

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

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

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15

16 **Abstract**

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

27  
28 Key words: biocontrol; non-target; safety; pest management; biopesticide.

29

## 30 **Introduction**

31 Among the diversity of the bacterial community living in the soil, the interest in *Pseudomonas*  
32 *protegens* is growing due to its ability to perform significant activities in favor of cultivated plants  
33 by occupying different ecological niches in the agroecosystem (Agaras et al., 2017). Such beneficial  
34 properties include the interaction with the plant root system, leading to increased access to nutrients  
35 in the soil through support in nitrogen fixation, siderophore secretion, phosphorus solubilization,  
36 phytohormone synthesis, and improvement in water uptake (Sivasakthi et al., 2014; Trivedi et al.,  
37 2020). Added to this, is the bacterium's ability to colonize the root, which results in both the  
38 formation of protective biofilms and the establishment of a more intimate relationship with the  
39 plant, up to endophytism (Morales-Cedeño et al., 2021). This leads to improved resistance to  
40 phytopathogens as a result of increased competitiveness for nutrients and space, release of  
41 antibiotics and various bioactive molecules (Ramamoorthy et al., 2001; Ramette et al., 2011).  
42 According to the production of several insect virulence factors and a significant pathogenic  
43 potential, *P. protegens* appears also to be a promising candidate for pest management  
44 (Kupferschmied et al., 2013; Keel, 2016; Pronk et al., 2022). Consistently, different strains of this  
45 bacterial species were reported to be active against a variety of insect pests, either orally and by  
46 intra-haemocoelic injection (Flury et al., 2016; Vesga et al., 2021). The insecticidal action by  
47 ingestion implies overcoming gut barriers, which can be supported by enzymes like chitinases  
48 possibly involved in the degradation of peritrophic matrix (Flury et al., 2016; Ruiu and Mura, 2021)  
49 and by toxins interacting with the epithelial cell membranes like the fluorescent insecticidal toxin  
50 FitD that was also found to harbor a pore-forming domain (Ruffner et al., 2015).  
51 While the expected multiple beneficial action of *P. protegens* in the agro-ecosystem is promising  
52 for the deriving application potential, still limited information on the possible side effects on non-  
53 target organisms, including natural pest parasites and predators, is available. It has in some cases  
54 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<sup>T</sup>), 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).

81

## 82 **2.2 Insect bioassays**

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

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

89

### 90 **2.2.1 Insect rearing**

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

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

96

### 97 **2.2.2 Direct feeding bioassays**

98 The possible direct toxicity of *P. protegens* to lacewing larvae was evaluated by feeding young  
99 larvae with a suspension containing bacterial cells. For this purpose, 1<sup>st</sup> instar larvae were  
100 maintained individually inside plastic jars (2 cm diameter x 3 cm high) into which a drop (4 µl) of a  
101 20% fructose suspension containing a variable bacterial cell concentration (treatments) or lacking  
102 bacterial cells (control), was placed. To mitigate possible effects of fructose on bacterial cell  
103 viability/pathogenicity, this mixture was prepared fresh just before being administered to the  
104 insects. On the other hand, no reduction in pathogenicity of *P. protegens* against susceptible targets  
105 was previously observed when bacterial cells were mixed with sugar (Ruiu et al., 2017).

106 Accordingly, a fresh drop was provided daily to each larva for a 5-day period. From the sixth day

107 on, the lacewing larvae were instead offered one larva per day of the artificial prey *T. molitor* which  
108 ensured that they were well fed until the end of their life cycle. The experimental design involved  
109 groups of ten individually reared larvae for each bacterial concentration assayed ( $10^9$ ,  $10^8$ ,  $10^7$ ,  $10^6$ ,  
110  $10^5$  CFU/ml) and for the control. Each treatment involved three replicates (10 individuals/replicate).  
111 Insects were examined daily to assess mortality, larval moults, pupation, and emergence of adults.  
112 Emerging adults were moved to new cages maintaining them within the same treatment group  
113 (respecting a male to female ratio of 1 to 1), where they could mate. These cages had a removable  
114 sheet of paper on their inner surface which allowed oviposition and egg counting during the  
115 subsequent 3-week period. Adult feeding in this period was based on the *ad libitum* administration  
116 of honeybee pollen and water. Insect death in the cage was also recorded.

117

### 118 **2.2.3 Tritrophic bioassays**

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

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

136

### 137 **2.3 Statistical analysis**

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

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

144

## 145 **3. Results**

### 146 **3.1 Direct feeding**

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

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

156 No significant differences were observed between adults emerging from 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

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

208 dipteran 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 hand, 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

234 chances of contact with significant doses of the pathogen (Cordeiro et al., 2010). On the other hand,  
235 the environmental conditions under which an insect pathogen could act in the ecosystem are more  
236 complex than a laboratory, and other factors, such as various stressors could increase the  
237 susceptibility of the host (Donegan and Lighthart, 2003).

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

243

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250

#### 251 **Declaration of Competing Interest**

252 Authors do not have any competing interests to declare.

253

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**Table 1** – Means ( $\pm$  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) <sup>a</sup>	Development time (days)		Adult emergence <sup>d</sup> %
	Larvae <sup>b</sup>	Pupae <sup>c</sup>	
10 <sup>9</sup>	21.4 $\pm$ 0.84 <sup>e</sup>	11.9 $\pm$ 0.10	90.0 $\pm$ 0.00
10 <sup>8</sup>	18.6 $\pm$ 0.48	11.8 $\pm$ 0.15	93.3 $\pm$ 3.33
10 <sup>7</sup>	19.0 $\pm$ 0.64	12.1 $\pm$ 0.28	83.3 $\pm$ 6.67
10 <sup>6</sup>	18.9 $\pm$ 0.68	11.7 $\pm$ 0.15	86.7 $\pm$ 3.33
10 <sup>5</sup>	20.3 $\pm$ 0.97	12.3 $\pm$ 1.31	80.0 $\pm$ 5.77
Control	20.9 $\pm$ 0.98	11.5 $\pm$ 0.20	80.0 $\pm$ 10.00

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<sup>a</sup> larvae were exposed to a cell suspension containing 20% fructose

<sup>b</sup> time from egg hatching to pupation

<sup>c</sup> time from pupation to adult emergence

<sup>d</sup> based on the initial number of larvae

<sup>e</sup> means in each column were not significantly different (2-ways ANOVA, P > 0.05).

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

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Bacterial concentration (CFU/ml) <sup>a</sup>	Eggs/female <sup>a</sup>
10 <sup>9</sup>	309.8 $\pm$ 88.51 <sup>b</sup>
10 <sup>8</sup>	249.8 $\pm$ 44.01
10 <sup>7</sup>	270.8 $\pm$ 39.31
10 <sup>6</sup>	253.1 $\pm$ 63.43
10 <sup>5</sup>	298.1 $\pm$ 3.33
Control	153.6 $\pm$ 49.14

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<sup>a</sup> Total number of eggs laid over a 3-week period.

<sup>b</sup> means in the column were not significantly different (ANOVA, P > 0.05).

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**Table 3** – Means ( $\pm$  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 <sup>a</sup>	Development time (days)		Adult Emergence <sup>d</sup> %
	Larvae <sup>b</sup>	Pupae <sup>c</sup>	
Treated	18.5 $\pm$ 0.95 <sup>e</sup>	11.7 $\pm$ 0.17	76.7 $\pm$ 8.82
Control	18.4 $\pm$ 0.89	11.6 $\pm$ 0.19	93.3 $\pm$ 6.67

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<sup>a</sup> Treated indicates *C. carnea* fed *T. molitor* larvae pre-treated with *P. protegens*

<sup>b</sup> time from egg hatching to pupation

<sup>c</sup> time from pupation to adult emergence

<sup>d</sup> based on the initial number of larvae

<sup>e</sup> means in each column were not significantly different (*t*-test,  $P > 0.05$ ).

402 **Table 4** –Fecundity (mean  $\pm$  SE) in *Chrysoperla carnea* females from larvae fed on insect preys  
403 treated with *Pseudomonas protegens* CHA0.  
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Treatment <sup>a</sup>	Eggs/female <sup>b</sup>
Treated	263.7 $\pm$ 70.47 <sup>c</sup>
Control	261.7 $\pm$ 39.18

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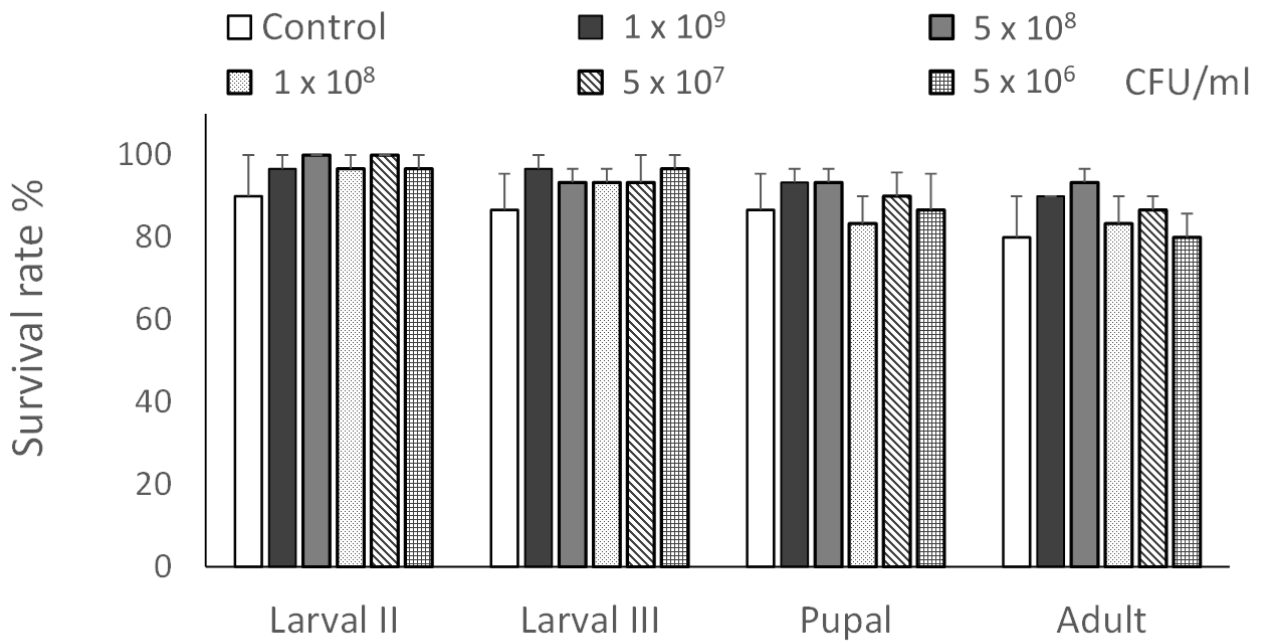
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406 <sup>a</sup> Treated indicates *C. carnea* fed *T. molitor* larvae pre-treated with *P. protegens*.

407 <sup>b</sup> Total number of eggs laid over a 3-week period.

408 <sup>c</sup> means in the column were not significantly different (*t*-test,  $P > 0.05$ ).

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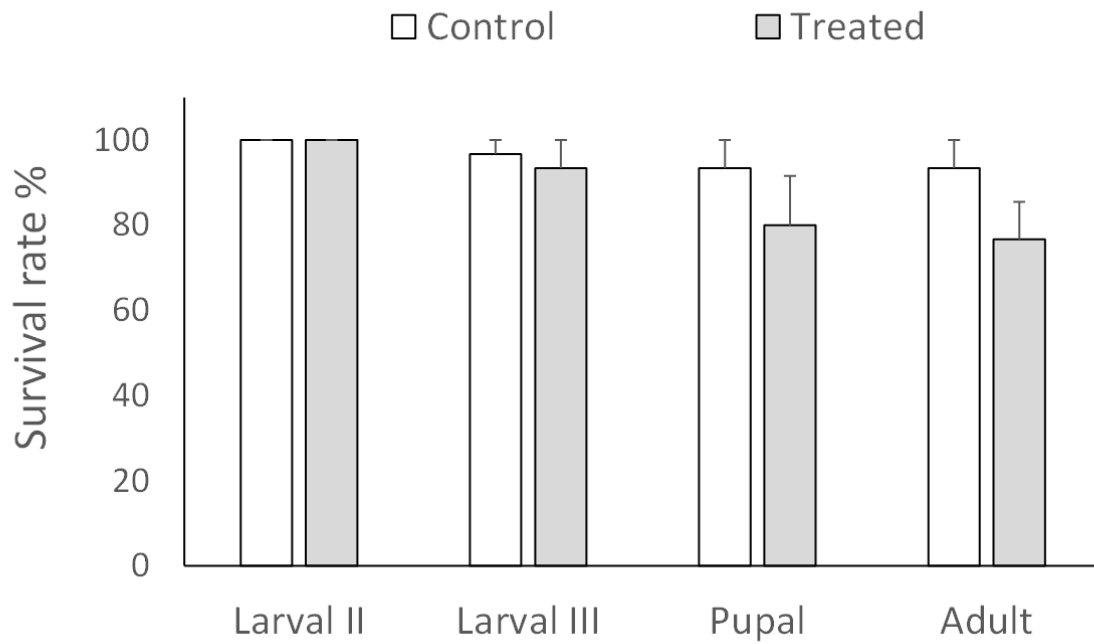


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413 Fig. 1 – Survival percentage (mean  $\pm$  SE) of different *Chrysoperla carnea* stages from 1<sup>st</sup> instar  
414 larvae fed with different concentrations (CFU/ml) of *Pseudomonas protegens* CHA0 cell  
415 suspensions. Means were not significantly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

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421 Fig. 2 - Survival percentage (mean  $\pm$  SE) of different *Chrysoperla carnea* stages from 1<sup>st</sup> instar422 larvae fed on mealworms pre-treated with *Pseudomonas protegens* CHA0. Means were not423 significantly different (ANOVA,  $P > 0.05$ )

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