Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures

Alizée Guérin, Helene Thorsen Rønning, Claire Dargaignaratz, Thierry Clavel, Veronique Broussolle, Jacques Mahillon, Per Einar Granum, Christophe Nguyen-The

► To cite this version:

Alizée Guérin, Helene Thorsen Rønning, Claire Dargaignaratz, Thierry Clavel, Veronique Broussolle, et al.. Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures. *Food Microbiology*, 2017, 65, pp.130-135. 10.1016/j.fm.2017.02.006 . hal-01602612

HAL Id: hal-01602612

<https://hal.science/hal-01602612>

Submitted on 12 Oct 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Accepted Manuscript

Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures

Alizée Guérin, Helene Thorsen Rønning, Claire Dargaignaratz, Thierry Clavel, Véronique Broussolle, Jacques Mahillon, Per Einar Granum, Christophe Nguyen-The

PII: S0740-0020(16)30884-X

DOI: [10.1016/j.fm.2017.02.006](https://doi.org/10.1016/j.fm.2017.02.006)

Reference: YFMIC 2739

To appear in: *Food Microbiology*

Received Date: 19 October 2016

Revised Date: 13 February 2017

Accepted Date: 15 February 2017

Please cite this article as: Guérin, A., Rønning, H.T., Dargaignaratz, C., Clavel, T., Broussolle, V., Mahillon, J., Granum, P.E., Nguyen-The, C., Cereulide production by *Bacillus weihenstephanensis* strains during growth at different pH values and temperatures, *Food Microbiology* (2017), doi: 10.1016/j.fm.2017.02.006.

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Cereulide production by *Bacillus weihenstephanensis* strains during growth
at different pH values and temperatures

Alizée Guérin¹, Helene Thorsen Rønning³, Claire Dargaignaratz¹, Thierry Clavel¹,

Véronique Broussolle¹, Jacques Mahillon², Per Einar Granum³ and Christophe Nguyen-the^{1*}

¹ UMR408 SQPOV « Sécurité et Qualité des Produits d'Origine Végétale », INRA,
University of Avignon, 84000 Avignon, France

² Laboratory of Food and Environmental Microbiology, Université catholique de Louvain,
Louvain-la-Neuve, Belgium

³ Norwegian University of Life Sciences, NMBU School of Veterinary Science, Oslo,
Norway

*Corresponding author. Mailing address: INRA, UMR408, site Agroparc, 84914 Avignon
cedex 9, France. Phone: +33 (0)4 32 72 25 21, Fax: +33 (0)4 32 72 24 92, Email:
christophe.nguyen-the@inra.fr

Abstract

Besides *Bacillus cereus*, some strains of the psychrotolerant, potentially foodborne pathogen *Bacillus weihenstephanensis* can produce the emetic toxin (cereulide). This toxin is a heat- and acid-stable cyclic dodecadeptide that causes food intoxication with vomiting. However, some severe clinical cases with lethal outcomes have been described. If cereulide can be produced during refrigerated storage, it will not be inactivated by reheating food, representing an important risk of food intoxication for consumers. In this paper, we determined the capacity of the *B. weihenstephanensis* strains BtB2-4 and MC67 to grow and produce cereulide on agar media at temperatures from 8 °C to 25 °C and at a pH from 5.4 to 7.0. At 8 °C, strain BtB2-4 produced quantifiable amounts of cereulide, whereas the limit of detection was reached for strain MC67. For BtB2-4, cereulide production increased 5-fold between 8 °C and 10-15 °C and by more than 100-fold between 15 °C and 25 °C. At temperatures of 10 °C and higher, cereulide concentrations were within the range of those reported by previous works in foods implicated in emetic poisoning. At 25 °C, decreasing the pH to 5.4 reduced cereulide production by strain BtB2-4 by at least 20-fold.

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

1 Introduction

Cereulide is the emetic toxin produced by some strains of the spore-forming pathogen *Bacillus cereus* (Ehling-Schulz et al., 2004; Stenfors Arnesen et al., 2008). Cereulide is a heat- and acid-stable cyclic dodecadepsipeptide, [D-*O*-Leu-D-Ala-*O*-Val-L-Val]₃, with a molecular mass of 1,153 Da (Agata et al., 1995; Teplova et al., 2006). Cereulide is synthesized by a non-ribosomal peptide synthase encoded by the cereulide synthase gene (*ces*) cluster (Ehling-Schulz et al., 2005). The cluster is located on the megaplasmid pCER270 which is related to the toxin plasmid pXO1 of *Bacillus anthracis* (Ehling-Schulz et al., 2015; Ehling-Schulz et al., 2006a; Hoton et al., 2005). The effects of cereulide on the human body have not yet been completely elucidated. In the duodenum, this toxin acts on the 5-HT₃ receptor and causes vomiting by stimulation of the afferent vagus nerve 0.5-6 h after the ingestion of contaminated food (Agata et al., 1995; Ehling-Schulz et al., 2004). It acts as a K⁺ ionophore with a mechanism of action similar to that of valinomycin. It interferes with oxidative phosphorylation on mitochondrial membranes (Hoton et al., 2005; Mikkola et al., 1999); (Paananen et al., 2002; Teplova et al., 2006). Cereulide has been involved in severe clinical cases, leading in some cases to death of the patient (Dierick et al., 2005; Mahler et al., 1997; Naranjo et al., 2011).

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

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

2 Materials and methods

2.1 Strains

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

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

2.2 Growth conditions

Growth conditions were adapted from (Rossvoll et al., 2014): briefly, the stock cultures consisted of exponential phase cells (OD₆₀₀ of 0.5) in 30 % glycerol, stored at -80 °C. Frozen suspensions were streaked onto plate count agar (PCA; Oxoid) and incubated at 30 °C overnight. Three colonies were transferred to 10 ml Tryptic Soy Broth (TSB; Oxoid) in KIMAX tubes and cultured for 18 to 20 h at 20 °C while shaking at 200 rpm. The cultures were then incubated at 4 °C for 18-21 h for cold adaptation prior to growth (Rossvoll et al., 2014).

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

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

2.3 Extraction of cereulide

For cereulide extraction (Ronning et al., 2015), 1.4 ml of all samples were transferred into 2-ml non-skirted microtubes with O-ring seal caps (Dutscher) with 0.3 g of 0.1 mm silica spheres (MP Biomedical), and the cells were disrupted for 45 s at 1500 beats per min (bpm) using a Mini-GTM (SPEX[®] SAMPLEPREP). After centrifugation (3 min at 19,000 × g), 1 ml of each sample supernatant was recovered in 10 ml glass tubes. Ten nanograms of an internal standard, ¹³C₆-cereulide (> 95% purity, 20 µg/ml in acetonitrile; Chiralix), and 1 ml of methanol LC-MS Grade (Fischer chemical) were added to each glass tubes. All samples were vigorously shaken for 30 s and evaporated to dryness by heating at 80 °C under a steady flow of nitrogen. Then, 1 ml of methanol was added, and all samples were vigorously shaken for 30 s and centrifuged 5 min at 5,000 × g. The supernatant was recovered in a new glass tube before a second evaporation step at 80 °C under a nitrogen flow. When the samples were completely dry, 100 µl of methanol/sterile water (3/1 vol/vol) was added to the glass tubes, which were immediately vortexed for 30 s. The 100-µl samples were spun down and transferred through a 0.2-µm nylon Micro centrifuge filter (Sigma-Aldrich) and centrifuged 1 min at 10,000 × g. Filtrates were then transferred into HPLC vials (screw vials 8-mm amber glass 12*32 mm with label; Interchim) containing a 0.1-ml micro insert (Sigma-Aldrich) and stored at -20 °C before analysis by LC-MS/MS.

2.4 Calibration curve

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

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

2.5 Quantification of cereulide by LC-MSMS

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

2.6 Expression of results

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

2.7 Statistical analysis

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

3 Results

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

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

3.1 Influence of growth temperature on cereulide production

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

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

9.81±0.01 log₁₀ CFU/ml on TSA plates, which was statistically significant ($p < 0.05$) (Fig. 1 and 2). The maximal cereulide production of the two strains on TSA plates at 10 °C, 6.83±1.3 ng/ml for BtB2-4 and 4.30±4.18 ng/ml for MC67, was not significantly different ($p > 0.05$) (Fig 1). While the cereulide production of strain BtB2-4 (9.01±2.91 ng/ml) was similar on TSA and on PCA plates ($p > 0.05$), the production of strain MC67 was increased on TSA plates (4.30±4.18 ng/ml) compared with PCA plates at 7 days (0.85±0.40 ng/ml). However, this difference was not significant ($p > 0.05$) and the cereulide production of that strain was similarly low on both TSA and PCA plates for other incubation times at 10 °C (< 0.6 ng/ml) (Fig 1). Therefore, the two strains produced the same amount of cereulide at 10°C on both TSA and PCA.

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

3.2 Influence of pH on cereulide production

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

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

4 Discussion

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

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

When MC67 grew at 25 °C, cereulide production did not exceed 13 ng/ml in the extract, corresponding to 22 ng/plate, which was low compared to previous results showing that MC67 produced up to 14 µg/cm² after 24 h at 25 °C (Thorsen et al., 2009a). This may be due to differences in the protocols used, such as preparation of the inoculum and/or growth media (Rossvoll et al., 2014), as previously suggested by (Thorsen et al., 2009a) for *B. weihenstephanensis* and (Dommel et al., 2010) for mesophilic *B. cereus*. In particular, after a pre-incubation of the inoculated PCA plates at 8 °C for 2 or 3 weeks, the MC67 cereulide production at 25 °C was approximately 10,000 times lower (0.001 µg/cm²) than without pre-incubation (Thorsen et al., 2009a). In the present study, the pre-incubation at 4 °C during the 18-21 h before growth experiments could explain the low cereulide production. The expression of the *ces* genes is activated by Spo0A and repressed by CodY, which linked cereulide production to the early stage of sporulation and to the nutritional and energy status

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

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

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

In our study, lowering the growth temperature markedly reduced cereulide production for both *B. weihenstephanensis* strains. This is particularly clear for strain BtB2-4, which 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. Less cereulide was produced by a mesophilic *B. cereus* on media rich in proteinaceous ingredients such as TSA (Dommel et al., 2010) and NaCl, which is absent in PCA, inhibited cereulide production (Dommel et al., 2011). The same low production of cereulide at low

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

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

313 Acknowledgements

314 This study is part of the OPTIFEL project that received funding from the European Union's
315 Seventh Framework Program for research, technological development and demonstration
316 under grant agreement n° 311754. AG's PhD grant is also from the EU Optifel project.

317

References

- Agata, N., Ohta, M., Mori, M., Isobe, M., 1995. A novel dodecadepsipeptide, cereulide, is an emetic toxin of *Bacillus cereus*. FEMS Microbiol. Lett. 129, 17-19.
- Agata, N., Ohta, M., Yokoyama, K., 2002. Production of *Bacillus cereus* emetic toxin (cereulide) in various foods. Int. J. Food Microbiol. 73, 23-27.
- Biesta-Peters, E.G., Reij, M.W., Blaauw, R.H., in't Veld, P.H., Rajkovic, A., Ehling-Schulz, M., Abee, T., 2010. Quantification of the Emetic Toxin Cereulide in Food Products by Liquid Chromatography-Mass Spectrometry Using Synthetic Cereulide as a Standard. Appl. Environ. Microbiol. 76, 7466-7472.
- Carlin, F., Fricker, M., Pielaat, A., Heisterkamp, S., Shaheen, R., Salkinoja Salonen, M., Svensson, B., Nguyen-the, C., Ehling-Schulz, M., 2006. Emetic toxin-producing strains of *Bacillus cereus* show distinct characteristics within the *Bacillus cereus* group. Int. J. Food Microbiol. 109, 132-138.
- Dierick, K., Van Coillie, E., Swiecicka, I., Meyfroidt, G., Devlieger, H., Meulemans, A., Hoedemaekers, G., Fourie, L., Heyndrickx, M., Mahillon, J., 2005. Fatal family outbreak of *Bacillus cereus*-associated food poisoning. J. Clin. Microbiol. 43, 4277 - 4279.
- Dommel, M.K., Frenzel, E., Strasser, B., Blochinger, C., Scherer, S., Ehling-Schulz, M., 2010. Identification of the Main Promoter Directing Cereulide Biosynthesis in Emetic *Bacillus cereus* and Its Application for Real-Time Monitoring of *ces* Gene Expression in Foods. Appl. Environ. Microbiol. 76, 1232-1240.
- Dommel, M.K., Lucking, G., Scherer, S., Ehling-Schulz, M., 2011. Transcriptional kinetic analyses of cereulide synthetase genes with respect to growth, sporulation and emetic toxin production in *Bacillus cereus*. Food Microbiol. 28, 284-290.
- Durack, J., Ross, T., Bowman, J.P., 2013. Characterisation of the Transcriptomes of Genetically Diverse *Listeria monocytogenes* Exposed to Hyperosmotic and Low Temperature Conditions Reveal Global Stress-Adaptation Mechanisms. PLoS One 8.
- Ehling-Schulz, M., Frenzel, E., Gohar, M., 2015. Food-bacteria interplay: pathometabolism of emetic *Bacillus cereus*. Front. Microbiol. 6:704.

- 347 Ehling-Schulz, M., Fricker, M., Grallert, H., Rieck, P., Wagner, M., Scherer, S., 2006a.
 348 Cereulide synthetase gene cluster from emetic *Bacillus cereus*: Structure and location on a
 349 mega virulence plasmid related to *Bacillus anthracis* toxin plasmid pXOI. BMC Microbiol.
 350 6:20.
- 351 Ehling-Schulz, M., Fricker, M., Scherer, S., 2004. *Bacillus cereus*, the causative agent of an
 352 emetic type of food-borne illness. Mol. Nutr. Food Res. 48, 479-487.
- 353 Ehling-Schulz, M., Vukov, N., Schulz, A., Shaheen, R., Andersson, M., Martlbauer, E.,
 354 Scherer, S., 2005. Identification and partial characterization of the nonribosomal peptide
 355 synthetase gene responsible for cereulide production in emetic *Bacillus cereus*. Appl.
 356 Environ. Microbiol. 71, 105-113.
- 357 Guérin, A., Dargaignaratz, C., Broussolle, V., Clavel, T., Nguyen-the, C., 2016. Combined
 358 effect of anaerobiosis, low pH and cold temperatures on the growth capacities of
 359 psychrotrophic *Bacillus cereus*. Food Microbiol. 59, 119-123.
- 360 Guinebretière, M.H., Thompson, F.L., Sorokin, A., Normand, P., Dawyndt, P., Ehling-Schulz,
 361 M., Svensson, B., Sanchis, V., Nguyen-The, C., Heyndrickx, M., De Vos, P., 2008.
 362 Ecological diversification in the *Bacillus cereus* Group. Env. Microbiol. 10, 851-865.
- 363 Guinebretiere, M.H., Velge, P., Couvert, O., Carlin, F., Debuyser, M.L., Nguyen-The, C.,
 364 2010. Ability of *Bacillus cereus* group strains to cause food poisoning varies according to
 365 phylogenetic affiliation (groups I to VII) rather than species affiliation. J. Clin. Microbiol. 48,
 366 3388-3391.
- 367 Hoton, F., Andrup, L., Swiecicka, I., Mahillon, J., 2005. The cereulide genetic determinants
 368 of emetic *Bacillus cereus* are plasmid-borne. Microbiology 151, 2121-2124.
- 369 Hoton, F., Fornelos, N., N'Guessan, E., Hu, X., Swiecicka, I., Dierick, K., Jaaskelainen, E.,
 370 Salkinoja-Salonen, M., Mahillon, J., 2009. Family portrait of *Bacillus cereus* and *Bacillus*
 371 *weihenstephanensis* cereulide-producing strains. Environ. Microbiol. Rep. 1, 177-183.
- 372 Kranzler, M., Stollewerk, K., Rouzeau-Szynalski, K., Blayo, L., Sulyok, M., Ehling-Schulz,
 373 M., 2016. Temperature Exerts Control of *Bacillus cereus* Emetic Toxin Production on Post-
 374 transcriptional Levels. Front. Microbiol. 7.
- 375 Mahler, H., Pasi, A., Kramer, J., Schulte, P., Scoging, A., Bar, W., Krahenbuhl, S., 1997.
 376 Fulminant liver failure in association with the emetic toxin of *Bacillus cereus*. N. Engl. J.
 377 Med. 336, 1142-1148.

- 378 Mei, X., Xu, K., Yang, L., Yuan, Z., Mahillon, J., Hu, X., 2014. The genetic diversity of
379 cereulide biosynthesis gene cluster indicates a composite transposon Tnces in emetic *Bacillus*
380 *weihenstephanensis*. BMC Microbiol. 14, 149.
- 381 Mikkola, R., Saris, N., Grigoriev, P., Andersson, M., Salkinoja-Salonen, M., 1999.
382 Ionophoretic properties and mitochondrial effects of cereulide - the emetic toxin of *B. cereus*.
383 Eur. J. Biochem. 263, 112-117.
- 384 Naranjo, M., Denayer, S., Botteldoorn, N., Delbrassinne, L., Veys, J., Waegenaere, J.,
385 Sirtaine, N., Driesen, R., Sipido, K., Mahillon, J., Dierick, K., 2011. Sudden death of a young
386 adult associated with *Bacillus cereus* food poisoning. J. Clin. Microbiol. 49, 4379-4381.
- 387 Paananen, A., Mikkola, R., Sareneva, T., Matikainen, S., Hess, M., Andersson, M., Julkunen,
388 I., Salkinoja-Salonen, M., Timonen, T., 2002. Inhibition of human natural killer cell activity
389 by cereulide, an emetic toxin from *Bacillus cereus*. Clin. Exp. Immunol. 129, 420-428.
- 390 Ronning, H.T., Asp, T.N., Granum, P.E., 2015. Determination and quantification of the
391 emetic toxin cereulide from *Bacillus cereus* in pasta, rice and cream with liquid
392 chromatography-tandem mass spectrometry. Food Add. Contam.: Part A 32, 911-921.
- 393 Rossvoll, E., Ronning, H.T., Granum, P.E., Moretro, T., Hjerpekkjon, M.R., Langsrud, S.,
394 2014. Toxin production and growth of pathogens subjected to temperature fluctuations
395 simulating consumer handling of cold cuts. Int. J. Food Microbiol. 185, 82-92.
- 396 Sorokin, A., Candelon, B., Guilloux, K., Galleron, N., Wackerow-Kouzova, N., Ehrlich, S.D.,
397 Bourguet, D., Sanchis, V., 2006. Multiple-locus sequence typing analysis of *Bacillus cereus*
398 and *Bacillus thuringiensis* reveals separate clustering and a distinct population structure of
399 psychrotrophic strains. Appl. Env. Microbiol. 72, 1569-1578.
- 400 Stenfors Arnesen, L.P., Fagerlund, A., Granum, P.E., 2008. From soil to gut: *Bacillus cereus*
401 and its food poisoning toxins. Fems Microbiol. Rev. 32, 579-606.
- 402 Teplova, V.V., Mikkola, R., Tonshin, A.A., Saris, N.E.L., Salkinoja-Salonen, M.S., 2006. The
403 higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium
404 at physiological plasma concentration. Toxicol. Appl. Pharm. 210, 39-46.
- 405 Thorsen, L., Budde, B.B., Henrichsen, L., Martinussen, T., Jakobsen, M., 2009a. Cereulide
406 formation by *Bacillus weihenstephanensis* and mesophilic emetic *Bacillus cereus* at
407 temperature abuse depends on pre-incubation conditions. Int. J. Food Microbiol. 134, 133-
408 139.

- Thorsen, L., Budde, B.B., Koch, A.G., Klingberg, T.D., 2009b. Effect of modified atmosphere and temperature abuse on the growth from spores and cereulide production of *Bacillus weihenstephanensis* in a cooked chilled meat sausage. *Int. J. Food Microbiol.* 130, 172-178.
- Thorsen, L., Hansen, B.M., Nielsen, K.F., Hendriksen, N.B., Phipps, R.K., Budde, B.B., 2006. Characterization of emetic *Bacillus weihenstephanensis*, a new cereulide-producing bacterium. *Appl. Environ. Microbiol.* 72, 5118-5121.
- Vilas-Boas, G., Sanchis, V., Lereclus, D., Lemos, M.V.F., Bourguet, D., 2002. Genetic differentiation between sympatric populations of *Bacillus cereus* and *Bacillus thuringiensis*. *Appl. Env. Microbiol.* 68, 1414-1424.

Figure Legends

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

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

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