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

Rim Hamze, Maria Tiziana Nuvoli, Carolina Pirino, Luca Ruiu*,

Dipartimento di Agraria, University of Sassari, Viale Italia 39, 07100 Sassari, Italy.

*Corresponding author: Luca Ruiu, Dipartimento di Agraria, University of Sassari, Viale Italia 39/A, 07100 Sassari, Italy.

Tel.: +39 079229326

E-mail address: lucaruiu@uniss.it

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.

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

55 soil, which further complicate the diversity of ecosystem relationships that this bacterial species
56 would have evolved (Flury et al., 2019).
57 A prominent biocontainment role against several insect pests such as aphids, thrips, whiteflies and
58 some lepidopterans, is played by diverse lacewing predatory species within the *Chrysoperla carnea*
59 Stephens group, in different agricultural systems (Principi and Canard, 1984, Senior and McEwen,
60 2001). In addition to their natural action as biological control agents (BCAs), these species are
61 commercially produced in biofactories and employed in augmentative (inundative) biocontrol
62 programs in different parts of the world (Pappas et al., 2011). The susceptibility of chrysopids to
63 some entomopathogenic bacteria has occasionally been reported even though most of the
64 entomopathogens usually employed for pest management are normally considered selective in
65 respect to these predators (Romeis et al., 2004).
66 This study aimed at investigating the possible lethal and sub-lethal effects that *P. protegens* may
67 cause to the lacewing *C. carnea*. For this purpose, the effects on survival of predatory larvae were
68 studied by both directly exposing the larvae to the bacterium through feeding and indirectly by
69 offering larvae a previously treated prey (tritrophic interaction). The potential effect on several
70 parameters such as immature developments, adult emergence and fecundity was examined.
71
72 **2 Materials and methods**
73 **2.1 Bacterial strain and preparations**
74 The entomopathogenic bacterium *P. protegens* strain CHA0 (CFBP 6595^{T}), whose
75 entomopathogenic properties are well known, was employed in this study (Stutz et al., 1986).
76 Bacterial cultures were routinely conducted in flasks at 30 °C in LB broth, shaken at 180 rpm, from
77 which bacterial cells were harvested by centrifugation at 15,000 x g at 4° C for 10 minutes after 48-
78 72 h, before being resuspended in water to make suspensions used in bioassays. Bacterial
79 concentration was assessed by plating serial dilutions on LB agar and determining the number of
80 colony forming units (CFUs).

2.2 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.

2.2.1 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).

2.2.2 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.

2.2.3 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.

2.3 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. Results

3.1 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.

156 No significant differences were observed between adults emerging form larvae treated with
157 different bacterial concentrations and untreated, in terms of both emergence rate ($F_{5,17}$ = 0.89; P =
158 0.5181) and average oviposition per female ($F_{5,27}$ = 0.67; P = 0.6535) (Table 2).
159
160 **3.2 Tritrophic interaction**
161 The lacewing survival rate after feeding larvae with mealworms pre-treated with *P. protegens*
162 CHA0, is shown in Fig. 2. A non-significant reduction in the survival rate was observed in the
163 transition from preimaginal to adult stage of treated insects ($F_{3,23}$ = 2.25; P = 0. 1219). However, no
164 differences in the overall survival were observed between treated and control groups ($F_{1,23}$ = 3.13; P
165 = 0. 0962).
166 Exposition to treated preys did not affect the development time of larvae (t = -0.010; df = 46; P =
167 0.4960) and pupae (t = -0.1557; df = 48; P = 0.4385) in respect to a control feeding on untreated
168 mealworms (Table 3). A slight reduction in adult emergence (18%) observed in treated insects
169 compared with control was not statistically significant (t = 1.508; df = 4; P = 0.1031).
170 The average oviposition rate per lacewing female was not affected by exposing them at the larval
171 stage to treated preys (Table 4) and no significant differences between treated and control groups
172 were detected (t = -0-025; df = 6; P = 0.4904).
173
174 **Discussion**
175 Studies on the insect pathogenesis potential of *Pseudomonas protegens* highlight its ability to
176 rapidly reproduce and develop in the insect haemocoel, once inside, which would therefore
177 represent a suitable and nutrient-rich environment (Ruffner et al., 2013). On the other hand, this
178 bacterium harbors an arsenal of virulence factors allowing its penetration inside the insect body
179 through the intestinal barriers (Péchy-Tarr et al., 2008; Loper et al., 2016; Vesga et al., 2020). Such
180 behavior has been observed on diverse target species in different orders, including Lepidoptera,
181 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

208 dipteron fed with *P. protegens*, were not affected and even behaved as vectors of the bacterium that
209 could spread to the roots of other plants exerting its beneficial action (Flury et al., 2019). This
210 therefore implies a complex evolutionary process that would explain variable susceptibility of
211 different hosts. In the case of other entomopathogenic bacteria, such as the most known *B.*
212 *thuringiensis*, insect toxicity is commonly related to a highly specific binding of Cry proteins with
213 epithelial cell membrane receptors of susceptible hosts (Jurat-Fuentes and Crickmore, 2017).
214 Accordingly, biopesticidal strains of *B. thuringiensis* were found to be generally nontoxic to *C.*
215 *carnea* and no specific interaction of Cry toxins with midgut receptors was observed (Romeis et al.,
216 2004; Rodrigo-Simón et al., 2006). On the other hands, some deleterious effects were occasionally
217 observed under specific laboratory conditions (Hilbeck et al., 1998), which, however, is not
218 necessarily representative of the natural environmental conditions under which chrysops could
219 come into contact with bacteria (Bourguet et al., 2002; Lövei et al., 2009). The absence of
220 susceptibility to *P. protegens* CHA0 by ingestion we observed in laboratory experiments would
221 therefore support the lack of a specific action toward this non-target insect. However, in addition to
222 the possibility of direct contact with the bacterium, the health of a predator could also be
223 undermined in the ecosystem by feeding on an entomopathogen-affected host, which may lead to
224 either infection or the availability of a prey with poorer nutritional characteristics (Ruiu et al., 2007;
225 Salama et al., 1991; Eubanks and Denno, 2000). No such detrimental effects on survival,
226 development or reproductive potential of *C. carnea* feeding on pre-treated mealworms were
227 observed, thus supporting the compatibility of *P. protegens* CHA0 within a tritrophic interaction
228 system involving predator, prey and pathogen.
229 According to the results of this study, neither contraindications nor indications of risk to the use of
230 *P. protegens* strain CHA0 as a biological control agent in the agroecosystem emerged. Moreover,
231 the conditions of artificial exposure of chrysopid larvae to the bacterium in the laboratory should be
232 considered extreme, considering that increased dilution in the environment is expected and that
233 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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Declaration of Competing Interest

Authors do not have any competing interests to declare.

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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, $P > 0.05$).

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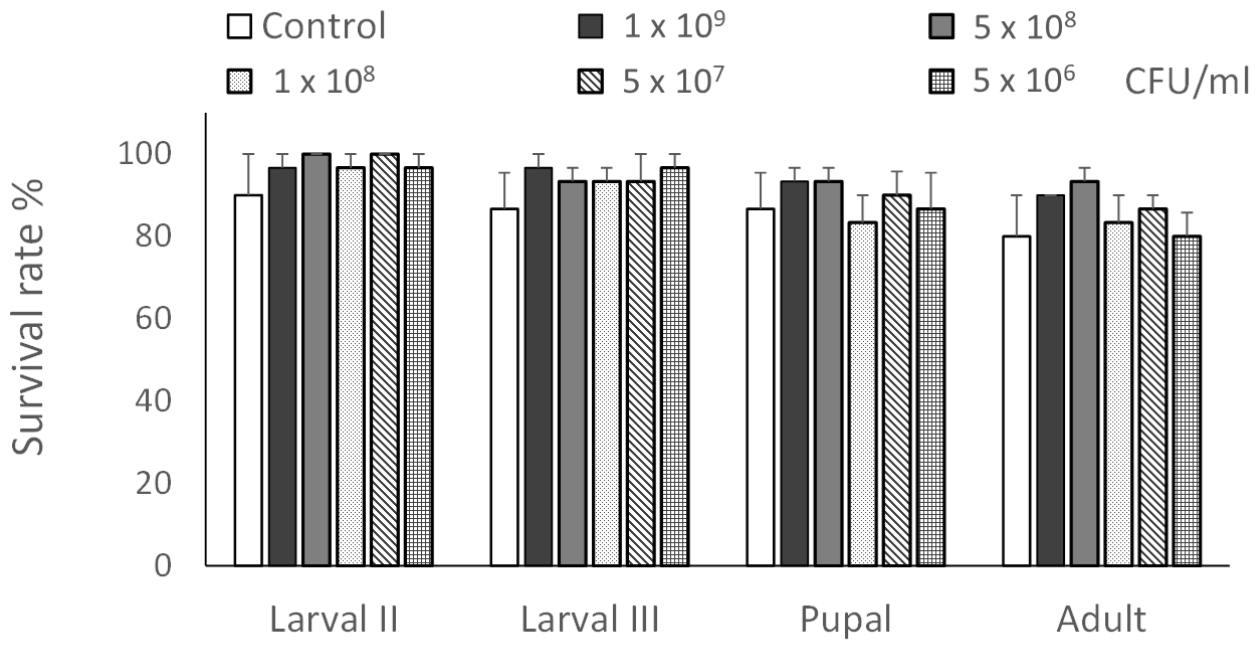

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413 Fig. 1 – Survival percentage (mean ± SE) of different *Chrysoperla carnea* stages from 1st instar
414 larvae fed with different concentrations (CFU/ml) of *Pseudomonas protegens* CHA0 cell
415 suspensions. Means were not significantly different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

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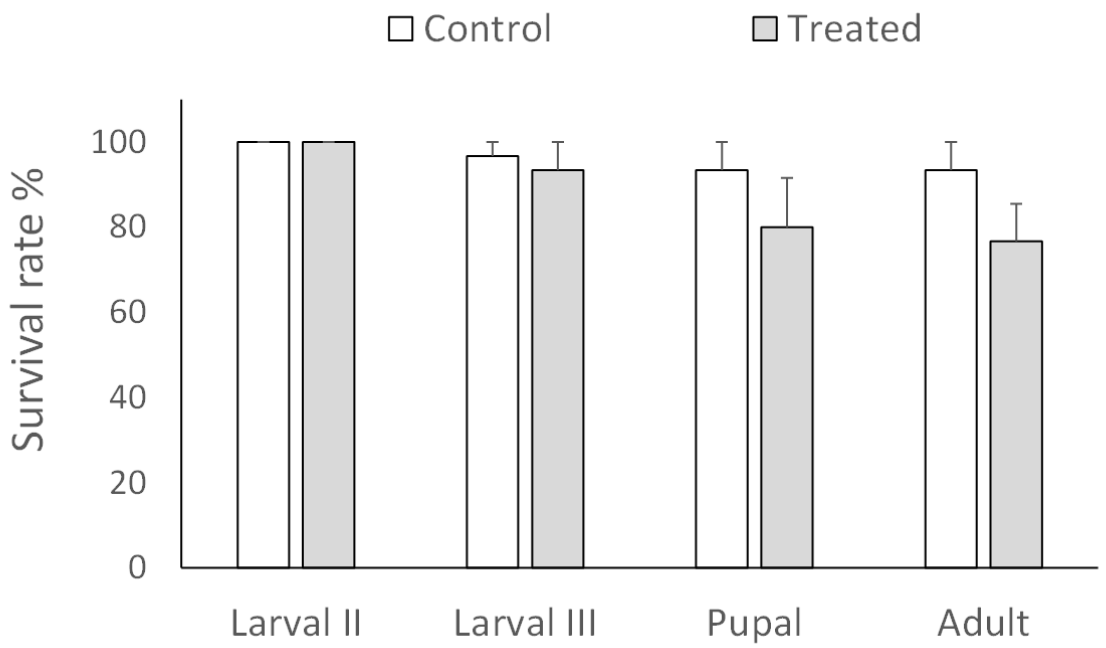

Fig. 2 - Survival percentage (mean ± SE) of different *Chrysoperla carnea* stages from 1st instar larvae fed on mealworms pre-treated with *Pseudomonas protegens* CHA0. Means were not significantly different (ANOVA, $P > 0.05$)