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2 **The cytotoxic potential of *Bacillus cereus* strains of various origins**

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Abstract

B. cereus is a human pathogen associated with food poisoning leading to gastrointestinal disorders, as well as local and severe systemic infections. The pathogenic spectrum of *B. cereus* ranges from strains used as probiotics in humans to lethal highly toxic strains. In this study, we gathered a collection of 100 strains representative of the pathological diversity of *B. cereus* in humans, and characterized these strains for their cytotoxic potential towards human cells. We analyzed the correlation between cytotoxicity to epithelial and macrophage cells and the combination of 10 genes suspected to play a role during *B. cereus* virulence. We highlight genetic differences among isolates and studied correlations between genetic signature, cytotoxicity and strain pathological status. We hope that our findings will improve our understanding of the pathogenicity of *B. cereus,* thereby making it possible to improve both clinical diagnosis and food safety.

40 **Introduction**

41 *B. cereus* sensus stricto belongs to a larger group of bacteria commonly named the « Bacillus
42 cereus group ». The group is constituted of eight species: *B. mycoides*, *B. pseudomycoides*,
43 *B. weihenstephanensis*, *B. anthracis*, *B. thuringiensis*, *B. cereus sensu stricto* (Bc)*, B.*
44 *cytotoxicus* and *B. toyonensis.* Differentiation of species within the *B. cereus* group is complex
45 due to the genetic proximity between the members of the group (Liu et al, 2015). Originally,
46 species in the group were classified on the basis of phenotypic differences, distinct virulence
47 trait and the presence of extrachromosomal elements that reflect the specie's virulence
48 spectrum. Currently, the most widespread classification system of the *B. cereus* group is
49 based on the sequencing of *panC* housekeeping gene, which encodes for the pantoate-beta
50 alanine ligase C. Using this classification, seven phylogenetic groups have been determined
51 based principally on their range of growth temperature (Guinebretière et al., 2002). *B. cereus*
52 can be found in several group among which the groups III and VII, which comprise species
53 associated with high toxicity (Guinebretière et al., 2010). However, a proper differentiation
54 between the group members is still difficult and the pathogenic potential of the Bc strains is
55 broad and diverse.

56 *Bacillus cereus* group species are sporulating ubiquitous bacteria that form biofilms.
57 These properties allow them to withstand most cleaning and decontamination processes
58 (Ramarao et al., 2015). It is not surprising to find them at all food processing steps and in
59 different hospital environments. Bc is the second causative agent of confirmed and
60 suspected foodborne illness in France and the third in Europe (Journal, 2009). Bc is found in
61 many raw and processed foods. The increasing use of refrigerated foods and the related
62 increase in the number of collective cases of food-borne poisoning involving Bc raise
63 questions about how far-reaching the danger actually is and how it could be controlled. Bc
64 causes two types of food-borne illnesses. The emetic form is caused by the ingestion of
65 cereulide, a peptide produced by the bacterium and already present in the ingested food
66 (Ehling-Schulz et al., 2004). The diarrheal form is generally associated with the ingestion of
67 bacteria producing toxins (ie: Nhe, Hbl, CytK) (Stenfors Arnesen et al., 2008). Symptoms

68 usually last less than 24 h (Decousser et al., 2013) but several fatal cases of bloody diarrhea
69 and emetic poisoning have been reported (Kotiranta et al., 2000; Lund, DeBuyser et al.,
70 2000; Naranjo et al., 2011).

71 Bc is also associated with severe local and systemic human infections, posing a public health
72 problem (Bottone, 2010). The increasing frequency with which such non-gastrointestinal
73 diseases are being reported highlights the importance to study this emerging pathogen
74 (Bottone, 2010). In particular, Bc induces systemic infections, especially in premature
75 newborns, leading to the patient death in about 10% of cases (Gaur et al., 2001; Glasset et
76 al., 2018; Lotte et al., 2017; Ramarao et al., 2014; Veysseyre et al., 2015).

77 Bc possess several toxin genes, which play a role during Bc virulence. Nhe complex is
78 composed of three different proteins, A, B and C that are encoded by the *nheA*, *nheB* and
79 *nheC* genes, respectively (Jeßberger et al., 2015). Nhe is recognized as the major diarrheal
80 toxin of *B. cereus.* Hbl is composed of a binding protein B encoded by the *hblA* gene and two
81 lytic components L1 and L2 encoded by *hblC* and *hblD* genes, respectively (Beecher and
82 Wong, 1994). The secreted enterotoxins Hbl and Nhe are more abundant in clinical and food
83 poisoning strains than in environmental strains (Guinebretière et al., 2002), and a correlation
84 has been shown between cytotoxicity and concentration of Nhe in Bc supernatant (Moravek
85 et al., 2006). These toxins provide an indication of the strain toxicity potential but are not,
86 alone, sufficient to discriminate hazardous from harmless strains (Glasset et al., 2018;
87 Guinebretière et al., 2002; Martinezl Blanch et al., 2009; Ramarao, 2020).

88 There are two variants of CytK that share 89 % of identity: CytK1, and CytK2. CytK1 was
89 identified from a strain that contributed to the death of three people (Lund, De Buyser et al.,
90 2000). CytK2 is five times less toxic than CytK1, but it seems more frequently associated

91 with strains causing Food Borne Outbreaks (FBO) (Ramarao and Sanchis, 2013). The Hemolysin
92 II (HlyII) toxin is encoded by the *hlyII* gene. It induces macrophage apoptosis and allows Bc
93 to bypass the host immune defenses (Cadot et al., 2010; Tran, Guillemet et al., 2011a;
94 Tran, Puhar et al., 2011). Finally, emetic Bc strains induce the emetic food intoxication
95 Form through the production of a cereulide toxin (Yabutani et al., 2009). The toxin is
96 synthetized by a non-ribosomal cereulide synthetase enzyme encoded by the plasmid-located
97 *ces* gene (Marxen et al., 2015).
98 Epithelial cells are the primary physical barriers preventing microbes from entering the host.
99 In addition, immune cells such as macrophages constitute a host defense against pathogenic
100 bacteria. The heterogeneity of the diseases associated with Bc infections suggests that the
101 ability of these bacteria to colonize their host, and to circumvent the host immune system
102 may differ between strains, although the basis of these differences mostly remains unclear.
103 In this work, we studied food-borne disease strains, strains isolated from human biological
104 samples following local or systemic infections, and non-pathogenic strains, for their capacity
105 to induce epithelial and macrophage cell toxicity, and we characterized the strains by the
106 analysis of ten virulence genes revealing various genetic signatures.

Methods

94 **Bacterial strains**

95 This study included 39 Bc strains causing foodborne illness (Glasset et al., 2016), 41 strains
96 isolated from human following systemic or local infection (Glasset et al., 2018) and 20 non-
97 pathogenic strains, isolated from food that did not cause infection in humans or animals
98 (Cadot et al., 2010; Guinebretière et al., 2002; Kamar et al., 2013) (supplementary Table 1).
99 The 39 strains isolated from foodborne illness and which caused gastrointestinal infections,
101 were characterized in a previous study (Glasset et al., 2016). These strains were isolated
102 between 2007 and 2014 in France. Each foodborne illness caused from 2 to 70 human cases,
103 from 30 min to 24 h after ingestion of bacterial doses between 4.00E+02 and 1.00E+09
104 CFU/g of food. Identification and numeration of Bc strains from FBO were conducted by
105 plating the strains on selective agar media (mannitol-phenol red- egg yolk medium
106 (Biokar) according to the International Organization for Standardization (ISO) 7932
107 standard Method, allowing for Bc identification.

104 The 41 strains of the clinical collection were isolated from patient samples (biopsy, blood
106 culture, etc) from nine French voluntary hospitals between 2008 and 2014 (Glasset et al.,
104 2018). 41% of the patients were newborns and 21% died. Bc were identified by plating on
105 specific agar media and confirmed by using 16S rDNA sequencing. The 20 non-pathogenic
106 strains were isolated from soil or food that did not cause infections in humans
107 (Guinebretière et al., 2002); They were also non-virulent in an insect infection model (Kamar
108 et al., 2013).

111 **Cell lines and cytotoxicity assay**

112 For cytotoxic studies, all strains were grown until the bacterial culture reached an optical

113 density (OD) at 600 nm from 1.3 to 1.7, corresponding to the middle of the exponential

114 growth phase. The cultures were centrifugated and supernatants were collected and

115 filtered. The eukaryotic cells viability was assessed following incubation with bacterial

116 culture supernatants. Tests were carried out on HeLa and Raw (Raw 264.7) cell lines (Sigma Aldrich).

117 Cell viability was measured by using the tetrazolium salt (MTS: (3- (4,5-dimethylthiazol-2-yl) -5- (3-

118 carboxymethoxyphenyl) -2-(4-sulfophenyl) -2H-tetrazolium)) which colors the solution

119 according to the mitochondrial activity. The eukaryotic cells were prepared in 96-well plates

120 and incubated until confluence for HeLa and Raw cells. Cells were incubated at 37°C + 5%

121 CO_2 with specific media (HeLa: RPMI + 10% FCS + 2% penicillin/streptavidin, Raw: DMEM + 10%

122 FCS + 2% penicillin/streptavidin). Cell medium was removed and the bacterial supernatants were

123 incubated with eukaryotic cells at dilutions 1/10 (V/V in fresh cell medium) for 1 h.

124 Then, 20 µl of MTS were added and the OD was measured at 490 nm after 20l60 min of incubation

125 at 37°C + 5% CO_2. The percentage of cytotoxicity was calculated by

126 normalizing the OD values between treated and untreated cells.

127 Results represent the average of at least three experiments done in duplicates.

128

129 **Molecular Characterization**

130 DNA was extracted after overnight incubation of the strains at 30°C on Trypticase Soy Agar with

131 0.6 Yeast Extract (TSAYE, Sigma-Aldrich) using the DNeasy Blood and Tissue Kit according to

132 the manufacturer’s recommendations (Qiagen). DNA was quantified by absorbance at 260

133 nm on a Nanodrop1000 spectrophotometer (Thermo scientific). The presence of ten virulence genes

134 *cytK1*, *cytK2*, *hblA*, *hblC*, *hblD*, *nheA*, *nheB*, *nheC*, *hlyII* and *ces* was evaluated by PCR as described

135 previously (Glasset et al., 2016). The combination of these genetic features allowed to attribute

136 to each strain one genetic signatures (GS) (table 1).

137

138 **Statistical analysis**

139 The cytotoxic activity results were analyzed by ANOVA and Tukey tests. ANOVA (analysis of

140 variance) was used to assess whether all the Bc strain collections follow the same normal

141 distribution. The Tukey test is a HSD test for "Honestly Significant Difference", and was done after

142 the ANOVA test. This paired comparison test compares each sample two by two and identifies

143 which ones differ from the normal distribution.

Results and discussion

147 **Cell lines susceptibility**

148 Each individual strain (n=100) was tested for its cytotoxic potential to HeLa and Raw cell lines
149 (sup Table 1). The average toxicity was then calculated for each collection of strains
150 (Figure 1). Bc strains involved in infections, foodborne illness or non-gastrointestinal
151 infections, showed a greater cytotoxic activity than non-pathogenic strains on HeLa
152 epithelial cells and mouse Raw macrophages. For HeLa cells, the difference is strongly
153 significant ($p < 0.001$). On the Raw cell line, the difference is strongly significant between the
154 foodborne illness strains and non-pathogenic strains (pvalue < 0.001) and significant between
155 the clinical and non-pathogenic strains (pvalue = 0.023).

156

157 **Genetic characterization**

158 All strains from our collection were characterized by detecting by PCR the presence of these
159 ten genes involved in virulence. According to the presence/absence of the genes, the strains
160 were assigned a genetic signature (GS) (Table 1, Supp Table 1). Among all the possible
161 combinations, all strains happened to spread in only twelve GS (Table 1).
162 The strains were then grouped into three collections according to their induced pathologies
163 (non-pathogenic, food-borne or clinical). We studied the distribution of GS in each collection
164 (Table 1, Figure 2). The distribution is not homogeneous. Six GS (GS1, 3, 6, 7, 9, 11) are
165 present in the foodborne illness and clinical strain collections and are absent from the non-
166 pathogenic collection. These signatures represent 52.4% of the foodborne illness strains and
167 55.4% of the clinical strains. Conversely, the GS8 signature is present only in the non-
168 pathogenic collection (52% of the total NP strains). Four signatures are present in all
169 collections (GS2, 4, 5 and 12).
170 GS1 represents 21% of the strains studied. This GS is the most frequent (34%) amongst the
171 clinical strains. This genetic signature is characteristic of strains having only the genes
172 encoding Nhe. However, 11% of the strains characterized by GS1 did not produce the toxin
173 *in vitro* (not shown). Analysis must be carried out to understand what would explain the
174 pathogenicity of these strains. A study has shown that the non-coding region upstream of
175 the *nhe* operon promoter has more or less conserved regions. A binding site for the
176 regulatory protein CodY has been identified and modifications in its sequence may explain
177 the differences in regulation and expression of *nhe* (Bohm et al., 2016). Other factors may
178 also be involved in the induction of clinical non gastro-intestinal symptoms.
179 GS3 and GS7 have the characteristics of the strains coding for the Cereulide and the
180 Cytotoxin K1, respectively. These factors are known for their toxicity potential and their
181 virulence on humans (Dierick et al., 2005; Lund, DeBuyser et al., 2000; Mahler et al., 1997).

182 The *cytK1* carrying strains were found only in FBO strains and represented 9,5% of this
183 collection. The GS3 strains, carrying *ces* and *nhe* genes were present in FBO (19%) but also
184 clinical (14%) strains.
185 GS6, GS9, GS11 were present only in pathogenic strains and GS11 was even represented only
186 among clinical strains. GS11 is characterized by the presence of *cytK2, ces* and *nhe* genes.
187 To our knowledge, the role of the *ces* gene in clinical outcome has not been shown and
188 further investigation are needed to correlate the pathogenic factors of these strains to their
189 clinical outcome.
190 GS2 was found in 27% of the strains but was involved in one-third of the food borne
191 outbreaks. This genetic signature is characteristic of strains possessing the genes encoding
192 Nhe, Hbl and CytK2 and produce strongly Nhe and Hbl *in vitro*. A synergistic action of these
193 toxins could explain its strong involvement in FBO (Beecher and Wong, 2000). However, this
194 GS was also found in 19% of the non-pathogenic strains.
195 Interestingly, only one GS, GS8 corresponding to strains carrying only *nheB* and *nheC* from
196 the *nhe* operon and only *hblA* and *hblD* from the *hbl* operon were specific to non-pathogenic
197 strains. This strongly suggests that the complete two operons need to be present in a strain
198 to give a potential pathogenic potential and that absence of a complete protein impairs the
199 strain virulence. Alternatively, this may indicate a specific role in toxicity of the NheA and HblC
subunits.
200 Characterization of the strains causing non-gastrointestinal infections by genetic signatures
201 has shown that 10/12 (83.3%) of the signatures are the same as those found in strains
202 causing FBO. Only the strains characterized by GS8, specific for non-pathogenic strains, and
203 GS7 specific for FBO associated with CytK1 were not found in clinical strains.
204

205 In general, the assignment of strains according to their genetic signature has made it
206 possible to show the genetic diversity of Bc strains, which can be involved in FBO and other
207 clinical outcomes. However, this did not make it possible to answer all the questions that
208 arise on the differences in virulence potential between the strains, or on their supposed
209 synergistic action. It therefore seems essential to look for other specific markers of
210 pathogenicity than those identified so far.
211

212 **Genetic signature and cytotoxicity correlation**

213 To further analyze the strains, the strains belonging to each GS were compared for their
214 cytotoxic potential towards human cells (Figure 3). The strains were divided in two groups:
215 the non-pathogenic strains (NP) and the pathogenic strains (FBO+clinical). Remarkably, for
216 the GS4 and 10, the pathogenic strains were significantly more cytotoxic to epithelial cells
217 than the non-pathogenic strains. This was however not the case regarding the cytotoxicity to
218 macrophage cells, indicating that the factors required for toxicity to epithelial and
219 macrophage cells are different.
220 For the GS2, the same trend was observed for HeLa although not significantly and by
221 contrast the cytotoxic potential of GS2 strains to Raw cell was significantly higher for
222 pathogenic strains compared to non-pathogenic strains (pvalue <0.001), further highlighting
223 a different tropism of the virulence factors for epithelial and macrophage cells. For other GS,
224 such as GS5, no significant difference in cell toxicity was observed for the two collections of
225 strains.
226 This is consistent with previous data showing the involvement of different factors depending
227 on the cell targeted. For instance, the role of Nhe, Hbl and CytK2 has been shown in the
228 toxicity to epithelial cells (Bohm et al., 2016; Bouillaut et al., 2005; Jessberger et al., 2015;

229 Ramarao and Lereclus, 2006), and HlyII is involved in the toxicity to phagocytic cells such as
230 macrophage (Tran, Guillemet et al., 2011b) .
231 We could consistently show that the strains characterized by GS5, GS6, GS9 and GS12
232 carrying the gene coding for HlyII are very toxic to Raw cells (82% average toxicity) compared
233 to the strains characterized by other GS (31% average toxicity) (Figure 4).
234 Similarly, the strains with the GS in which the *cytk2* gene is present (GS2, GS4 and GS11)
235 showed a higher toxic potential on epithelial cells compared to the other strains, i.e. 73 %
236 versus 55%, as has already been described (Jessberger et al., 2015). The strains of GS8
237 having the genes coding for NheB, NheC, HblA and HblD, belong only to the collection of
238 non-pathogenic Bc and are the least toxic on the two cell lines with an average toxicity of
239 13%.
240 Therefore, the significantly lower toxicity of the collection of non-pathogenic strains (Figure
241 1) is largely due to the strains characterized by GS8. The non-pathogenic strains
242 characterized by the other genetic signatures did not show differences in toxicity compared
243 to the pathogenic strains sharing these same genetic signatures.
244
245 **Conclusions**

The emergence of Bc as a foodborne pathogen and as an opportunistic pathogen has intensified the need to distinguish strains of public health concern. Over the years, new methods have been developed with the leading principle to detect and distinguish Bc from others Bacillus group members by a time-saving and in-situ analysis (Ramarao et al 2020). For example, Manzano et al., (Manzano et al 2009) compared different molecular methods that use specific probes and primers as recognition elements to distinguish Bc from *B. thuringiensis* from different sources (food, clinical and bio-pesticide). Another method used to distinguish Bc from other members of the *B. cereus* group is the genotyping using high-resolution melting analysis (Antolinos et al, 2012). The use of multi-locus sequence data allowed identification of several clades within the *B. cereus* group. The classification of the strains according to their affiliation to a phylogenetic group (I to VII) also offers a first useful indicator of risk (Guinebretière et al, 2008).

The need to better assess the *B. cereus* pathogenic potential has also led to the development of several molecular tools for Bc toxin quantification including Nhe and Hbl (Ramarao et al 2020). All these tests give indication on the genetic and toxin production of the strains but are not sufficient to accurately predict their pathogenic potential.

In this study, 12 genetic signatures (GS) were defined allowing to classify the strains of various origins from our collection. Cytotoxic activity tests showed significant differences between Bc strains that led to infections and that have higher toxicity than non-pathogenic strains. The combination of GS and cytotoxicity provides a next step in the strain characterization, with some GS being more associated with cytotoxic and pathogenic strains than others. A complementary method associating MLST, phylogeny and GS identification may be an innovative alternative to current characterization and differentiation methods.

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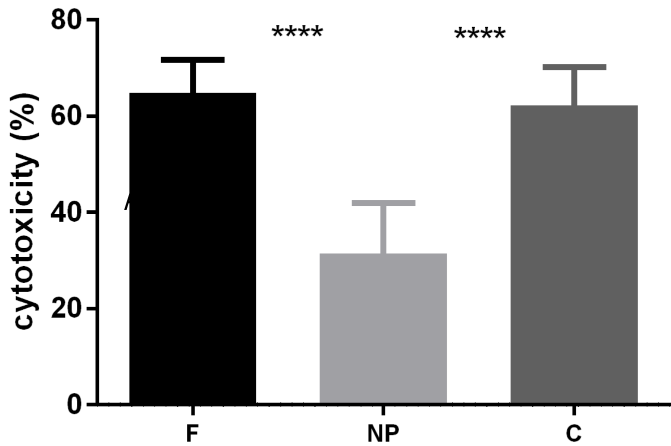
A.



B.

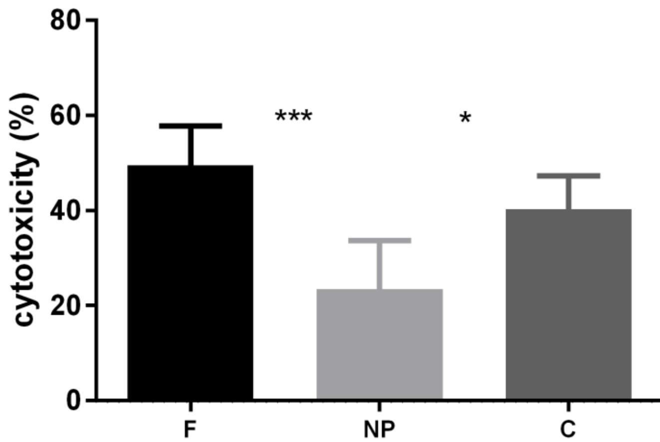


Figure 1: Results of the toxicity of the bacterial culture supernatants belonging to the "foodborne illness", "Clinical" and "non-pathogenic" collections. The percentage of cytotoxicity corresponds to the percentage of dead cells in the sample compared to the control. A.: Cytotoxic activity on the HeLa cell line. B.: Cytotoxic activity on the Raw cell line. Statistical analysis were performed and the significant difference is indicated as: ***$p<0.001$ and * $p<0.03$.

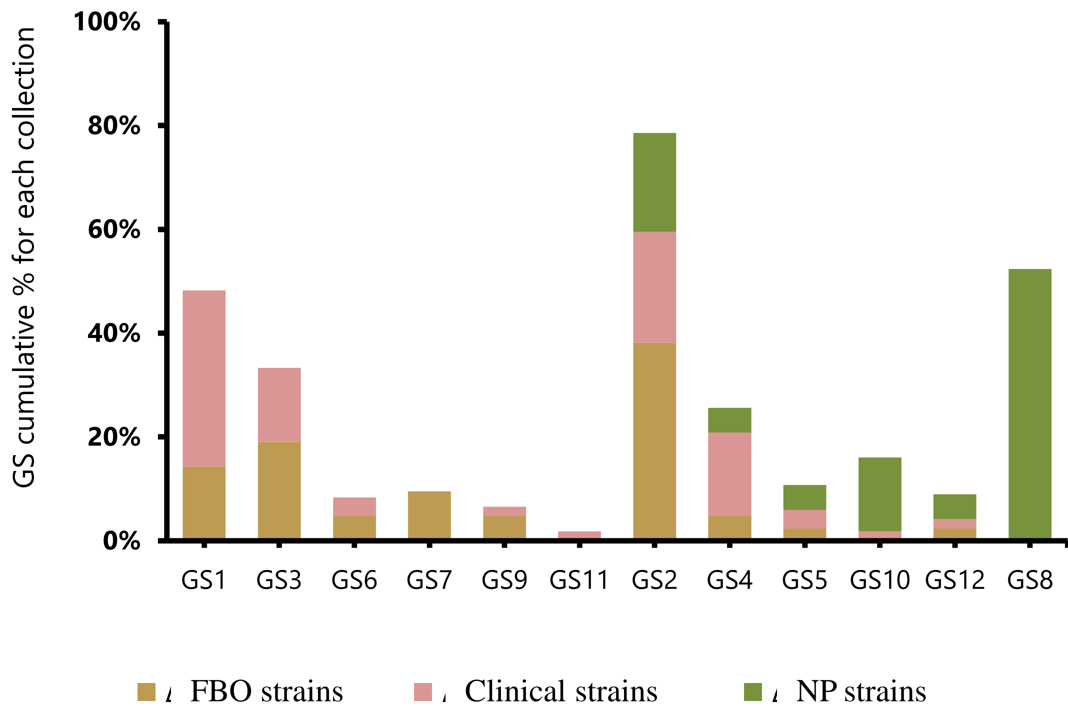


Figure 2: Cumulative genetic signature (GS) distribution according to the "foodborne illness", "Clinical" and "non-pathogenic" collections.

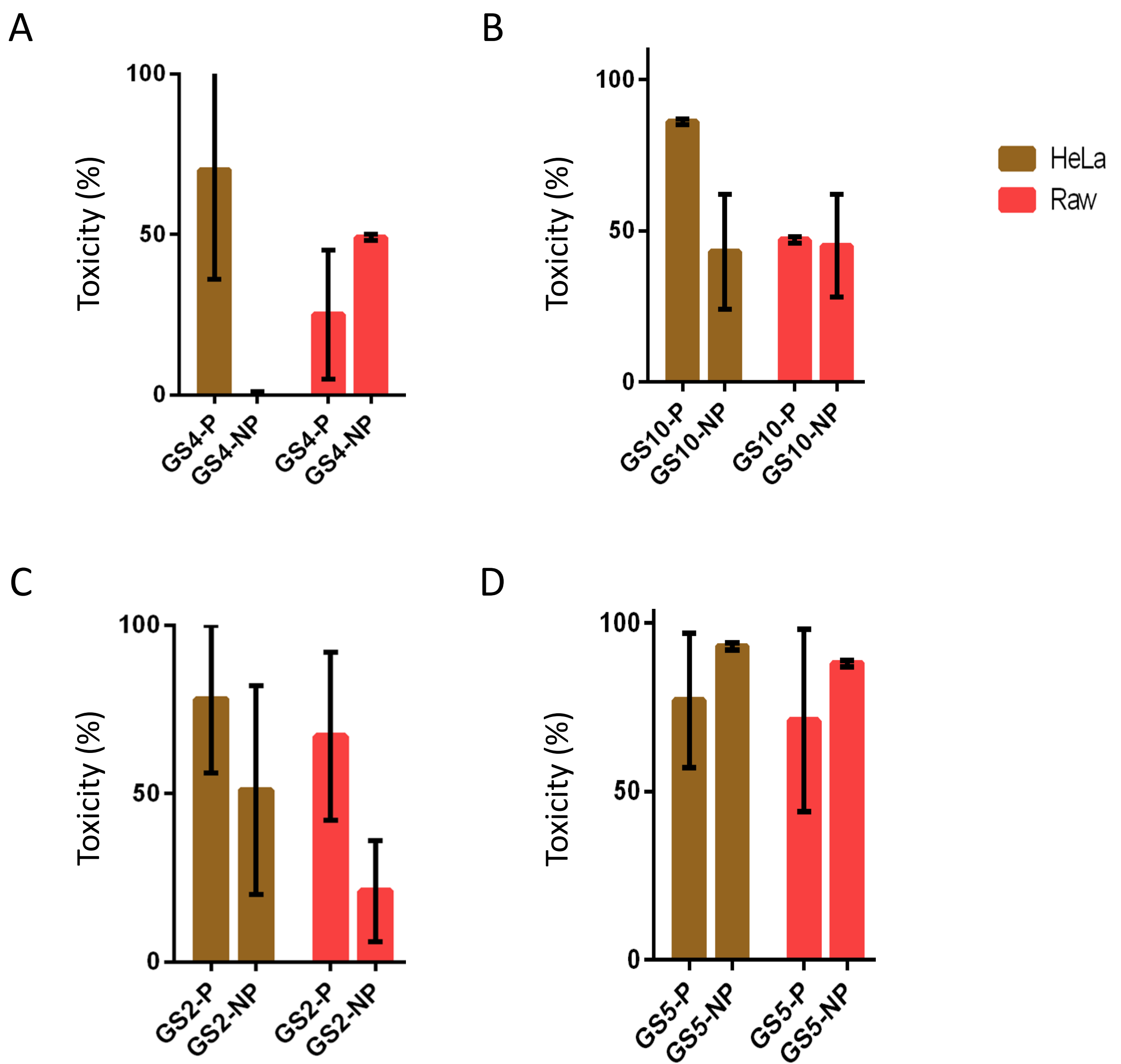


Figure 3. Toxicity of culture supernatants of strains of *B. cereus* characterized by GS4 (A), GS10 (B), GS 2 (C) or GS 5 (D) and belonging to the pathogenic (-P) or non-pathogenic (-NP) collection.

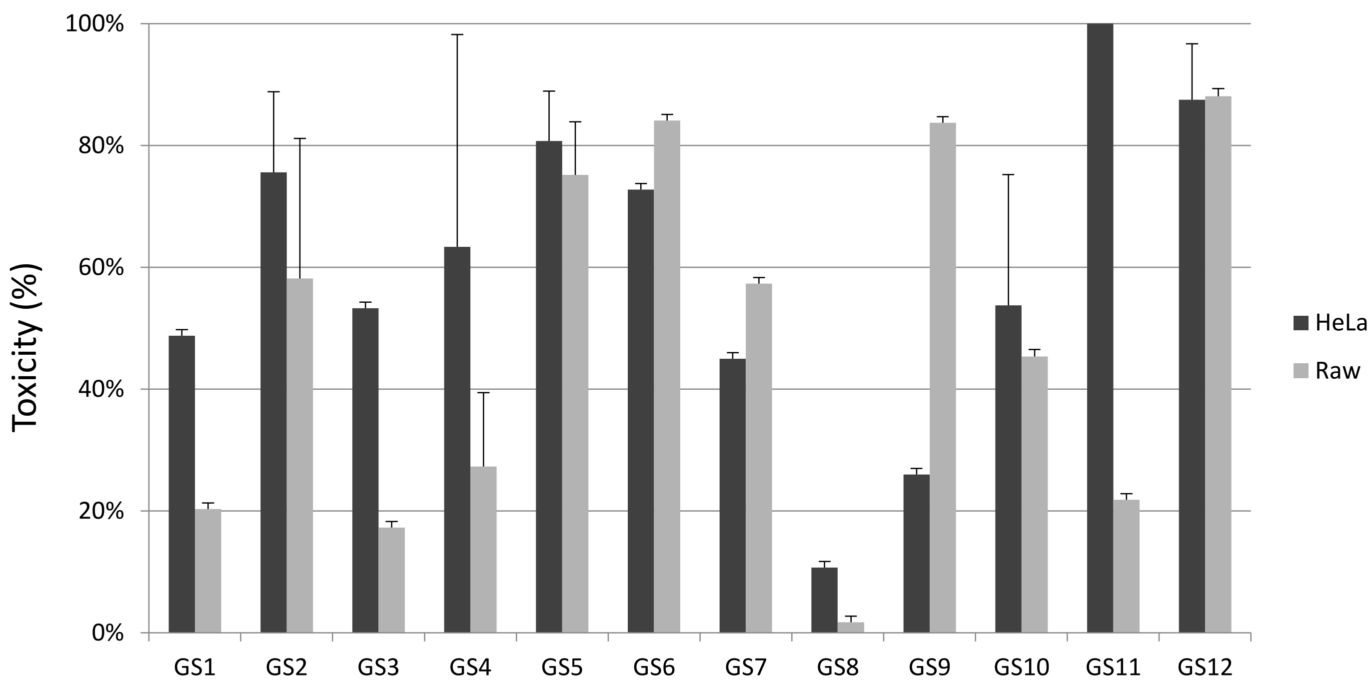


Figure 4. Average toxicity of culture supernatants of strains of *B. cereus* belonging to the GS 1 to 12.

Genetic signature	Number of strains	Genes detected						% total	F (n=42) In %	C (n=45) In %	NP (n=21) In %
		cytk-1	*cytk-2*	*ces*	*hlyII*	*nheABC*	*hblCDA*				
GS1	34	-	-	-	-	+	-	21,0	14,3	33,9	-
GS2	28	-	+	-	-	+	+	26,9	38,1	21,4	19,0
GS3	25	-	-	+	-	+	-	13,4	19,0	14,3	-
GS4	18	-	+	-	-	+	-	10,1	4,8	16,1	4,8
GS5	18	-	-	-	+	+	+	3,4	2,4	3,6	4,8
GS6	10	-	+	-	+	+	+	3,4	4,8	3,6	-
GS7	8	+	/	/	/	/	/	3,4	9,5	-	-
GS8	6	-	-	-	-	BC	AD	9,2	-	-	52,4
GS9	4	-	+	-	+	+	-	2,5	4,8	1,8	-
GS10	5	-	-	-	-	+	+	3,4	-	1,8	14,3
GS11	1	-	+	+	-	+	-	0,8	-	1,8	-
GS12	2	-	-	-	+	+	-	2,5	2,4	1,8	4,8

/ : primers used are unable to detect the genes in GS7 group except Cytk-1

BC: only *nheB* and *nheC* detected by PCR designed

AD: only *hblA* and *hblD* detected by PCR designed

Table 1: Genetic signatures (GS) of *B. cereus* strains according to gene detection and repartition in the FBO (F), Clinical (C) and non-pathogenic (NP) strain collections.