Insecticidal potential of Brevibacillus laterosporus against dipteran pest species in a wide ecological range

Insecticidal potential of *Brevibacillus laterosporus* against dipteran pest species in a wide ecological range

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Abstract

In order to increase our understanding of the insecticidal potential of the entomopathogenic bacterium *Brevibacillus laterosporus* strain UNISS 18 against insect pests, investigations were conducted on a selection of dipteran species including fruit flies, house flies, blow flies, and mosquitoes, characterized by adaptations to very diverse habitats.

According to lethal concentration (LC_{50}) values, the common house mosquito *Culex pipiens* (LC_{50} = 0.10 x 10^6 spores/mL) and the yellow fever mosquito *Aedes aegypti* (LC_{50} = 0.18 x 10^6 spores/mL) were significantly more susceptible than the flies. The blow flies were the second taxon in term of susceptibility to *B. laterosporus* spores, with *Calliphora vomitoria* achieving a higher mortality (LC_{50} = 78.84 x 10^6 spores/mL) than *Lucilia caesar* (LC_{50} = 148.30 x 10^6 spores/mL). The effectiveness of *B. laterosporus* spores was reduced by half in the case of the house fly *Musca domestica* (LC_{50} = 82.41 x 10^6 spores/mL), while, the lowest susceptibility was observed in the fruit files among which the Spotted wing drosophila (SWD) *Drosophila suzukii* was the most susceptible (LC_{50} = 217.51 x 10^6 spores/mL) in comparison with the medfly *C. capitata* and the olive fly *B. oleae* (LC_{50} = 2567.32 and 2567.36 x 10^6 spores/mL, respectively). The present study demonstrated that significantly different degrees of susceptibility are associated with diverse dipteran species including plant and animal parasites, that suggest that *B. laterosporus* established different relationships with dipteran species living in different ecosystems.

Key words: bioinsecticide; pest management; flies, mosquitoes, bacteria.

Introduction

Brevibacillus laterosporus is an emerging biological control agent with significant potential against invertebrate pests and several phytopathogens affecting various crop species (Ruiu, 2013). The pesticidal and antimicrobial activity of this bacterium is due to the production of an arsenal of toxins, antibiotics and other virulence factors. Despite remarkable differences among strains, most of the pesticidal potential appears to be well conserved in the genome of this species (Glare et al., 2020). Within a complex insecticidal mechanism of action, an important role is played by certain enzymes (i.e., chitinases, proteases) (Marche et al., 2018), Cry toxin homologues (Bowen et al., 2017), spore-associated proteins (Marche et al., 2017), polyketides, nonribosomal peptides, and other putative toxins (Djukic et al., 2011). Normally, after ingesting bacterial spores, the insect goes into progressive midgut epithelium deterioration, followed by paralysis and death (Ruiu et al., 2012). Although there are differences between *B. laterosporus* strains, the target range includes pests in the orders Coleoptera, Lepidoptera and Diptera (Ruiu, 2013). On the other hand, this bacterium has been observed to have no detrimental activity against certain Hymenopteran species like the honeybee with which it has an endosymbiotic relationship (Marche et al., 2016), or parasitic wasps (Ruiu et al., 2007a). A lack of significant toxicity was also observed against chrysopids (Ruiu et al., 2020). The insecticidal activity so far found against dipterans includes some species of mosquitoes and muscoid flies (Pereira et al., 2018; Zubasheva et al., 2010). However, several studies on different bacterial strains and preparations, including vegetative or sporulating cells, sporangia and spores, led to a high variability of results in terms of specificity and effectiveness. Moreover, a narrow range of dipteran species has so far been considered, while this order is wide and includes a variety of families characterized by crop pests living in very different ecosystems and species of medical and veterinary interest. Due to this still limited knowledge, the pathogen-host relationship between *B. laterosporus* and Diptera remains unclear. In order to increase our understanding of *B. laterosporus* target range, investigations were conducted on a selection of dipteran species characterized by adaptations to very diverse habitats. These include house flies and

71 blow flies adapted to develop on organic matter, polyphagous and monophagous fruit flies, and
72 mosquito species.
73
74 **2 Materials and methods**
75 **2.1 Bacterial preparations**
76 The bioassays were conducted employing pure spore suspensions of *Brevibacillus laterosporus*
77 strain UNISS 18 (= NCIMB 41419), an entomopathogenic strain previously reported to be active
78 against dipters (Marche et al., 2018). Pure spores were selected as the bacterial fraction to be used
79 in bioassays for standardization purposes and because they are known to have a significantly higher
80 insecticidal activity, in comparison with vegetative cells and culture supernatant (Ruiu et al., 2007b;
81 Marche et al., 2017). The strain was cultured on LB broth in a shaking incubator (180 rpm) at 30
82 °C. In order to produce pure spore suspensions, culture synchronization was obtained as described
83 by Marche et al. (2017). Briefly, heat-activated spore suspension (1 mL) was inoculated in a pre-
84 culture rich medium (25 mL, LB broth). An aliquot of this culture at the exponential phase was then
85 used to inoculate a sporulation medium (200 mL, T3). After 48 h of growth, the spores were
86 harvested by centrifugation at 15,000 × g at 4° C for 15 min and resuspended in water to adjust the
87 concentration at 2×10^9 or at 1×10^9 spores/mL to be stored at -20 °C until being used in insect
88 bioassays.
89
90 **2.2 Bioassays**
91 Bioassays were conducted exposing dipteran larvae or adults to bacterial spores added to their diet
92 or developmental substrate. Based on the feeding behaviour, a different bioassay method was
93 employed for each target species, in order to simulate their natural intake of a spore suspension
94 possibly applied to their habitat. In this way, all insects were forced to feed spores while satisfying
95 their daily food needs. The bioassays were performed under laboratory conditions at 25° C, 65%

RH and natural photoperiod with insects provided by University rearing facilities or collected in the field. Four replicates were performed for each experiment that was repeated twice.

2.2.1 Muscoid flies

House fly

Because the insecticidal activity of the strain on *Musca domestica* (L.) (Diptera: Muscidae) is known (Ruiu et al., 2007b), these bioassays had the main purpose to confirm the bioinsecticidal potential of bacterial preparations used in the experiments. Lab reared flies (0-24 h after emergence) were fed with 30% saccharose solution drops incorporating *B. laterosporus* spores at a dose of 10 µl/fly/day. Flies were exposed to different concentrations ranging between 10^7 and 10^9 spores/mL. Treated and untreated flies (control) were maintained in groups of 10 individuals inside plastic cages and mortality was assessed after 72 h (Mura and Ruiu, 2017).

Blow fly

Newly emerged (0-24 h) *Lucilia caesar* (L.) and *Calliphora vomitoria* (L.) (Diptera: Calliphoridae) were fed with 30% saccharose solution drops incorporating *B. laterosporus* spores at a dose of 20 µl/fly/day. Control was performed with drops of 30% saccharose water solution. Bacterial drops were administered into plastic cages containing 10 individuals that were checked daily for 72 h to assess mortality. The following range of bacterial concentrations was assayed: 1.7×10^9, 9.0×10^8, 4.5×10^8, 2.3×10^8, 9.0×10^8, 1.0×10^8, 5.8×10^7, 2.9×10^7 spores/mL.

2.2.2 Fruit flies

Spotted wing drosophila (SWD)

Drosophila suzukii Matsumura (Diptera: Drosophilidae) was reared under laboratory conditions following the method described in Bedini et al. (2020). Briefly, ten newly emerged adults (0-24 h) were exposed to drops including bacterial spores at different concentrations: 2.0×10^8, 1.0×10^8,

122 5.0×10^7, 2.0×10^7, 2.0×10^6, 2.0×10^5 spores/mL. Control was performed with drops of 30%
123 saccharose water solution. The bioassay was conducted into plastic cages containing 10 individuals
124 that were checked daily for 72 h to assess mortality.
125
126 *Mediterranean Fruit Fly*
127 Lab reared *Ceratitis capitata* Wied (Diptera: Tephritidae) adults aged of 0-24 h were maintained in
128 groups of 10 individuals and fed daily by capillary tubes (50 µl) containing a saccharose solution
129 (30%) including bacterial spores at different concentrations: 2.0×10^9, 1.0×10^9, 7.5×10^5, $5.0 \times$
130 10^8 spores/mL. Mortality was assessed after 72 h.
131
132 *Olive fly*
133 Field collected *Bactrocera oleae* (Diptera: Tephritidae) olive fly pupae were maintained in the
134 laboratory up to emergence of adults that were employed in bioassays. Newly emerged adults (0-24
135 h) were kept in plastic containers in groups of 10 and were administered daily by capillary tubes (50
136 µl) a saccharose solution (30%) including bacterial spores at the following concentrations: $2.0 \times$
137 10^9, 1.0×10^9, 7.5×10^5, 5.0×10^8 spores/mL. Control was performed with capillary tubes (50 µl)
138 containing only 30% saccharose water solution. Mortality was assessed after 72 h.
139
140 2.2.3 Mosquitoes
141 *Common house mosquito* and *Yellow fever mosquito*
142 Groups of 10 coetaneous second instar larvae of *Culex pipiens* L and *Aedes aegypti* L. (Diptera:
143 Culicidae), obtained from laboratory reared colonies, were maintained in plastic cups containing 50
144 mL of the appropriate dilution in dechlorinated tap water (Lacey, 1997). The following range of
145 concentrations was assayed: 1.0×10^6, 7.5×10^5, 5.0×10^5, 2.5×10^5, 1.2×10^5, 0.6×10^5, $0.3 \times$
146 10^5. Control was performed with dechlorinated tap water only. Mortality was assessed after 48 h.
147

148 **2.3 Statistical analysis**

149 Fly adults and mosquito larvae median and lethal concentration to 95% (LC_{50}, LC_{95}) were
150 calculated by probit regression. The differences among species susceptibility were assessed by
151 relative median potency analyses (rmp) and considered statistically significant when values in the
152 95% confidence interval of were ≠ 1.0. Data were processed by SPSS 22.0 software (IBM SPSS
153 Statistics, Armonk, North Castle, New York, USA)

154

155 **3. Results**

156

157 3.1 Muscoid flies

158 The *B. laterosporus* spores were found to have a clear toxic effect on the blow flies *C. vomitoria*
159 and *L. caesar*, and on the house fly *M. domestica*. According to the Probit analysis, *C. vomitoria*
160 was the most susceptible species with an LC_{50} value of 78.836×10^6 spores/mL while the most
161 resistant appeared to be *L. caesar* whose LC_{50} value was 148.296×10^6 spores/mL (Table 1). The
162 RMP analysis showed no significant difference in toxicity between *C. vomitoria* and *M. domestica*,
163 while *L. caesar* was significantly more resistant than *C. vomitoria* and *M. domestica*, (*L. caesar vs*
164 *C. vomitoria* RMP = 1.881 (1.332-2.807) (Table 2). The effectiveness of *B. laterosporus* spores
165 appeared to be reduced by half in the case of *L. caesar* compared to *C. vomitoria* and *M. domestica*
166 (Fig. 1).

167

168 3.2 Fruit flies

169 Similarly to what has been observed on muscoid flies, significant insecticidal action was also
170 observed on the fruit flies *D. suzukii*, *C. capitata*, and *B. oleae,* fed a saccharose suspension
171 containing different amounts of *B. laterosporus* spores. The most susceptible species was *D.*
172 *suzukii,* with an LC_{50} value of 217.508×10^6 spores/mL, while *B. oleae* and *C. capitata* showed
173 about the same LC_{50} value (2567.364 and 2567.320×10^6 spores/mL for *B. oleae* and *C. capitata*,

174 respectively) (Table 3). As expected, the RMP analysis showed no significant difference in toxicity
175 between *B. oleae* and *C. capitata*, while *D. suzukii* was significantly more susceptible (Table 4)
176 (Fig. 2).
177
178 3.3 Mosquitoes
179 A clear mosquitocidal activity was associated with *B. laterosporus* spores, achieving significant
180 larval mortality at low concentrations against different species. More specifically, the LC_{50} values
181 for *Ae. aegypti* and *C. pipiens* were 0.179 and 0.097 × 10^6 spores/mL, respectively (Table 5). When
182 compared (Fig. 3), *C. pipiens* appeared significantly more susceptible to *B. laterosporus* spores than
183 *Ae. aegypti* larvae (*Ae. aegypti vs C. pipiens* RMP = 1.839; CI, 1.288 – 2.739).
184
185 **4. Discussion**
186 *Brevibacillus laterosporus* is a ubiquitous bacterium that has developed different ways to interact
187 with invertebrates and soil-dwelling microorganisms including plant pathogens (Ruiu, 2013). Due
188 to its remarkable antimicrobial properties, the antagonistic potential of this bacterium is also
189 considerable with regard to several human pathogenic agents (Choopan et al., 2008; Desjardine et
190 al., 2007).
191 Several studies have highlighted the entomopathogenicity of various *Brevibacillus* strains, and this
192 bacterial genus is emerging as an important source of bacterial toxin genes with potential against
193 noxious pests (Glare et al., 2020). Among the putative virulence factors there are a variety of
194 enzymes like chitinases and proteases, homologous Cry proteins, polyketides, and nonribosomal
195 peptides (Marche et al., 2018). These molecules are implied in midgut epithelium degeneration,
196 following ingestion of bacterial spores (Ruiu et al., 2012). Although the involvement of specific
197 bacterial compounds against certain targets has been documented, the insecticidal action appears to
198 be complex and not fully understood. Alongside the availability of *B. laterosporus* strains especially

promising for applications in pest management, the evolutionary relations of this entomopathogen with the arthropod world are still little known.

B. laterosporus has been reported to have insecticidal activity against insects belonging to different orders, and in particular Coleoptera (De Oliveira et al., 2004), Lepidoptera (Narciso et al., 2019), and Diptera (Zubasheva et al., 2010), in addition to other invertebrates like nematods (Singer, 1996) and mollusks (Ruiu et al., 2013). Although the wide range of targets, *B. laterosporus* appears to be a selective microbe, being not significantly active against non-target Hymenoptera (Ruiu et al., 2007a) and chrysopids (Ruiu et al., 2020). The lack of toxicity observed in these cases makes it even more complex to understand the ecological role of this species. Moreover, *B. laterosporus* is a common resident of the honeybee body in which it appears to play a role in health preservation, alongside the well-known bacterial symbiont community of this insect species (Marche et al., 2016; Marche et al., 2019).

Based on the information available, it could be inferred that the insect species belonging to certain taxa tend to be more susceptible, while others have possibly established beneficial relationships with this bacterium. Although the effectiveness of an entomopathogenic agent may in many cases be ascribed to one or a few orders of insects, specific studies should be conducted for a more appropriate assessment of actual activity within each taxon. Diptera is a good example of an insect order characterized by species living in distinct habitats and establishing significantly diverse relationships with the species communities inhabiting the ecosystems of which they are part (Pape et al., 2011). Accordingly, the present study demonstrated that significantly different degrees of susceptibility are associated with diverse dipteran species including plant and animal parasites (Fig. 4). Young larvae of the common house mosquito *C. pipiens* and of the yellow fever mosquito *Ae. aegypti* appeared to be the most affected by *B. laterosporus* treatment, achieving median lethal concentrations below one million spores per mL. This is in line with studies employing crystal forming strains of this bacterium against mosquitoes, in which the insecticidal activity was associated with crystal proteins (Zubasheva et al., 2010). On the other hand, the susceptibility of the

house fly and the blow flies *C. vomitoria* and *L. caesar* was much lower than mosquitoes, corroborating previous studies conducted with *M. domestica* (Marche et al., 2017.) and different Calliphoridae species, including *Chrysomya megacephala* (Fabricius, 1794) (Carramaschi et al.,2015) and *Lucilia cuprina* (Wiedemann, 1830) (Pessanha et al., 2015). The present study also demonstrated the susceptibility of different fruit fly species to *B. laterosporus*. Among them *D. suzukii* appeared to be significantly more susceptible, than *B. oleae* and *C. capitata*. However, the susceptibility of fruit flies to the bacterium appeared to be lower when compared to muscoid flies and mosquitoes. The reasons for these differences are not known, but it can be assumed that the bacterium may express different degrees of virulence against diverse targets as a consequence of the differential action of a variety of insecticidal factors (Glare et al., 2020). Although different susceptibility among targets is reported in the literature, the data were obtained from different studies conducted with different bacterial strains and preparations (i.e, vegetative cells, sporangia, sporulated cultures, pure spores, parasporal bodies, purified toxins) and therefore such a different susceptibility may be biased by many variables. In this study, all target insect species were exposed to the same spore preparation, which allowed to highlight the species different susceptibility to the strain that it is known to express several insecticidal protein genes and virulence factors (Marche et al., 2018). On the other hand, we cannot exclude that a different comparative susceptibility could be associated with diverse bacterial stages of growth. Increased resistance to spore ingestion may result from different conditions in the insect intestine, that is the first barrier to *B. laterosporus* infection (Mura and Ruiu, 2017) and the environment in which the activation of bacterial toxins normally takes place (Bravo et al., 2002). Different diets and eating habits could be the first causes of such differences. Accordingly, the highest susceptibility was found in mosquito larvae normally feeding on solid particles suspended in water, followed by muscoid fly species that feed on organic matter. A lower susceptibility has been detected in insects that feed on plant species, and in particular on fruit. Such result is in line with the previously observed reduced susceptibility of fruit fly species to other entomopathogenic bacteria, including *Bacillus thuringiensis* and other *Bacillus cereus* group

species (Ruiu et al., 2015), although the potential of some strains against Tephritid fruit flies has been reported (Alberola et al., 1999; Robacker et al., 1996).

Based on previous knowledge and the results in this study, *B. laterosporus* appears to be a selective microbial species that show a high potential only against specific targets, supporting its use in their management. However, further research is needed to understand the specific mechanisms leading to different degrees of susceptibility of insects belonging to the same order and the lack of pathogenicity against certain non-target species.

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351

352 **Table 1 - Virulence of *Brevibacillus laterosporus* to the synanthropic flies *Calliphora vomitoria*,**

353 ***Lucilia caesar*, and *Musca domestica*.**

354

Species	LC_{50}[6]	95% CI	LC_{95}[b]	95% CI	Intercept ± SE	*P*[d]
C. vomitoria	78.836	65.105 - 96.440	689.865	494.168 - 1045.522	-3.312 ± 0.214	< 0.001
L. caesar	148.296	117.060 - 187.274	1297.685	939.709 - 1919.676	-3.791 ± 0.267	< 0.001
M. domestica	82.408	63.705 - 105.758	721.119	522.050 - 1061.960	-3.345 ± 0.249	< 0.001

355 [a] concentration of *B. laterosporus* spores that kills 50% of adult flies.
356 [b] concentration of *B. laterosporus* spores that kills 95% of adult flies; CI, confidence interval. Data are calculated on the
357 number of dead flies after 72 h from contact with the pathogen by Probit regression analysis and given as 10^6 spores/mL.
358 Model: Slope = 1.746 ± 0.114; $\chi 2$ = 53.142; df = 64; Pearson Goodness-of-Fit Test $P = 0.832$; Parallelism Test $P < 0.001$.
359

360

361 **Table 2 - Relative susceptibility of the synanthropic flies *Calliphora vomitoria*, *Lucilia caesar*,**

362 **and *Musca domestica* to *Brevibacillus laterosporus***

363

Species (X) / Species (Y)	*C. vomitoria*	*L. caesar*	*M. domestica*
C. vomitoria	-	**1.881(1.332-2.807)**	1.045(0.762-1.465)
L. caesar	**0.532 (0.356-0.751)**	-	**0.556 (0.378-0.790)**
M. domestica	0.957 (0.682-1.313)	**1.800(1.266-2.648)**	-

364 [a] Relative median potency analyses (rmp) values of probits (Species in column vs Species in row): Values
365 < 1 indicates lower susceptibility Values > 1 indicates higher susceptibility. Bold indicates significant
366 values (95% CI ≠ 1).
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Table 3 - Virulence of *Brevibacillus laterosporus* to the fruit flies *Batrocera oleae*, *Ceratitis capitata*, *and Drosophila suzukii.*

Species	LC_{50}[6]	95% CI	LC_{95}[b]	95% CI	Intercept ± SE	*P*[d]
B. oleae	2567.364	1776.508- 4140.198	28769.216	13897.229- 94327.171	-5.344 ± 0.652	< 0.001
C. capitata	2567.320	1776.480- 4140.110	28768.718	13897.033- 94325.008	-5.344 ± 0.652	< 0.001
D. suzukii	217.508	167.818- 313.975	2437.338	1231.512- 7625.594	-3.664± 0.426	< 0.001

[a] concentration of *B. laterosporus* spores that kills 50% of adult flies.
[b] concentration of *B. laterosporus* spores that kills 95% of adult flies; CI, confidence interval. Data are calculated on the number of dead flies after 72 h from contact with the pathogen by Probit regression analysis and given as 10^6 spores/mL. Model: Slope = 1.567 ± 0.208; $\chi 2$ = 26.077; df = 36; Pearson Goodness-of-Fit Test *P* = 0.888; Parallelism Test *P* = 0.268.

Table 4 - Relative susceptibility of the fruit flies *Batrocera oleae*, *Ceratitis capitata*, *and* *Drosophila suzukii* to *Brevibacillus laterosporus*

Species (X) / Species (Y)	*B. oleae*	*C. capitata*	*D. suzukii*
B. oleae	-	1.00(0.595-1.680)	**0.085(0.014-0.250)**
C. capitata	1.00(0.595-1.680)	-	**0.085(0.014-0.250)**
D. suzukii	**11.804 (3.995-71.181)**	**11.803 (3.995-71.179)**	-

[a], Relative median potency analyses (rmp) values of probits (Species in column vs Species in row): Values < 1 indicates lower susceptibility Values > 1 indicates higher susceptibility. Bold indicates significant values (95% CI ≠ 1).

Table 5 - Virulence of *Brevibacillus laterosporus* to the mosquitoes *Aedes aegypti*, and *Culex pipiens*

Species	LC_{50}[6]	95% CI	LC_{95}[b]	95% CI	Intercept ± SE	*P*[d]
Ae. aegypti	0.179	0.139 – 0.229	1.935	1.326 – 3.152	-7.934 ± 0.658	< 0.001
C. pipiens	0.097	0.074 - 0.125	1.052	0.746 – 1.629	-8.355± 0.672	< 0.001

[a], concentration of *B. laterosporus* spores that kills 50% of mosquito larvae. [b], concentration of *B. laterosporus* spores that kills 95% of larvae; CI, confidence interval. Data are calculated on the number of dead larvae after 24 h from contact with the pathogen by Probit regression analysis and given as 10^6 spores/mL. Model: Slope = 1.591 ± 0.126; $\chi 2$ = 26.077; df = 56; Pearson Goodness-of-Fit Test $P = 0.969$; Parallelism Test $P = 0.007$.

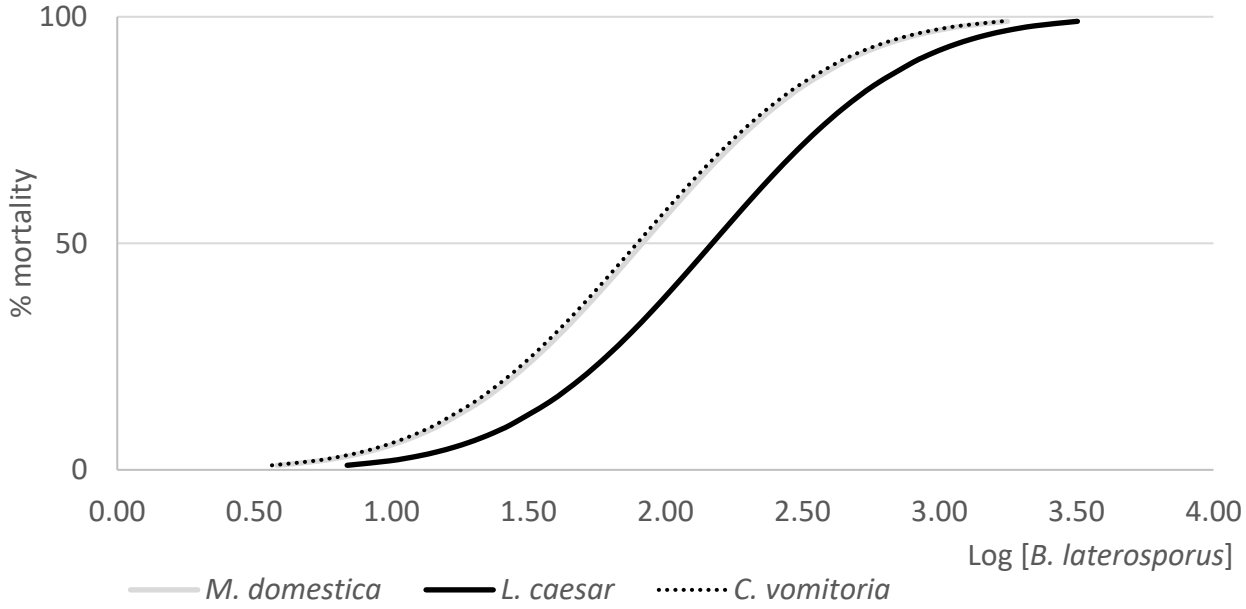

Fig. 1 - Probability of mortality (mortality %) of the synanthropic flies *Calliphora vomitoria*, *Lucilia caesar*, and *Musca domestica* exposed to *Brevibacillus laterosporus* spores calculated by Log-Probit regression.

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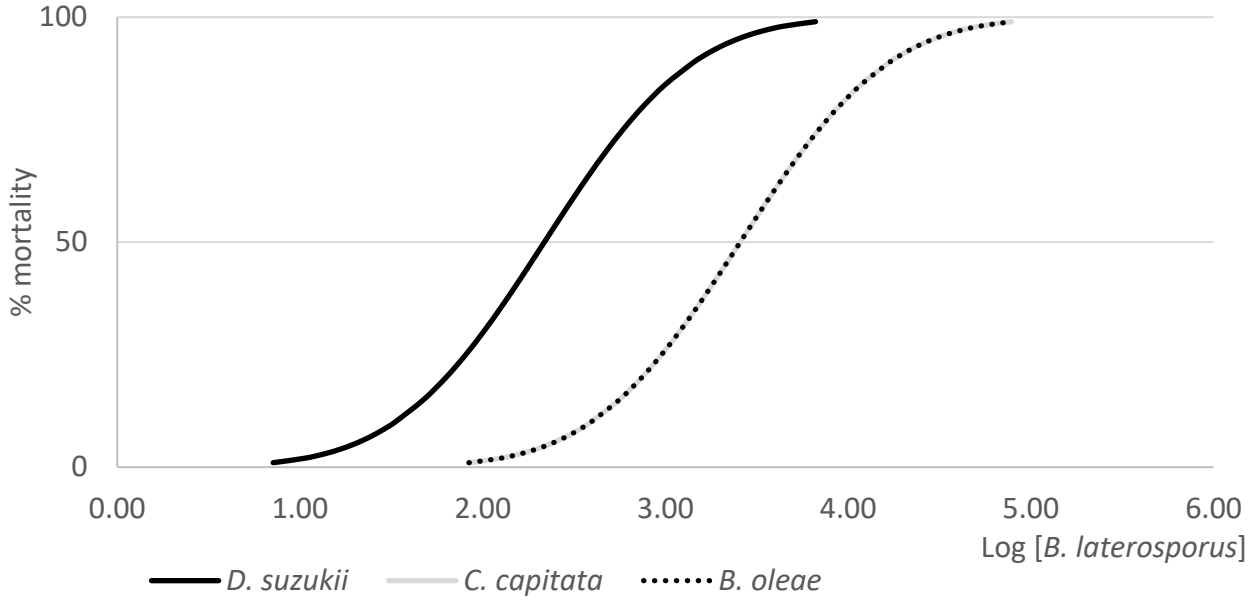

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406 Fig. 2 - Probability of mortality (mortality %) of the fruit flies *Batrocera oleae*, *Ceratitis capitata*,
407 and *Drosophila suzukii* exposed to *Brevibacillus laterosporus* spores calculated by Log-Probit
408 regression.

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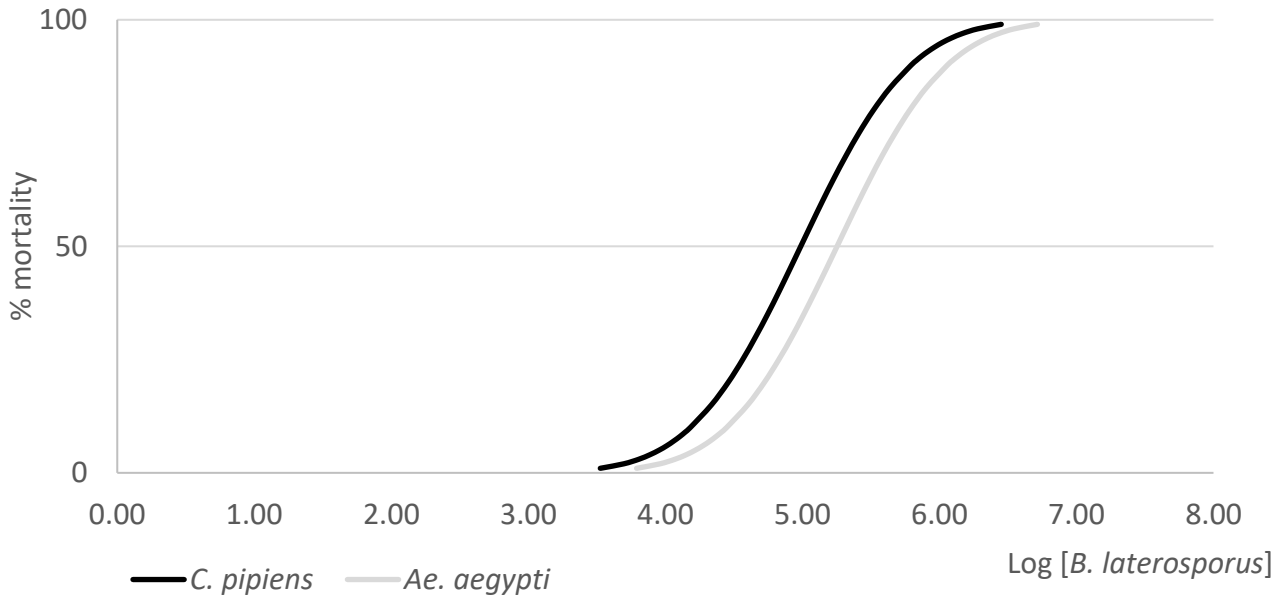

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414 Fig. 3 - Probability of mortality (mortality %) of the larvae of the mosquitoes *Culex pipiens* and
415 *Aedes aegypti* exposed to *Brevibacillus laterosporus* spores calculated by Log-Probit regression.

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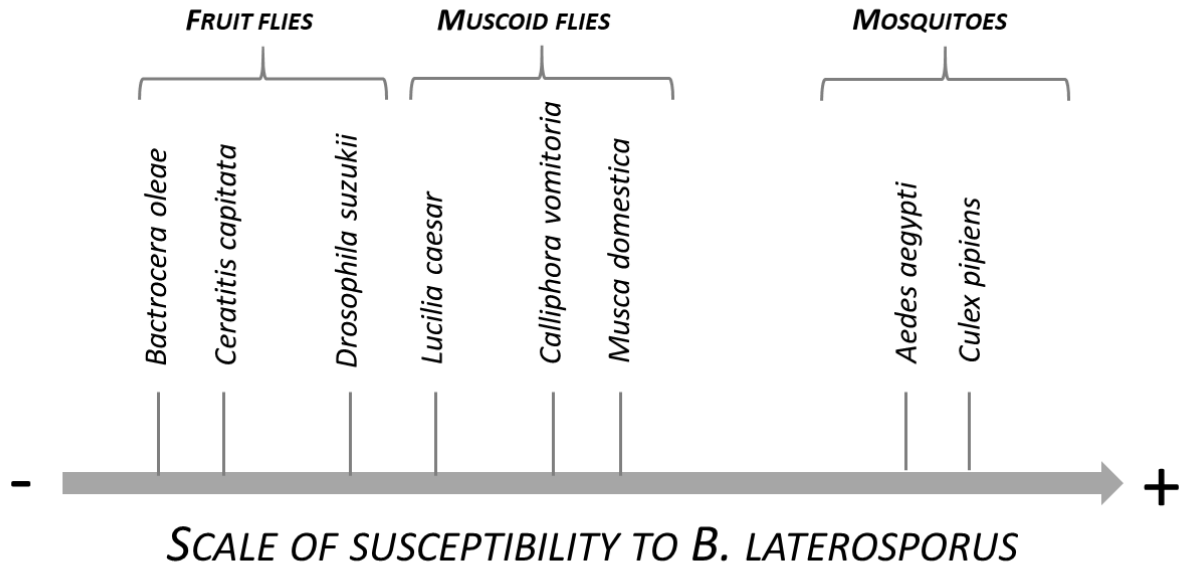

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420 Fig. 4 - Relative susceptibility (increases to the right) of different Diptera species exposed to
421 *Brevibacillus laterosporus* spores.

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