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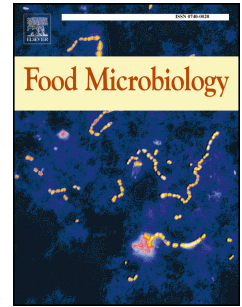
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1 Cereulide production by *Bacillus weihenstephanensis* strains during growth  
2 at different pH values and temperatures  
3  
4

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18

19 **Abstract**

20

21 Besides *Bacillus cereus*, some strains of the psychrotolerant, potentially foodborne pathogen22 *Bacillus weihenstephanensis* can produce the emetic toxin (cereulide). This toxin is a heat-

23 and acid-stable cyclic dodecadepsipeptide that causes food intoxication with vomiting.

24 However, some severe clinical cases with lethal outcomes have been described. If cereulide

25 can be produced during refrigerated storage, it will not be inactivated by reheating food,

26 representing an important risk of food intoxication for consumers. In this paper, we

27 determined the capacity of the *B. weihenstephanensis* strains BtB2-4 and MC67 to grow and

28 produce cereulide on agar media at temperatures from 8 °C to 25 °C and at a pH from 5.4 to

29 7.0. At 8 °C, strain BtB2-4 produced quantifiable amounts of cereulide, whereas the limit of

30 detection was reached for strain MC67. For BtB2-4, cereulide production increased 5-fold

31 between 8 °C and 10-15 °C and by more than 100-fold between 15 °C and 25 °C. At

32 temperatures of 10 °C and higher, cereulide concentrations were within the range of those

33 reported by previous works in foods implicated in emetic poisoning. At 25 °C, decreasing the

34 pH to 5.4 reduced cereulide production by strain BtB2-4 by at least 20-fold.

35

36

37 Key words: *Bacillus cereus*; psychrotolerant; cold; acid; emetic toxin

38

## 39 1 Introduction

40 Cereulide is the emetic toxin produced by some strains of the spore-forming pathogen  
41 *Bacillus cereus* (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Cereulide is a  
42 heat- and acid-stable cyclic dodecadepsipeptide, [D-O-Leu-D-Ala-O-Val-L-Val]<sub>3</sub>, with a  
43 molecular mass of 1,153 Da (Agata et al., 1995; Teplova et al., 2006). Cereulide is  
44 synthesized by a non-ribosomal peptide synthase encoded by the cereulide synthase gene  
45 (*ces*) cluster (Ehling-Schulz et al., 2005). The cluster is located on the megaplasmid pCER270  
46 which is related to the toxin plasmid pXO1 of *Bacillus anthracis* (Ehling-Schulz et al., 2015;  
47 Ehling-Schulz et al., 2006a; Hoton et al., 2005). The effects of cereulide on the human body  
48 have not yet been completely elucidated. In the duodenum, this toxin acts on the 5-HT<sub>3</sub>  
49 receptor and causes vomiting by stimulation of the afferent vagus nerve 0.5-6 h after the  
50 ingestion of contaminated food (Agata et al., 1995; Ehling-Schulz et al., 2004). It acts as a K<sup>+</sup>  
51 ionophore with a mechanism of action similar to that of valinomycin. It interferes with  
52 oxidative phosphorylation on mitochondrial membranes (Hoton et al., 2005; Mikkola et al.,  
53 1999); (Paananen et al., 2002; Teplova et al., 2006). Cereulide has been involved in severe  
54 clinical cases, leading in some cases to death of the patient (Dierick et al., 2005; Mahler et al.,  
55 1997; Naranjo et al., 2011).

56 The majority of the *B. cereus* strains producing cereulide are mesophilic (Carlin et al., 2006)  
57 and belong to phylogenetic group III, growing between 15 °C and 45 °C (Guinebretière et al.,  
58 2008). These strains are not able to grow and produce cereulide during cold storage of food.  
59 However, some psychrotolerant *Bacillus weihenstephanensis* strains, belonging to the *B.*  
60 *cereus* group and classified into phylogenetic group VI (Guinebretière et al., 2008;  
61 Guinebretiere et al., 2010), can produce cereulide (Hoton et al., 2009; Thorsen et al., 2006).  
62 These psychrotolerant cereulide-producing strains represent a potentially serious food safety  
63 risk for consumers of cooked-refrigerated foods. Indeed, if these strains grow and produce

64 cereulide during food storage at low temperatures, the emetic toxin will not be inactivated by  
65 food reheating before consumption. However, the production of cereulide at low temperatures  
66 by these psychrotolerant strains is still debated. For instance, a recent study showed that  
67 *B. weihenstephanensis* MC67 grew at 8 °C and produced approximately 230 ppb of cereulide  
68 per bacterial wet biomass after 9 days on PCA (Rossvoll et al., 2014). In contrast, a previous  
69 study showed that this strain produced cereulide at a level close to the limit of detection at  
70 8 °C after 1, 2 or 3 weeks on BHI-agar (Thorsen et al., 2009a). Furthermore, another  
71 cereulide-producing strain, *B. weihenstephanensis* M118, did not produce cereulide in cooked  
72 meat sausages stored at 8 °C (Thorsen et al., 2009b). At higher temperatures, the quantity of  
73 cereulide produced varied according to the food products and seemed to depend on the food  
74 pH. For example, a mesophilic emetic *B. cereus* strain produced 2.4 µg/g of cereulide in liver  
75 sausages at pH 6.2 and 1.0 µg/g in a quark dessert at pH 5.1 (Dommel et al., 2010).  
76 All studies to date regarding cereulide production by *B. weihenstephanensis* have been  
77 performed using the psychrotolerant MC 67 and MC118 strains, which are phylogenetically  
78 very similar, both harbouring the *ces* genes on a comparable plasmid (Mei *et al.* 2014).  
79 The goal of the present study was to determine the impact of growth temperature and pH on  
80 cereulide production by phylogenetically distant *B. weihenstephanensis* strains.(Hoton et al.,  
81 2009; Rossvoll et al., 2014; Thorsen et al., 2006)(Hoton et al., 2009; Mei et al., 2014)

82

## 83 **2 Materials and methods**

### 84 **2.1 Strains**

85 The cereulide-producing strains used in this study were *B. weihenstephanensis* MC67,  
86 isolated from soil in a sandy loam on the island of Møn, Denmark, and *B. weihenstephanensis*  
87 BtB2-4, isolated from a forest soil (Hoton et al., 2009; Thorsen et al., 2006). Among the  
88 cereulide producing *B. weihenstephanensis*, these two strains are phylogenetically distant

89 (Hoton et al., 2009; Mei et al., 2014). *B. weihenstephanensis* KBAB4, which was isolated  
90 from forest soil and belongs to phylogenetic group VI, was used as a non cereulide-producing  
91 strain (Sorokin et al., 2006; Vilas-Boas et al., 2002).

## 92 2.2 Growth conditions

93 Growth conditions were adapted from (Rossvoll et al., 2014): briefly, the stock cultures  
94 consisted of exponential phase cells (OD<sub>600</sub> of 0.5) in 30 % glycerol, stored at -80 °C. Frozen  
95 suspensions were streaked onto plate count agar (PCA; Oxoid) and incubated at 30 °C  
96 overnight. Three colonies were transferred to 10 ml Tryptic Soy Broth (TSB; Oxoid) in  
97 KIMAX tubes and cultured for 18 to 20 h at 20 °C while shaking at 200 rpm. The cultures  
98 were then incubated at 4 °C for 18-21 h for cold adaptation prior to growth (Rossvoll et al.,  
99 2014).

100 Bacterial growth occurred on PCA plates and on tryptic soy agar plates at 10 °C (TSA;  
101 Sigma-aldrich). The pH of the PCA plates was  $7.0 \pm 0.2$  and was adjusted to 6.0, 5.6 or 5.4  
102 with 1 N or 6 N HCl when necessary, 5.4 being the lowest pH permitting growth of *B.*  
103 *weihenstephanensis* at 8-10°C (Guérin et al., 2016).

104 Each PCA plate was inoculated with 100 µl of a  $10^3$  CFU/ml dilution of the inoculum stored  
105 at 4 °C, which resulted in approximately  $10^2$  cells per plate. The agar plates were incubated at  
106 temperatures varying between 8 °C and 25 °C. For each condition, the test was performed  
107 three times with an independently prepared inoculum (biological triplicates). Plates were  
108 removed daily for the determination of viable cells. As 95% of cereulide is associated with the  
109 bacterial biomass (Thorsen et al., 2009a), the cells were collected from the agar surface using  
110 2 ml of sterile water and a sterile Drigalski spatula. An aliquot of 100 µl of each suspension  
111 was used and plated in serial dilutions on Luria Bertani plates (LB; Biokar), and then the cells  
112 were incubated at 30 °C. The rest of the suspension was stored at -20 °C for cereulide  
113 extraction.

## 114 2.3 Extraction of cereulide

115 For cereulide extraction (Ronning et al., 2015), 1.4 ml of all samples were transferred into 2-  
116 ml non-skirted microtubes with O-ring seal caps (Dutscher) with 0.3 g of 0.1 mm silica  
117 spheres (MP Biomedical), and the cells were disrupted for 45 s at 1500 beats per min (bpm)  
118 using a Mini-G<sup>TM</sup> (SPEX<sup>®</sup> SAMPLEPREP). After centrifugation (3 min at 19,000 × g), 1 ml  
119 of each sample supernatant was recovered in 10 ml glass tubes. Ten nanograms of an internal  
120 standard, <sup>13</sup>C<sub>6</sub>-cereulide (> 95% purity, 20 µg/ml in acetonitrile; Chiralix), and 1 ml of  
121 methanol LC-MS Grade (Fischer chemical) were added to each glass tubes. All samples were  
122 vigorously shaken for 30 s and evaporated to dryness by heating at 80 °C under a steady flow  
123 of nitrogen. Then, 1 ml of methanol was added, and all samples were vigorously shaken for  
124 30 s and centrifuged 5 min at 5,000 × g. The supernatant was recovered in a new glass tube  
125 before a second evaporation step at 80 °C under a nitrogen flow. When the samples were  
126 completely dry, 100 µl of methanol/sterile water (3/1 vol/vol) was added to the glass tubes,  
127 which were immediately vortexed for 30 s. The 100-µl samples were spun down and  
128 transferred through a 0.2-µm nylon Micro centrifuge filter (Sigma-Aldrich) and centrifuged 1  
129 min at 10, 000 × g. Filtrates were then transferred into HPLC vials (screw vials 8-mm amber  
130 glass 12\*32 mm with label; Interchim) containing a 0.1-ml micro insert (Sigma-Aldrich) and  
131 stored at -20 °C before analysis by LC-MS/MS.

## 132 2.4 Calibration curve

133 The calibration curve, for quantification of cereulide in the samples, was performed by adding  
134 cereulide (cereulide of > 95% purity, 50 µg/ml in acetonitrile; Chiralix) (Biesta-Peters et al.,  
135 2010) to the fresh cell biomass of the non-cereulide producer *B. weihenstephanensis* KBAB4  
136 at final concentrations of 0.1, 1.0, 5.0, 10.0, 50.0 and 100.0 ng/ml (Ronning et al., 2015).  
137 KBAB4 cells were grown on PCA plates at 30 °C overnight as described above. The cells  
138 were collected from the plates, disrupted and centrifuged as described above for the emetic



139 strains. One millilitre of this supernatant was supplemented with cereulide and underwent the  
140 subsequent steps of the cereulide extraction protocol described above, including the addition  
141 of  $^{13}\text{C}_6$ -cereulide as an internal standard. The 18 samples of KBAB4 biomass were divided  
142 into the six cereulide concentration levels. Several controls were used for analysis, including  
143 extracts of KBAB4 cells alone (negative control) and extracts of KBAB4 cells with either  
144 cereulide at 10 ng/ml or  $^{13}\text{C}_6$ -cereulide.

#### 145 2.5 Quantification of cereulide by LC-MSMS

146 The LC-MS/MS analysis was performed according to Rønning et al (2015) using an Agilent  
147 1200 SL LC-system (Agilent Technologies, Waldbronn, Germany) coupled with an Agilent  
148 G6460 MS/MS (Agilent Technologies, Santa Clara, CA, USA) (Ronning et al., 2015). The  
149 limit of detection (LOD) and the limit of quantification (LOQ) were calculated as 3 times and  
150 10 times the standard deviation of the signal obtained with the negative control, respectively  
151 (Ronning et al., 2015). They were determined as 0.1 ng/ml (LOD) and 0.33 ng/ml (LOQ).

#### 152 2.6 Expression of results

153 The amount of cereulide was expressed as ng per ml of the bacterial suspension washed off  
154 the agar plate, with the whole biomass from one agar plate ( $19.6\text{ cm}^2$ ) corresponding to 2 ml.

#### 155 2.7 Statistical analysis

156 The results were expressed as the means of three independent biological replicates. A  
157 Student's T-test was used to compare mean values. The null hypothesis was rejected for  
158  $p < 0.05$

159

### 160 3 Results

161 *B. weihenstephanensis* BtB2-4 and MC67 strains were able to grow under all tested  
162 conditions (Fig. 1 and 2), except at 8 °C with a pH 5.6 (data not shown). In artificially  
163 inoculated food samples or media, cereulide production usually started at the end of the

164 exponential phase (Dommel et al., 2011; Thorsen et al., 2009a). For this reason, extraction of  
165 cereulide was not done during the earlier phase of cell growth, as in (Rossvoll et al., 2014).

### 166 3.1 Influence of growth temperature on cereulide production

167 *B. weihenstephanensis* BtB2-4 and MC67 strains grew between 8 °C and 25 °C on PCA  
168 plates at pH 7.0. At 8°C the stationary growth phase was reached after 7 days and 9-10 days  
169 for strain BtB2-4 and strain MC67, respectively, with a maximal population ( $N_{max}$ ) of  
170  $9.42 \pm 0.12 \log_{10}$  CFU/ml for strain BtB2-4 and  $8.50 \pm 0.02 \log_{10}$  CFU/ml for strain MC67  
171 (Fig 1). The time to reach the stationary phase decreased with the increase of incubation  
172 temperature (Fig 1).

173 The MC67 strain produced cereulide at 8 °C just above the detection limit (0.1 ng/ml) at a  
174 level not quantifiable. It clearly produced emetic toxin between 10° and 25 °C. The BtB2-4  
175 strain produced cereulide beginning at 8 °C and at all tested temperatures. For both strains,  
176 the production of cereulide increased with increasing temperature (up to 25°C). Furthermore,  
177 BtB2-4 cereulide production was always higher than that of MC67 (Fig 1). At 8 °C, the  
178 maximal cereulide production ( $2.17 \pm 0.69$  ng/ml) by BtB2-4 was reached at the beginning of  
179 stationary growth phase (7 days), while cereulide production was near the detection limit  
180 ( $0.16 \pm 0.03$  ng/ml) for MC67 over the 11 days of the experiment (Fig. 1). At 10°C growth and  
181 cereulide production of strains BtB2-4 and MC67 were also measured on TSA, in addition to  
182 PCA. TSA differs from PCA by higher peptone content, presence of NaCl and absence of  
183 glucose, compounds known to influence cereulide production (Dommel et al., 2010; Dommel  
184 et al., 2011). Growth at 10 °C and pH 7.0 for these two *B. weihenstephanensis* strains on PCA  
185 and TSA was similar, with an entry into stationary phase after 7 days (Fig. 1). The maximal  
186 population of BtB2-4 cells on PCA plates and TSA plates were not significantly different  
187 ( $p > 0.05$ ) at  $9.32 \pm 0.02$  and  $9.95 \pm 0.14 \log_{10}$  CFU/ml, respectively. However, the maximal  
188 population for strain MC67 was  $9.23 \pm 0.09 \log_{10}$  CFU/ml on PCA plates and

189 9.81±0.01 log<sub>10</sub> CFU/ml on TSA plates, which was statistically significant ( $p < 0.05$ ) (Fig. 1  
190 and 2). The maximal cereulide production of the two strains on TSA plates at 10 °C, 6.83±1.3  
191 ng/ml for BtB2-4 and 4.30±4.18 ng/ml for MC67, was not significantly different ( $p > 0.05$ )  
192 (Fig 1). While the cereulide production of strain BtB2-4 (9.01±2.91 ng/ml) was similar on  
193 TSA and on PCA plates ( $p > 0.05$ ), the production of strain MC67 was increased on TSA  
194 plates (4.30±4.18 ng/ml) compared with PCA plates at 7 days (0.85±0.40 ng/ml). However,  
195 this difference was not significant ( $p > 0.05$ ) and the cereulide production of that strain was  
196 similarly low on both TSA and PCA plates for other incubation times at 10 °C ( $< 0.6$  ng/ml)  
197 (Fig 1). Therefore, the two strains produced the same amount of cereulide at 10°C on both  
198 TSA and PCA.

199 At 15 °C, the maximal cereulide concentration produced by strain BtB2-4 was not higher than  
200 that at 10 °C on PCA plates (Fig 1). The MC67 strain produced between 0.74±0.23 ng/ml and  
201 2.45±1.46 ng/ml of cereulide, with a maximal concentration obtained after 7 days (Fig 1). At  
202 25 °C, the production of cereulide on PCA plates at pH 7.0 was high for the BtB2-4 strain,  
203 with the maximal cereulide concentration ( $> 1,000$  ng/ml) reached at the end of the stationary  
204 growth phase (Fig.1). The same kinetic of cereulide production was observed for the MC67  
205 strain, with however a lower maximal cereulide concentration, not exceeding  
206 13.20±4.67 ng/ml after 4 days (Fig 1). Cereulide production increased during the stationary  
207 phase at 25 °C, whereas it was maximal at the onset of the stationary phase for the lower  
208 temperatures tested.

### 209 3.2 Influence of pH on cereulide production

210 The growth of strains MC67 and BtB2-4 on PCA plates at pH 6.0 at 25 °C (Fig 2) was similar  
211 to that observed on PCA plates at pH 7.0 at 25 °C (Fig. 1). The growth of both strains on PCA  
212 plates at pH 5.4 was slower than that observed at pH 6.0 or 7.0 (Fig 1 and 2). Cereulide  
213 production increased during the stationary growth phase for both strains at pH 6.0 (Fig 2), as

214 was observed for pH 7.0 (Fig 1). At these pH levels, BtB2-4 cereulide production was greater  
215 than MC67 cereulide production, as observed at pH 7.0 ( $p < 0.05$ ). The maximal cereulide  
216 production of strain BtB2-4 at pH 6.0 and 25 °C was approximately 400 ng/ml, lower than the  
217 production at pH 7.0 (Fig 1 and 2), while the maximal cereulide production of the MC67  
218 strain at pH 6.0 ( $11 \pm 0.50$  ng/ml) was not significantly different ( $p > 0.05$ ) from that observed  
219 at pH 7.0 ( $13.20 \pm 4.67$  ng/ml) (Fig 1 and 2). When the pH of PCA plates was reduced to 5.4,  
220 the maximal cereulide production of the BtB2-4 strain was  $45.23 \pm 29.56$  ng/ml, with  
221  $5.53 \pm 1.11$  ng/ml of cereulide production for MC67 (Fig 2). The maximal production occurred  
222 after 3 days and declined thereafter. However, cereulide production by BtB2-4 at pH 5.4 and  
223 25 °C was still significantly higher than that of MC67 at pH 7.0 and 25 °C ( $p < 0.05$ ) (Fig 1  
224 and 2).

225

#### 226 4 Discussion

227 In our study, the growth phase corresponding to the maximal cereulide production varied  
228 with the growth temperature and pH. At 25 °C, cereulide production increased during the  
229 stationary growth phase as previously observed for various strains of *B. cereus* at 25-30°C  
230 (Dommel et al., 2011; Thorsen et al., 2009a). For *B. weihenstephanensis* BtB2-4, between  
231 8 °C and 15 °C, maximum cereulide production occurred at the beginning of the stationary  
232 phase and then decreased. At these low temperatures, it is possible that the expression of the  
233 cereulide synthase genes (*ces*) is initially induced at the end of the exponential phase and  
234 thereafter repressed during the stationary phase. The expression of the *ces* genes is activated  
235 by Spo0A and repressed by CodY (Ehling-Schulz et al., 2015). Activation by Spo0A occurs  
236 transiently at an early stage of its phosphorylation (Ehling-Schulz et al., 2015) which could  
237 explain an initial production of cereulide at the end of the exponential phase. Then, it is  
238 possible that at low temperatures repression by CodY was not lifted, explaining the

239 interruption of cereulide production during the stationary phase, in contrast to the  
240 accumulation observed at higher temperatures. Indeed, cold has been shown to increase  
241 production of CodY in *Listeria monocytogenes* (Durack et al., 2013), but the effect of cold on  
242 CodY in *B. weihenstephanensis* is not known. Alternatively, temperatures of 8-10 °C might  
243 impact on cereulide production by *B. weihenstephanensis* at the post transcriptional level, as  
244 proposed for mesophilic strains of *B. cereus* (Kranzler et al., 2016). In such case, after an  
245 initial production at the end of the exponential phase, it is possible that the cereulide synthase  
246 loses its activity, due to e.g. a lower stability and a poor translation at low temperatures. At pH  
247 5.4, cereulide production occurred during the exponential growth phase. However, the impact  
248 of pH on the expression of the *ces* gene cluster, on its effectors, and on the activity of the  
249 cereulide synthase, is unknown. Initiation of cereulide production during the exponential  
250 growth phase was previously shown by Ronning et al. (2015), in food samples naturally  
251 contaminated with a very low number of *B. cereus*.

252 When MC67 grew at 25 °C, cereulide production did not exceed 13 ng/ml in the extract,  
253 corresponding to 22 ng/plate, which was low compared to previous results showing that  
254 MC67 produced up to 14 µg/cm<sup>2</sup> after 24 h at 25 °C (Thorsen et al., 2009a). This may be due  
255 to differences in the protocols used, such as preparation of the inoculum and/or growth media  
256 (Rossvoll et al., 2014), as previously suggested by (Thorsen et al., 2009a) for *B.*  
257 *weihenstephanensis* and (Dommel et al., 2010) for mesophilic *B. cereus*. In particular, after a  
258 pre-incubation of the inoculated PCA plates at 8 °C for 2 or 3 weeks, the MC67 cereulide  
259 production at 25 °C was approximately 10,000 times lower (0.001 µg/cm<sup>2</sup>) than without pre-  
260 incubation (Thorsen et al., 2009a). In the present study, the pre-incubation at 4 °C during the  
261 18-21 h before growth experiments could explain the low cereulide production. The  
262 expression of the *ces* genes is activated by Spo0A and repressed by CodY, which linked  
263 cereulide production to the early stage of sporulation and to the nutritional and energy status

264 of the cells, respectively (Ehling-Schulz et al., 2015). Although cold stress has been shown to  
265 increase production of CodY in *Listeria monocytogenes* (Durack et al., 2013), the potential  
266 influence of pre-incubation at low temperatures on *B. weihenstephanensis* CodY production  
267 remains unknown. Nevertheless, this possible effect of pre-incubation is strain dependent,  
268 because in the same conditions, *B. weihenstephanensis* BtB2-4 produced much higher levels  
269 of cereulide than MC67.

270 In MC67, the cereulide gene cluster is located on a plasmid and is part of a composite  
271 transposon (Mei et al., 2014). Whether this peculiar genetic location of *ces* genes in MC67  
272 could explain important variations in their expression is not known.

273 Environmental conditions of growth influenced the amount of cereulide produced by  
274 *B. weihenstephanensis*. Lowering the medium pH reduced cereulide production, particularly  
275 at pH 5.4, while *B. weihenstephanensis* reached a maximal population of approximately  
276  $10^9$  CFU/plate as at pH 7.0. Cereulide production at various pH levels has been previously  
277 reported for mesophilic *B. cereus* strains in food matrices (Dommel et al., 2010). For instance,  
278 cereulide production by *B. cereus* F4810/72 was higher in béarnaise sauce at pH 5.8  
279 (8.0  $\mu\text{g/g}$ ) than in cooked rice at pH 7.0 (1.8  $\mu\text{g/g}$ ) or in camembert cheese at pH 7.9  
280 (0.6  $\mu\text{g/g}$ ). However, one could hypothesize that the food matrix may also have an influence  
281 on cereulide production (Ehling-Schulz et al., 2015).

282 In our study, lowering the growth temperature markedly reduced cereulide production for  
283 both *B. weihenstephanensis* strains. This is particularly clear for strain BtB2-4, which  
284 produced 1,000 ng/ml of cereulide at 25 °C, 10 ng/ml at 15 °C or 10 °C, and 2 ng/ml at 8 °C.  
285 Less cereulide was produced by a mesophilic *B. cereus* on media rich in proteinaceous  
286 ingredients such as TSA (Dommel et al., 2010) and NaCl, which is absent in PCA, inhibited  
287 cereulide production (Dommel et al., 2011). The same low production of cereulide at low

288 temperatures by *B. weihenstephanensis* was measured at 10 °C on the two media (TSA and  
289 PCA), indicating that the effect of cold presumably prevails over the effect of growth media.  
290 Storing foods at 4 °C prevents the growth of *B. weihenstephanensis* (Guinebretière et al.,  
291 2008). Yet, at low temperatures allowing the growth of *B. weihenstephanensis*, it is important  
292 to assess if the amount of cereulide produced can cause food poisoning. It has been shown  
293 that 10 to 1,280 ng/g of cereulide were present in 13 samples from foods identified as being  
294 the source of vomiting-type food poisoning due to cereulide produced by *B. cereus* (Agata et  
295 al., 2002). Based on these observations and the results reported in the present study, the risk  
296 of an emetic poisoning from food stored below 8 °C seems very low. In contrast, the amount  
297 of cereulide produced by *B. weihenstephanensis* BtB2-4 at 10 °C-15 °C and 25 °C and pH  
298 7.0, or at 25 °C and pH 5.7, could represent a risk for consumers. However, the low  
299 prevalence of cereulide producing *B. weihenstephanensis* must be taken into account. Only  
300 three of these strains were found among approximately 2000 strains from *B. cereus sensu lato*  
301 (Hoton et al., 2009) and no cereulide producers were found among 143 strains of *B.*  
302 *weihenstephanensis* (Guinebretière et al., 2008). This may explain why so far no *B.*  
303 *weihenstephanensis* has been linked to emetic foodborne poisoning. However, more strains of  
304 *B. weihenstephanensis* might acquire the cereulide genes by horizontal transfer in the future  
305 (Mei et al., 2014).

306 The present study and previous works showed that the environmental conditions of growth  
307 (temperature, pH, oxygen, NaCl concentration), the inoculum history (temperature,  
308 sporulation), the food composition (proteins versus carbohydrates) and the strain strongly  
309 influence cereulide production by mesophilic *B. cereus* or psychrotolerant  
310 *B. weihenstephanensis*. Therefore, it is important to consider this information to assess  
311 cereulide production in food products.

312

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317

ACCEPTED MANUSCRIPT



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422 **Figure Legends**

423 **Figure 1.** Growth curves of *B. weihenstephanensis* BtB2-4 and MC67 (log CFU/plate) (◆)  
424 and cereulide (crl) production (ng/ml) (○) at 8 °C, 10 °C, 15 °C and 25 °C on PCA plates and  
425 on TSA plates at pH 7.0. Error bars indicate standard deviations of results obtained with  
426 independent triplicates and can be smaller than symbols.

427

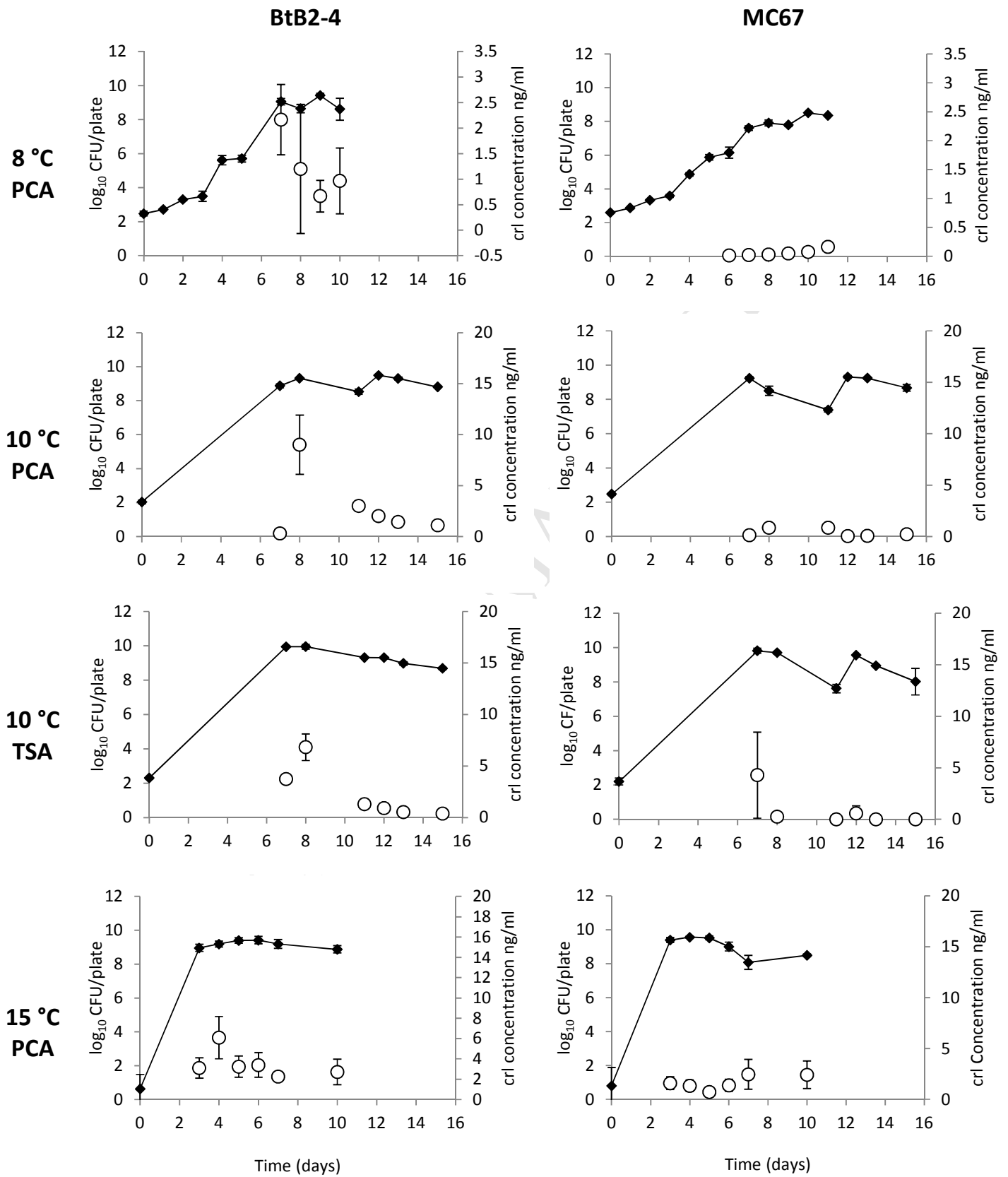
428

429 **Figure 2.** Growth curves of *B. weihenstephanensis* BtB2-4 and MC67 (log CFU/plate) (◆)  
430 and cereulide (crl) production (ng/ml) (○) on PCA plates at pH 6.0 or at pH 5.4 at 25 °C.  
431 Error bars indicate standard deviations of results obtained with independent triplicates and can  
432 be smaller than symbols.

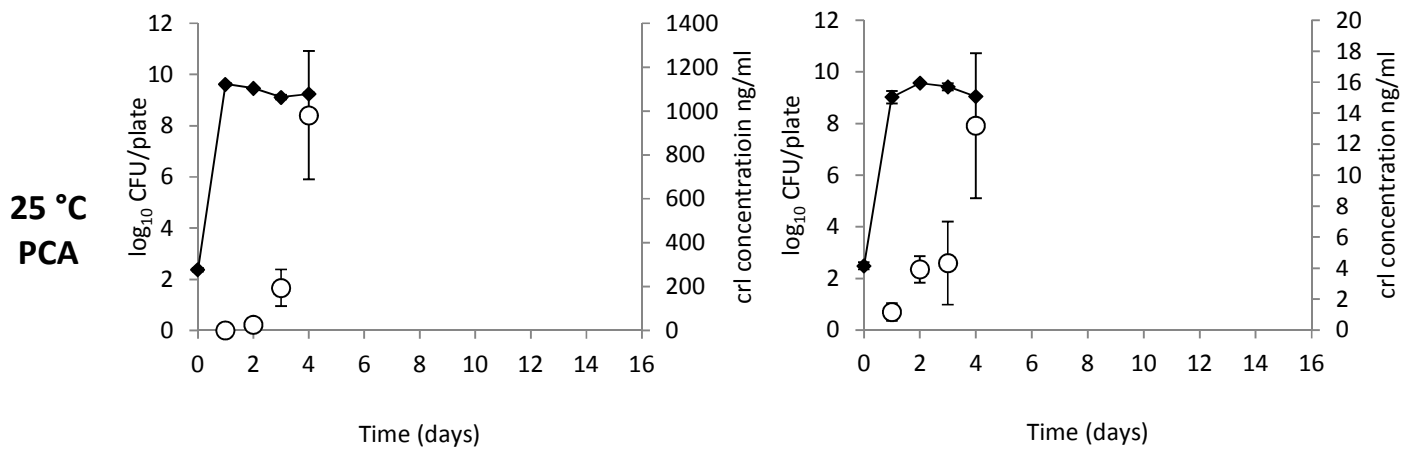
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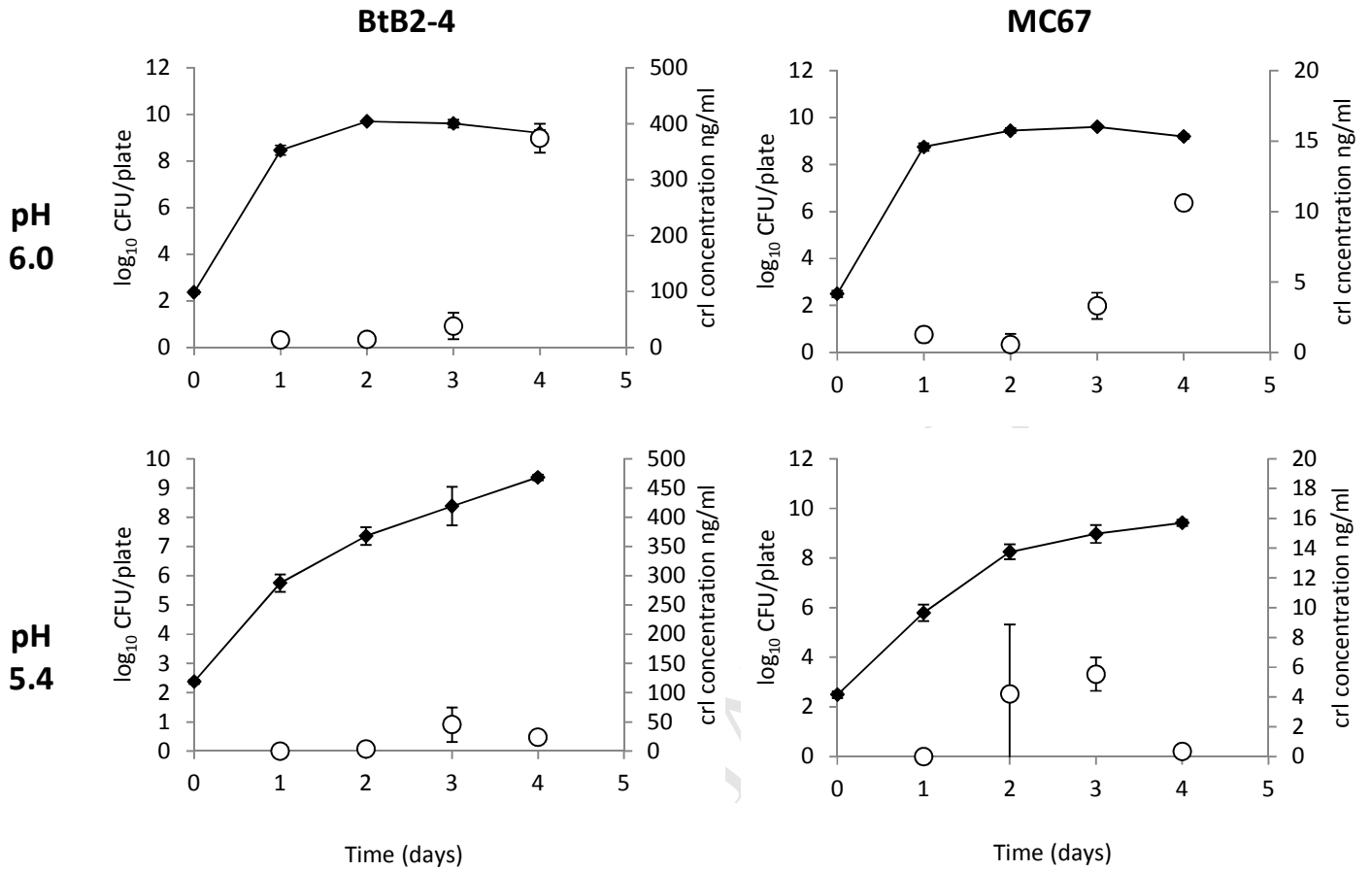
Guérin et al. R1 Figure 1



Guérin et al. R1 Figure 1



Guérin et al. R1 Figure 2





**Highlights**

- *B. weihenstephanensis* produced quantifiable cereulide at 8°C and pH7
- *B. weihenstephanensis* produced cereulide at pH 5.4 and 25°C
- Cereulide production increased 5-fold between 8°C and 10°C
- Amount of cereulide produced was markedly different between the strains tested