

Safety evaluation of the entomopathogenic bacterium *Brevibacillus laterosporus* for the green lacewing *Chrysoperla agilis* (Neuroptera: Chrysopidae)

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1 **Safety evaluation of the entomopathogenic bacterium *Brevibacillus laterosporus* for the green**  
2 **lacewing *Chrysoperla agilis* (Neuroptera: Chrysopidae)**

3

4

5 **Luca Ruiu<sup>\*</sup>, Anna Marta Lazzeri, Maria Tiziana Nuvoli, Ignazio Floris, Alberto Satta**

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

7

8 \*Corresponding author: Luca Ruiu, Dipartimento di Agraria, University of Sassari, Viale Italia 39,  
9 07100 Sassari, Italy.

10 Tel.: +39 079229326

11 E-mail address: [lucaruiu@uniss.it](mailto:lucaruiu@uniss.it)

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16 **Abstract**

17 The safety of the entomopathogenic bacterium *Brevibacillus laterosporus* for the natural insect  
18 predator *Chrysoperla agilis* was evaluated in this study. For this purpose, laboratory bioassays were  
19 conducted exposing different larval instars and adults of the chrysopid to bacterial spore preparations,  
20 in order to evaluate the possible effects on survival, longevity, immature development, and adult  
21 reproductive performance. The sub-lethal effects were investigated by feeding the bacterium directly  
22 to adults and larvae of *C. agilis* or to mealworm beetles (*Tenebrio molitor*) used as hosts for  
23 chrysopids (tritrophic interaction). Direct feeding of *B. laterosporus* spores to different lacewing  
24 larvae instars and to adults did not cause mean mortality levels significantly different from untreated  
25 control, and slight though not significant effects of treatments were generally observed on insect  
26 longevity, development, fecundity and egg hatching. In the case of lacewing larvae feeding on treated  
27 mealworm beetles, adult emergence percentage was reduced approximately 12 %, in comparison with  
28 untreated control. Based on these results, the use of *B. laterosporus* for pest management in the  
29 agroecosystem, appears to be compatible with chrysopids.

30

31 Key words: bioinsecticide; side-effects; sub-lethal effects; pest management; bacteria.

32

### 33 **Introduction**

34 The need to identify effective and eco-sustainable active substances for the management of crop pests  
35 is leading to the discovery of new species and strains of entomopathogenic bacteria, fungi, virus,  
36 microsporidia, and nematodes, with novel bioinsecticidal properties and target range (Ruiu, 2018).  
37 On the other hand, for a more complete evaluation of a new entomopathogen and its potential, effects  
38 in the agricultural ecosystem should be considered in the broadest sense. This involves the evaluation  
39 of the possible side-effects against non-target organisms, including natural predators and parasites  
40 (Lacey et al., 2001).

41 *Brevibacillus laterosporus* is a bacterial species represented by several strains exhibiting varying  
42 levels of pathogenicity against a variety of insect targets in different orders, including Coleoptera,  
43 Lepidoptera, and Diptera (Ruiu, 2013). The insecticidal action is mostly related to the production of  
44 diverse toxins, most of which act in the insect gut after ingestion (Marche et al., 2018; Glare et al.,  
45 2019).

46 While future applications of this microorganism and its products in agriculture appear promising,  
47 knowledge about the possible effects on non-target organisms is still limited. Previous studies have  
48 demonstrated a slight susceptibility of the muscoid fly parasitoid wasp *Muscidifurax raptor* Girault  
49 and Sanders (Hymenoptera: Pteromalidae) exposed to higher doses than those active against target  
50 pests (Ruiu et al., 2007), but no information on the possible effects on other beneficial insects is  
51 available.

52 Chrysopid species are primary predators against several plant pests including aphids, lepidopterans,  
53 mites, thrips, and whiteflies (Principi and Canard, 1984, Senior and McEwen, 2001). In addition to  
54 predation by natural populations, different species belonging to the *Chrysoperla carnea* group have  
55 been employed successfully in pest management programs that involve augmentation releases in the  
56 field (Pappas et al., 2011). Appropriate integrated pest management strategies should ensure  
57 protection and enhancement of their populations in the agricultural ecosystem (Cordeiro et al., 2010)  
58 and the safety of plant protection products, including entomopathogens, for chrysopids is therefore

59 necessary. While several studies considering the side-effects of several chemically active substances  
60 against these beneficial insects have been conducted (Michaud and Grant, 2003), knowledge about  
61 the possible non-target toxicity or pathogenicity associated with entomopathogenic microbials is still  
62 limited. On the other hand, some studies have been dedicated to evaluating the safety of some insect  
63 pathogens for chrysopids, including fungi, for example, *Beauveria bassiana* (Donegan and Lighthart,  
64 1989) and *Metharizium anisopliae* (Ríos-Moreno et al., 2018), and bacteria, including *Bacillus*  
65 *thuringiensis* (Rodrigo-Simón et al., 2006; Lövei et al., 2009). No information on susceptibility of  
66 chrysopids to *B. laterosporus* is available.

67 *Chrysoperla agilis* Henry et al. (Neuroptera: Chrysopidae) is a natural biocontrol agent belonging to  
68 the *Chrysoperla carnea* group. *C. agilis* populations are distributed in Western Europe, overlapping  
69 with other lacewing species within the same phylogenetic group, including *C. carnea sensu stricto*  
70 (Stephens), *Chrysoperla lucasina* (Lacroix), *Chrysoperla mediterranea* (Hölzel) and *Chrysoperla*  
71 *pallida* Henry et al. (Noh and Henry, 2010). Because of its wide distribution in agricultural  
72 ecosystems and a broad range of hosts, the predatory role of *C. agilis* in the bio-containment of pests  
73 in agriculture is considered significant (Pappas et al., 2013).

74 The purpose of this study was to evaluate the susceptibility of *C. agilis* to a *B. laterosporus* strain that  
75 shows significant pathogenicity against different insect pests. In particular, our work investigated the  
76 lethal and sublethal effects (development of immatures, adult emergence and reproductive  
77 performance) in lacewings exposed to a diet containing bacterial spores. To evaluate possible indirect  
78 effects on *C. agilis* larvae feeding on insect prey previously exposed to *B. laterosporus*, the tritrophic  
79 interaction of host-predator-bacterium was also considered.

80

## 81 **2 Materials and methods**

### 82 **2.1 Bacterial preparations**

83 Entomopathogenic *B. laterosporus* strain UNISS 18 (= NCIMB 41419) was selected for this work  
84 because of its well documented insect pathogenic properties (Marche et al., 2018). Spore

85 suspensions used in bioassays were routinely produced by cultivation in LB broth, shaken at 180  
86 rpm at 30 °C for 48-72 h to achieve culture sporulation.  
87 For this purpose, a pre-culture (25 ml) was inoculated with a heat-activated spore suspension (1 ml),  
88 and then used to inoculate a second culture in sporulation medium T3, as described elsewhere  
89 (Marche et al., 2017). Spores were harvested by centrifugation at 15,000 x g at 4° C for 15 minutes  
90 and resuspended in water to achieve the concentration needed in bioassays.

91

## 92 **2.2 Insect bioassays**

93 All insect bioassays were conducted in a climatic room at 25° C with a photoperiod of L16:D8.  
94 Lacewings used in bioassays were provided by the insect rearing facility of the University of  
95 Sassari where a colony of *C. agilis* established in 2016 from field-collected individuals is  
96 maintained according to methods described by Pasqualini (1975) with some adaptations (Loru et al.,  
97 2013). *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) larvae (mealworm beetles) and *Musca*  
98 *domestica* L. (Diptera: Muscidae) adults employed in bioassays were furnished by the same insect  
99 facility.

100

### 101 **2.2.1 Toxicity bioassays**

102 Ingestion assays were conducted exposing lacewing adults and larvae to bacterial suspension drops  
103 because it represents the way they may contact a bioinsecticide sprayed on plants against pests.

104 *C. agilis* larvae of each instar (1<sup>st</sup>, 2<sup>nd</sup>, and 3<sup>rd</sup>), immediately after moult, were placed individually  
105 into plastic jars (2 cm diameter x 3 cm high) where a 4-µl drop of a 20% fructose solution  
106 incorporating *B. laterosporus* spores (10<sup>9</sup> spores/ml) was administered daily for 5 d. From the sixth  
107 day on, larvae of *T. molitor* (1 larva per day) were provided to lacewing larvae as food. Mortality  
108 was assessed daily for 10 d.

109 Newly emerged male and female *C. agilis* adults were maintained individually in plastic jars (2 cm  
110 diameter and 3 cm high) and exposed daily to a 4-µl drop of a 20% fructose solution incorporating

111 *B. laterosporus* spores ( $10^9$  spores/ml). Honeybee pollen was provided *ad libitum* to each adult  
112 beginning on the sixth day and insect viability was verified daily for 10 d. These experiments  
113 included 50-60 individual *C. agilis* adults and larvae.  
114 Experiments also were conducted on *M. domestica* adults in order to verify the pathogenicity of the  
115 spore suspensions used in experiments with lacewings. Newly emerged *M. domestica* adults were  
116 grouped into four groups of 10 individuals per cage (10 x 10 x10 cm) and exposed to drops (10  
117  $\mu$ l/fly/day) of a 30% saccharose solution containing *B. laterosporus* spores ( $10^9$  spores/ml) from the  
118 same spore culture used in the lacewing experiments. Insect mortality was checked daily for 7 d,  
119 comparing treated flies and untreated controls (Mura and Ruiu, 2017).

120

### 121 **2.2.2 Bioassays testing for sublethal effects**

122 Lacewing larvae and adults were challenged for 5 d with *B. laterosporus* spores as previously  
123 described in the toxicity bioassays. Surviving individuals were maintained in the laboratory to  
124 investigate the possible sub-lethal effects.

125 To test immature stages, second and third instar larvae surviving treatments ( $n = 20-30$ ), were  
126 maintained individually in jars, fed on *T. molitor* larvae (1 larva/individual/day), through pupation  
127 and adult emergence. Treated and control insects were checked daily, and the dates of larval moult,  
128 pupation, adult emergence, and death during the bioassay, were recorded.

129 Individual adult insects surviving *B. laterosporus* challenge ( $n = 20$ ) were transferred into larger  
130 plastic cages (10 cm diameter x 10 cm high) with two sides covered with gauze to allow ventilation.  
131 Treated and control females were paired with treated and control males (1 pair per cage),  
132 respectively, to allow mating and oviposition. The inner surface of the cage was covered with paper  
133 on which females could lay their eggs, which were removed and counted daily. During this period,  
134 lacewing adults in the cage were provided *ad libitum* with honeybee pollen and a piece of cotton  
135 soaked in water. Death of each male or female individual in a cage was recorded during the  
136 bioassay to assess insect longevity. A group of eggs ( $n=10$ ) from each control and treated cages was

137 analysed to evaluate hatching rate. For this purpose, eggs were kept individually in a jar for a week  
138 up to hatching. This analysis was repeated 5 times during the bioassay with different cohorts of  
139 eggs.

140

### 141 **2.2.3 Tritrophic bioassays**

142 In this experiment, mealworm beetles were used to evaluate the possible indirect effects on *C. agilis*  
143 larvae feeding on an insect prey previously exposed to *B. laterosporus*. Mealworm beetles were  
144 maintained for minimum 1 week on sterile dry wheat bran without a source of water to encourage  
145 their subsequent feeding on a 2-cm diameter ball of bran moistened with a *B. laterosporus*  
146 suspension ( $10^9$  spores/ml) or water (untreated control), replaced daily. Treated mealworm beetles  
147 were maintained under these conditions for at least 3 d before being administered to lacewing  
148 larvae.

149 Second instar *C. agilis* larvae (n = 30) were maintained individually in plastic jars and fed treated or  
150 untreated (control) mealworm beetles, replaced daily (1 larva/individual/day) for 7 days. From the  
151 eighth day on, larvae were fed on untreated mealworm beetles and were inspected daily to assess  
152 mortality, date of moult, pupation, and adult emergence. Pupal weight was also recorded.

153

### 154 **2.3 Statistical analysis**

155 Data were analysed using R software (R Development Core Team, 2016).

156 We used *t*-tests to compare data means of treated and control groups of different experiments.

157 General Linear Models (GLM) of ANOVA, followed by Least Significant Difference

158 (LSD) tests for post-hoc comparison of means when needed, were used to analyse data on immature  
159 (larval and pupal) development time and percentage of adult emergence in experiments involving  
160 direct feeding of *B. laterosporus* to either second or third instar lacewing larvae.

161

## 162 **3. Results**

### 163 3.1 Toxicity bioassays

164 Direct feeding by different lacewing larvae instars and adults for 5 d on a fructose solution  
165 containing *B. laterosporus* spores did not result in mean mortality levels that were significantly  
166 different from untreated controls ( $F_{7,33} = 0.53$ ;  $P = 0.8091$ ). Larval mortality ranged on average  
167 between 7 and 10% for treated insects and between 3 and 6% for the controls, while mean adult  
168 mortality was 9–13%, for both treated and control insects (Fig. 1).

169 The insecticidal properties of the spore suspensions were confirmed in bioassays with *M.*  
170 *domestica*, producing 100% adult mortality within 5 d in comparison with the control mortality of  
171 approximately 5% ( $t = 39.19$ ;  $df = 4$ ;  $P < 0.001$ ).

172

### 173 3.2 Sublethal bioassays

174 The sublethal effects on lacewings that survived exposure to *B. laterosporus* for 5 d during the second  
175 or third larval instar are shown in Table 1. Larval development from egg hatch to pupation was not  
176 affected by treatment in comparison with control ( $F_{3,59} = 0.24$ ;  $P = 0.8699$ ). Pupal development was  
177 significantly influenced by exposure of second instar larvae to *B. laterosporus* ( $F_{3,59} = 5.04$ ;  $P =$   
178  $0.0037$ ). An average reduction in pupal development of approximately 1 d was observed (Treatment:  
179  $F_{1,59} = 6.00$ ;  $P = 0.0176$ ; interaction Instar x Treatment:  $F_{1,59} = 9.11$ ;  $P = 0.0039$ ). Adult emergence  
180 of treated insects did not differ from control ( $F_{3,12} = 0.12$ ;  $P = 0.9458$ ).

181 Viability and reproductive performance of lacewing *C. agilis* adults fed *B. laerosporus* spores  
182 incorporated in a fructose solution are shown in Table 2. Exposure to the bacterium did not cause  
183 significant changes in the longevity of either sex (males:  $t = 1.088$ ;  $df = 30$ ;  $P = 0.1426$ ); females:  $t =$   
184  $-0.56$ ;  $df = 24$ ;  $P = 0.2900$ ). Treated lacewing females that survived exposure to the bacterium  
185 exhibited fecundity levels comparable with control ( $t = 0.939$ ;  $df = 15$ ;  $P = 0.1955$ ). Similarly, no  
186 differences in egg hatch rate were observed in treated and control groups ( $t = 0.138$ ;  $df = 10$ ;  $P =$   
187  $0.4466$ ).

188

### 189 3.3 Tritrophic bioassays

190 The sub-lethal effects on immature lacewing development after second instar larvae were exposed to  
191 mealworm beetles treated with *B. laterosporus* are reported in Table 3. Treatment did not result in  
192 significant changes in development time of second ( $t = 0.535$ ;  $df = 23$ ;  $P = 0.2988$ ) and third ( $t =$   
193  $0.713$ ;  $df = 20$ ;  $P = 0.2421$ ) instar larvae, nor were there differences in pupal weight ( $t = -1.141$ ;  $df =$   
194  $17$ ;  $P = 0.1349$ ). A slight though not significant slowing of pupal development time was observed in  
195 treated lacewings ( $t = -1.637$ ;  $df = 11$ ;  $P = 0.0649$ ). Treatment of larvae reduced the average  
196 percentage of adult emergence by approximately 12% in comparison with the untreated control ( $t =$   
197  $1.964$ ;  $df = 6$ ;  $P = 0.0400$ ).

198

### 199 Discussion

200 *Brevibacillus laterosporus* showed only slight effects on *C. agilis* larvae and adults directly exposed  
201 to bacterial spores at concentrations that cause high mortality in Diptera (Lethal Concentration<sub>100</sub>)  
202 (Ruiu et al., 2007a; Marche et al., 2017). The bioassay methods used in this study involved the  
203 administration of spores incorporated in a fructose solution to simulate the way in which chrysopids  
204 would contact *B. laterosporus* as an insecticide sprayed in the field. Additionally, the continuous  
205 exposure to bacterial drops in the laboratory can be considered an extreme condition, given that  
206 natural feeding behavior of *C. agilis* larvae and adults may significantly reduce possible contacts with  
207 a bioinsecticide applied in the field. Chrysopid adults typically feed on plant resources such as pollen,  
208 nectar, and honeydew, so could be attracted by sugary baits, but the insecticidal formulations might  
209 be less attractive for these non-target insects (Wang et al., 2011). In addition to food preferences,  
210 contact between non-target insects and the active ingredient in the field may be affected by specific  
211 behaviors related to repellency or, more generally, to avoidance (Cordeiro et al., 2010).

212 Lacewing larvae feed on insect prey, which may pose an additional barrier to direct contact between  
213 the beneficial insect and the entomopathogen. When *C. agilis* larvae were offered mealworm beetles  
214 treated with *B. laterosporus* as food, no significant effects on development until pupation were

215 observed. However, slight changes in adult emergence rate were detected, suggesting a possible  
216 indirect effect of treated mealworms, due to the bacterium or, possibly due to deteriorated quality of  
217 the treated prey as food (Eubanks and Denno, 2000). Previous laboratory studies showed that  
218 development and survival of beneficial arthropods feeding on hosts that were pre-fed with a diet  
219 containing entomopathogenic bacteria like *Bacillus thuringiensis* could be affected under specific  
220 conditions (Blumberg et al., 1997; Ruiu, et al., 2007b; Salama et al., 1991; Sterk et al., 1999).

221 The general safety of *B. laterosporus* for *C. agilis* we recorded for this study is in line with results of  
222 several investigations conducted with *B. thuringiensis* and its insecticidal toxins against different  
223 chrysopid species. Given its highly specific mode of action, crystal toxin Cry1Ab from *B.*  
224 *thuringiensis* was found to have no direct effects on *C. carnea* after ingestion (Romeis et al., 2004),  
225 which is corroborated with no detected interaction of Cry toxins with midgut receptors of this non-  
226 target species (Rodrigo-Simón et al., 2006). On the other hand, significant differences between treated  
227 and control insects were observed in different laboratory conditions (Hilbeck et al., 1998). Similarly,  
228 several laboratory experiments assessing the safety of genetically modified (GM) plants, mostly  
229 expressing Cry proteins from *Bt*, for beneficial arthropods, have led to variable and sometimes  
230 conflicting results, probably in relation to the use of different non-target species and experimental  
231 conditions (Lövei et al., 2009). However, such effects observed in the laboratory are expected to be  
232 attenuated in natural field conditions (Bourguet et al., 2002).

233 The entomopathogenic action of bacteria on their targets is often complex and involves the combined  
234 effect of several toxins and virulence factors (Glare et al., 2019). Accordingly, the pathogenicity of  
235 *B. laterosporus* against invertebrate pests may rely on a wide range of molecules including enzymes  
236 (i.e., chitinases, proteases), insecticidal toxins homologous to Cry proteins, polyketides, and  
237 nonribosomal peptides (Glare et al., 2019). The insecticidal potential of the *B. laterosporus* strain  
238 employed in this study and the range of toxins and virulence factors it can express are well  
239 documented (Marche et al., 2018). The lack of toxicity and only slight sublethal effects we observed  
240 on *C. agilis* fed extreme spore concentrations support the compatibility of *B. laterosporus* with this

241 non-target species. According to our findings, the use of this bacterium in the pest management  
242 context appears promising.

243 It is not fully understood how *B. laterosporus* specifically evolved as a pathogen for certain insect  
244 species, while it is weakly active or inactive in others (Ruiu et al., 2007b). Among beneficial  
245 insects, microbiome studies revealed that *B. laterosporus* is a common resident of the honeybee  
246 body (Marche et al., 2017) and a beneficial role in favour of bee health was proposed, as a result of  
247 its antagonism against honeybee pathogens (Bartel et al., 2018; Marche et al., 2019 a and b).  
248 Based on the present knowledge, *B. laterosporus* can be considered a selective microbial species  
249 with potential for integrated pest management programs. Further research in the laboratory and in  
250 the field is needed to screen a wider range of non-target species in order to evaluate the safety of  
251 this microorganism in different agroecosystem contexts.

252

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257

### 258 **Declaration of Competing Interest**

259 Authors do not have any competing interests to declare.

260

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**Table 1** – Means ( $\pm$  SE) of larval development time, pupal development time, and percentage of adult emergence of *Chrysoperla agilis* exposed to *Brevibacillus laterosporus* at different developmental stages.

Treated lacewing larval instar	Development time (days)		Adult emergence <sup>c</sup> %
	Larvae <sup>a</sup>	Pupae <sup>b</sup>	
<i>2<sup>nd</sup> instar</i>			
Treated	18.4 $\pm$ 0.47 a <sup>d</sup>	9.8 $\pm$ 0.16 a	82.5 $\pm$ 4.74 a
Control	18.8 $\pm$ 0.37 a	11.1 $\pm$ 0.37 b	80.0 $\pm$ 2.36 a
<i>3<sup>rd</sup> instar</i>			
Treated	18.9 $\pm$ 0.64 a	10.7 $\pm$ 0.33 ab	82.5 $\pm$ 3.03 a
Control	18.7 $\pm$ 0.58 a	10.2 $\pm$ 0.25 ab	85.0 $\pm$ 3.73 a

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<sup>a</sup> calculated from egg hatching to pupation

<sup>b</sup> calculated from pupation to adult emergence

<sup>c</sup> calculated on the initial number of larvae

<sup>d</sup> Different letters in a column indicate significantly different means (GLM ANOVA, followed by LSD test,  $P < 0.05$ ).

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369 **Table 2** - Means ( $\pm$  SE) of longevity, fecundity, and percentage of egg hatching of *Chrysoperla*  
370 *agilis* adults surviving exposure to *Brevibacillus laterosporus*

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Treatment <sup>a</sup>	Longevity (days) <sup>b</sup>		n <sup>c</sup>	Eggs/female	Egg hatching <sup>d</sup> %
	Male	Female			
Treated	26.5 $\pm$ 2.02 a <sup>e</sup>	27.9 $\pm$ 1.98 a	19	352.9 $\pm$ 32.85 a	77.1 $\pm$ 5.65 a
Control	24.7 $\pm$ 2.46 a	31.1 $\pm$ 2.28 a	19	291.9 $\pm$ 56.14 a	78.3 $\pm$ 6.54 a

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373 <sup>a</sup> Newly emerged adults were exposed for 5 days to a 20% fructose solution containing *B. laterosporus*.

374 <sup>b</sup> Days from adult emergence to death.

375 <sup>c</sup> Number of ovipositing females.

376 <sup>d</sup> Egg hatching was evaluated at different time intervals during the oviposition period. Mean values are reported.

377 <sup>e</sup> Means in each column followed by different letters, are significantly different (*t*-test,  $p < 0.05$ ).

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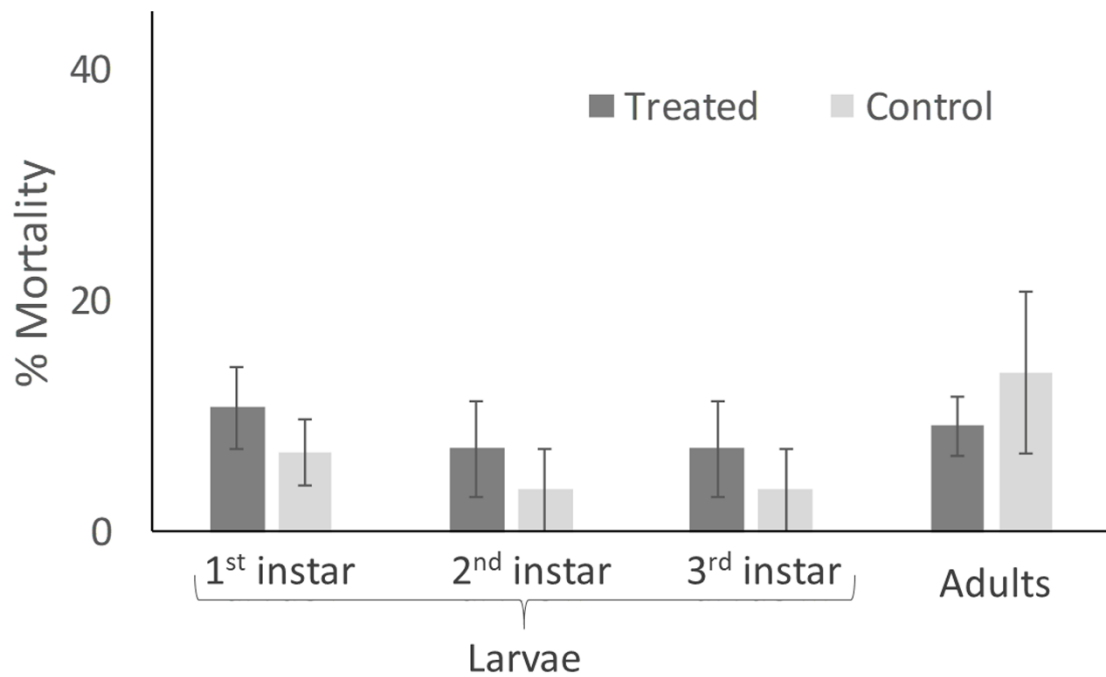
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381 Table 3 - Means ( $\pm$  SE) of larval instar and pupal development time, pupal weight, and  
 382 percentage of emergence of *Chrysoperla agilis* exposed to mealworm beetles treated with  
 383 *Brevibacillus laterosporus*  
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Treatment	Larval development time <sup>a</sup> (days)		Pupal weight (mg)	Pupal development time <sup>b</sup> (days)	Adult emergence <sup>c</sup> %
	2 <sup>nd</sup> instar	3 <sup>rd</sup> instar			
Treated	4.8 $\pm$ 0.22 a <sup>d</sup>	4.2 $\pm$ 0.20 a	7.6 $\pm$ 0.20 a	11.2 $\pm$ 0.32 a	72.5 $\pm$ 1.58 a
Control	4.9 $\pm$ 0.24 a	4.4 $\pm$ 0.23 a	7.1 $\pm$ 0.36 a	10.6 $\pm$ 0.15 a	82.5 $\pm$ 1.44 b

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 386 <sup>a</sup> time between moults  
 387 <sup>b</sup> calculated from pupation to adult emergence  
 388 <sup>c</sup> calculated on the initial number of larvae  
 389 <sup>e</sup> Means in each column followed by different letters, are significantly different (*t*-test,  $p < 0.05$ ).

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395 **Fig. 1** – Mean ( $\pm$  SE) percentage mortality of *Chrysoperla agilis* larvae and adults after 5 days  
 396 exposure to *Brevibacillus laterosporus* spores. No significant differences between means were  
 397 found (GLM ANOVA, followed by LSD test:  $P > 0.05$ ).

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# *Brevibacillus laterosporus* safety evaluation

