent mosquitocidal effects were observed in our bioassays involving the use of the reference strain CHA0, which would support a more general potential of this bacterial species toward these target insects, rather than a prerogative of a specific strain. Consistently, *P. protegens* strain CHA0, which is also the most studied, is characterized by an arsenal of genes encoding for toxins and virulence factors that can act in the insect pathogenic process at different levels (Vesga et al., 2020). According to a recently proposed pathogenesis model, the initial action of the bacterium in the gut leverages the adhesion properties of the lipopeptide orfamide A followed by the chitinolytic action on the peritrophic matrix of chitinases (Flury et al., 2017). Other virulence factors such as phospholipase, exopolysaccharides, and two-partner secretion proteins (TPS) might then act to disrupt the midgut epithelium paving the way for the bacterium to haemocoel (Job et al., 2022). While mortality of mosquito larvae occurs rapidly after exposure to appropriate doses of *P. protegens*, at lower concentrations we found that surviving individuals showed a reduction in the over-time survival rate and also a slight but significant slowdown in the timing of pupation and adult emergence. Such effects have commonly been observed in laboratory experiments with entomopathogenic bacteria administered at sublethal doses on a variety of insects, including mosquitoes (Bedini et al., 2021). On the other hand, such effects on immature development were not observed on non-target species such as lacewings exposed to *P. protegens* strain CHA0, which, however, were found to be poorly susceptible to the bacterium (Hamze and Ruiu, 2022).

Previous investigations with *Pseudomonas fluorescens* Migula against other *Culex* and *Aedes* species showed a significant larvicidal and pupicidal activity, highlighting the role of an exotoxin (Preethi and Pandian, 2009). On the other hands *Pseudomonas* species have been found to be common inhabitants of mosquito midgut, and their presence has occasionally been reported to be related with development regulation (Roy et al., 2010) and possibly with insecticide resistance (Wang et al., 2021).

Unlike pseudomonad species that may have developed evolutionary relationships with mosquitoes, *P. protegens* seems rather to be a microorganism closely related to the agroecosystem and the plant rhizosphere (Agaras et al., 2017; Ruiu, 2020). Despite the hypothesized evolution on divergent pathways, the insecticidal activity of this bacterium on insects living in the aquatic environment appears noteworthy, confirming the opportunity to find new potential biological control agents looking in environments different from those typical of the target pest. It remains of vital importance to verify the compatibility of such microbial insecticides within the specific ecosystem, safeguarding the environment and non-target fauna health (Bedini et al., 2021).

According to the present study, *P. protegens* shows a significant biocidal potential against *C. pipiens* and *Ae. albopictus*, which hints at the prospect of future practical applications. However, the behaviour of this bacterium in water is poorly known and specific studies should be conducted to observe and predict the interactions it may establish with other living forms in such ecosystem. In addition, the actual application potential in field conditions may also depend on its compatibility and competitiveness with other available biocidal products that are normally applied against mosquitoes.

5.7 Acknowledgements

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5.9 Figures and tables

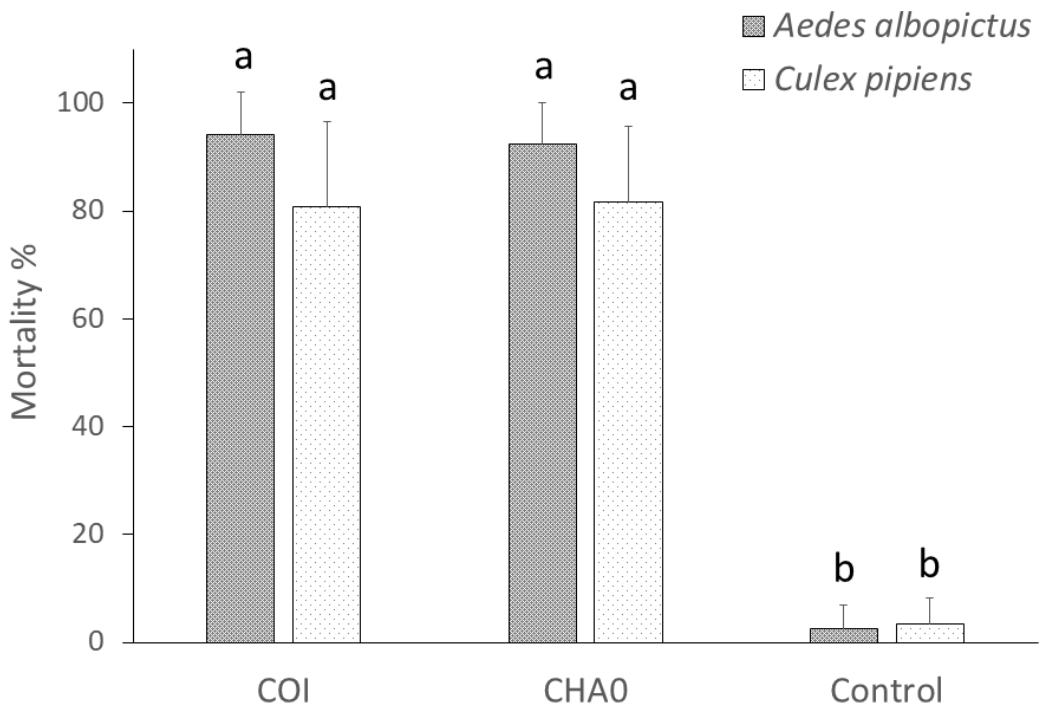

Fig. 1. Mortality (mean ± s.d.) of *C. pipiens* and *Ae. albopictus* 2nd instar larvae exposed for 72 h to a concentration of 10^8 CFU/ml of *P. protegens* strains COI and CHA0. Different letters above bars indicate significantly different means (2-ways ANOVA, followed by Tukey test, $p < 0.05$).

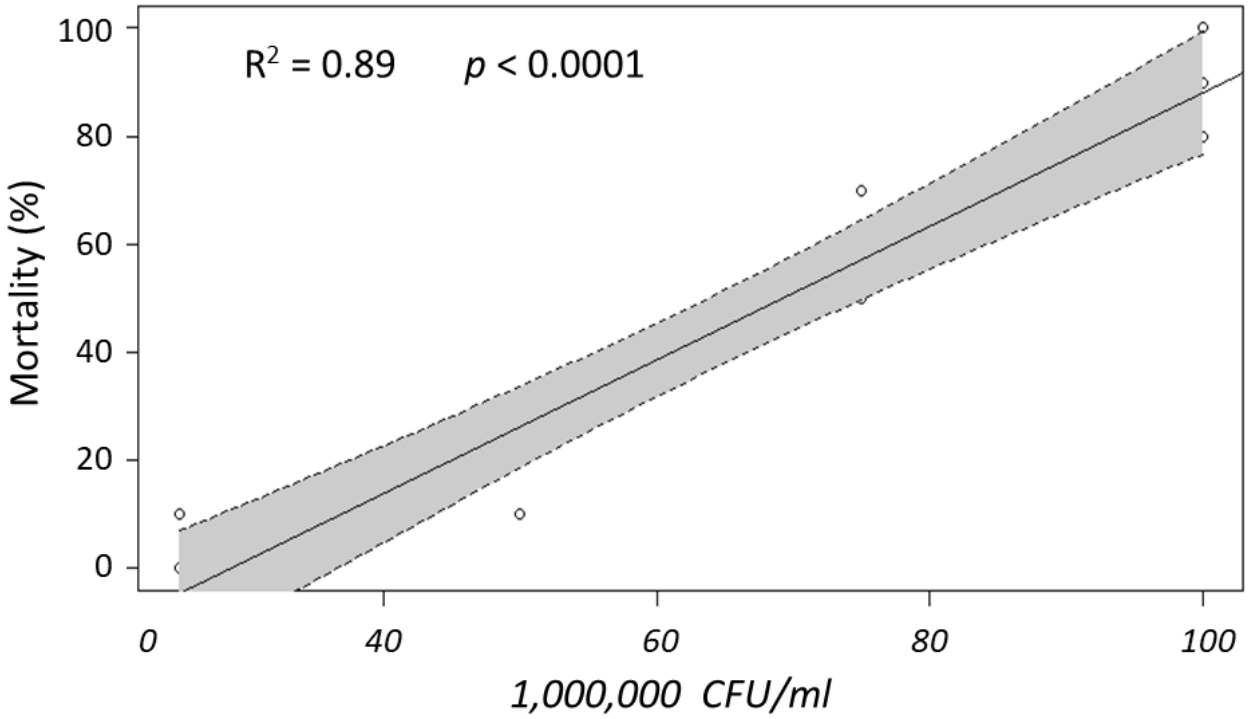

Fig. 2. Linear regression plot representing the relationship between *Culex pipiens* larval mortality and *P. protegens* COI concentration within 95% confidence intervals (shaded areas).

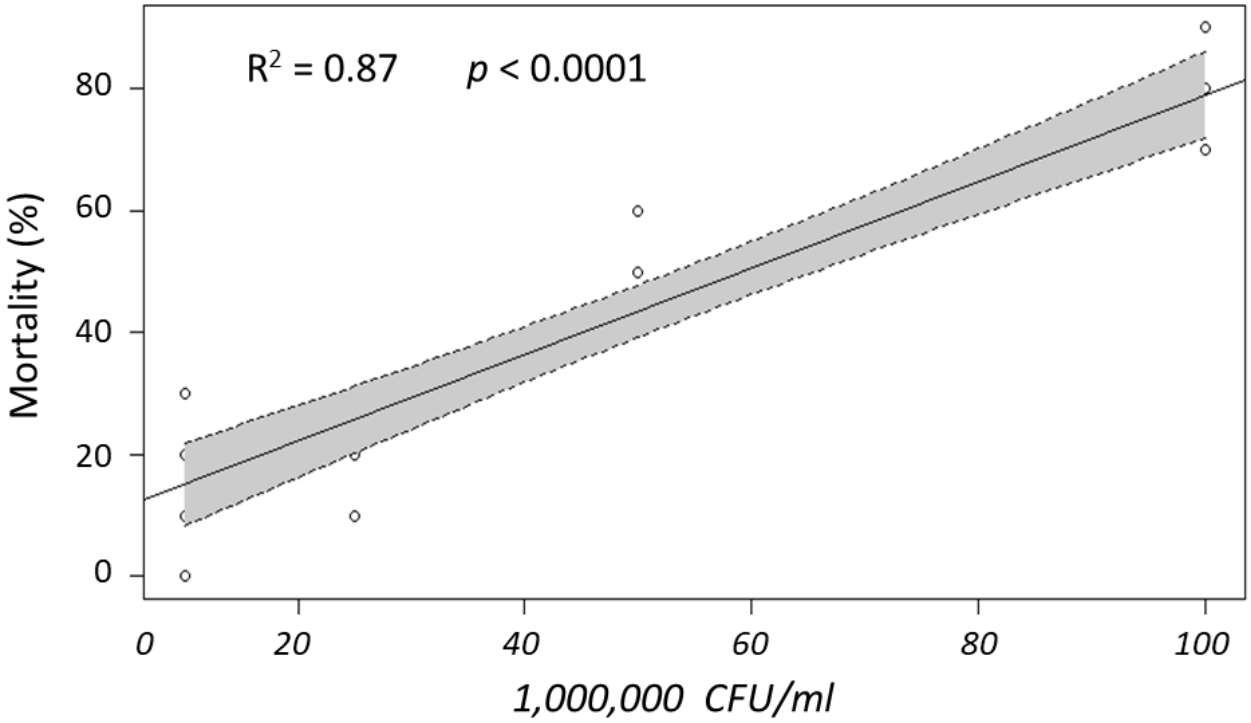

Fig. 3. Linear regression plot representing the relationship between *Aedes albopictus* larval mortality and *P. protegens* COI concentration within 95% confidence intervals (shaded areas).

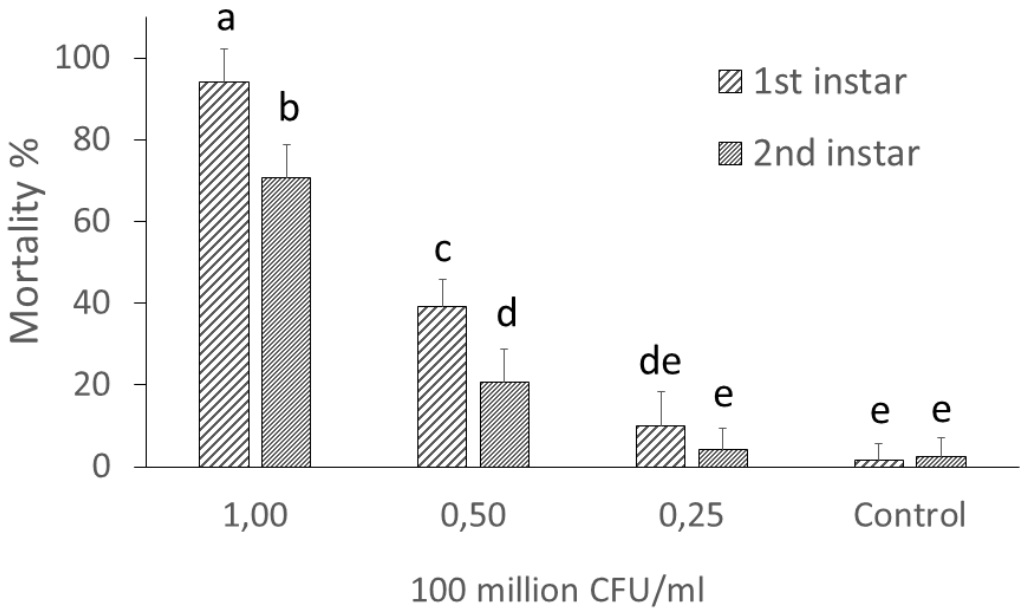

Fig. 4. Mortality (mean ± s.d.) of *C. pipiens* larvae exposed to different concentrations of *P. protegens* COI. Different letters above bars indicate significantly different means (1-way ANOVA, followed by Tukey test, $p < 0.05$).

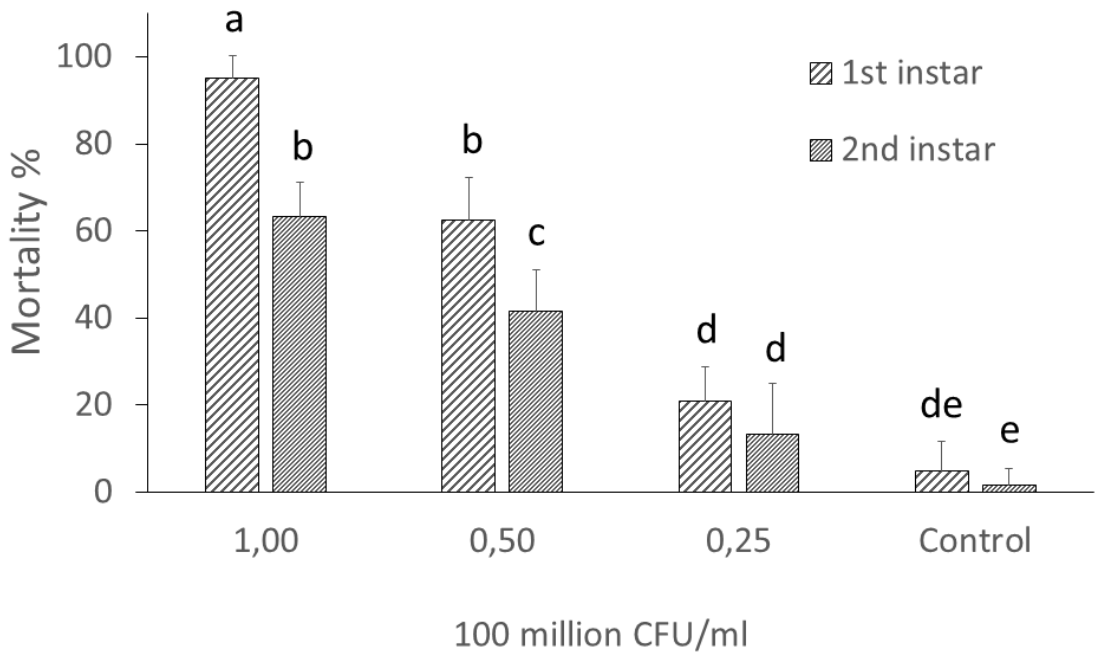

Fig. 5. Mortality (mean ± s.d.) of *Ae. albopictus* larvae exposed to different concentrations of *P. protegens* COI. Different letters above bars indicate significantly different means (1-way ANOVA, followed by Tukey test, $p < 0.05$).

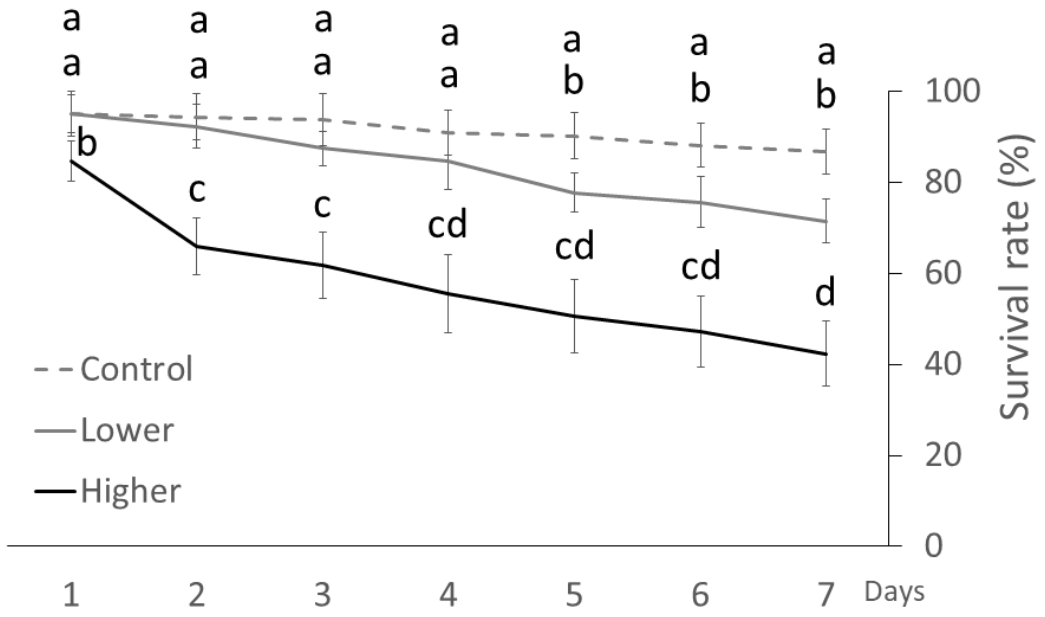

Fig. 6. Over time survival (mean ± s.d.) of *C. pipiens* larvae exposed to different concentrations (Higher = 5 x 10^7, and Lower = 2.5 x 10^7 CFU/ml) of *P. protegens* COI. Different letters indicate significantly different means (ANOVA proc. mixed, LSMEANS adjust=Tukey, $p < 0.05$).

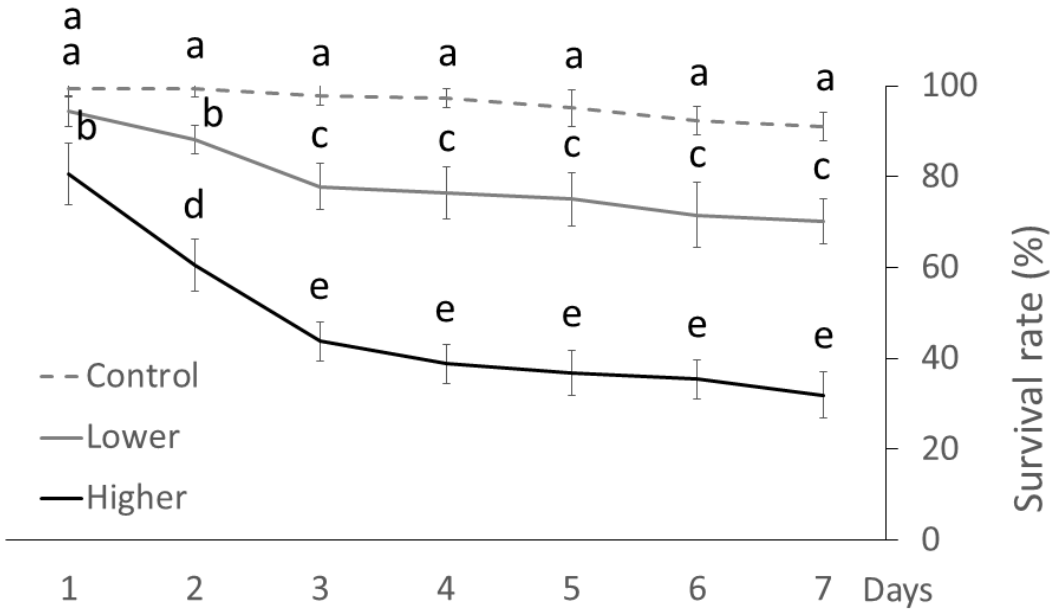

Fig. 7. Over time survival (mean ± s.d.) of *Ae. albopictus* larvae exposed to different concentrations (Higher = 5 x 10^7, and Lower = 2.5 x 10^7 CFU/ml) of *P. protegens* COI. Different letters indicate significantly different means (ANOVA proc. mixed, LSMEANS adjust=Tukey, $p < 0.05$).

Table 1. Immature (larval and pupal) development time and adult emergence rate (mean ± s.d.) of *C. pipiens* surviving sublethal concentrations of *P. protegens* COI.

Bacterial concentration (CFU/mL)	n[a]	Larval development time[b] (days)	n[c]	Pupal development time[d] (days)	Adult emergence[e] (%)
5.0×10^7	61	8.2 ± 0.40 [a]*	39	2.4 ± 0.4 [a]	64.6 ± 9.9 [a]
2.5×10^7	103	7.3 ± 0.7 [ab]	69	2.0 ± 0.4 [ab]	63.3 ± 8.9 [a]
Control	125	6.9 ± 0.4 [b]	96	2.0 ± 0.1 [b]	76.8 ± 8.7 [b]

[a] number of pupated larvae,

[b] days from egg hatching to pupation,

[c] number of emerged adults,

[d] days from pupation to adult emergence,

[e] calculated on the number of pupated insects.

*Different letters in each column indicate significantly different means (ANOVA, Tukey test, $p < 0.05$)

Table 2. Immature (larval and pupal) development time and adult emergence rate (mean ± s.d.) of *Ae. albopictus* surviving sublethal concentrations of *P. protegens* COI.

Bacterial concentration (CFU/mL)	n[a]	Larval development time[b] (days)	n[c]	Pupal development time[d] (days)	Adult emergence[e] (%)
5.0×10^7	46	8.1 ± 0.4 [a]*	24	2.3 ± 0.3 [a]	53.3 ± 12.5 [a]
2.5×10^7	101	7.6 ± 0.6 [ab]	80	2.0 ± 0.2 [ab]	79.4 ± 14.5 [b]
Control	131	7.0 ± 0.3 [b]	99	2.0 ± 0.1 [b]	75.8 ± 9.3 [b]

[a] number of pupated larvae,

[b] days from egg hatching to pupation,

[c] number of emerged adults,

[d] days from pupation to adult emergence,

[e] calculated on the number of pupated insects.

*Different letters in each column indicate significantly different means (ANOVA, Tukey test, $p < 0.05$)

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