

Pathogenicity and characterization of a novel *Bacillus cereus* sensu lato isolate toxic to the Mediterranean fruit fly *Ceratitis capitata* Wied.

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1 **Pathogenicity and characterization of a novel *Bacillus cereus sensu lato* isolate toxic to**  
2 **the Mediterranean fruit fly *Ceratitis capitata* Wied.**

3

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14

15 **Abstract**

16 The lethal and sub-lethal effects of sporulated cultures of a novel *Bacillus cereus sensu lato*  
17 strain lacking detectable *cry* genes and identified through morphological and genetic analyses,  
18 have been studied on the Mediterranean fruit fly *Ceratitis capitata*. The lethal effects on  
19 young larvae were concentration dependent, with a median lethal concentration (LC<sub>50</sub>) of 4.48  
20 x 10<sup>8</sup> spores/g of diet. Sporulated cultures of this strain significantly extended development  
21 time and reduced immature survival, and the size of emerging fly adults. Besides spores, the  
22 toxicity has been associated to the insoluble extra-spore fraction characterized through a  
23 proteomic approach. The profile of the extra-spore protein fraction (ES) showed major protein  
24 bands within the 35-65 kDa range. The results of mass spectrometry analysis highlighted the  
25 presence of putative virulence factors, including members of protein families previously  
26 associated to the insecticidal action of other microbial entomopathogens. These proteins  
27 include metalloproteases, peptidases and other enzymes.

28

29

30 *Keywords:* Fruit fly; Tephritidae; bioinsecticide, entomopathogenic bacteria; *Bacillus*  
31 *thuringiensis*; toxicity.

32

33

## 34 **1. Introduction**

35 The Mediterranean fruit fly *Ceratitis capitata* Wiedemann (Diptera: Tephritidae) is a  
36 polyphagous species affecting more than 250 species of fruits and vegetables worldwide  
37 (White and Elson-Harris, 1994). In the Mediterranean basin, its host range includes several  
38 economically important crops, such as sour and sweet orange, grapefruit, loquat, apricot,  
39 peach, fig, pear, persimmon, prickly, pear and clementine (Liquido et al., 1991).

40 Due to its high reproductive potential, the management of this multivoltine pest is always  
41 necessary to protect fruits from the destructive action of larvae (Papadopoulos, 2008).

42 Most conventional management methods involve the application of various synthetic  
43 insecticidal formulations as foliage baiting or cover spraying. The negative impact of  
44 chemicals on non-target organisms (Damalas and Eleftherohorinos, 2011) has over time  
45 encouraged the research and development of safer methods of control including the use of low  
46 impact insecticides, mass trapping and the release of natural enemies. Efforts have been made  
47 to explore the opportunity to find and employ entomopathogenic microorganisms like fungi  
48 and bacteria against *C. capitata*. In this prospect, the potential of specific strains of fungal  
49 species *Beauveria bassiana* and *Metarhizium anisopliae* (Quesada-Moraga et al., 2006; Ortiz-  
50 Urquiza et al., 2010; Beris et al., 2013; Falchi et al., 2015), and of the entomopathogenic  
51 bacterium *Bacillus thuringiensis* (*Bt*) (Gingrich, 1987; Karamanlindou et al., 1991; Martinez  
52 et al., 1997) on diverse fly stages has been investigated. Similarly, the effects of *B.*  
53 *thuringiensis* isolates have been studied on other Tephritid species such as the olive fly  
54 *Bactrocera oleae* Gmelin (Alberola et al., 1999) and the Mexican fruit fly *Anastrepha ludens*  
55 (Loew) (Robacker et al., 1996). In this context, the insecticidal action of bacterial protein  
56 toxins produced by *B. thuringiensis* strains have been demonstrated, thus envisioning their  
57 possible employment in bait sprays against adults or as a resource to develop transgenic plants  
58 resistant to endophytic larval stages (Vidal-Quist et al., 2009 and 2010). A main concern with

59 different *B. thuringiensis* strains showing toxicity against flies is the production of unwanted  
60 secondary metabolites like the  $\beta$ -exotoxin, whose use in agriculture is prohibited (Liu et al.,  
61 2014). However, Vidal-Quist et al. (2010) have demonstrated the activity of Cyt1Aa protein  
62 from *B. thuringiensis* serovar *israelensis* against *C. capitata*.

63 The infectivity of other bacterial species to the Mediterranean fruit fly has been in a few cases  
64 reported (Sarakatsanou et al., 2011; Molina et al., 2010), even if the molecular implications  
65 and the role of specific bacterial metabolites in the interaction with the host have not been  
66 explained.

67 The *Bacillus cereus* phylogenetic cluster, which includes *B. thuringiensis*, is represented by  
68 close related bacterial species whose pathogenic properties are highly divergent. While their  
69 genetic relationships are still under debate, the insecticidal potential of new isolates in this  
70 group is of actual interest. Due to the production of typical parasporal crystals, *B.*  
71 *thuringiensis* can generally be phenotypically distinguished from other *B. cereus* group  
72 members (Vega and Kaya, 2012).

73 The present study investigates the effects of a recently isolated acrySTALLIFEROUS *Bacillus*  
74 *cereus sensu lato* strain on immature *C. capitata* survival and development in relation to  
75 specific bacterial fractions characterized through a proteomic approach.

76

## 77 **2. Materials and methods**

78

### 79 *2.1. Bacterial strain identification and preliminary characterization*

80 The present study was conducted with a bacterial isolate selected for its toxicity against *C.*  
81 *capitata* larvae in a screening program including several *B. thuringiensis*-like isolates. Among  
82 more than 300 isolates assayed, this strain was the most effective against *C. capitata* larvae,  
83 and no toxicity was observed against adults. In addition, no effects were detected on other fly

84 species like the olive fly *Bactrocera oleae* Gmelin (Diptera: Tephritidae) and the house fly  
85 *Musca domestica* L. (Diptera: Muscidae). Pathogenic effects were associated to whole spore  
86 suspensions and not to bacterial vegetative cells (Floris et al., 2007).  
87 Spores of this strain were found in soil samples from Zimbabwe (Southern Africa) and were  
88 permanently stored in glycerol at -80°C in the *B. thuringiensis*-like collection of the  
89 Dipartimento di Agraria (University of Sassari, Italy). For taxonomic identification,  
90 preliminary morphological examinations under light and transmission electron microscopy  
91 (TEM) were conducted. Subsequently, different genes were amplified and sequenced. These  
92 include the *16S rRNA* gene encoding for the Small subunit ribosomal RNA, the two house-  
93 keeping genes *gyrB* and *aroE* encoding for the subunit B protein of DNA gyrase  
94 (topoisomerase type I) and for the shikimate dehydrogenase, respectively. Specific flagellin  
95 (H antigen) genes (*hag*) were also used to complement phylogenetic analyses.  
96 Genomic DNA of the bacterium was routinely isolated from overnight cultured cells  
97 employing the DNeasy Blood & Tissue Kit (QIAGEN GmbH, Hilden, Germany) in  
98 compliance with the manufacturer's instructions. PCRs were performed using a Veriti™  
99 Thermal Cycler (Applied Biosystems) in a total volume of 25 µl containing 1x reaction buffer  
100 (supplied with 2.0 mM Mg<sup>2+</sup>); approximately 100 ng DNA; 0.3 µM of each primer; 0.3 mM  
101 of each dNTPs; and 0.5 U of KAPA HiFi HotStart DNA polymerase (KAPABIOSYSTEMS,  
102 Boston, USA). The following sets of primers were used: BcF 5'-  
103 GGATTAAGAGCTTGCTCTTAT-3' (forward) and 16S2 5'-  
104 AAGCCCTATCTCTAGGGTTT-3' (reverse) for *16S rRNA* (Chen and Tsen, 2002); *gyrB*-F1  
105 5'-ATGGAACAAAAGCAAATGCA-3' (forward) and *gyrB*-R1 5'-  
106 TTAAATATCAAGGTTTTTCA-3' (reverse) for *gyr*; *aroE*-F1 5'-  
107 ATCGGAAATCCAATTGGACA-3' (forward) and *aroE*-R1 (5'-  
108 CCTGTCCACATTTCAAAYGC-3' (reverse) for the *aroE* gene (Soufiane and Côté, 2009);

109 *slt*-F1, 5'-ATATGCAAGCACTTCTTTTACT-3' (forward) and *fliC*-R6, 5'-  
110 ATTHGCDGGATTATCMGAAGC-3' (reverse) for the amplification of the flagellin  
111 sequences between *slt* and *fliC* genes (Xu and Côté, 2008). To amplify the *hag* allele internal  
112 sequences, the following primers pairs were used: BtHag-F1, 5'-  
113 AGTACATGCGCCAAAACCAAG-3' (forward) and BtHag-R1, 5'-  
114 GTTTGCTTGAGAAAGCATGCT-3' (reverse); BtHag-F2, 5'-  
115 GGGGTTCTTAATCATGAGAA-3' (forward) and BtHag-R2, 5'-  
116 TAACTCAAATGGCTTATTGT -3' (reverse) (Xu and Côté, 2006).

117 PCR conditions were set according to the above mentioned references and PCR products were  
118 routinely analyzed by agarose gel electrophoresis using SYBR® Safe DNA stain (Life  
119 Technologies Europe BV, Bleiswijk, The Netherlands) for DNA visualization using UV  
120 transillumination. The bands of interest were excised from gel, and the DNA was extracted  
121 using a QIAquick gel extraction kit (Qiagen). Purified amplicons were supplied to the Sanger  
122 sequencing facilities of BMR Genomics (Padova, Italy) and output sequences were analyzed  
123 with the NCBI Basic Local Alignment Search Tool  
124 (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>).

125 In addition to taxonomic studies, genomic DNA was enriched with the bacterial plasmid  
126 isolated with PureYield™ Plasmid Midiprep System (Promega®, Madison, USA) in  
127 compliance with manufacturer's instructions, and used as a template for the detection of  
128 possible *cry* genes, using the PCR primer system designed by Noguerra and Ibarra (2010) on  
129 conserved regions of the *cry* family. These analyses were conducted using *B. thuringiensis*  
130 *kurstaky* HD1 as a positive control.

131

132 *2.2. Bacterial preparations*

133 Bacteria were routinely grown in conical flasks containing 1 l T3 medium (Travers et al.,  
134 1987) incubated at 30 °C with shaking at 180 rpm. An aliquot (10 ml) of an overnight LB pre-  
135 culture inoculated with 1 ml heat-activated spore suspension (70°C for 30 min) was used as  
136 *inoculum* to facilitate culture synchronization. In these conditions, culture sporulation and  
137 sporangia lysis usually occurred within 72 hrs, as monitored by phase microscopy. Sporulated  
138 cultures were harvested by centrifugation at 10,000 x g for 10 min at 4 °C, washed three times  
139 in sterile water to eliminate main cell debris, and quantified using a Thoma chamber (E.  
140 Hartnack, Berlin, Germany). Culture supernatants and these spore suspensions at a  
141 concentration of  $2 \times 10^9$  spores/ml were stored at -20 °C until use in bioassays. As confirmed  
142 by phase microscopy observations, no parasporal crystals were detected in these spore  
143 suspensions employed in bioassays.

144 At a later stage, an additional cleaning step was included in preparation procedures, in order  
145 to separate spores from other insoluble components that could be harvested by centrifugation.  
146 For this purpose, whole spore suspensions obtained as previously described, were submitted  
147 to five consecutive centrifugation cycles at 5,000 x g for 3 min at 4 °C. The resulting  
148 supernatant was ultra-centrifuged for 1 h at 50,000 x g to collect the insoluble extra-spore  
149 (ES) fraction, while the remaining pellet was represented by pure spores.

150

### 151 2.3. Bioassays

152 Experiments were conducted with insects from a *C. capitata* colony established in the  
153 entomology laboratory of the Dipartimento di Agraria of the University of Sassari (Italy).  
154 Insect rearing methods and conditions have been described elsewhere (Falchi et al., 2015).

155 Dose-response bioassays were conducted to determine the lethal effects caused by different  
156 bacterial preparations, including whole spore suspensions, culture supernatants and, at a later  
157 stage, pure spores and the insoluble extra-spore fraction. For this purpose, the bioassay design

158 involved four replications consisting of four groups of 24 second instar larvae to be  
159 maintained in 96-well polystyrene microplates (one larva per well) filled with an artificial diet  
160 made as described in Falchi et al. (2015). The diet was incorporated with the different  
161 bacterial preparations to be assayed or was left untreated (control). Insects were routinely  
162 incubated inside a growth chamber at 25 °C and 60% relative humidity, and were inspected  
163 daily. In line with similar experiments conducted with fly larvae, mortality data were assessed  
164 after 5 days (Floris et al., 2007; Ruiu et al., 2007).

165 In preliminary experiments, the spore suspension obtained harvesting and washing the whole  
166 sporulated culture was assayed at a standard concentration of  $10^9$  spores per g of diet, while  
167 culture supernatant was mixed with the diet at a 1:1 rate. Subsequently, bioassays were  
168 conducted with a range of 7 concentrations used to estimate the median lethal concentration  
169 ( $LC_{50}$ ) value:  $1 \times 10^9$ ,  $7.5 \times 10^8$ ;  $5 \times 10^8$ ,  $2.5 \times 10^8$ ,  $1.2 \times 10^8$ ,  $0.6 \times 10^8$  and  $0.3 \times 10^8$  spores per g of  
170 diet. This experiment was repeated three times with different batches of flies and bacterial  
171 preparations.

172 Additional observations were conducted to study the sub-lethal effects of sporulated cultures  
173 on *C. capitata* larvae exposed to a selected range of concentrations ( $5 \times 10^8$  and  $1 \times 10^8$  spores/g  
174 of diet). In this case, four replicated groups of 24 larvae were individually maintained until  
175 pupation in 96 wells microplate as previously described. The pupal development time, the  
176 pupal weight and the percentage of adult emergence calculated on the number of pupated  
177 insects were determined.

178 In a separate experiment, four replicated groups of 10 adults (5 males and 5 females),  
179 emerging from larvae reared on a diet treated with a concentration of  $5 \times 10^8$ ,  $1 \times 10^8$  spores per  
180 g of diet or untreated (control), were maintained in transparent plastic boxes where food was  
181 provided *ad libitum* as a solid powder containing sugar and brewer's yeast hydrolysate (1:1),  
182 while water was supplied by a cotton thread soaked with water. On a lateral face, each box

183 had a window (3 x 2 cm) covered with gauze that allowed females to insert the ovipositor and  
184 to lay eggs that were collected into a water-containing plate underneath. From the 5<sup>th</sup> day on,  
185 the number of laid eggs were recorded for a 3 weeks period. During the same period and until  
186 death of all flies, insect mortality was assessed daily.

187 Egg viability for each treatment group and replicate was determined counting the number of  
188 larvae emerging from 20 eggs collected in three different dates during the bioassay (after 6,  
189 10 and 15 days). For this purpose eggs were kept at 25 °C in a plate onto wet filter paper and  
190 at 90% relative humidity until hatching.

191 Further dose-response experiments were conducted to assay pure spores and the ES fraction  
192 obtained from the sporulated culture as previously described. This fraction was assayed at a  
193 concentration of 1 mg/g of diet, which was in line with the amount of proteins normally  
194 associated to 10<sup>9</sup> spores. Each experiment involved four replications (24 larvae each) and  
195 was repeated three times with different batches of insects and of bacterial preparations.

196

### 197 *2.3. Statistical analysis*

198 Data were analyzed using SAS software (version 9.1) with significance level set at  $\alpha = 0.05$   
199 (SAS Institute, 2004).

200 Probit procedure (Finney, 1971) was used to analyze dose-response data to determine the  
201 median lethal concentration (LC<sub>50</sub>).

202 General Linear Models (GLM) of ANOVA were used to analyze data on immature  
203 development (larval and pupal development time, pupal weight and percentage of adult  
204 emergence). Data on larval mortality, on adult longevity and fecundity, and on egg viability,  
205 were compared across treatments using one-way ANOVA (factor: concentration). When  
206 significant treatment effects were detected, ANOVAs were followed by Least Significant  
207 Difference (LSD) tests for post-hoc comparison of means.

208

### 209 2.3. Proteomic studies

210 Proteins extracted from the ES fraction were analyzed by polyacrylamide gel electrophoresis  
211 and subsequent mass spectrometry. Protein concentration was routinely determined using the  
212 Folin-phenol reagent (Lowry et al., 1951) and bovine serum albumin (Sigma) as a standard.

213 For protein extractions, ES was resuspended in 1% SDS and 0.01% 2-mercaptoethanol,  
214 followed by boiling for 5 min before centrifugation for 5 min at 10,000 x g. The supernatant  
215 was resuspended in Laemmli buffer and analyzed by 10% SDS-PAGE (Laemmli and Favre,  
216 1973). Gels were routinely stained with Coomassie and digitized with an ImageScanner III  
217 (GE Healthcare). Three biological replicates were analyzed for each group.

218 Individual regions including major protein bands (molecular weight ranging between 35 and  
219 65 kDa) obtained from the extra-spore fraction were manually excised from the gels,  
220 destained and subjected to *in situ* tryptic digestion before analysis by MS/MS (Biosa et al.,  
221 2011). Tryptic peptides were supplied to the proteomic facilities of Porto Conte Ricerche Srl  
222 (Tramariglio, Alghero, Italy) where samples were analyzed using an LTQ-Orbitrap Velos  
223 (Thermo Scientific, San Jose, CA, USA) interfaced with an UltiMate 3000 RSLCnano LC  
224 system (Dionex, Sunnyvale, CA, USA, now part of Thermo Scientific) as described in Tanca  
225 et al. (2013). Mass spectrometry output data were analyzed on a protein discoverer (v.1.4i,  
226 Thermo Scientific, Bremen, Germany) for protein identification employing an in-house  
227 Mascot server (Matrix Science, London, UK) and processed against the NCBI database  
228 (<http://www.ncbi.nlm.nih.gov>).

229

## 230 3. Results

231

### 232 3.1. Bacterial strain identification and preliminary characterization

233 The *16S rRNA* gene sequence showed the highest percentage sequence similarity (99%) for  
234 bacterial strains belonging to the *B. cereus* cluster including *B. thuringiensis*, *B. cereus sensu*  
235 *stricto*, *B. mycoides*, and *B. weihenstephanensis*. The *gyrB* and *aroE* gene nucleotide  
236 sequences confirmed the highest percentage similarity (99%) for *B. thuringiensis* and *B.*  
237 *cereus sensu stricto*. DNA sequences of these genes were deposited in the GenBank database  
238 (National Centre for Biotechnology Information, Bethesda, Maryland) under accession  
239 numbers KP297917, KP318802, KP318803, respectively.

240 Complementary observations on flagellin sequences showed at most 95% similarity with  
241 different *B. thuringiensis* serovars including *jegathesan*, *monterrey*, *canadensis*,  
242 *darmstadiensis*, *londrina*, and *galleriae*, and 94 % with *B. cereus*.

243 Besides, no *cry* genes were detected by PCR and no parasporal bodies were observed by  
244 phase or electron microscopy (Fig. 1).

245 Given the results of our observations and in line with the taxonomic relationships among  
246 members of the *B. cereus* cluster, the new isolate is considered as *B. cereus sensu lato (s.l.)*.

247

### 248 3.2. Bioassays

249 As a result of preliminary observations, significant levels (> 80%) of larval mortality were  
250 observed when 24-hr-old *C. capitata* larvae were reared on an artificial diet incorporated with  
251 a sporulated culture ( $10^9$  spores/g of diet) of the *B. cereus s.l.* isolate ( $F_{2,33} = 385.27$ ,  $P <$   
252  $0.0001$ ). In the same bioassays, no significant lethal effects were detected for the culture  
253 supernatant. The lethal effects were concentration dependent with a  $LC_{50}$  of  $4.48 \times 10^8$   
254 spores/g of diet (FL = 4.03-4.96, Slope  $\pm$  SE =  $1.91 \pm 0.12$ ,  $\chi^2 = 235.66$ ,  $P < 0.0001$ ).

255 The sub-lethal effects of *B. cereus s.l.* on immature development are shown in table 1.

256 Development time from egg hatching to pupation significantly increased in larvae reared on

257 treated diets ( $F_{2,225} = 66.96$ ,  $P < 0.0001$ ), with higher effects on larvae exposed to the highest  
258 concentration assayed.

259 Average pupal weight was affected by treatments at the highest concentration assayed ( $F_{2,9} =$   
260  $4.98$ ,  $P = 0.0349$ ), with a 27.3% reduction in comparison to the control, but it was not  
261 significantly reduced for the low concentration treatment.

262 Pupal development time was not significantly influenced by treatments ( $F_{2,186} = 1.82$ ,  $P =$   
263  $0.1656$ ), while the percentage of adult emergence was reduced in pupae from treated diets  
264 ( $F_{2,9} = 11.93$ ,  $P = 0.0029$ ) with a significant decrease (25.3%) for the highest concentration  
265 assayed.

266 The sub-lethal effects observed on *C. capitata* adults emerging from larvae reared on *B.*  
267 *cereus s.l.* treated diets are shown in table 2. Differences between treated and control groups  
268 were not significant for male ( $F_{2,57} = 1.85$ ,  $P = 0.1664$ ) and female ( $F_{2,57} = 0.35$ ,  $P = 0.7097$ )  
269 longevity, and for egg viability ( $F_{2,33} = 0.13$ ,  $P = 0.8754$ ). A significant decrease in fecundity  
270 was observed in the group treated with the highest bacterial concentration in comparison to  
271 the control ( $F_{2,9} = 8.22$ ,  $P = 0.0093$ ).

272 Significant mortality was determined by both pure spores and the ES fraction even if at a  
273 lower degree compared to the whole spore suspensions ( $F_{3,44} = 174.27$ ,  $P < 0.0001$ ) (Table 3).

274

### 275 3.3. Proteomic studies

276 The proteome of the insoluble ES fraction obtained from sporulated cultures of the *B. cereus*  
277 *s.l.* strain showed major proteins within the 35-65 kDa range (Fig. 2).

278 Based on the results of mass spectrometry, we successfully analyzed more than 700 internal  
279 peptide sequences from 5 main gel regions (Fig. 2). The most significant results of protein  
280 identification are summarized in Table 4. These proteins include metalloproteases, aldehyde  
281 dehydrogenases, molecular *chaperones* (GroEL), peptidases, and other enzymes.

282

## 283 **Discussion**

284 The present study demonstrated that the Mediterranean fruit fly larvae are susceptible to a  
285 newly isolated *B. cereus s.l.* strain. The potential of *B. thuringiensis* against the Mediterranean  
286 fruit fly was formerly reported by Gingrich (1987), followed by different studies highlighting  
287 the susceptibility of other Tephritid fruit flies such as the Olive fruit fly *Bactrocera oleae*  
288 (Gmelin) (Karamanlidou et al., 1991; Alberola et al., 1999) and the Mexican fruit fly  
289 *Anastrepha ludens* (Loew) (Robacker et al., 1996) to different *Bt* isolates. In a screening  
290 program involving a wide variety of *B. thuringiensis* strains from citrus orchards in Spain,  
291 Vidal-Quist et al. (2009) observed a maximum toxicity against *C. capitata* adults of barely  
292 30%. Other studies reported a higher susceptibility of larvae (Floris et al., 2007; Aboussaid et  
293 al., 2011). In these studies the insecticidal action against fruit flies was associated to both  
294 spore-crystal mixtures or to the soluble and heat-stable  $\beta$ -exotoxin, a metabolite certain *Bt*  
295 strains produce and release in the culture broth. Robacker et al. (2000) demonstrated the  
296 effectiveness of pure spore-crystal suspensions against *A. ludens*, which supported a possible  
297 direct action of Cry toxins in the gut. Later on, Vidal-Quist et al. (2010) for the first time  
298 demonstrated the activity of Cyt1Aa delta-endotoxin from *B. thuringiensis* serovar *israelensis*  
299 against *C. capitata* larvae, thus envisioning possible future biotechnological approaches for  
300 the development of new management strategies for this pest. In this case, authors showed that  
301 *Bti* spores and crystal mixtures were not active, while solubilized and activated toxins were  
302 significantly toxic. In the case of our *B. cereus s.l.* strain showing toxicity against *C. capitata*  
303 larvae, no parasporal crystals were observed by electron microscopy, and neither Cry proteins  
304 nor *cry* genes were detected through proteomic and genomic analyses, respectively. The  
305 insecticidal action was never associated to the culture supernatant, thus excluding a major  
306 implication of possible soluble metabolites produced by the bacterium. On the contrary,

307 toxicity was associated to spores and to the insoluble extra-spore protein fraction harvested  
308 from sporulated cultures. This is different from the results obtained by Molina et al. (2010) in  
309 bioassays with a *Bacillus pumilus* strain toxic to *C. capitata* larvae. In that case, a low  
310 temperature treatment of the whole bacterial culture (cell fractions and supernatant) is  
311 required to activate virulence factors.

312 In line with other studies involving the entomopathogenic bacterium *Brevibacillus*  
313 *laterosporus* and the house fly *Musca domestica* L. (Ruiu et al. 2007), the insecticidal effects  
314 of the *B. cereus s.l.* isolate were concentration dependent with a median lethal concentration  
315 of spores in larval diet comparable to values observed in studies with environmental isolates  
316 of *Bacillus thuringiensis* against Diptera (Lonc et al., 2001).

317 When administered at sub-lethal concentrations, sporulated cultures of this strain significantly  
318 reduced immature survival, extended larval development time, reduced pupal weight, adult  
319 emergence rate, and fecundity. Also these effects were concentration dependent, which  
320 corroborates the outcomes of former studies with diptera such as the house fly (Ruiu et al.,  
321 2006) and the mosquito *Aedes aegypti* L. (Hare and Nasci, 1986). The mechanism causing  
322 such reduction in adult reproduction fitness has not been explained, however it is known that  
323 a lower fecundity of fly females emerging from smaller pupae is associated to a reduced  
324 number of ovarioles (Bennetova et al., 1981).

325 Effects on survival and reproduction of *C. capitata* were also observed in studies with the  
326 insect endosymbiont *Wolbachia pipientis* Hertig (Sarakatsanou et al., 2011). Transinfection  
327 events with some strains of this bacterium reduced survival in egg and pupal stage and caused  
328 a slight decrease in female fecundity on specific Mediterranean fruit fly lines.

329 Both the lethal and sub-lethal effects caused by *B. cereus s.l.* on *C. capitata* can provide an  
330 important contribution to the prospect of the eventual use of the bacterium or its virulence  
331 factors as pest management active ingredients.

332 Toxic effects of sporulated cultures against *C. capitata* larvae are partly associated to a  
333 proteinaceous extra-spore fraction produced by the bacterium, which contains putative  
334 virulence factors. The characterization of this fraction focused on the identification of major  
335 proteins using electrophoresis combined with mass spectrometry. These useful proteomics  
336 tools allowed us to identify the most abundant proteins that were represented by functional  
337 molecules like *chaperones* (GroEL), proteins with primary metabolic functions like aldehyde  
338 dehydrogenases, and degradative enzymes like metalloproteases and various peptidases.  
339 Some of these proteins have been found also as component of *B. cereus* exosporium (Charlton  
340 et al., 1999) and extracellular proteome (Gohar et al., 2005), and belong to families that have  
341 already been associated to the insecticidal action of diverse entomopathogens. Interestingly,  
342 toxicity of the *B. cereus s.l.* was associated to both pure spores and the extra-spore fraction,  
343 even if at a lower degree compared to the whole sporulated culture. This might be in relation  
344 to the presence of the same virulence factors in the exosporium and in the extra-spore  
345 proteome, in addition to a synergistic action of spore germination (Liu et al., 1998). Besides,  
346 it is not excluded that the insoluble extra-spore fraction may contain fragments of the  
347 exosporium.

348 Among putative virulence factors are bacterial metalloproteases, with special regard to zinc  
349 containing proteases, that are known to act occasionally as toxins in many pathogenic bacteria  
350 or they may play indirect roles in pathogenicity (Miyoshi and Shinoda, 2000). This is the case  
351 of the 51.8 kDa metalloprotease released by the nematode symbiont *Photorhabdus*  
352 *luminescens* and showing oral toxicity against the diamondback moth *Plutella xylostella* L.  
353 (Chang et al., 2013). A significant role in virulence of zinc metalloproteases produced by  
354 acrySTALLIFEROUS *B. thuringiensis* strains when the host is infected via the oral route, was also  
355 observed (Fedhila et al., 2002). Although specific investigations are required to demonstrate  
356 the actual role of the different proteases produced by the *B. cereus s.l.* strain, their possible

357 implication in the observed effects on *C. capitata* is in line with a well known potential of a  
358 variety of microbial proteases to act as insect toxins targeting different sites like the cuticle,  
359 the peritrophic matrix in the gut, the hemocoel, haemocytes and fat bodies (Harrison and  
360 Bonning, 2010; Manachini et al., 2013; Castagnola and Stock, 2014). Similarly, other bacteria  
361 showing pathogenicity against *C. capitata* larvae, like *B. pumilus*, are known to produce  
362 abundant extracellular proteases (Yu et al., 2014).

363 Among other proteins identified in the ES fraction produced by the *B. cereus s.l.* strain there  
364 is a 53 kDa protein showing high homology with a *B. cereus* betaine-aldehyde dehydrogenase  
365 whose insecticidal potential still need to be demonstrated, even though the contribution of this  
366 group of proteins to bacterial virulence and pathogenesis has recently been reported (Lee et  
367 al., 2014).

368 The extra-spore proteome of our strain contained a patatin-like phospholipase homolog,  
369 which may display insect inhibitory properties, as demonstrated against larvae of Coleoptera  
370 (Alibhai et al., 2006).

371 Among other major proteins identified in the protein profile of the *B. cereus s.l.* strain there  
372 are molecular *chaperones* (GroEL), normally involved in protein folding. Some studies show  
373 the implication of chaperone-like proteins in enhancing Cry toxins synthesis and  
374 crystallization (Crickmore and Ellar, 1992; Diaz-Mendoza et al., 2012). More recent studies  
375 highlighted an insecticidal 58-kDa GroEL homolog with chitin binding activity, secreted by  
376 *Xenorhabdus nematophila* in the culture medium through outer membrane vesicles (Joshi et  
377 al., 2008). This might not be our case, but we could speculate that these proteins could help  
378 the synthesis of other bacterial virulence factors.

379 In conclusion, the present study determined the lethal and sub-lethal effects caused by  
380 sporulated cultures of a new *B. cereus s.l.* strain against *C. capitata* larvae. As a result of  
381 bioassays combined with proteomic analysis, bacterial toxicity is associated both to spores

382 and to the extra-spore proteome. The characterization of the latter fraction highlighted major  
383 proteins, whose possible implication in the pathogenesis mechanisms has been discussed.  
384 Further studies are needed to determine the specific role of each of these compounds and to  
385 evaluate their possible use for the development of new bio-based pest management  
386 ingredients.

387

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541 **Table 1 Means ( $\pm$  SEM) of larval development time, pupal weight, pupal development**  
542 **time and percentage of emergence of *Ceratitis capitata* larvae reared on *Bacillus***  
543 ***cereus s. l.* treated diets.**

544

Treatment						
concentration (spore/g)	n <sup>a</sup>	Larval development time <sup>b</sup> (days)	Pupal weight (mg)	n <sup>c</sup>	Pupal development time <sup>d</sup> (days)	Adult emergence (%)
5x10 <sup>8</sup>	52	12.18 $\pm$ 0.76 a <sup>e</sup>	7.48 $\pm$ 0.76 a	43	5.84 $\pm$ 0.36 a	66.68 $\pm$ 1.65 a
1x10 <sup>8</sup>	83	10.26 $\pm$ 0.72 b	9.58 $\pm$ 0.65 b	73	5.83 $\pm$ 0.39 a	85.41 $\pm$ 1.06 b
Control	93	9.73 $\pm$ 0.41 c	10.29 $\pm$ 0.52 b	83	6.03 $\pm$ 0.31 a	89.21 $\pm$ 0.81 b

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546 <sup>a</sup> Number of surviving pupae from 96 treated larvae.

547 <sup>b</sup> Days from egg hatching to pupation.

548 <sup>c</sup> Number of emerged *C. capitata* adults.

549 <sup>d</sup> Days from pupation to adult emergence.

550 <sup>e</sup> Means followed by different letters are significantly different (GLM followed by LSD test:  $P < 0.05$ ).

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570 Table 2 Means ( $\pm$  SEM) of longevity, fecundity, percentage of egg hatching of  
 571 ovipositing females of *Ceratitis capitata* emerging from larvae reared on *Bacillus*  
 572 *cereus s. l.* treated diets.

573

574

Treatment concentration (spore/g)	Longevity (days) <sup>a</sup>		Fecundity (min-max) n	Egg hatching <sup>b</sup> %
	Male	Female		
5x10 <sup>8</sup>	19.95 $\pm$ 2.31 a <sup>c</sup>	26.35 $\pm$ 1.06 a	164.5 $\pm$ 21.02 a	96.67 $\pm$ 0.42a
1x10 <sup>8</sup>	23.45 $\pm$ 1.03 a	27.10 $\pm$ 1.08 a	258.2 $\pm$ 22.97 b	97.50 $\pm$ 1.25 a
Control	24.45 $\pm$ 1.82 a	28.15 $\pm$ 1.13 a	296.3 $\pm$ 26.60 b	97.08 $\pm$ 1.10 a

575

576 <sup>a</sup> Days from adult emergence to adult death.

577 <sup>b</sup> Mean values of egg hatching recorded after 6, 10 and 15 days from adult emergence.

578 <sup>c</sup> Means in each column followed by different letters, are significantly different (ANOVA followed by LSD test:  
 579  $P < 0.05$ ).

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583 **Table 3**  
 584 **Mortality (mean  $\pm$  SEM) of *Ceratitis capitata* larvae reared on a diet incorporated**  
 585 **with different fractions of the *Bacillus cereus s. l.* strain.**

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Treatment <sup>a</sup>	Mortality (%)
Whole spore suspension	82.64 $\pm$ 2.61 a <sup>b</sup>
Pure spores	55.56 $\pm$ 3.64 b
Extra-spore fraction (ES)	40.63 $\pm$ 1.99 c
Control (untreated)	3.47 $\pm$ 1.00 d

588

589 <sup>a</sup> Whole spore suspensions and pure spores were applied at a concentration of 10<sup>9</sup> spores/ g of diet, while the  
 590 extra-spore fraction was applied at a concentration of 1 mg/g of diet.

591 <sup>b</sup> Means in each column followed by different letters are significantly different (LSD test, *P* < 0.05).

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594 **Table 4**  
 595 Major proteins of the *B. cereus s. l.* insoluble extraspore fraction (ES).

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Gel region No. <sup>a</sup>	Accession number	Protein	Coverage (%)	Peptides	MOWSE Score	PI	MW (kDa)
1	218235093	Molecular chaperone GroEL	99.63	84	21382.08	4.84	57.4
	446746191	Peptide ABC transporter substrate-binding protein	78.37	75	4395.26	8.46	63.4
	434374375	Oligoendopeptidase F	68.97	56	2997.81	4.94	66.1
	218231104	Succinate dehydrogenase flavoprotein subunit	80.74	35	1924.00	6.02	65.9
2	449090484	Aldehyde dehydrogenase	98.18	66	8162.62	5.66	53.7
	30020418	2-methylcitrate dehydratase	94.98	53	3813.71	5.63	53.4

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	445956496	Penicillin-binding protein	62.47	39	2618.60	5.64	54.6
	446410102	Aminopeptidase A	58.50	30	1251.16	4.88	53.6
3	296501054	Homogentisate 1,2-dioxygenase	94.10	43	6997.79	6.68	45.1
	446773703	Aminotransferase	89.09	44	3291.32	6.00	50.1
	30021879	Zinc protease	91.04	44	2347.43	5.36	48.9
4	402559902	Acetoin dehydrogenase E1 component	85.17	46	5057.10	5.19	37.0
	446954252	Peptidase M28	94.40	32	2117.55	6.55	39.2
5	446457953	Formamidase	95.48	31	1970.29	5.30	36.7
	449090436	TPR domain protein	93.80	39	1699.33	5.85	43.9
	218231626	Patatin phospholipase	51.26	13	512.15	6.16	40.7

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<sup>a</sup> Numbers indicate regions that were excised from SDS-polyacrylamide gels for Mass Spectrometry analysis.

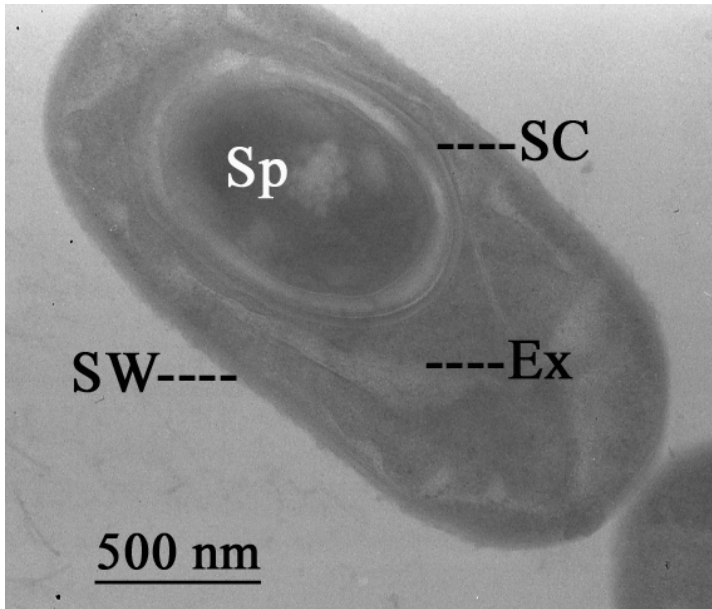
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603 **Figure legends**

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605 Fig. 1 – Electron micrograph showing a sporangium of the *Bacillus cereus s.l.* isolate. SW,

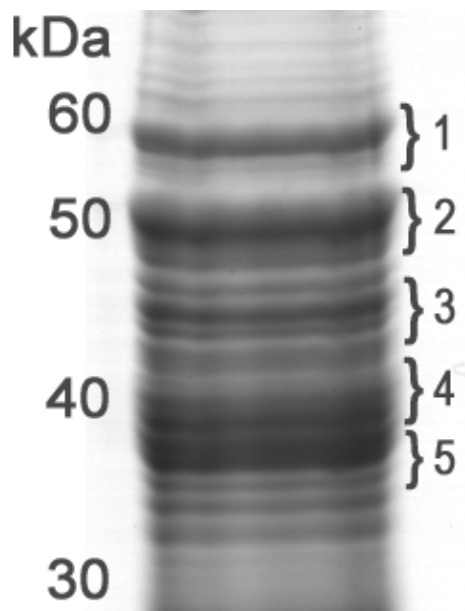
606 sporangium wall; Sp, spore; SC, spore coat; Ex, exosporium.



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608 Fig. 2 – Representative protein profile on 10% SDS-PAGE of the *Bacillus cereus s.l.* isolate

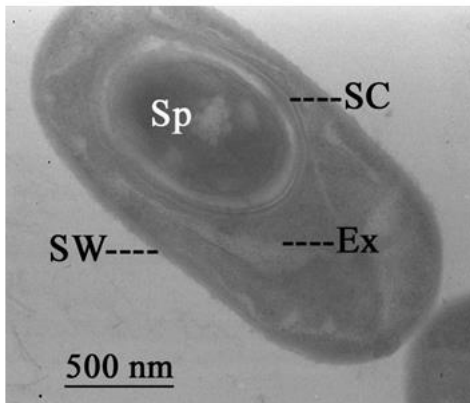
609 ES fraction. Numbers indicate gel regions that were analyzed by mass spectrometry.



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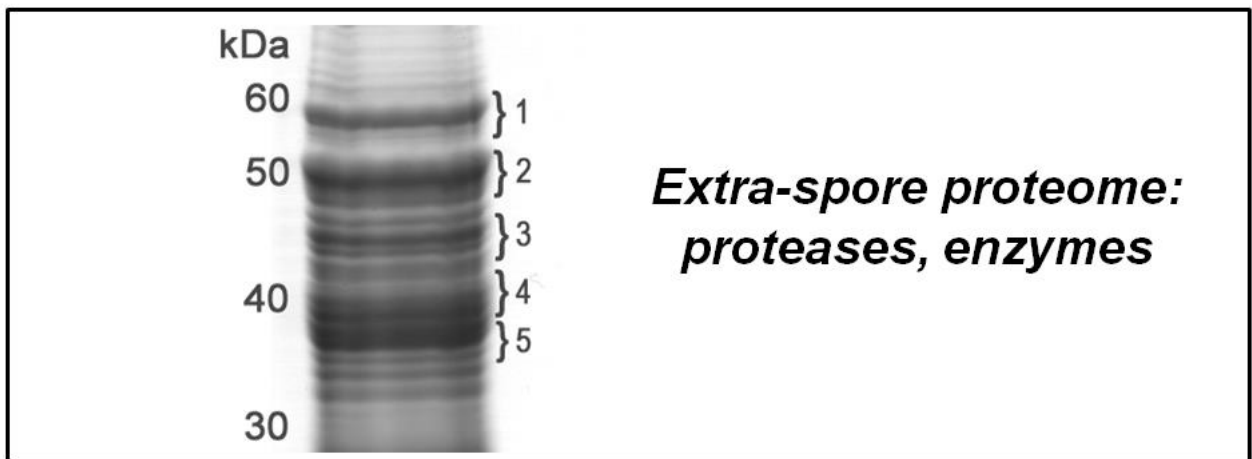
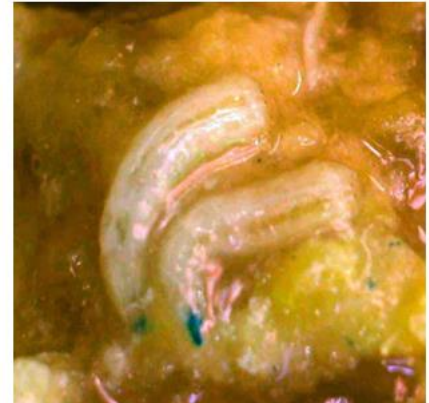
***B. cereus s.l.* strain**



**Lethal  
and  
sub-lethal  
effects**



***C. capitata* larvae**



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615 **Highlights**

- 616 • A new *Bacillus cereus s.l.* isolate is toxic to *Ceratitidis capitata* larvae.
- 617 • Spores of *B. cereus s.l.* ( $10^9$ /g of diet) cause sub-lethal effects on the Medfly.
- 618 • The insecticidal action is partly associated to an extra-spore fraction.
- 619 • The extra-spore proteome contains putative virulence factors.

620