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Emetic toxin production of *Bacillus cereus* in a biofilm

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ABSTRACT

Bacillus cereus sensu stricto (*B. cereus*) belongs to the *B. cereus* group, and is a well-known foodborne pathogen causing human disease including emesis which is caused by an emetic toxin, cereulide, with 10^5-10^8 cells per gram required to cause disease. The presence of this highly heat, pH and protease-resistant toxin presents a serious challenge to the food industry, as the bacteria itself may be eliminated during processing but the cereulide toxin will survive most food processing techniques. This study shows that cereulide toxin is associated with cells and biofilm structures rather than suspended in the surrounding liquid phase or environment. This is the first report investigating the cereulide toxin production in the presence of biofilms of *B. cereus*, showing that the cereulide toxin produced is associated with biofilm complex and also attaches to the substrate such as glass and stainless-steel on which the biofilm grows. The RT-qPCR showed that the expression of *cesA* and *cesB* were comparable between planktonic cells and biofilms. This study contributes a better understanding of food safety issues in the industry caused by cereulide toxin produced by *B. cereus*, and provides valuable information for developing control methods for cereulide toxin in the food industry.

1. Introduction

Bacillus cereus sensu stricto (*B. cereus*), belongs to *Bacillus cereus sensu lato* (*B. cereus s.l.*), is an opportunistic human pathogen causing foodborne diseases, presenting in different raw materials as well as processed food, such as rice, vegetables, dairy products etc. (Burgess, Flint, & Lindsay, 2014; Kim et al., 2014; Park et al., 2009; Yibar, Çetinkaya, Soyutemiz, & Yaman, 2017). Emetic *B. cereus* strains can produce a highly heat- and acid-resistant emetic toxin called "cereulide". The cereulide toxin is of special concern, it will stay in the food and processing line, although the bacteria itself may be eliminated (Rouzeau-Szynalski, Stollewerk, Messelhäusser, & Ehling-Schulz, 2020). The emesis syndrome is caused by the cereulide toxin, which is usually formed in the contaminated food before ingestion (Ehling-Schulz, Fricker, & Scherer, 2004).

Cereulide is a small dodecadepsipeptide toxin (1.2 KDa) (Agata et al., 1994), synthesized by a non-ribosomal peptide-synthetase (NRPS), called CesNRPS, and its gene cluster (*ces*) comprises seven genes (*cesHPTABCD*) with different responsibilities in the synthetic process, such as *cesA* and *cesB* that are responsible for the assembly (Ehling-Schulz et al., 2006). Cereulide is a potassium ionophore and has a structural similarity with the antibiotic valinomycin (Agata et al., 1994). The toxin can cause the disturbance of mammalian cell membranes and is also known to inhibit mitochondrial activity that can lead to vomiting, liver damage or multi-organ failure and even death (Mahler et al., 1997; Mikkola, Saris, Grigoriev, Andersson, & Salkinoja-Salonen, 1999; Tschiedel et al., 2015). Cereulide production starts at the late exponential phase of growth and continues throughout the stationary phase (Häggblom, Apetroaie, Andersson, & Salkinoja-Salonen, 2002). However, the toxin production is affected by strain variability as well as environmental conditions such as nutrient availability, temperature and oxygen (Ehling-Schulz, Frenzel, & Gohar, 2015; Häggblom et al., 2002; Jääskeläinen, Häggblom, Andersson, & Salkinoja-Salonen, 2004; Kranzler et al., 2016).

Once *B. cereus* is introduced to a food processing line, it can be difficult to eliminate due to the heat and chemical resistance of its spores. In addition, the spores of *B. cereus* are generally hydrophobic enabling them to adhere strongly to stainless steel surfaces commonly

Abbreviations: B. cereus, *Bacillus cereus sensu stricto*; *B. cereus s.l.*, *Bacillus cereus sensu lato*; NRPS, non-ribosomal peptide-synthetase; LC-MS/MS, liquid chromatography coupled to mass spectrometry; RT-qPCR, real-time quantitative PCR; TSA, tryptic soy agar; TSB, tryptic soy broth; GW, glass wool; SS, stainless-steel.

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found in food manufacturing plants. On attachment to surfaces, the spores can germinate and form biofilms, which enhances the resistance of bacteria to heating treatments or cleaning agents (Andersson, Rönner, & Granum, 1995; Kumari & Sarkar, 2016). Joaquín Caro-Astorga et al. (2019) showed that enterotoxin gene expression was reduced in biofilm cells compared to planktonic cells. However, the relationship between emetic toxin and biofilm for *B. cereus* is unclear. This study aimed to investigate the cereulide toxin production in biofilms of *B. cereus* using liquid chromatography coupled to mass spectrometry (LC-MS/MS). Both glass and stainless steel materials were used in this study to investigate the effect of biofilm-grown substrates on cereulide production. Real-time PCR was also used to predict the cereulide producing ability of biofilm cells. This study aimed to provide useful information to help design control measures to reduce *B. cereus* food poisoning.

2. Materials and methods

2.1. Bacterial culture conditions

B. cereus emetic reference strain F4810/72 (DSMZ, Germany) was used in this study. Stock cultures were streaked on tryptic soy agar (TSA, DifcoTM, Becton, Dickinson and Company, USA) plates and incubated for 24 h at 30 °C to obtain single colonies. The overnight culture was obtained by inoculating a single colony into tryptic soy broth (TSB, DifcoTM, Becton, Dickinson and Company, USA) and incubating overnight (17–18 h) at 30 °C with 120 rpm shaking. The overnight culture was used for subsequent experiments. The TSB media was used throughout this study.

2.2. Biofilm and planktonic growth conditions

Two types of substrates (glass and stainless-steel) were used for biofilm growth in this study. Biofilms were grown on stainless-steel (SS) coupons (304-2B; 10 mm × 10 mm x 1 mm; Advanced Sheetmetals Ltd., Palmerston North) as described by Huang, Flint, Yu, Ding, and Palmer (2021). Briefly, the coupon was placed vertically into the well of 48-well plates (Costar®, Corning, USA) filled with TSB broth and inoculated with 1% overnight culture. Biofilm cells developed on SS coupons were swabbed and resuspended in saline (0.85% NaCl solution). Glass wool (GW) and stainless steel (SS) wool were used to provide a large surface area for bacterial attachment and biofilm growth. Approximately 0.5 g GW and 1 g SS wool were added into a 500 mL shaking flask filled with 50 mL TSB and 1% inoculum. Biofilm cells on GW or SS wool were detached using glass beads and resuspended in 50 mL saline. All the SS materials were treated with nitric acid and sterilized (autoclaved at 121 °C for 15 min). The planktonic culture was grown in the absence of wool in the flask. The toxin was also measured in planktonic cell pellets, by adding extraction solvents to the pelleted centrifuged cells as well as the supernatant after centrifugation at 11,000×*g* for 1 min at 4 °C. All the 48-well plates and flasks were incubated at 30 °C with 120 rpm agitation for 48 h incubation before toxin quantification. The numbers of bacterial cells were quantified using spread plating. Serial 10-fold dilutions were made and spread on TSA plates, and incubated at 30 °C for 18 h, then colony-forming units (CFU) were counted.

2.3. Toxin attachment on glass or stainless-steel wool

The planktonic culture was centrifuged at 11,000×*g* for 2 min at 4 °C and the supernatant was used as the toxin-containing solution. Either 0.5 g or 3 g of GW or SS wool was added into 30 mL planktonic supernatant and shaken at 120 rpm for 30 min to allow the attachment of toxin, followed by extracting and quantifying the toxin in the supernatant after attachment and extraction of the toxin on the wools. Planktonic supernatant without adding GW or SS wool was used as a control.

2.4. HPLC-MS/MS quantification of cereulide production

2.4.1. Standard solutions and calibration curves

The synthetic cereulide standard was purchased from Chiralix B.V. (Nijmegen, the Netherlands) in the form of a powder. The valinomycin (HPLC grade; ≥ 90%; Merck, USA) was used as an internal standard. Other reagents including acetonitrile, methanol, water, formic acid and ammonium formate were all purchased from Thermo Fisher Scientific (Optima™ LC/MS grade, USA). Both the synthetic cereulide and valinomycin were dissolved in methanol to concentrations of 0.01, 0.05, 0.1, 1, 5, 10 ng/mL. All of the stocks and standard solutions were stored at −20 °C until testing. The standard curves are shown in the supplementary file. The correlation effect of valinomycin and cereulide and the recovery rate of valinomycin in bacterial cultures were assessed (provided in supplementary file).

2.4.2. HPLC-MS/MS conditions

Liquid chromatographic separation was performed using an UltiMate 3000 HPLC system (Thermo Fisher Scientific, USA) equipped with an Accucore 150-C18 column (100 × 2.1 mm, 2.6 μm, 150 Å) and a matching Accucore Defender Guard Column (Thermo Fisher Scientific, Lithuania) maintained at 40 °C. The gradient used was according to Hiroshi Koike et al. (2018). Briefly, the water phase (buffer A) was applied using 1 mmol/l ammonium formate in water containing 0.1% formic acid and the organic phase was methanol containing 0.1% formic acid (buffer B). The detailed gradient elution conditions are shown in Table 1. The flow rate was 0.35 mL/min and the injection volume was 2 μl.

The mass spectrometric detection was performed using a Q Exactive™ Focus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, USA). The mass spectrometer was operated according to the parameters listed and the expected retention time for cereulide and valinomycin detection were listed in Table 2. The product ion for peak integration (quantification) used was 1125.7 (m/z) and 1083.6 (m/z) for cereulide and valinomycin, respectively (Hiroshi Koike et al., 2018).

2.4.3. Sample preparation

Cereulide in bacterial culture or on wools was extracted by acetonitrile to optimize the recovery of the toxin (Hiroshi Koike et al., 2018). One ml of culture was added with 9 mL acetonitrile and shaken for 1 h at 150 rpm, followed by centrifugation at 15,000×*g* at 4 °C using a high-speed refrigerated centrifuge (Himac CR22GII, Japan). The upper clear liquid was collected into an Eppendorf tube and centrifuged again at 4 °C. The upper one mL liquid was used for cereulide quantification. All of the tested bacterial cultures had 25 ng/mL valinomycin added as an internal standard before extraction.

2.5. Real-time PCR

To compare the toxin-producing ability between planktonic and biofilm cells, real-time quantitative PCR (RT-qPCR) was used to determine the expression of *cesA* and *cesB*. RNA was extracted from planktonic cells, biofilm on GW, biofilm on SS wool and SS coupons, in three independent preparations using the following procedure. Cultures were centrifuged at 12,000×*g* for 30 s at 4 °C and the pellets were

Table 1
LC (gradient elution) conditions for the quantification of cereulide in this study.

Time	Flow rate (ml/min)	A (%)	B (%)
0.00	0.35	85	15
2.00	0.35	5	95
4.00	0.35	5	95
6.00	0.35	85	15
10.00	Stop Run		

Table 2
Mass spectrometer settings.

Scan parameters	
Survey scan range	350–1500 *m/z*
Resolution	70,000 (MS1), 35,000 (MS2)
Polarity	Positive
MS2 isolation window	3.0 *m/z*
Default charge	1
AGC target	1e6 (MS1), 5e4 (MS2)
Max IT (ms)	auto
Microscans	1
Spectrum data type	Profile
HESI source	
Sheath gas flow rate	35 psi
Aux gas flow rate	6 psi
Spray voltage	4.5 kV
Capillary temperature	350 °C
S-lens RF level	85%
Aux gas heater temperature	275 °C
Inclusion list	
Chemical Mass (*m/z*) CS (z) Polarlity	Start-End (min) CE
Cereulide 1170.712 1	6.3–7.8 53
Valinomycin 1128.665 1	6.5–8.0 50

immediately put on ice. Nucleospin RNA Plus kit (Macherey-Nagel, Germany) was used to extract RNA from cells according to the manufacturer's instructions. Approximately 0.3–0.4 g ice-cold acid-washed glass beads (Sigma Aldrich, USA) was added to the cell pellets together with lysis buffer in the extraction kit, followed by bead beating (FastPrep® −24 Tissue, USA) for 4 min (1 min per time and 20 s paused on ice) to allow the maximum disruption of cells. The RNA concentration, purity and integrity were checked by RNA Labchip® Assay (Massey Genome Service, Massey University).

RT-qPCR was performed using the Light Cycle 480 platform (Roche Diagnostics, USA). Luna® Universal One-step RT-qPCR kit (BioLabs, UK) was used and the cycling conditions were conducted according to the manufacturer's instructions. The primers and annealing temperatures used were according to (Dommel, Lücking, Scherer, & Ehling-Schulz, 2011) as follows: *cesA* (forward: GATTACGTTCGATTATTTGAAG; reverse: CGTAGTGGCAATTTCGCAT; annealing temperature: 53 °C); *cesB* (forward: TTAGATGGTATTCTTCACTTGGC; reverse: TTGATACAAATCGCATTCTTATAACC; annealing temperature: 57 °C). *16S* genes (forward: GGAGGAAGGTGGGGATGACG; reverse: ATGGTGTGACGGGCGGTGTG; annealing temperature: 63 °C) used for normalization (Dommel et al., 2011). The relative gene expression analysis was performed applying the $2^{-\Delta\Delta Ct}$ Livak method (Livak & Schmittgen, 2001). The data shown in this study was indicated as fold changes compared to the planktonic cells.

2.6. Statistical analysis

The statistical analysis was done by GraphPad Prism 7. The data expressed were generated from the average value of three independent biological repetitions. Standard deviations represent the variation among replicates. One-way ANOVA (Tukey's multiple comparison test) by GraphPad Prism 7 was used to determine the statistically significant ($P < 0.05$) differences between conditions.

3. Results

3.1. Standard curves for cereulide and valinomycin

Quantification values were obtained from the standard curves of the set of diluted synthetic cereulide solutions without adding valinomycin as internal standard, and a similar standard curve was prepared for valinomycin (Supplementary file). The calibration equation for cereulide toxin production was followed:

$$Y = 288057 \times X\text{-}20501 \ (R^2 = 0.9975)$$

where X is the concentration of toxin and Y is the peak absolute area, R^2 determined the coefficient of the linear regression.

3.2. Toxin production in planktonic growth

The toxin was measured in planktonic cell pellets and supernatant to determine the binding relationship between the toxin and cells (Table 3). The planktonic culture and its resuspended cell pellets contained approximately 8.8 Log CFU/mL cells. The resuspended cell pellets contained the majority of the toxin compared to the total planktonic culture, containing 852.23 ± 111.03 ng per mL culture. The supernatant contained only 88.31 ± 19.43 ng per mL culture, indicating the cereulide toxin was associated with cells instead of releasing into the surrounding medium.

3.3. Toxin production in biofilm growth

3.3.1. Biofilm grown on stainless-steel coupon

The toxin was measured in the presence of biofilms grown on the SS coupon, and the result is shown in Table 4. Cereulide toxin in the planktonic culture surrounding SS coupon contained 1.95 ± 0.74 ng/10^8 cells of cereulide toxin, which is significantly ($P < 0.05$) less than in the planktonic culture without the insertion of the SS coupon (10.59 ± 4.12 ng/10^8 cells), although the cell counts were similar in two cultures. Biofilm cells were removed by swabbing the SS coupons (20 coupons in total) and the cells were resuspended into the saline solution and contained 22.04 ± 5.07 ng per 10^8 cells of cereulide toxin associated with the biofilm cells removed by swabbing.

3.3.2. Biofilm grown on glass and stainless steel wool

Glass (GW) and stainless-steel (SS) wool were used to create a larger surface area to support biofilm development, and the cereulide toxin quantification is shown in Tables 5 and 6, respectively. The planktonic cultures surrounding GW contained similar amounts of cells (8.76 ± 0.22 Log CFU/mL) compared with the pure planktonic culture (8.82 ± 0.09 Log CFU/mL), however, cereulide toxin was significantly ($P < 0.05$) less in the presence of GW (61.85 ± 4.75 ng/10^8 cells). Biofilms developed on GW were detached and resuspended in saline followed by toxin quantification to determine the toxin associated with the biofilm complex. The resuspension of detached biofilm from 0.5 g GW contained significantly higher amounts of cereulide (234.86 ± 2.64 ng/10^8 cells) than planktonic culture (143.89 ± 39.04 ng/10^8 cells).

Similar to biofilm grown on GW, approximately 1 g of SS wool was added in the media to allow biofilm growth, and toxin quantification as shown in Table 6. Planktonic culture influenced by the presence SS wool contained similar amounts of cereulide (176.28 ± 69.15 ng/10^8 cells) to planktonic cultures (143.89 ± 39.04 ng/10^8 cells), and the CFU numbers for the two cultures were similar, containing 8.84 ± 0.06 Log CFU/mL and 8.82 ± 0.09 Log CFU/mL, respectively. Detached biofilm cells resuspension from 1 g SS wool contained significantly ($P < 0.05$) higher amounts of toxin (871.18 ± 31.57 ng/10^8 cells) than the planktonic culture, although only 7.31 ± 0.01 Log CFU/mL cells detached from the

Table 3
Cereulide toxin production in planktonic growth. One-way ANOVA (Tukey's multiple comparison test) was performed, and different alphabets within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/mL)
Planktonic culture	932.79 ± 115.60 [a]
Planktonic cell pellets	852.23 ± 111.03 [a]
Planktonic supernatant	88.31 ± 19.43 [b]

Table 4
Cereulide toxin production in the presence of biofilms grown on stainless-steel coupons. The amounts of cereulide in cultures or biofilm resuspensions were normalized to the same amounts of cells (8 log CFU/mL). One-way ANOVA (Tukey's multiple comparison test) was performed, and different alphabets within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8 cells)	Cell counts (log CFU/mL)
Planktonic culture in 48-well plate	10.59 ± 4.12 [a]	8.69 ± 0.15
Planktonic influenced by SS coupons	1.95 ± 0.74 [b]	8.89 ± 0.14
SS coupons swabbed	22.04 ± 5.07 [c]	7.77 ± 0.10

Table 5
Cereulide toxin production in the presence of biofilms grown on glass wool. Approximately 0.5 g GW were added to the medium. The amounts of cereulide in cultures or biofilm resuspensions were normalized to the same amounts of cells (8 log CFU/mL). One-way ANOVA (Tukey's multiple comparison test) was performed, and different alphabets within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (log CFU/mL)
Planktonic culture	143.89 ± 39.04 [a]	8.82 ± 0.09
Planktonic influenced by 0.5 g GW biofilm	61.85 ± 4.75 [b]	8.76 ± 0.22
Detached 0.5 g GW biofilm	234.86 ± 2.64 [c]	7.44 ± 0.02

Table 6
Cereulide toxin production in the presence of biofilms grown on stainless-steel wool. Approximately 1 g of SS wool was added to the medium. The amounts of cereulide in cultures or biofilm resuspensions were normalized to the same amounts of cells (8 log CFU/mL). One-way ANOVA (Tukey's multiple comparison test) was performed, and different alphabets within each type of culture indicate a significant difference ($P < 0.05$).

	Cereulide (ng/10^8)	Cell counts (log CFU/mL)
Planktonic culture	143.89 ± 39.04 [a]	8.82 ± 0.09
Planktonic culture influenced by 1 g SS wool	176.28 ± 69.15 [a]	8.84 ± 0.06
Detached 1 g SS wool	871.18 ± 31.57 [b]	7.31 ± 0.01

SS wool.

3.4. Toxin attachment on glass and stainless-steel wool

Less cereulide toxin was measured in the planktonic cells surrounding GW or SS wool than planktonic culture alone (Tables 5 and 6). From this result, it was speculated that the toxin was associated with or attached to the GW or SS wool. To investigate this, the supernatant of a planktonic culture was used as a toxin-containing solution and GW and SS wool were added to this and then removed after 30 min, and the cereulide was extracted from GW and SS wool. There were significant ($P < 0.05$) losses in the planktonic supernatants after adding either GW or SS wool (9.44 ± 0.75 to 16.86 ± 1.1 ng/mL), compared with the supernatant alone (90.02 ± 9.99 ng/mL). The toxin attached to wools was measured with similar amounts (outlined in Table 7) of the toxin compared with the original planktonic supernatant.

3.5. RT-qPCR of cereulide toxin-related genes

The expression of *cesA* and *cesB* which are responsible for synthesizing cereulide toxin (Ehling-Schulz et al., 2006) was measured in planktonic cells and three types (grown on glass wool, stainless-steel wool and stainless-steel coupon) of biofilm cells using RT-qPCR. There were no statistically significant differences in the expression levels of the two tested genes in planktonic and biofilm cells, suggesting a similar amount of toxin synthesis for planktonic and biofilm cells (Fig. 1).

Table 7
The attachment of cereulide toxin on glass or stainless-steel wool. Either 0.5 g or 3 g wool was served as attaching substrate for toxin which was contained in the planktonic supernatant. The toxin on the wool was also measured after 30 min attachment. One-way ANOVA (Tukey's multiple comparison test) was performed, and different alphabets within each type of culture indicate a significant difference ($P < 0.05$).

		Cereulide (ng/mL) or (ng/wool)
Planktonic supernatant after	Planktonic supernatant	90.02 ± 9.99 [a]
	Added 0.5 g GW	15.51 ± 0.6 [b]
	Added 3 g GW	9.44 ± 0.75 [c]
	Added 0.5 g SS wool	16.86 ± 1.1 [b]
	Added 3 g SS wool	13.07 ± 0.64 [b]
Toxin on	0.5 g GW after attachment	63.12 ± 9.37 [d]
	3 g GW after attachment	60.27 ± 13.89 [a]
	0.5 g SS wool after attachment	103.39 ± 6.94 [a]
	3 g SS wool after attachment	108.34 ± 4.59 [a]

4. Discussion

Cereulide is the potent toxin produced by emetic *B. cereus* strains, challenging safety in the food industry. Although *B. cereus* cells may be eliminated during processing, the emetic toxin can remain behind and is extremely difficult to destroy. Foodborne intoxication after consuming the cereulide toxin-containing food products can result in emesis (vomiting) (Ehling-Schulz et al., 2004; Rouzeau-Szynalski et al., 2020). This study showed that the cereulide toxin produced by planktonic cells grown in liquid culture was mainly associated with cells rather than secreted into the surrounding environment. Past studies on cereulide detection generally used the pelleted cultures or collected biomass from agar plates (Altayar & Sutherland, 2006; Ulrich, Gottschalk, Dietrich, Märtlbauer, & Gareis, 2019; Yamaguchi, Kawai, Kitagawa, & Kumeda, 2013), however, they did not specify if the toxin was associated with cells. The current study has demonstrated that the emetic toxin is closely associated with vegetative cells.

Intensive studies regarding the prevalence of cereulide in food products, development of quantification methods and extrinsic factors influencing toxin production have been conducted and recently reviewed by Rouzeau-Szynalski et al. (2020). In the present study, the toxin measured in the liquid culture in a stationary well-plate (10.59 ±

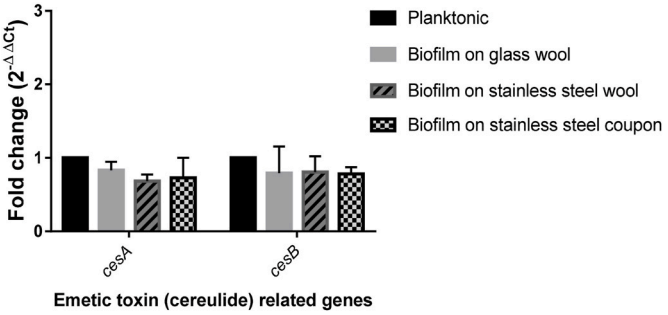

Fig. 1. Real-time PCR assay for relative quantification of two cereulide synthesis-related genes (*cesA* and *cesB*) in planktonic cells and three types of biofilm cells (on glass wool, stainless-steel wool and stainless-steel coupon). Planktonic samples were treated as the calibrator (value = 1); value > 1 presents gene up-regulation while 0 < value < 1 presents down-regulation compared with the gene expression in planktonic cells. The results are expressed as the mean ± SD of three biological preparations.

4.12 ng/10^8) was significantly ($P < 0.05$) less than culture grown in a shaking flask (143.89 ± 39.04 ng/10^8) containing similar amounts of cells, which is in agreement with Dommel et al. (2011) illustrating that the gene expression level and toxicity of cereulide was not simply associated with cells numbers, but also affected by extrinsic conditions. In the present trial, the reduced amount of toxin present in a stationary well-plate may be caused by the reduced oxygen availability compared to a shaking flask (Jääskeläinen et al., 2004).

The emetic toxin production in the biofilms of *B. cereus* is unknown. In the present study, the swabbed biofilm resuspension from SS coupons was quantified with almost double the amount of toxin (22.04 ± 5.07 ng/10^8) compared to the planktonic culture (10.59 ± 4.12 ng/10^8), however, significantly ($P < 0.05$) less toxin was detected in the planktonic cells the SS coupon culture (1.95 ± 0.74 ng/10^8). This suggests that biofilm cells are either suppressing cereulide production and/or the cereulide toxin is becoming associated with biofilm cells or the substrate. The hydrophobic property of cereulide (Agata et al., 1994) may support the attachment to hydrophobic surfaces such as SS coupons. Cereulide is the secondary metabolite via NRPS which is a costly metabolism, however, the mechanism of cereulide biosynthesis is largely unknown. The possible regulation of cereulide biosynthesis was discussed by Dietrich, Jessberger, Ehling-Schulz, Märtlbauer, and Granum (2021). As the effect of biofilms on cereulide production has been reported in the present study, it would be worthwhile to explore the role of biofilm in regulating cereulide synthesis. RNA sequencing and nuclear magnetic resonance (NMR) -based methods would be useful to investigate the transcriptomic and metabolomic profile of cereulide synthesis in biofilm forming *B. cereus* (Wu, Zhao, Lai, & Yang, 2021). Furthermore, the combined NMR and MS method may also improve the accuracy of metabolite identification (Li et al., 2021), which may be used in the future studies regarding cereulide quantification.

To create larger surfaces for biofilm development, GW and SS wool were used. Lower and comparable amounts of the toxin were measured in the planktonic cultures surrounding GW (61.85 ± 4.75 ng/10^8) and SS wool (176.28 ± 69.15 ng/10^8), respectively, while, the detached biofilm resuspension from GW and SS wool had significantly ($P < 0.05$) higher amounts of toxin (234.86 ± 2.64 and 871.18 ± 31.57 ng/10^8, respectively) than planktonic cultures (143.89 ± 39.04 ng/10^8), suggesting that cereulide toxin produced by biofilm cells is associated with the biofilm or the substrate. The toxin measured in the detached biofilm resuspension from SS wool (871.18 ± 31.57 ng/10^8) was higher than it's from GW (234.86 ± 2.64 ng/10^8), indicating a substrate effect on cereulide production. SS surfaces contain high levels of chromium oxide and iron availability (>70% of SS composition) which has been shown to enhance biofilm formation by *B. cereus* (Hayrapetyan, Muller, Tempelaars, Abee, & Nierop Groot, 2015; Rajasekar & Ting, 2011), and may also affect the cereulide production by biofilms.

The attachment of cereulide toxin on either glass or SS surface was confirmed (Table 7), supporting the previous observations in this study, which may be explained by the hydrophobic property of cereulide (Agata et al., 1994). The higher metal availability on SS may also support this attachment, as cereulide has ionophoretic properties and binds potassium ions (Mikkola et al., 1999; Teplova, Mikkola, Tonshin, Saris, & Salkinoja-Salonen, 2006), however, this needs to be confirmed. The attachment and accumulation of toxins may increase the risk of *B. cereus* in the food industry where hydrophobic surfaces such as SS are commonly used. The bacterial cells may be removed from processing lines but the toxin may remain to cause contamination of food products. This leads to the question of whether the toxin can attach to food products without the presence of the *B. cereus* cells. This needs to be clarified in the future.

The cereulide toxin-producing ability of biofilm and planktonic cells of *B. cereus* was compared using the RT-qPCR assay. It showed that the expression of *cesA* and *cesB* genes, which are structural encoding genes for cereulide toxin synthesis, were comparable between different types of biofilm cells (grown on GW, SS wool and SS coupon) and planktonic cells, suggesting a similar toxin-producing ability between the two biofilm cells and planktonic cells. The observations made in this study are not due to changes in gene expression.

5. Conclusion

This study shows that cereulide toxin produced by *B. cereus* is associated with cells or biofilm complex instead of being released into the surrounding environment. To the best of our knowledge, this is the first report of cereulide toxin associated with biofilms and will help us to better understand the food safety issues caused by cereulide and biofilms of *B. cereus*.

CRediT authorship contribution statement

Yiying Huang: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Steve H. Flint:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Trevor S. Loo:** Methodology, Writing – review & editing. **Jon S. Palmer:** Conceptualization, Funding acquisition, Methodology, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lwt.2021.112840.

References

Agata, N., Mori, M., Ohta, M., Suwan, S., Ohtani, I., & Isobe, M. (1994). A novel dodecadepsipeptide, cereulide, isolated from Bacillus cereus causes vacuole formation in HEp-2 cells. *FEMS Microbiology Letters, 121*, 31–34. http://10.1111/j.1574-6968.1994.tb07071.x.

Altayar, M., & Sutherland, A. D. (2006). Bacillus cereus is common in the environment but emetic toxin producing isolates are rare. *Journal of Applied Microbiology, 100*, 7–14. http://10.1111/j.1365-2672.2005.02764.x.

Andersson, A., Rönner, U., & Granum, P. E. (1995). What problems does the food industry have with the spore-forming pathogens Bacillus cereus and Clostridium perfringens? *International Journal of Food Microbiology, 28*, 145–155. http://10.1016/0168-1605(95)00053-4.

Burgess, S. A., Flint, S. H., & Lindsay, D. (2014). Characterization of thermophilic bacilli from a milk powder processing plant. *Journal of Applied Microbiology, 116*, 350–359. http://10.1111/jam.12366.

Dietrich, R., Jessberger, N., Ehling-Schulz, M., Märtlbauer, E., & Granum, P. E. (2021). The food poisoning toxins of Bacillus cereus. *Toxins, 13*, 98. https://doi.org/10.3390/toxins13020098

Dommel, M. K., Lücking, 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 Microbiology, 28*(2), 284–290. http://10.1016/j.fm.2010.07.001.

Ehling-Schulz, M., Frenzel, E., & Gohar, M. (2015). Food–bacteria interplay: Pathometabolism of emetic Bacillus cereus. *Frontiers in Microbiology, 6*, 704. http://10.3389/fmicb.2015.00704.

Ehling-Schulz, M., Fricker, M., & Scherer, S. (2004). Bacillus cereus, the causative agent of an emetic type of food-borne illness. *Molecular Nutrition & Food Research, 48*(7), 479–487. http://10.1002/mnfr.200400055.

Ehling-Schulz, M., Guinebretiere, M. H., Monthán, A., Berge, O., Fricker, M., & Svensson, B. (2006). Toxin gene profiling of enterotoxic and emetic Bacillus cereus. *FEMS Microbiology Letters, 260*, 232–240. http://10.1111/j.1574-6968.2006.00320.x.

Häggblom, M. M., Apetroaie, C., Andersson, M. A., & Salkinoja-Salonen, M. S. (2002). Quantitative analysis of cereulide, the emetic toxin of Bacillus cereus, produced under various conditions. *Applied and Environmental Microbiology, 68*, 2479–2483. http://10.1128/AEM.68.5.2479-2483.2002.

Hayrapetyan, H., Muller, L., Tempelaars, M., Abee, T., & Nierop Groot, M. (2015). Comparative analysis of biofilm formation by Bacillus cereus reference strains and undomesticated food isolates and the effect of free iron. *International Journal of Food Microbiology, 200*, 72–79. http://10.1016/j.ijfoodmicro.2015.02.005.

Hiroshi Koike, M. K., Hayashi, H., Matsushima, Y., Ohba, Y., Nakagawa, Y., Nagano, C., et al. (2018). Identification and quantification of cereulide in cow's milk using liquid chromatography-tandem mass spectrometry. *Food Additives & Contaminants, 35*(12), 2424–2433. http://10.1080/19440049.2018.1544722.

Huang, Y., Flint, S. H., Yu, S., Ding, Y., & Palmer, J. S. (2021). Phenotypic properties and genotyping analysis of Bacillus cereus group isolates from dairy and potato products. *LWT-Food Science and Technology, 140*, 110853. http://10.1016/j.lwt.2021.110853.

Jääskeläinen, E. L., Häggblom, M. M., Andersson, M. A., & Salkinoja-Salonen, M. S. (2004). Atmospheric oxygen and other conditions affecting the production of cereulide by Bacillus cereus in food. *International Journal of Food Microbiology, 96*, 75–83. http://10.1016/j.ijfoodmicro.2004.03.011.

Joaquín Caro-Astorga, E. F., Perkins, J. R., Ana Álvarez-Mena, Antonio de Vicente, Ranea, Juan A. G., Kuipers, Oscar P., et al. (2019). Biofilm formation displays intrinsic offensive and defensive features of Bacillus cereus. *Npj Biofilms and Microbiomes, 6*(3). https://doi.org/10.1038/s41522-019-0112-7

Kim, B., Bang, J., Kim, H., Kim, Y., Kim, B.s., Beuchat, L. R., et al. (2014). Bacillus cereus and Bacillus thuringiensis spores in Korean rice: Prevalence and toxin production as affected by production area and degree of milling. *Food Microbiology, 42*, 89–94. http://10.1016/j.fm.2014.02.021.

Kranzler, M., Stollewerk, K., Rouzeau-Szynalski, K., Blayo, L., Sulyok, M., & Ehling-Schulz, M. (2016). Temperature exerts control of Bacillus cereus emetic toxin production on post-transcriptional levels. *Frontiers in Microbiology, 7*(1640). http://10.3389/fmicb.2016.01640.

Kumari, S., & Sarkar, P. K. (2016). Bacillus cereus hazard and control in industrial dairy processing environment. *Food Control, 69*, 20–29. http://10.1016/j.foodcont.2016.04.012.

Li, S., Tian, Y., Jiang, P., Lin, Y., Liu, X., & Yang, H. (2021). Recent advances in the application of metabolomics for food safety control and food quality analyses. *Critical Reviews in Food Science and Nutrition, 61*, 1448–1469. https://doi.org/10.1080/10408398.2020.1761287

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method. *Methods, 25*(4), 402–408. http://doi:10.1006/meth.2001.1262.

Mahler, H., Pasi, A., Kramer, J. M., Schulte, P., Scoging, A. C., Bär, W., et al. (1997). Fulminant liver failure in association with the emetic toxin of Bacillus cereus. *New England Journal of Medicine, 336*(16), 1142–1148. http://10.1056/nejm199704173361604.

Mikkola, R., Saris, N. E. L., Grigoriev, P. A., Andersson, M. A., & Salkinoja-Salonen, M. S. (1999). Ionophoretic properties and mitochondrial effects of cereulide. The emetic toxin of B. cereus. *European Journal of Biochemistry, 263*, 112–117. http://10.1046/j.1432-1327.1999.00476.x.

Park, Y.-B., Kim, J.-B., Shin, S.-W., Kim, J.-C., Cho, S.-H., Lee, B.-K., et al. (2009). Prevalence, genetic diversity, and antibiotic susceptibility of Bacillus cereus strains isolated from rice and cereals collected in Korea. *Journal of Food Protection, 72*(3), 612–617. http://10.4315/0362-028X-72.3.612.

Rajasekar, A., & Ting, Y.-P. (2011). Role of inorganic and organic medium in the corrosion behavior of Bacillus megaterium and Pseudomonas sp. in stainless steel SS 304. *Industrial & Engineering Chemistry Research, 50*(22), 12534–12541. http://10.1021/ie200602a.

Rouzeau-Szynalski, K., Stollewerk, K., Messelhäusser, U., & Ehling-Schulz, M. (2020). Why be serious about emetic Bacillus cereus: Cereulide production and industrial challenges. *Food Microbiology, 85*, 103279. http://10.1016/j.fm.2019.103279.

Teplova, V. V., Mikkola, R., Tonshin, A. A., Saris, N.-E. L., & Salkinoja-Salonen, M. S. (2006). The higher toxicity of cereulide relative to valinomycin is due to its higher affinity for potassium at physiological plasma concentration. *Toxicology and Applied Pharmacology, 210*(1–2), 39–46. http://10.1016/j.taap.2005.06.012.

Tschiedel, E., Rath, P.-M., Steinmann, J., Becker, H., Dietrich, R., Paul, A., et al. (2015). Lifesaving liver transplantation for multi-organ failure caused by Bacillus cereus food poisoning. *Pediatric Transplantation, 19*(1), E11–E14. http://10.1111/petr.12378.

Ulrich, S., Gottschalk, C., Dietrich, R., Märtlbauer, E., & Gareis, M. (2019). Identification of cereulide producing Bacillus cereus by MALDI-TOF MS. *Food Microbiology, 82*, 75–81. http://10.1016/j.fm.2019.01.012.

Wu, J., Zhao, L., Lai, S., & Yang, H. (2021). NMR-based metabolomic investigation of antimicrobial mechanism of electrolysed water combined with moderate heat treatment against Listeria monocytogenes on salmon. *Food Control, 125*, 107974. https://doi.org/10.1016/j.foodcont.2021.107974

Yamaguchi, M., Kawai, T., Kitagawa, M., & Kumeda, Y. (2013). A new method for rapid and quantitative detection of the Bacillus cereus emetic toxin cereulide in food products by liquid chromatography-tandem mass spectrometry analysis. *Food Microbiology, 34*(1), 29–37. http://10.1016/j.fm.2012.11.010.

Yibar, A., Çetinkaya, F., Soyutemiz, E., & Yaman, G. (2017). Prevalence, enterotoxin production and antibiotic resistance of Bacillus cereus i?solated from milk and cheese. *Kafkas Universitesi Veteriner Fakultesi Dergisi, 23*, 635–642. http://10.9775/kvfd.2017.17480.