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1 *Bacillus cereus* in powdered foods

2

3 **Characterization of *Bacillus cereus* group isolates from powdered**
4 **food products**

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26 **ABSTRACT**

27 Mashed potato powder as well as powdered infant formula (PIF) are frequently
28 contaminated with *Bacillus cereus sensu lato* (*B. cereus s.l.*), mainly with its spores. These
29 products have also been implicated in foodborne illnesses. Here, we characterized *B. cereus*
30 *s.l.* isolates originating from powdered products based on sporulation assays, toxin gene
31 profiling, and *panC* typing combined with a SplitsTree analysis. Furthermore, cytotoxicity
32 assays with *B. cytotoxicus* isolates were performed. 78% of PIF tested positive for *B. cereus*
33 *s.l.*, whereas 92% of all mashed potato powders were positive. In total, 43 isolates were
34 further characterized. The *nhe* and *cytK2* genes were most frequently detected. Moreover, a
35 cereulide-producer was detected from PIF. Most isolates were assigned to *panC* group III, but
36 members of group II, IV, V, and VII could also be found. Nine *B. cytotoxicus* were isolated
37 out of nine mashed potato powders. All *panC* group VII isolates were positive for *cytK1*.
38 Cytotoxicity assays of these nine isolates revealed one highly cytotoxic strain, while all other
39 isolates exhibited no detectable cytotoxicity, underpinning that cytotoxicity of a certain *B.*
40 *cereus* group strain cannot be deduced from the sole presence or absence of toxin genes.

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42

43 **Keywords:** *Bacillus cytotoxicus*; *Bacillus cereus* group; Vero cell assay; mashed potato;
44 powdered infant formula

45

46 **1. Introduction**

47 *Bacillus cereus sensu lato* (*B. cereus s.l.*), a group of Gram-positive spore-forming bacteria, is
48 ubiquitous in nature and can therefore widely be found as part of the microflora of agricultural
49 products (Stenfors Arnesen *et al.*, 2008). The group comprises several genetically closely
50 related species, with *B. cereus sensu stricto* as well as *B. anthracis*, *B. mycoides*, *B.*
51 *pseudomycooides*, *B. thuringiensis*, *B. weihenstephanensis*, *B. cytotoxicus*, and *B. toyonensis* as
52 the most prominent members.

53 *B. cereus* is known as an important foodborne pathogen that can cause two distinct
54 forms of illness (Stenfors Arnesen *et al.*, 2008). Firstly, the diarrheal syndrome that is linked
55 to three enterotoxins - Hbl, Nhe and CytK - and secondly, the emetic syndrome caused by
56 cereulide toxin preformed in food. *B. thuringiensis* forms characteristic parasporal crystals
57 with insecticidal activity, enabling the use of *B. thuringiensis*-based insecticides in agriculture
58 (Chattopadhyay *et al.*, 2004). *B. thuringiensis* is known as a common contaminant of milk
59 (Bartoszewicz *et al.*, 2008). However, its relevance as a causative agent of foodborne disease
60 has been controversially discussed (EFSA, 2016, Jackson *et al.*, 1995, McIntyre *et al.*, 2008).
61 The thermotolerant species *Bacillus cytotoxicus*, which has been described 2013,
62 characteristically harbors the *cytKI* variant of the cytotoxin K gene (Guinebretière *et al.*,
63 2013). The description of this novel *B. cereus* group member was based on five strains, four
64 of which were linked to food poisoning, including an outbreak caused by strain NVH 391-98^T
65 that led to three fatalities of diarrheal disease in France in 1998 (Guinebretière *et al.*, 2013,
66 Lund *et al.*, 2000).

67 The *B. cereus* group species do not show a clear phylogenetic separation and generally
68 form three major clades, in which species are intermingled. SpoAB typing allows the
69 assignment of a strain to a certain clade (Ehling-Schulz *et al.*, 2005; Fricker *et al.*, 2011). For
70 gaining a deeper insight into the population structure of the *B. cereus* group, an AFLP system
71 has been established by Guinebretiere *et al.* (2008), which allows for assignment of *B. cereus*

72 group strains to 7 phylogenetic subtypes. *panC* has been found to be a suitable housekeeping
73 gene to assign new strains to these subtypes (Guinebretière *et al.*, 2010). The ability of strains
74 to cause food poisoning was suggested to vary depending on phylogenetic affiliation with
75 *panC* groups I to VII rather than species affiliation (Guinebretière *et al.*, 2010). To date,
76 strains causing emetic illness have exclusively been associated with *panC* group III
77 (Guinebretière *et al.*, 2010).

78 According to EFSA, *B. cereus* holds fourth place as a cause of foodborne outbreaks in
79 the European Union (EFSA, 2015). It has been stated by the Food and Agriculture
80 Organization (FAO) and the World Health Organization (WHO) that *B. cereus s.l.* is an
81 organism of concern in PIF with regard to the strength of evidence of a causal association
82 between the presence of the microorganism in PIF and illness in infants (FAO/WHO, 2006).
83 *B. cereus s.l.* is a frequent contaminant of dried milk products (Becker *et al.*, 1994; Di Pinto *et*
84 *al.*, 2013; Reyes *et al.*, 2007). Powdered infant formula (PIF) could also represent a source for
85 isolates of the *B. cereus* group, which could have severe consequences as neonates are highly
86 susceptible for infections. *B. cytotoxicus* has been detected in infant foods in China, showing
87 that the possibility of food poisoning outbreaks due to *B. cytotoxicus* is a risk in this
88 particularly vulnerable consumer group (Zhang *et al.*, 2017).

89 Although the production of powdered products involves heating and drying processes, which
90 pose harsh living conditions for most bacteria, isolates of the *B. cereus* group and in particular
91 *B. cytotoxicus* have mainly been isolated from dehydrated potato products (Contzen *et al.*,
92 2014) (Kim and Goepfert, 1971; King *et al.*, 2007; Turner *et al.*, 2006). *B. cereus* group
93 isolates are capable of producing spores, which are able to survive stress conditions
94 encountered in the production of powdered products. Foodborne illnesses caused by isolates
95 of the *B. cereus* group in association with potato products have been reported (Doan and
96 Davidson, 2000; Lindqvist *et al.*, 2000). Especially the newly described species *B. cytotoxicus*

97 that was discovered during an outbreak in France with three fatalities (Lund *et al.*, 2000) has
98 gained attention in recent times. First studies attributed its high cytotoxicity to the possession
99 of *cytKI* (Fagerlund *et al.*, 2007; Lund *et al.*, 2000) and provided phylogenetic data
100 (Guinebretière *et al.*, 2013; Sorokin *et al.*, 2006). Though the number of characterized *B.*
101 *cytotoxicus* strains is low to date, many of them originated from mashed potatoes and have
102 been linked to food poisoning cases (Guinebretière *et al.*, 2013). A recent study by Contzen *et*
103 *al.* has shown that *B. cytotoxicus* can frequently be detected in different dehydrated potato
104 products and occurs far more wide-spread than previously suggested (Contzen *et al.*, 2014).
105 Although *B. cytotoxicus* is generally assumed to be highly cytotoxic (Fagerlund *et al.*, 2004;
106 Guinebretière *et al.*, 2010; Hardy *et al.*, 2001), Fagerlund *et al.* suggested that presence of the
107 *cytKI* gene does not correlate with cytotoxic activity (Fagerlund *et al.*, 2007). As cytotoxicity
108 data has so far only been published for three *B. cytotoxicus* isolates (Fagerlund *et al.*, 2007),
109 further cytotoxicity testing is crucial to assess the food poisoning risk related to this new *B.*
110 *cereus* group species.

111 Therefore, the objective of the present study was to isolate and characterize *B. cereus* species
112 out of powdered food products including PIF, mashed potato powder, and fruit powder. In
113 addition, we aimed to determine the cytotoxic potential of all isolated *B. cytotoxicus* strains.

114

115 **2. Materials and methods**

116 *2.1 Sampling material and enrichment procedure*

117 A total of 13 powdered mashed potato products and nine PIF from different brands were
118 bought in supermarkets in Switzerland. Furthermore, 11 *B. cereus* group isolates originating
119 from self-control of a powdered infant formula producer were included in the study. In
120 addition, four strains of *B. cereus s.l.* were included in this study that had been isolated out of
121 fruit powders. Two different approaches of enrichment were used for the purchased products.
122 First, 10 g of powder was mixed with 90 ml buffered peptone water (Oxoid, Basel, CH) in a

123 stomacher bag using the Stomacher® 400 Circulator (Seward, Worthing, UK) for 30 s. The
124 samples were subsequently incubated at 37°C overnight. After overnight incubation, one loop
125 of the overnight culture was streaked onto Mossel (Mossel *et al.*, 1967) and sheep blood agar
126 plates (BD Difco™ Columbia Blood Agar Base) that were incubated at 37°C overnight.
127 Second, an approach was used that has already been described by Contzen *et al.* in order to
128 detect *B. cytotoxicus* (Contzen *et al.*, 2014). This included enrichment of the powder in 90 ml
129 CGY medium (Beecher and Wong, 1994) followed by incubation at 50°C overnight. The next
130 day, a loopful of the enriched culture was streaked onto Mossel and blood agar plates that
131 were subsequently incubated at two different temperatures, 37°C (Mossel) and 50°C (blood
132 agar), respectively. In the present study, the minor modification was made that the culture and
133 the blood plates were incubated at 46°C instead of 50°C. Mossel plates were checked for
134 colonies showing an egg-yolk lecithinase-positive and mannitol-negative phenotype
135 characteristic for isolates of the *B. cereus* group.

136

137 *2.2 DNA extraction and toxin gene profiling*

138 DNA was extracted from all isolates using the GenElute Bacterial Genomic DNA Kit
139 according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO). Toxin gene
140 profiles were determined using a PCR approach as previously described by Ehling-Schulz *et*
141 *al.* (Ehling-Schulz *et al.*, 2006) with minor modifications: The GoTaq PCR system (Promega
142 AG, Dübendorf, Switzerland) was used at (i) 2 min at 95°C, (ii) 30x [45 s at 95°C, 45 s at
143 51°C, 2 min at 72°C]; (iii) 5 min at 72°C. The respective forward primer used for detection of
144 the *nhe* complex is located in *nheA* while the reverse primer is located in *nheB*, thus enabling
145 detection of the first and second gene of the *nhe* operon. The respective primers for *hbl* are
146 located in *hblD* and *hblA*, thus allowing for detection of the second and third gene of the *hbl*
147 operon. Moreover, a duplex PCR was carried out to distinguish between *cytK1* and *cytK2* as
148 previously described (Guinebretière *et al.*, 2006).

149

150 *2.3 Genotyping using panC*

151 A PCR-based genotyping approach targeting *panC* was performed (Guinebretière et al.,
152 2008). In cases in which previously published *panC* primers did not result in an amplicon,
153 additional primers designed in this study were used (see Table 1). The following cycling
154 conditions were used: (i) 2 min at 95°C, (ii) 30x [45 s at 95°C, 45 s at 60°C, 50 s at 72°C];
155 (iii) 5min at 72°C. The PCR products were purified with the GenElute™ PCR Clean-Up Kit
156 according to the manufacturer's instructions. Subsequently, the sample's concentration and
157 purity were measured using a NanoDrop™ Fluorospectrometer (Witec AG, Luzern, CH).
158 Sequencing was outsourced (Microsynth™, Balgach, CH). Sequences of *panC* were assigned
159 to seven (I-VII) phylogenetic groups as previously described
160 (<https://tools.symprevius.org/Bcereus/english.php>) (Guinebretière et al., 2008, 2010).

161 Cluster analysis of *panC* sequences was performed with the SplitsTree™ software
162 (<http://www.splitstree.org>). Several reference strains were included in the SplitsTree analysis
163 (*panC* type I: DSM 12442; *panC* type II: WSBC10311; *panC* type III: Ames; *panC* type IV:
164 ATCC 14579; *panC* type V: BCT-7112; *panC* type VI: WSBC 10204; *panC* type VII:
165 NVH391-98).

166

167 *2.4 Detection of B. thuringiensis parasporal crystal*

168 A sporulation assay was performed to identify *B. thuringiensis* isolates. To this end, all
169 isolates were streaked onto T3 plates (Travers et al., 1987), which were incubated for three
170 days at 30°C to promote sporulation. A tiny amount of colony material was mixed with
171 double distilled water on a microscope slide until a homogenous suspension resulted. All
172 strains were checked for the presence of parasporal crystals with diamond, bipyramidal, or
173 spherical shape using a phase contrast microscope (1000 x, oil immersion) (EFSA, 2016).

174

175 2.5 *Vero cell cytotoxicity assay*

176 A Vero cell assay was used to determine cytotoxicity of all isolated *B. cytotoxicus*. Assays
177 were performed using WST-1 bioassay as described elsewhere (Moravek et al., 2006).
178 Reference strains for low (RIVM Bc90) and high-level toxin production (NVH 0075-95) were
179 included in every run. In order to obtain cell-free culture supernatants, strains were grown in
180 30 ml CGY broth in an Erlenmeyer flask and were adjusted to an OD₆₀₀ of 0.05 using an
181 overnight culture of the isolate. The day cultures were incubated at 30°C (120 rpm shaking)
182 until an OD of 7 was reached. After centrifugation at 11000 rpm for 10 min and filtration
183 through 0.2 µm sterile filters, aliquots of 1 ml supernatants were supplemented with 10 µL 0.1
184 M Na₂ EDTA and stored at -80°C.

185

186 **3. Results**187 *3.1 Identification of B. cereus group species and toxin profiling*

188 We detected *B. cereus s.l.* in 78% of purchased PIF and 92% of mashed potato
189 powders. In total, 28 strains were isolated out of the purchased PIF and mashed potato
190 samples. Six products harbored *B. cereus s.l.* of two or more different colony morphologies
191 on blood agar. Including the 11 strains provided by a PIF producer and the four strains that
192 originated from fruit powder, a total of 43 strains have been characterized.

193 Parasporal crystals were detected in one of the 43 isolates (P21), which exhibited small,
194 round-shaped crystals and originated from powdered infant formula. Nine isolates were
195 classified as *B. cytotoxicus* based on presence of *cytK1* and their affiliation to *panC* group VII.
196 These isolates originated from mashed potato powder from nine different brands. An
197 overview of all other toxin genes detected by PCR is provided in Table 2. All isolates
198 displayed one or more enterotoxin genes, and seven strains carried all three enterotoxin genes
199 (*nheA/B*, *hblD/A*, and *cytK*). One cereulide-producer was isolated out of a PIF product
200 collected on retail level.

201

202 *3.2 Affiliation of isolates to panC groups and visualization of genetic relatedness in SplitsTree*

203 The 43 isolates represented five different *panC* groups (Table 3). No representatives of
204 group I and VI could be found. All *panC* group VII isolates were positive for *cytKI*. The *B.*
205 *thuringiensis* isolate belonged to *panC* group III. Most of the strains were affiliated with
206 group III, including the strain positive for *ces* (strain P22). In addition to *panC* typing was
207 performed. The similarity of *panC* nucleotide sequences of the isolates was depicted by a
208 SplitsTree (Figure 1). The isolates formed clusters consistent with the results of *panC* typing.
209 Apart from *B. cytotoxicus* isolates, all isolates from mashed potato powder formed a highly
210 homogeneous group and belonged to the cluster exclusively comprising *panC* group III
211 isolates, while strains that originated from PIF and fruit powder showed a higher degree of
212 heterogeneity.

213 *3.3 Cytotoxicity testing of B. cytotoxicus isolates*

214 Cytotoxicity in a Vero cell assay was determined for all *B. cytotoxicus* isolates. Seven
215 out of nine isolates exhibited no detectable cytotoxic effect. One isolate showed very low
216 cytotoxicity and another isolate exhibited cytotoxicity 4.5 times as toxic as the highly toxic
217 reference strain (Figure 2).

218

219 **4. Discussion**

220 The present study revealed a high prevalence of *B. cereus* group species in mashed
221 potato powder and PIF products. *B. cereus* group species were detected in 92% of tested
222 mashed potato powders. Based on varying sample sizes, prevalence rates for *B. cereus* in
223 dehydrated potato products of 74% (Turner *et al.*, 2006) and 10 to 40% (King *et al.*, 2007)
224 have been previously reported. The prevalence in PIF in the current study (78%) is similar to
225 a large study from Becker *et al.* who stated that 70% of the powdered infant formula in

226 Germany were positive for *B. cereus s.l.* (Becker *et al.*, 1994). Results obtained by *panC*
227 typing were consistent with clusters formed by SplitsTree based on *panC* sequences. A
228 correlation of toxin patterns and *panC* types could however not be seen, except for *panC*
229 group IV, which exclusively comprised isolates positive for *nhe*, *hbl*, and *cytK2*. Toxin gene
230 profiling of all isolates investigated in frame of this study revealed that all *B. cereus s.s.*
231 harbor *nheA/B*, consistent with previous publications reporting that *nhe* is present in almost all
232 *B. cereus s.s.* (Ehling-Schulz *et al.*, 2011).

233 Only one *B. thuringiensis* strain was detected by screening for parasporal crystals (data not
234 shown). However, this method may not be fully reliable, as tiny or irregular crystals can be
235 missed (EFSA, 2016). The strain detected in our study was isolated from PIF and assigned to
236 *panC* group III, consistent with previous assignments of *B. thuringiensis* to this *panC* group
237 (Guinebretière *et al.*, 2008). While there were no reports of *B. thuringiensis* in PIF, they are
238 known to be a common contaminant of milk (Bartoszewicz *et al.*, 2008). *B. thuringiensis*-
239 based insecticides are used worldwide in agriculture and are highly effective against different
240 groups of insects (Chattopadhyay *et al.*, 2004) including the Colorado potato beetle - the most
241 destructive insect pest of potato - that is also widespread in Switzerland (Wang *et al.*, 2017).
242 Still, no *B. thuringiensis* strains were detected in mashed potato powder samples investigated
243 in this study.

244 The cluster analysis and *panC* typing revealed that most of the isolated strains belonged to
245 group III, which has previously been suggested to harbor cytotoxic strains (Guinebretière *et al.*
246 *et al.*, 2010). To date, outbreaks of emetic illness due to *B. cereus s.l.* have exclusively been
247 associated with this *panC* type (Guinebretière *et al.*, 2010). Notable, apart from *B. cytotoxicus*
248 isolates, this cluster included all isolates obtained from mashed potato powders, while isolates
249 originating from PIF showed much higher phylogenetic heterogeneity.

250 Mashed potatoes are often served in child-care institutions or hospitals, where they are likely
251 to be held at temperatures promoting growth of germinated bacteria (Turner *et al.*, 2006),
252 before being served to particularly vulnerable groups of humans. To prevent becoming ill with
253 diarrheal or emetic syndrome when eating mashed potatoes, it is essential to keep the food
254 above 60°C or to dispose of it within 2 h as Turner *et al.* have shown (Turner *et al.*, 2006).

255 FAO and WHO classified *B. cereus s.l.* as an organism of concern in PIF with regard to the
256 strength of evidence of a causal association between the presence of the microorganism in PIF
257 and illness in infants (FAO/WHO, 2006). Indeed, several studies reported high contamination
258 levels of *B. cereus s.l.* in PIF (Rowan *et al.*, 1997; Zhang *et al.*, 2017). Due to the increasing
259 numbers of *B. cereus* infections in infants (Gaur *et al.*, 2001; Hilliard *et al.*, 2003; Wang *et al.*,
260 2009), EFSA suggests the numbers of *B. cereus s.l.* spores in PIF should be as low as possible
261 (EFSA, 2005). Lequin *et al.* reported three preterm infants with fatal hemorrhagic
262 meningoencephalitis due to *B. cereus* infections (Lequin *et al.*, 2005).

263 Although *ces*-positive strains have been rarely reported from food samples, their occurrence
264 often resulted in fatalities (Dierick *et al.*, 2005; Naranjo *et al.*, 2011; Takabe and Oya, 1976).
265 In the present study, one cereulide-producer was isolated out of a PIF product which is
266 consistent with other studies (Andersson *et al.*, 2004; Zhang *et al.*, 2017). The presence of a
267 cereulide-producing strain in PIF raises concern, given the fact that this toxin can be
268 preformed in the reconstituted PIF. It was shown by Shaheen *et al.* that PIF containing cereal
269 as well as dairy ingredients are especially conducive for cereulide production (Shaheen *et al.*,
270 2006).

271 In contrast to mashed potato powders, no *B. cytotoxicus* could be detected in PIF. Nine
272 isolates were found in mashed potato powder that harbored the *cytK1* variant, which is known
273 to have necrotic and hemolytic activity and whose toxic potential is stated to be higher
274 compared to *cytK2* (Fagerlund *et al.*, 2004). Up to now, only few strains of *B. cytotoxicus*

275 have been further characterized (Guinebretière *et al.*, 2013). This low number could be due to
276 the fact that isolated *B. cereus s.l.* strains are normally summarized under the term of
277 “presumptive *B. cereus*” comprising all different group members (Ehling-Schulz and
278 Messelhäusser, 2013). The present study revealed a high prevalence of *B. cytotoxicus* in
279 mashed potato powders. This is in accordance with the study of Contzen *et al.* who found a
280 prevalence of 88% in mashed potato powder, flakes and granules (Contzen *et al.*, 2014). All
281 nine *B. cytotoxicus* isolated in the present study could be assigned to *panC* group VII, which
282 is known to exclusively comprise *B. cytotoxicus* (Guinebretière *et al.*, 2008, 2013). Depicting
283 the isolates in a SplitsTree has shown that *B. cytotoxicus* isolates (M12-M20) represent a very
284 remote cluster within the *B. cereus* group, consistent with other phylogenetic analyses using
285 MLST (Fagerlund *et al.*, 2007; Sorokin *et al.*, 2006). It stays unclear why *B. cytotoxicus* has
286 been mostly associated with mashed potato powders (Contzen *et al.*, 2014) or potato purée
287 (Guinebretière *et al.*, 2013), considering that also PIF contain a high level of carbohydrates
288 like starch, sucrose or lactose. Contzen *et al.* hypothesized that soil may be the source of
289 contamination for mashed potato powders, as they had found *B. cytotoxicus* on a raw potato
290 (Contzen *et al.*, 2014).

291 The results of the performed cytotoxicity assays in this study suggest that there are few strains
292 which are highly cytotoxic, and which could lead to food poisoning outbreaks, while most *B.*
293 *cytotoxicus* seem to be non-toxic. However, up to now, cytotoxicity assays have – with one
294 exception - only been performed with strains related to food poisoning cases, thus leading to
295 an overestimation of the cytotoxicity of *B. cytotoxicus* (Fagerlund *et al.*, 2007). The results of
296 the present study support the assumption of Fagerlund *et al.* that harboring the *cytKI* gene is
297 not a sufficient criterion for highly cytotoxic strains (Fagerlund *et al.*, 2007). Fagerlund *et al.*
298 have also shown that the different levels of expression of *cytKI* could not be due to
299 differences in the PlcR-PapR quorum sensing system, which acts as key transcriptional
300 regulator for extracellular virulence factors in *B. cereus* group strains (Fagerlund *et al.*, 2007).

301 Furthermore, YvrGH and YvfTU two-component systems have also been studied and neither
302 seem to be responsible for the differences in the expression of *cytK1*.

303 In conclusion, this study shows that *B. cereus s.l.* in mashed potato powders as well as PIF
304 pose a potential food safety risk. Further research is needed to extend the hitherto very limited
305 knowledge on the ecological niches of *B. cytotoxicus* and mechanism of its cytotoxicity. Due
306 to the ubiquity, resistance, and persistence of *B. cereus s.l.* and colonization of processing
307 facilities with spores (Carlin, 2011), contamination of food products is almost impossible to
308 avoid. It is therefore essential that producers uphold highest quality control standards, while
309 consumers should assure good practices such as proper holding times and storage
310 temperatures to protect especially vulnerable consumer groups such as infants or hospital
311 inpatients.

312

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317

318 **Competing interests**

319 The authors declare that they have no competing interests.

320

321 **References**

- 322 Andersson, M.A., Jääskeläinen, E.L., Shaheen, R., Pirhonen, T., Wijnands, L.M., Salkinoja-
323 Salonen, M.S., 2004. Sperm bioassay for rapid detection of cereulide-producing *Bacillus*
324 *cereus* in food and related environments. *Int. J. Food Microbiol.* 94(2), 175– 183.
- 325 Bartoszewicz, M., Hansen, B., Swiecicka, I., 2008. The members of the *Bacillus cereus* group
326 are commonly present contaminants of fresh and heat-treated milk. *Food Microbiol.* 25(4),
327 588–596.
- 328 Becker, H., Schaller, G., Von Wiese, W., Terplan, G., 1994. *Bacillus cereus* in infant foods
329 and dried milk products. *Int. J. Food Microbiol.* 23(1), 1-15.
- 330 Beecher, D., Wong, A., 1994. Improved purification and characterization of hemolysin BL, a
331 hemolytic dermonecrotic vascular permeability factor from *Bacillus cereus*. *Infect. Immun.*
332 62(3), 980-986.
- 333 Carlin, F., 2011. Origin of bacterial spores contaminating foods. *Food Microbiol.* 28(2), 177-
334 182.
- 335 Chattopadhyay, A., Bhatnagar, N., Bhatnagar, R., 2004. Bacterial Insecticidal Toxins. *Crit.*
336 *Rev. Microbiol.* 30(1), 33-54.
- 337 Contzen, M., Hailer, M., Rau, J., 2014. Isolation of *Bacillus cytotoxicus* from various
338 commercial potato products. *Int. J. Food Microbiol.* 174, 19-22.
- 339 Di Pinto, A., Bonerba, E., Bozzo, G., Ceci, E., Terio, V., Tantillo, G., 2013. Occurrence of
340 potentially enterotoxigenic *Bacillus cereus* in infant milk powder. *Eur. Food Res. Technol.*
341 237, 275-279.
- 342 Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A.,
343 Hoedemaekers, G., Fourie, L., Heyndrickx, M., Mahillon, J., 2005. Fatal family outbreak of
344 *Bacillus cereus*-associated food poisoning. *J. Clin. Microbiol.* 43(8), 4277–4279.
- 345 Doan, C., Davidson, P., 2000. Microbiology of potatoes and potato products: A Review. *J.*
346 *Food Prot.* 63(5), 668–683.

- 347 EFSA, 2016. Risks for public health related to the presence of *Bacillus cereus* and other
348 *Bacillus* spp. including *Bacillus thuringiensis* in foodstuffs. EFSA J. 14.
- 349 Ehling-Schulz, M., Guinebretière, M.H., Monthán, A., Berge, O., Fricker, M., Svensson, B.,
350 2006. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol. Lett.
351 260(2), 232-240.
- 352 Ehling-Schulz, M., Knutsson, R., Scherer, S. 2011. *Bacillus cereus*. In: Fratamico, P., Liu, Y.,
353 Kathariou, S. (Eds) Genomes of Foodborne and Waterborne Pathogens. Washington, DC, pp.
354 147-164.
- 355 Ehling-Schulz, M., Messelhäusser, U., 2013. *Bacillus* 'next generation' diagnostics: Moving
356 from detection towards sub-typing and risk related strain profiling. Front. Microbiol. 4, 1-8.
- 357 Ehling-Schulz, M., Svensson, B., Guinebretière, M.H., Lindbäck, T., Andersson, M., Schulz,
358 A., Fricker, M., Christiansson, A., Granum, P.E., Märklbauer, E., Nguyen-The, C., Salkinoja-
359 Salonen, M., Scherer, S., 2005. Emetic toxin formation of *Bacillus cereus* is restricted to a
360 single evolutionary lineage of closely related strains. Microbiology 151, 183-197.
- 361 EFSA, 2015. The European Union summary report on trends and sources of zoonoses,
362 zoonotic agents and food-borne outbreaks in 2013. EFSA Journal 13(1).
- 363 EFSA, 2005. Opinion of the Scientific Panel on Biological Hazards on *Bacillus cereus* and
364 other *Bacillus* spp in foodstuffs. EFSA Journal 175, 1-48.
- 365 EFSA, 2016. *Bacillus cereus* and other *Bacillus* spp. including *B. thuringiensis* in foodstuffs.
366 EFSA Journal 14(7).
- 367 Fagerlund, A., Brillard, J., Fürst, R., Guinebretière, M.H., Granum, P.E., 2007. Toxin
368 production in a rare and genetically remote cluster of strains of the *Bacillus cereus* group.
369 BMC Microbiol. 7.
- 370 Fagerlund, A., Ween, O., Lund, T., Hardy, S., 2004. Genetic and functional analysis of the
371 *cytK* family of genes in *Bacillus cereus*. Microbiology 150, 2689–2697.

- 372 Gaur, A., Patrick, C.C., McCullers, J.A., Flynn, P.M., Pearson, T.A., Razzouk, B.I.,
373 FAO/WHO. 2006. *Enterobacter sakazakii* and *Salmonella* in powdered infant formula.
374 <http://www.who.int/foodsafety/publications/micro/mra10/en/>
- 375 Jackson, S. G., Goodbrand, R. B., Ahmed, R., Kasatiya, S., 1995. *Bacillus cereus* and *Bacillus*
376 *thuringiensis* isolated in a gastroenteritis outbreak investigation. Lett. Appl. Microbiol., 21(2),
377 103-105.
- 378 Fricker M., Ågren, J., Segerman, B., Knutsson, R., Ehling-Schulz, M. 2011. Evaluation of
379 *Bacillus* strains as model systems for the work on *Bacillus anthracis* spores. Int. J. Food
380 Microbiol., 145, 129-136. Guinebretière, M.H., Fagerlund, A., Granum, P.E., Nguyen-The,
381 C., 2006. Rapid discrimination of *cytK-1* and *cytK-2* genes in *Bacillus cereus* strains by a
382 novel duplex PCR system. FEMS Microbiol. Lett. 259(1), 74-80.
- 383 Guinebretière, M.H., Auger, S., Galleron, N., Contzen, M., De Sarrau, B., De Buyser, M.L.,
384 Lamberet, G., Fagerlund, A., Granum, P.E., Lereclus, D., De Vos, P., Nguyen-The, C.,
385 Sorokin, A., 2013. *Bacillus cytotoxicus* sp. nov. is a novel thermotolerant species of the
386 *Bacillus cereus* group occasionally associated with food poisoning. Int. J. Syst. Evol.
387 Microbiol. 63, 31–40.
- 388 Guinebretière, M.H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz,
389 M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., De Vos, P., 2008.
390 Ecological diversification in the *Bacillus cereus* group. Environmental Microbiology, 851–
391 865.
- 392 Guinebretière, M.H., Velge, P., Couvert, O., Carlin, F., Debuyser, M.L., Nguyen-The, C.,
393 2010. Ability of *Bacillus cereus* group strains to cause food poisoning varies according to
394 phylogenetic affiliation (Groups I to VII) rather than species affiliation. J. Clin. Microbiol. 48
395 (9), 3388–3391.
- 396 Hardy, S., Lund, T., Granum, P.E., 2001. *CytK* toxin of *Bacillus cereus* forms pores in planar
397 lipid bilayers and is cytotoxic to intestinal epithelia. FEMS Microbiol. Lett. 197(1), 47-51.

- 398 Hilliard, N., Schelonka, R., Waites, K., 2003. *Bacillus cereus* bacteremia in a preterm
399 neonate. *J. Clin. Microbiol.* 41(7), 3441–3444.
- 400 Kim, H., Goepfert, J., 1971. Occurrence of *Bacillus cereus* in selected dry food products.
401 *Journal of Milk and Food Technology*, 12-15.
- 402 King, N., Whyte, R., Hudson, J., 2007. Presence and significance of *Bacillus cereus* in
403 dehydrated potato products. *J. Food Prot.* 70(2), 514-520.
- 404 Lequin, M.H., Vermeulen, J.R., Van Elburg, R.M., Barkhof, F., Kornelisse, R.F., Swarte, R.,
405 Govaert, P.P., 2005. *Bacillus cereus* meningoencephalitis in preterm infants: neuroimaging
406 characteristics. *AJNR Am. J. Neuroradiol.* 26(8), 2137–2143.
- 407 Lindqvist, R., Andersson, Y., De Jong, B., Norberg, P., 2000. A summary of reported
408 foodborne disease incidents in Sweden, 1992 to 1997. *J. Food Prot.* 63(10), 1315-1320.
- 409 Lund, T., De Buyser, M., Granum, P.E., 2000. A new cytotoxin from *Bacillus cereus* that may
410 cause necrotic enteritis. *Mol. Microbiol.* 38(2), 254-261.
- 411 McIntyre, L., Bernard, K., Beniac, D., Isaac-Renton, J. L., Naseby, D. C., 2008. Identification
412 of *Bacillus cereus* group species associated with food poisoning outbreaks in British
413 Columbia, Canada. *Appl. Environ. Microbiol.* 74 (23), 7451-7453.
- 414 Moravek, M., Dietrich, R., Buerk, C., Broussolle, V., Guinebretière, M.H., Granum, P.E.,
415 Nguyen-The, C., Märtlbauer, E., 2006. Determination of the toxic potential of *Bacillus cereus*
416 isolates by quantitative enterotoxin analyses. *FEMS Microbiol. Lett.* 257(2), 293–298.
- 417 Mossel, D., Koopman, M., Jongerius, E., 1967. Enumeration of *Bacillus cereus* in foods.
418 *Appl. Microbiol.* 15(3), 650–653.
- 419 Naranjo, M., Denayer, S., Botteldoorn, N., Delbrassinne, L., Veys, J., Waegenaere, J., Sirtaine,
420 N., Driesen, R.B., Sipido, K.R., Mahillon, J., Dierick, K., 2011. Sudden death of a young adult
421 associated with *Bacillus cereus* food poisoning. *J. Clin. Microbiol.* 49(12), 4379–4381.
- 422 Reyes, J., Bastias, J., Gutiérrez, M., Rodríguez, M.L., 2007. Prevalence of *Bacillus cereus* in
423 dried milk products used by Chilean school feeding program. *Food Microbiol.* 24(1), 1-6.

- 424 Rowan, N., Anderson, J., Anderton, A., 1997. The bacteriological quality of hospital-prepared
425 infant feeds. *J. Hosp. Infect.* 35(4), 259-267.
- 426 Shaheen, R., Andersson, M.A., Apetroaie, C., Schulz, A., Ehling-Schulz, M., Ollilainen,
427 V.M., Salkinoja-Salonen, M.S., 2006. Potential of selected infant food formulas for
428 production of *Bacillus cereus* emetic toxin, cereulide. *Int. J. Food Microbiol.* 107(3), 287-294.
- 429 Sorokin, A., Candelon, B., Guilloux, K., Galleron, N., Wackerow-Kouzova, N., Ehrlich, S.D.,
430 Bourguet, D., Sanchis, V., 2006. Multiple-locus sequence typing analysis of *Bacillus cereus*
431 and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of
432 psychrotrophic strains. *Appl. Environ. Microbiol.* 72(2), 1569–1578.
- 433 Stenfors Arnesen, L., Fagerlund, A., Granum, P.E., 2008. From soil to gut: *Bacillus cereus*
434 and its food poisoning toxins. *FEMS Microbiol. Rev.* 32(4), 579-606.
- 435 Takabe, F., Oya, M., 1976. An autopsy case of food poisoning associated with *Bacillus*
436 *cereus*. *Forensic Sci.* 7(2), 97-101.
- 437 Thompson, S.J., Shenep, J.L., 2001. *Bacillus cereus* bacteremia and meningitis in
438 immunocompromised children. *Clin. Infect. Dis.* 32(10), 1456–1462.
- 439 Travers, R., Martin, P., Reichelderfer, C., 1987. Selective process for efficient isolation of soil
440 *Bacillus* spp.. *Appl. Environ. Microbiol.* 53(6), 1263-1266.
- 441 Turner, N., Whyte, R., Hudson, A., Kaltovei, S., 2006. Presence and growth of *Bacillus*
442 *cereus* in dehydrated potato flakes and hot-held, ready-to-eat potato products purchased in
443 New Zealand. *J. Food Prot.* 69(5), 1173-1177.
- 444 Wang, C., Hawthorne, D., Qin, Y., Pan, X., Li, Z., Zhu, S., 2017. Impact of climate and host
445 availability on future distribution of Colorado potato beetle. *Sci. Rep.* 7(1), 1-9.
- 446 Wang, M., Cao, B., Gao, Q., Sun, Y., Liu, P., Feng, L., Wang, L., 2009. Detection of
447 *Enterobacter sakazakii* and other pathogens associated with infant formula powder by use of a
448 DNA Microarray. *J. Clin. Microbiol.* 47(10), 3178–3184.

449 Zhang, Y., Chen, J., Feng, C., Zhan, L., Zhang, J., Li, Y., Yang, Y., Chen, H., Zhang, Z.,
450 Zhang, Y., Mei, L., Li, H., 2017. Quantitative prevalence, phenotypic and genotypic
451 characteristics of *Bacillus cereus* isolated from retail infant foods in China. Foodborne
452 Pathog. Dis. 14(10), 564-572.
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TABLES AND FIGURES

456 **Table 1:** Primers used in this study.

Target gene	Primer	Primer sequence (5' → 3')	Reference
<i>panC</i>	panC_Cyto_for	CGTTATCCAAGGGATATAAAGCGA	This study
	panC_Cyto_rev	TCTACATAATCAACTATACCGTTTG	This study
<i>panC</i>	panC_fwd	CGATATCCTCGTGATATTGATAGA	Sorokin <i>et al.</i> (2006)
	panC_rev	TCCGCATAATCTACAGTGGCTTTC	Sorokin <i>et al.</i> (2006)
<i>nhe</i>	NA2F	AAGCIGCTCTTCGIATTC	Ehling-Schulz <i>et al.</i> (2006)
	NB1R	ITIGTTGAAATAAGCTGTGG	Ehling-Schulz <i>et al.</i> (2006)
<i>hbl</i>	HD2F	GTAAATTAIGATGAICAATTTC	Ehling-Schulz <i>et al.</i> (2006)
	HA4R	AGAATAGGCATTCATAGATT	Ehling-Schulz <i>et al.</i> (2006)
<i>ces</i>	CesF1	GGTGACACATTATCATATAAGGTG	Ehling-Schulz <i>et al.</i> (2006)
	CesR2	GTAAGCGAACCTGTCTGTAACAACA	Ehling-Schulz <i>et al.</i> (2006)
<i>cytK1</i>	CK1F	CAATTCCAGGGGCAAGTGTC	Guinebretiere <i>et al.</i> (2006)
	CK1R	CCTCGTGCATCTGTTTCATGAG	Guinebretiere <i>et al.</i> (2006)
<i>cytK2</i>	CK2F	CAATCCCTGGCGCTAGTGCA	Guinebretiere <i>et al.</i> (2006)
	CK2R	GTGIAGCCTGGACGAAGTTGG	Guinebretiere <i>et al.</i> (2006)

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463 **Table 2:** Toxin genes detected by PCR in a total of 43 *B. cereus s.l.* isolates collected from
 464 powdered infant formula (PIF), mashed potato powder, and fruit powder.

	<i>nhe</i>	<i>hbl</i>	<i>cytK1</i>	<i>cytK2</i>	<i>ces</i>
PIF ^P isolates (n = 11)	11	4	0	8	0
PIF ^R isolates (n = 8)	8	1	0	5	1
Mashed potato powder isolates (n = 20)	12	2	9	6	0
Fruit powder isolates (n = 4)	4	2	0	4	0

465 PIF^P Samples obtained at the level of production

466 PIF^R Samples obtained at retail level

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468 **Table 3:** Assignment of 43 *B. cereus s.l.* isolates originating from different food sources to
 469 *panC* groups

<i>panC</i> group	PIF ^P isolates (n = 11)	PIF ^R isolates (n = 8)	Mashed potato powder isolates (n = 20)	Fruit powder isolates (n = 4)
II	2	1	0	0
III	6	6	10	2
IV	2	1	0	2
V	1	0	0	0
VII	0	0	9	0
NS	0	0	1	0

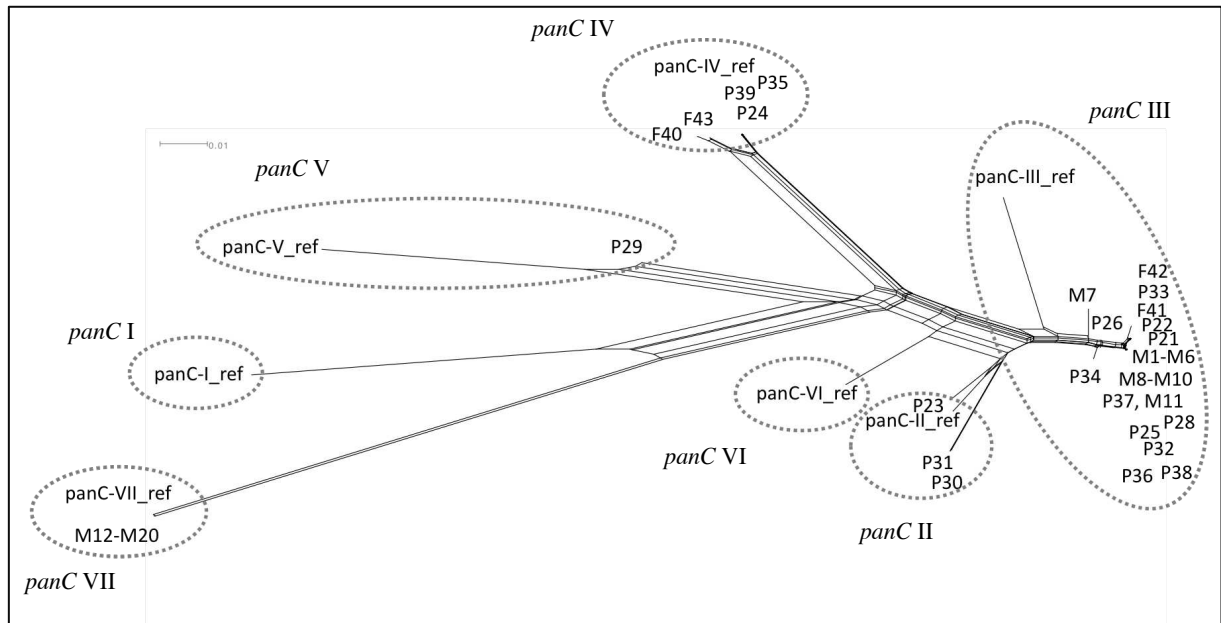
470 NS = no assignment to any of the *panC* groups I-VII.

471 PIF^P Samples obtained at the level of production

472 PIF^R Samples obtained at retail level

473 **Figure 1:** SplitsTree depicting the degree of similarity of the *panC* sequences. (a) Overview
 474 over the full SplitsTree depicting all isolates as well as one reference strain per *panC* type
 475 (*panC* type I: DSM 12442; *panC* type II: WSBC10311; *panC* type III: Ames; *panC* type IV:
 476 ATCC 14579; *panC* type V: BCT-7112; *panC* type VI: WSBC 10204; *panC* type VII:
 477 NVH391-98); (b) Detail zooming in on the region depicting the *panC* type III cluster, while
 478 omitting isolates assigned to other *panC* groups. M = isolate originating from mashed potato
 479 powder, P = isolate originating from PIF, F = isolate originating from fruit powder.

480 (a)



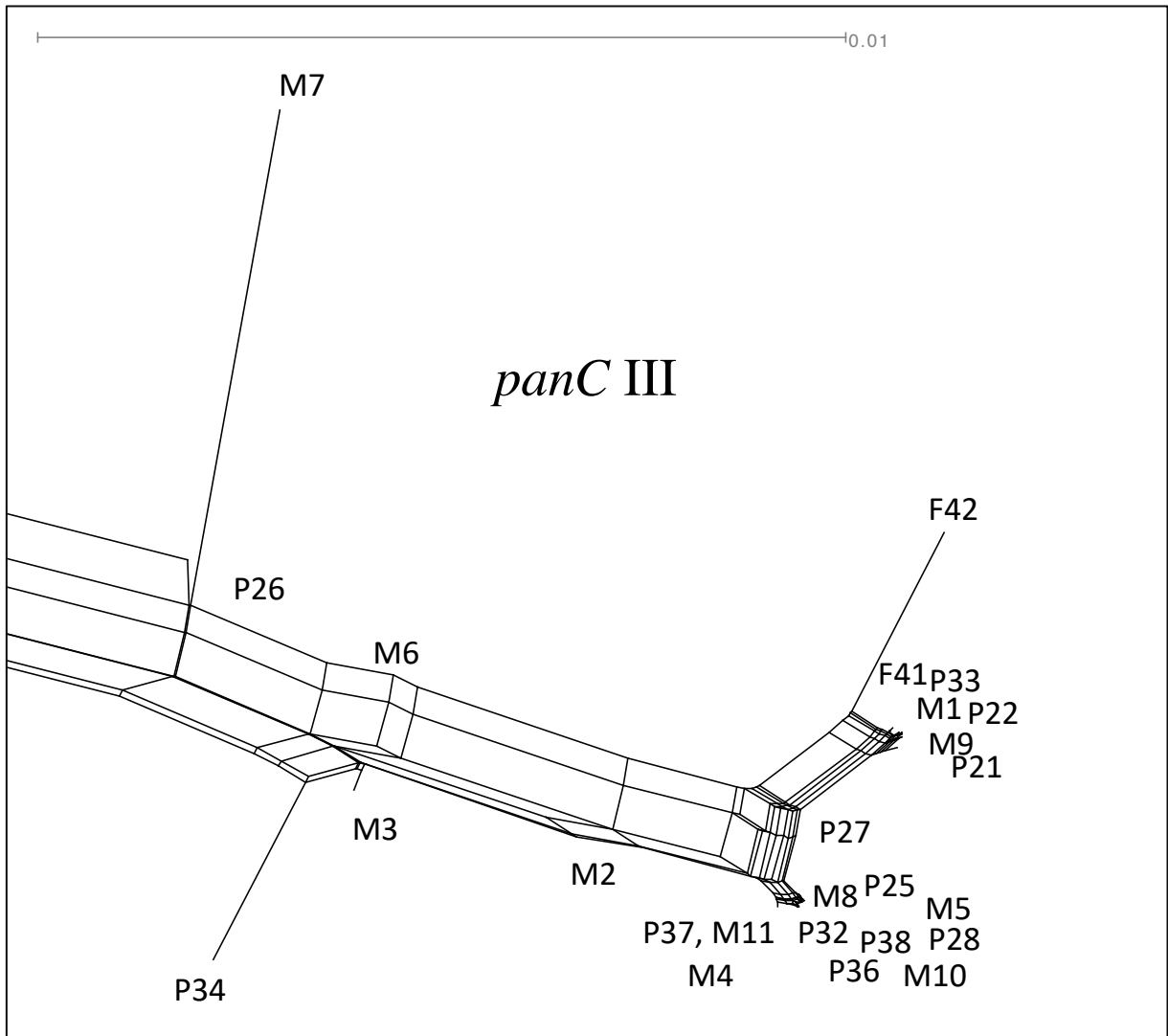
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485 (b)



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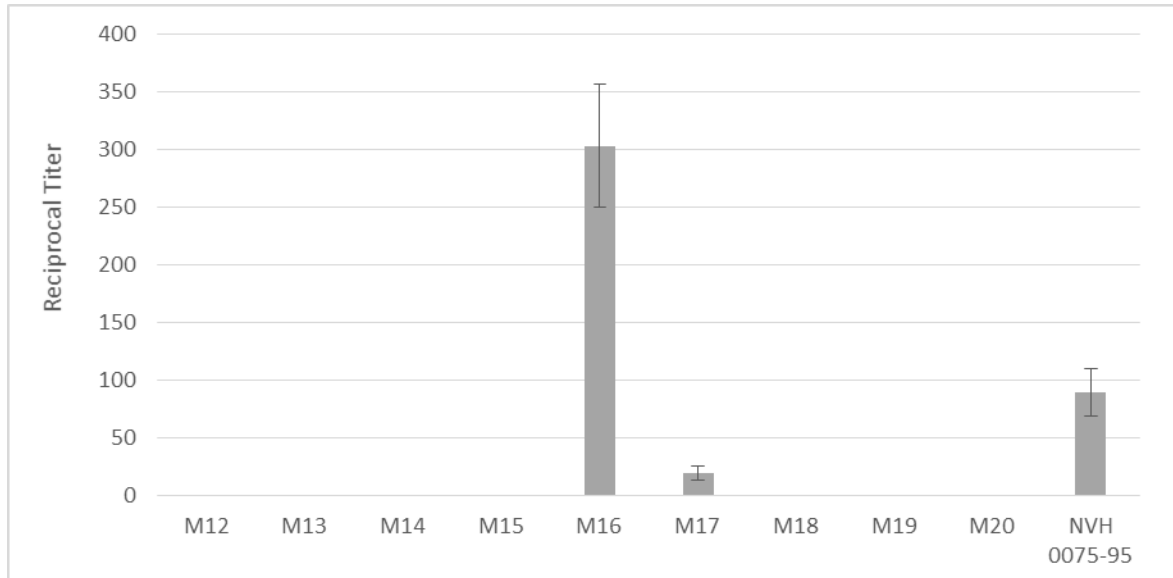
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497 **Figure 2:** Reciprocal cytotoxicity titers of *B. cytotoxicus* isolates M12-M20 and a reference
498 strain for high level toxin production (food poisoning strain *B. cereus* NVH 0075-95). Values
499 indicated are based on supernatants tested in two Vero cell cytotoxicity assays with each
500 dilution of the supernatant tested in duplicate. Error bars represent one standard deviation of
501 the mean.



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