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The toxicological spectrum of the *Bacillus cereus* toxin cereulide points towards niche-specific specialisation

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Abstract

Most microbes share their environmental niches with very different forms of life thereby engaging in specialised relationships to enable their persistence. The bacterium *Bacillus cereus* occurs ubiquitously in the environment with certain strain backgrounds causing foodborne and opportunistic infections in humans. The emetic lineage of *B. cereus* is capable of producing the toxin cereulide, which evokes emetic illnesses. Although food products favouring the accumulation of cereulide are known, the ecological role of cereulide and the environmental niche of emetic *B. cereus* remain elusive. To better understand the ecology of cereulide-producing *B. cereus*, we systematically assayed the toxicological spectrum of cereulide on a variety of organisms belonging to different kingdoms. As cereulide is a potassium ionophore, we further tested the effect of environmental potassium levels on the action of cereulide. We found that adverse effects of cereulide exposure are species-specific, which can be exacerbated with increased environmental potassium. Additionally, we demonstrate that cereulide is produced within an insect cadaver indicating its potential ecological function for a saprophytic lifestyle. Collectively, distinct cereulide susceptibilities of other organisms may reflect its role in enabling competitive niche specialization of emetic *B. cereus*.

INTRODUCTION

Microorganisms live in complex, multispecies environments. To ensure persistence and survival, bacteria employ diverse mechanisms to ensure competitive fitness, such as toxins, which often function in interspecies warfare (Gonzalez & Mavridou, 2019; Granato et al., 2019). The endospore-forming bacterium *Bacillus cereus*, which is the name giving species of a group of closely related species, displays a wide array of phenotypic and pathogenicity traits, reflecting its open pan genome (Okinaka & Keim, 2016; Tourasse et al., 2023). Generally, the *B. cereus* group strain are grouped in three to five major phylogenetic clades (Ehling-Schulz et al., 2011; Ehling-Schulz et al., 2019; Priest et al., 2004), characterised by distinct physiological traits indicative of different ecological niches (Guinebretière et al., 2008; Manktelow et al., 2021; Raymond et al., 2010). For instance, *Bacillus anthracis*, the causative agent of anthrax in humans and animals, belongs to clade I while *Bacillus weihenstephanensis*, known for its psychrotolerance and high food spoilage potential, belongs to clade III. By contrast, *B. cereus* is distributed throughout all clades, highlighting its adaptability to broad range of environments and ecological niches (Guinebretière et al., 2008; White et al., 2022). The latter species causes different types of food poisoning, mainly manifesting in diarrheal and emetic

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symptoms. Thereby, different sets of toxins are the causative agent (Dietrich et al., 2021; Ehling-Schulz et al., 2019). The emetic syndrome is triggered by the cyclic non-ribosomal peptide (NRP) toxin cereulide, which is synthesized via non-ribosomal peptide synthase (NRPS) modules (Agata et al., 1995; Ehling-Schulz, Vukov et al., 2005). Cereulide is a potassium ionophore that interferes with cellular and mitochondrial membranes, with high cytotoxic potential (Andersson et al., 2007; Jääskeläinen et al., 2003; Mikkola et al., 1999). Besides emesis, cereulide can occasionally evoke severe intoxications linked to acute liver failures (Mahler et al., 1997; Schreiber et al., 2021; Tschiedel et al., 2015).

B. cereus is frequently isolated from various environmental niches, including soil, plant material or insect guts (Ehling-Schulz et al., 2015). Given the ubiquitous environmental occurrence of *B. cereus*, it may encounter a myriad of environmental conditions including the niche-specific inhabitants, which requires tailored adaptation to facilitate bacterial persistence. Indeed, *B. cereus* may reside as a symbiont in the rhizosphere of certain plants (Dutta et al., 2013; Halverson et al., 1993; Shang et al., 1999; Wang et al., 2017) and adapts its behaviour and morphology in response to different densities of bactivorous amoeba (Beeton et al., 2013). In addition, filamentous *B. cereus* ('Arthromitus') could be isolated from the gut lumen of termites (Margulis et al., 1998) and as a commensal in other arthropods such as sow bugs, millipedes and cockroaches (Feinberg et al., 1999). Notably, a later report using culture-independent approaches did not confirm that *Arthromitus* is a life stage of *B. cereus* (Thompson et al., 2012). Further, cereulide production is restricted to *B. cereus* strains with a specific genotypic background (Ehling-Schulz, Svensson et al., 2005). This emetic *B. cereus* lineage is equipped with the pCER270 megaplasmid, which harbours the genetic elements, the *ces* gene cluster, required for cereulide biosynthesis (Ehling-Schulz, Vukov et al., 2005; Ehling-Schulz et al., 2006). The proportion of emetic *B. cereus* soil isolates is rather low (Altayar & Sutherland, 2006; Bağcıoğlu et al., 2019; Hoton et al., 2009), but its incidence increases in food materials with a propensity to starch-rich goods, like rice or potato (Messelhäusser et al., 2014). In addition, emetic strains have been isolated from potato tubers and rice paddy fields (Altayar & Sutherland, 2006; Hoornstra et al., 2013; Ueda & Kuwabara, 1988). Hence, it is thought that cereulide and pCER270 provide an adaptive advantage in certain environments (Ehling-Schulz et al., 2015; Ekman et al., 2012). One remarkable feature of cereulide is its high stability against environmental parameters such as heat (150°C), extreme pH and proteases (Mikkola et al., 1999; Rajkovic et al., 2008). Once produced, cereulide possesses high stability and it is difficult to inactivate, which makes it especially problematic for the food industry (Rouzeau-Szynalski et al., 2020). Additionally, because of its high hydrophobicity and strong adherence to surfaces, an absorbance by other organisms is likely. Indeed, inhibitory activity of cereulide on Gram-positive bacteria (Tempelaars et al., 2011) and extracts prepared from emetic *B. cereus* on filamentous fungi has been demonstrated on a limited number of strains (Ladeuze et al., 2011). In addition, emetic strains have a competitive growth advantage when potassium is scarce (Ekman et al., 2012). However, the biological function of cereulide is still poorly characterized. Thus, our current study aimed to assess systematically the biological effect of cereulide on a broad range of organisms, which emetic *B. cereus* may encounter within its potential ecological niches. Based on the high affinity of cereulide to potassium (Teplova et al., 2006), we additionally assayed the effect of environmental potassium levels on the action of cereulide. Thereby, we found species-specific cereulide susceptibility profiles, further implying that cereulide exerts its function in a targeted and niche-specific manner. Hence, this study serves as a starting to point to dissect the yet ill-defined ecological niches of emetic *B. cereus*.

EXPERIMENTAL PROCEDURES

Microbial strains and standard culture conditions

All strains used in this study are listed in Tables 1 and S1. Bacterial species were routinely grown on LB agar (10 g/L peptone, 5 g/L yeast extract, 10 g/L NaCl [Roth], 15 g/L agar), unless otherwise stated. *Lactobacillus* spp were cultured in MRS medium (De Mas, Rogasa and Sharp; Oxoid) and *Listeria monocytogenes* was grown in brain-heart-infusion medium (37 g/L; Merck). Bacterial culture conditions are further indicated in Table 1. The emetic reference strain *B. cereus* F4810/72 (Ehling-Schulz, Vukov et al., 2005; Turnbull et al., 1979), originally isolated from vomit by Public Health Laboratory Service (London, United Kingdom), was routinely grown on LB agar or in LB broth with 150-rpm rotary shaking at 30°C. Strains harbouring pMDX[P1/luxABCDE] and pMDX[PcspA/luxABCDE] were cultured in LB medium containing 5 μg/mL chloramphenicol (CM).

The ammonia oxidising archaeon *Nitrososphaera viennensis* EN76 (Tourna et al., 2011) was grown in sterile 30 mL polystyrene screw cap tubes (Greiner Bio-One, GB201170) containing 5 mL freshwater medium supplemented with 2 mM ammonium chloride, 2 mM sodium bicarbonate, 1 mM sodium pyruvate, 10 mM HEPES pH 7.5, 5 μL non-chelated trace element solution, 7.5 μM FeNaEDTA, 5 μL vitamin solution and 50 μg/mL kanamycin (for detailed medium

TABLE 1 Antibacterial effects of cereulide.

Gram-negative bacteria					
ID/reference	**Species**	**Medium**	**Temp**	**Inhibition (μg/mL)**	**Delay (μg/ml)**
WS 1844	*Pseudomonas stutzeri*	LBK$^+$	37°C	>50	>50
WS 2201	*Pseudomonas fluorescens*	LBK$^+$	30°C	>50	>50
WS 3316	*Proteus mirabilis*	LBK$^+$	37°C	>50	>50
WS 4200	*Escherichia coli* MG1655	LBK$^+$	37°C	>50	>50
ATCC 14028	*Salmonella enterica*	LBK$^+$	37°C	>50	>50
Cornelis and Colson (1975)	*Yersinia enterolitica*	LBK$^+$	30°C	>50	>50
Gram-positive bacteria					
Group 1: Resistant					
ID	Species	Medium	Temp	Inhibition (μg/ml)	Delay (μg/ml)
WS 2095	*Lactobacillus brevis*	MRS	30°C	>50	>50
WS 1023	*Lactobacillus rhamnosus*	MRS	30°C	>50	>50
WS 1937	*Micrococcus luteus*	LBK$^+$	30°C	>50	>50
Schlech et al. (1983)	*Listeria monocytogenes EGDe*	BHI$^+$	37°C	>50	>50
Novick (1967)	*Staphylococcus aureus*	LBK$^+$	37°C	>50	>50
F4810/72	*Bacillus cereus*	LBK$^+$	30°C	>50	50
Group 2: Moderately sensitive					
WS 1084	*Corynebacterium glutamicum*	LBK$^+$	30°C	>50	12.5
WSBC 28001	*Bacillus thuringiensis*	LBK$^+$	30°C	>50	12.5
WS 2892	*Staphylococcus epidermidis*	LBK$^+$	30°C	>50	12.5
ATCC14579	*Bacillus cereus*	LBK$^+$	30°C	>50	6.25
WSBC10204	*Bacillus weihenstephanensis*	LBK$^+$	30°C	>50	6.25
WSBC 25001	*Bacillus pumilus*	LBK$^+$	30°C	>50	6.25
WS 1998	*Microbacterium oxidans*	LBK$^+$	30°C	50	12.5
WSBC 23001	*Bacillus licheniformis*	LBK$^+$	30°C	50	3.13
WS 1073	*Arthrobacter nicotianae*	LBK$^+$	30°C	50	0.39
WSBC 26001	*Bacillus subtilis*	LBK$^+$	30°C	50	0.39
WS 3023	*Brevibacterium casei*	LBK$^+$	30°C	25	0.78
WS 1943	*Arthrobacter aurescens*	LBK$^+$	30°C	25	0.78
Group 3: Sensitive					
WS 1914	*Microbacterium lacticum*	LBK$^+$	30°C	12.5	6.25
WS 1744	*Arthrobacter viscosus*	LBK$^+$	30°C	12.5	0.78
WS 1723	*Propionibacterium freudenreichii*	LBK$^+$	30°C	12.5	0.78
WS 3539	*Corynebacterium casei*	LBK$^+$	30°C	3.13	0.78
WS 1061	*Arthrobacter oxydans*	LBK$^+$	30°C	1.56	0.78
WS 1481	*Micrococcus aurantiacus*	LBK$^+$	30°C	1.56	0.78
Rattray et al. (1999)	*Brevibacterium linens*	LBK$^+$	30°C	0.39	0.2
WS 1106	*Rhodococcus fascians*	LBK$^+$	30°C	0.39	0.2
WS 1457	*Arthrobacter globiformis*	LBK$^+$	30°C	0.2	0.1

Note: 'Growth inhibition' signifies no growth, while 'Growth delay' a delayed lag-phase within the assessed time period (20 h) determined by empirical assessment of growth curves from two experiments. Group1: Resistant (no growth inhibition; delay at a minimum of 50 μg/mL cereulide); Group 2: Moderately sensitive (growth inhibition at a minimum of 25 μg/mL cereulide; delay with lower concentrations); Group 3: Sensitive (growth inhibition at a minimum of 12.5 μg/mL cereulide; delay with lower concentrations). Temp = cultivation temperature. BHI$^+$: brain-heart-infusion medium supplemented with 0.171 M KCl.

composition see Reyes et al., 2020). Cultures were inoculated with 5% (v/v) and incubated aerobically in the dark shaking at 80 rpm.

Fungal species were routinely grown on YPD agar plates (20 g/L yeast extract [Oxoid], 10 g/L peptone [Roth], 2% glucose [Roth], 20 g/L agar [BD Difco]) at 30°C, unless otherwise stated. All fungal strains are listed in Table S1 and were retrieved from the University of Natural Resources and Life Sciences (BOKU), Vienna with the exception of *Candida albicans* and

Candida glabrata, which were a gift from Karl Kuchler from the Medical University of Vienna.

Chemicals

Cereulide was produced biosynthetically by *B. cereus* strain F4810/72 in LB broth with the addition of 0.2% glucose. For cultivation conditions and purification see Bauer et al. (2010). The purity of the dried cereulide was analysed using NMR spectroscopy. Valinomycin was obtained from Sigma Aldrich. Alternatively, cereulide was commercially purchased (Chiralix, Netherlands). Cereulide stock solutions were prepared in ethanol (>99.8%, Baker; for fungal growth inhibition, *G. mellonella* and *O. dentatum* assays) or DMSO (for bacterial growth inhibition and HEp-2 assays) as 1–3 mg/mL solutions.

Bacterial reporter strains

B. cereus transformed with pMDX[P1/luxABCDE] (Dommel et al., 2010) was employed to study *ces* gene cluster expression in vivo (described below). To additionally monitor the vegetative growth of *B. cereus*, the luminescent reporter plasmid pMDX[PcspA/luxABCDE] was created. Briefly, the promoter of cold shock protein A (*cspA*, locus tag BCAH187_A1289) was cloned upstream of the luciferase cassette *luxABCDE* in the promoterless plasmid pXen1 (Francis et al., 2001) and 1 μg plasmid was electroporated into *B. cereus* to generate P*cspA*-lux (0.2 cm cuvette, 2 kV, 25 μF, 200 Ω). Cells were regenerated in LB medium for 2 h at 30°C and plated on LB supplemented with 5 μg/mL CM. Strains harbouring pMDX[P1/luxABCDE] and pMDX[PcspA/luxABCDE] were cultured in LB medium containing 5 μg/mL CM.

Antimicrobial activity

Assessment of cereulide antimicrobial activity of the tested organisms engaged different assays owing to their specific growth requirements, cereulide susceptibilities and replication times.

The antibacterial activity of cereulide was determined using growth inhibition assays. Bacterial cultures were grown overnight at the temperature and medium indicated in Table 1, adjusted to an optical density (OD_{600}) of 0.1–0.12 and 1:1 mixed with growth medium containing cereulide in a 100-well Honeycomb plate (ThermoFisher Scientific). The cereulide stock solution (3 mg/mL in DMSO) was serially 2-fold diluted in the respective culture medium, resulting in a final cereulide test concentration ranging from 0.1 to 50 μg/mL. Plates were then incubated at the desired temperature and OD_{600} values were recorded every 30 min using a Bioscreen C instrument (Bioscreen). The medium alone and DMSO in final concentrations of 3%, corresponding to the highest cereulide concentration served as control. Cereulide concentrations leading to a complete growth inhibition or a growth delay with respect to the solvent control were then assessed empirically and summarized in Table 1 and Figure S1.

To investigate the influence of different chlorides on the antimicrobial activity of cereulide, *Brevibacterium casei* was incubated with cereulide in LB broth without NaCl, but supplemented with 0.171 M LiCl, KCl, $MgCl_2$, NH_4Cl or CsCl instead. To analyse the barrier function of the outer membrane of Gram-negative bacteria in response to cereulide, *Escherichia coli* was incubated with different concentrations of cereulide as described above but with and without addition of 10 μg/mL polymyxin B nonapeptide (PMBN). Duration of lag phase (λ) and maximal growth rate (μmax) were determined after growth fitting via a modified Gompertz function according to Kleer and Hildebrandt (Kleer & Hildebrandt, 2002). Bacterial strains, culture medium and growth temperature are displayed in Table 1.

The effect of cereulide on ammonia oxidising archaea was assessed by a growth inhibition assay in liquid cultures. Therefore, *Nitrososphaerea viennensis* was grown in triplicates as described above in medium supplemented with 3.125 μg/mL, 6.25 μg/mL or 12.5 μg/mL cereulide, starting from a 10 mg/mL cereulide stock dissolved in DMSO and leading to a final DMSO concentration of 0.03%, 0.06% and 0.125% (v/v), respectively, in the culture medium. Growth was followed daily by measuring nitrite production colorimetrically with the Griess reagent as described previously (Tourna et al., 2011). The standard medium as described above and DMSO in final concentrations of 0.03%, 0.06% and 0.125% (v/v) served as controls.

Antifungal activity against *Candida albicans*, *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* was tested using a minimal inhibitory concentration assay. Therefore, a cereulide working dilution of 50 μg/mL was prepared starting from a 2 mg/mL cereulide stock dissolved in ethanol; and 100 μL of the working dilution were dispensed in a well of a 96-well plate. From this dilution, 50 μL was taken and mixed with 50 μL YPD or YPDK+ (YPD medium supplemented with 0.171 M KCl) medium to achieve the next dilution step. As 100% growth control, wells with the respective ethanol concentration (solvent control), corresponding to the cereulide dilution factor, were prepared in addition. Fungal cultures were grown overnight in YPD or YPDK + medium, diluted to an OD_{600} of 0.1 and further grown to the logarithmic growth phase (~OD_{600} 1). Cultures were then diluted to an OD_{600} of 0.005 and 50 μL of cells were mixed with 50 μL of the medium containing either cereulide or the solvent control. The maximum final cereulide concentration was 25 μg/mL. Plates

were incubated at 30°C and OD_{600} values were measured at the indicated time. The raw OD_{600} values were blanked with wells containing only medium and the relative growth represents OD_{600} medium containing cereulide versus medium containing the respective ethanol concentration. Growth inhibition of fungal species was then further tested as described for bacteria using a Bioscreen C instrument, except that YPDK+ medium supplemented with 0.171 M KCl was used. Growth curves were quantified by employing a logistic model used for area under the curve (AUC) calculation using the 'growthcurver' package (Sprouffske & Wagner, 2016) in RStudio (RStudio Team, 2020).

Cell toxicity assays (HEp-2)

The influence of cereulide toxicity on HEp-2 cells in response to different KCl concentrations (5, 25, 50 mM) was analysed using HEp-2 cytotoxicity assay (Lücking et al., 2009). Dilutions of cereulide (0.1–50 ng/mL in 5 and 25 mM; 0.01–5 ng/mL in 50 M KCL) were made in 96 well microtiter plates using Eagle's minimum essential medium (MEM) with Earl salts supplemented and addition of 1% foetal calf serum, 1% sodium pyruvate, 0.4% penicillin–streptomycin, as well as, 2% ethanol to ensure solubility of cereulide. HEp2-cells were added to a final concentration of 2.8×10^5 cells/mL, plates were incubated for 48 h at 37°C and 5% CO_2. Cytotoxicity was determined by adding the cell proliferation reagent WST-1 (Roche). The produced formazan product was measured in a microplate reader at 450 nm (A450) after 20 min incubation at 37°C. Fifty percent inhibitory concentration was calculated from three independent experiments with a two-fold approach on the basis of a dose response curve (R Dietrich et al., 1999).

Nematode assays

A monospecific laboratory strain of *Oesophagostomum dentatum* was propagated continuously at the Institute of Parasitology of the Vetmeduni Vienna for the production of worm eggs (by permission of the Austrian Ministry of Science and Research, GZ BMWF-68.205/242/3b/2010). Third-stage larvae (L3) were harvested and purified from coprocultures of experimentally infected pigs as described previously (Joachim et al., 1997) and cleaned worms were stored in distilled water at 11°C for a maximum of 6 months. For culture experiments, the L3 larvae (100 L3/mL medium) were purified using a small-scale agar-gel migration and exsheathed with sodium hypochlorite (10%–14% free chlorine) to 1 mL of a larval suspension (Joachim et al., 1997). A defined larval population of exsheathed L3 was thus obtained and further maintained in 24-well culture plates in 1 mL medium containing 75% LB-medium (Roth), 25% inactivated pig serum and 1% antibiotics and antifungals (100,000 U penicillin, 10 mg streptomycin and 25 μL amphotericin B per 50 mL of medium; PAA). Plates were incubated at 38.5°C and 10% CO_2 for 14 days with medium change on day 4 and 11.

The antinematodal activity of cereulide was determined through in vitro developmental experiments by adding 10, 4 or 2 μg/mL cereulide to the culture medium. To avoid precipitation of cereulide, sodium chloride (NaCl) in the LB culture medium was substituted with potassium chloride (KCl). The rate of L3 development to fourth-stage larvae (L4) was determined on day 5, 7, 11 and 13 of culture. The effects on the nematode development and viability were observed and assessed visually using an inverted light microscope (Diaphot 300, Nikon Corporation). Each treatment group consisted of three replicate wells and appropriate solvent controls were used for each assay.

For electron microscopy, the larvae were fixed in glutaraldehyde and transferred to ascending acetone concentrations ranging from 30% to 90%. Critical point drying (Balzers Bal-Tec® CPD030) was performed and the larvae were transferred onto a microscope slide. Objects were sputtered with a gold–palladium target (Labco, Brake) and transferred to the raster electron microscope (JEOL) with low-vacuum function. Exposure was performed at 1000× magnification and 5 kV.

Insect assays

Last instar larvae of the greater wax-moth *Galleria mellonella* (Kerf-Terraristik, Hamm, Germany) were stored with wood shavings in the dark at 15°C and used within 3 weeks upon reception. Five microlitre of cereulide dissolved in 10% or 30% (for 10 μg/g bodyweight [bw] treatment) were injected into larvae (300–350 mg) as described previously (Cotter et al., 2000; Ratcliffe & Walters, 1983). Three controls were used in all assays: uninfected larvae, larvae with 5 μL 10% ethanol or 30% ethanol. After injection, the larvae were incubated in petri dishes with wood shavings at 24°C and observed over a period of 2 days. Larvae were considered paralysed if they kept their yellow colour and failed to respond to tweezers stimulation. Dead larvae could be differentiated from the paralysed phenotype by brownish discoloration.

To analyse cereulide synthesis and in vivo growth of *B. cereus* in *G. mellonella*, larvae (400–450 mg) were injected with 5 μL of bacterial cultures cultivated in LB for 14 h at 24°C, 120 rpm as described previously (Frenzel et al., 2012). In vivo produced cereulide concentrations were determined by stable isotope dilution analysis (SIDA) (Bauer et al., 2010). Therefore, *B. cereus* infected-larvae were collected per time point and frozen at −20°C. Samples were autoclaved (121°C, 20 min), freeze dried for up to 3 days and

stored at −20°C. Larvae were crushed into powder and 5 μg $^{13}C_6$ cereulide dissolved in EtOH was added. After incubation for 2 h, samples were extracted by addition of 4.5 mL EtOH and incubated on a rocking table for 15 h. After pelletizing (8600×*g*; room temperature; 10 min), supernatant was concentrated to a volume of 1 mL. For C18-SPE cartridge cleanup, supernatant was 1:5 diluted with water and applied onto the top of a cartridge (6 mL; 1000 mg; Strata C18-E, Phenomenex). After sample rinsing with water (4 mL) and methanol/water (3 mL; 70/30, v/v), the target compounds were eluted with ethanol (1.5 mL) and 5 μL of an 1:1000 dilution of the membrane filtered aliquot (0.2 μm, PTFE, Phenex, Phenomenex) was analysed by means of LC–MS/MS analysis (see Bauer et al., 2010).

In vivo promotor activity of P1*ces* and P*cspA* was assessed by larval injection of P1*ces*-lux and P*cspA*-lux strains as described above and bioluminescence was recorded with a photon-counting intensified-charge-coupled-device (ICCD) camera (Xenogen IVIS Lumina 100, Caliper Life Science, USA). Images were acquired for 10 s with a binning factor of 3 (without filter; relative aperture, 1), and the bioluminescence intensity was superimposed as false-colour rendering. Editing of pictures was performed by the Living Image software (Caliper Life Science, USA).

Statistical analysis

Plotted data represent mean values with error bars indicating standard deviations (SD). The sample number is stated in each figure legend. Kaplan–Meier survival curves were plotted and analysed using a log-rank test in GraphPad Prism 9 (Prism).

RESULTS

Antibacterial activity

Since cereulide is an ionophore with high affinity to potassium (Teplova et al., 2006), an initial screen using the susceptible bacterium *Brevibacterium casei* was used to test the effect of potassium. Therefore, LB medium was modulated by exchanging NaCl with KCl (0.171 M each). In conventional LB broth, growth of *B. casei* was not significantly influenced by cereulide (Figure 1). However, the exchange of NaCl to KCl in LB medium (LBK+) enhanced cereulide toxicity, as indicated by the prolonged lag phases in a concentration dependent manner, while growth in LBK+ without cereulide was unaffected (Figure 1A,B). This was further reflected in the synergistic action of increasing KCl and cereulide concentration on lag phase prolongation and the maximum growth rate (Figure 1C,D). Notably, LB supplementation with other chlorides (MgCl, LiCl, NH4Cl, CsCl; 0.171 M each) did not augment *B. casei* growth inhibition in response to cereulide, with the presence of CsCl allowing no growth of *B. casei* (data not shown). To extend the knowledge on the antibacterial activity of cereulide, we tested 33 bacterial species, including *B. casei*, mainly derived from similar environmental habitats as *B. cereus*. Growth curves were then assessed empirically for the cereulide concentration at which no growth occurred anymore (i.e., 'growth inhibition') and which caused a prolonged lag-phase (i.e., 'growth delay'). All tested Gram-negative bacteria ($n = 6$) were completely resistant to cereulide (maximum test concentration 50 μg/mL; Figure S1, Table 1), while Gram-positive bacteria displayed species-specific susceptibilities (Table 1, Figure S1). Out of 27 tested Gram-positive bacteria, growth of six species was not affected or only marginally delayed upon cereulide treatment (Group 1 in Table 1 and Figure S1) and hence, considered resistant. This group included the cereulide-producer strain *B. cereus* F4810/72, *Staphylococcus aureus* and *Listeria monocytogenes*. Further, 12 species showed moderate growth inhibition, meaning a lag-phase delay in response to 12.5 μg/mL cereulide or lower concentrations (Group 2 in Table 1 and Figure S1). This group encompassed for instance other *B. cereus* group members, such as non-emetic *B. cereus*, *B. weihenstephanensis* and *B. thuringiensis*. Lastly, growth of nine bacterial species was markedly affected upon cereulide treatment, meaning complete growth inhibition was observed at a minimum of 12.5 μg/mL cereulide (Group 3 in Table 1 and Figure S1). The most sensitive Group 3 bacterium was *Arthobacter globiformes*, which was completely inhibited in response to 0.2 μg/mL cereulide (Table 1). These data demonstrate that the antibacterial action of cereulide is species-specific and may further depend on the environmental context.

To investigate the role of the outer membrane (OM) in the cereulide resistance of Gram-negative bacteria, *E. coli* was cultivated in LBK+ supplemented with different concentrations of cereulide and polymyxin B nonapeptide (PMBN) to enhance the OM permeability. PMBN disrupts the OM of Gram-negative bacteria leading to a weak leakage of periplasmic proteins without significantly affecting cell growth (Sahalan & Dixon, 2008; Vaara, 1992). Addition of PMBN to cereulide treated *E. coli* caused an elongation of the lag-phase in a cereulide concentration-dependent manner (Figure 1E), with 25 μg/mL cereulide prolonging the lag-phase 2-fold with respect to *E. coli* treated without PMBN.

Effect of cereulide on ammonia-oxidising archaea (AOA)

AOA are major players in environmental nitrogen cycling and even outnumber their bacterial counterparts

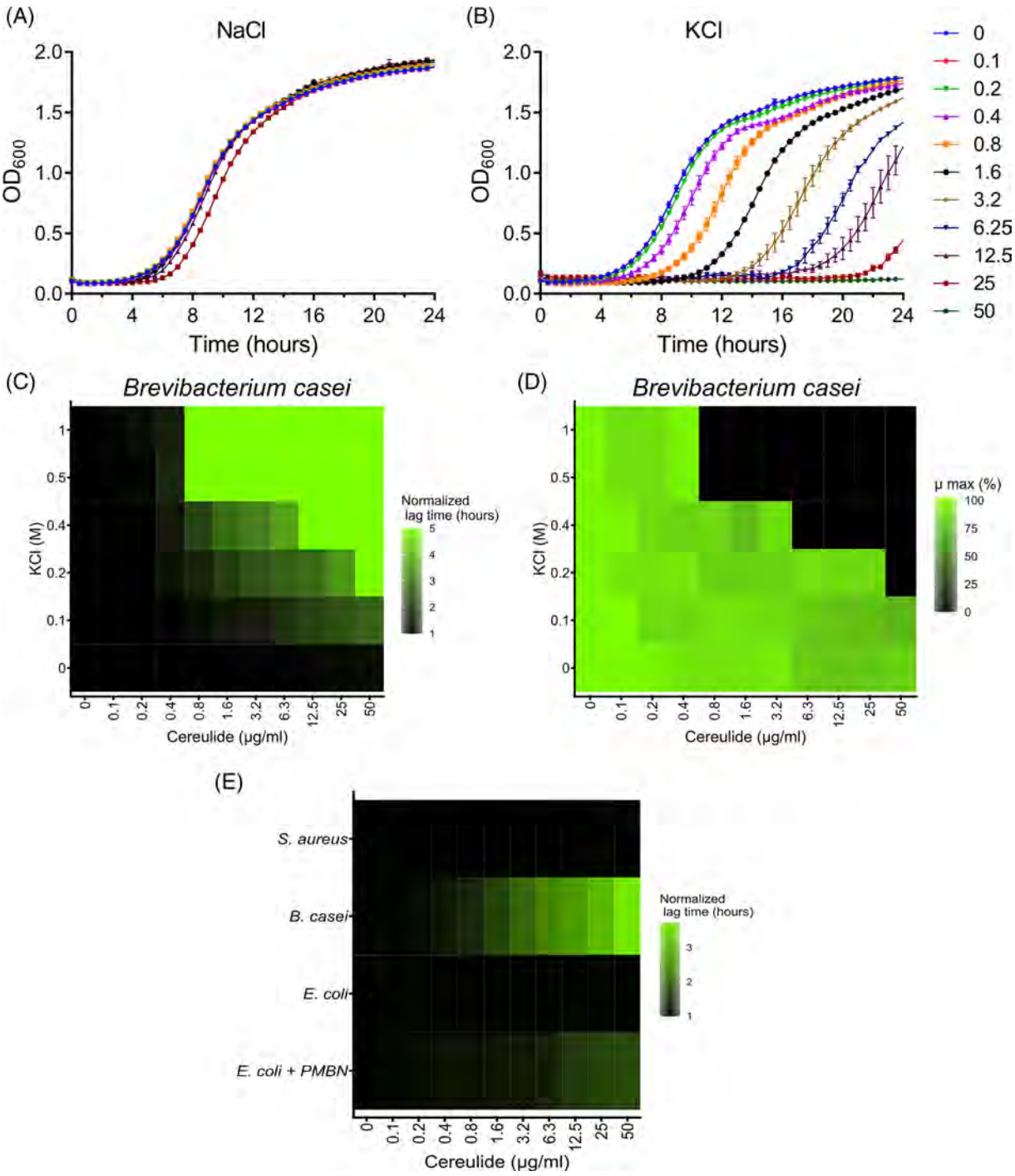

FIGURE 1 Extracellular potassium exaggerates antibacterial activity of cereulide against *Brevibacterium casei*. (A, B) Growth inhibition assay in standard LB medium (0.171 M NaCl; A) and modified LB medium containing KCl instead of NaCl (LBK+ medium; B) in response to the indicated cereulide concentrations. OD_{600} values were recorded during growth at 30°C every 30 min in the presence of the indicated cereulide concentrations. Mean and SD values of two replicates are indicated. (C, D) Lag time (C) and maximum growth rate (µ max; D) relative to the solvent control of *B. casei* cultured at 30°C in LBK+ medium containing the indicated KCl concentrations and cereulide. Mean values from biological duplicates are shown. Lag time and µ max values were derived from growth curves using a modified Gompertz function and normalised to cells treated with the cereulide solvent control (see experimental procedures). (E) Relative lag time of the indicated bacterial species in response to increasing cereulide concentration in LBK+. Lag time values were calculated as in D. Mean values from biological duplicates are shown. PMBN, polymyxin B nonapeptide.

in soil (Leininger et al., 2006; Prosser & Nicol, 2012). Hence, we tested whether cereulide affects the growth of the soil AOA, *Nitrososphaera viennensis*. By measuring nitrite production during AOA batch culture, we observed no effect of cereulide on the growth of *Nitrososphaera viennensis* (Figure S2).

Antifungal activity of cereulide

First, the impact of KCl supplementation on cereulide sensitivity was assessed using the human fungal pathogen *Candida albicans* (Pappas et al., 2018). Growth of *C. albicans* was inhibited by roughly 40% in response to 25 μg/mL cereulide after 20 hours of incubation in standard yeast medium YPD (Figure 2A). Supplementation of 0.171 M KCl (in line with LBK+) had no significant effect on cereulide susceptibility (Figure 2A). We further included eight additional yeast-like fungi, which may share environmental niches with *B. cereus* (Table S1) and assessed the antifungal effect of cereulide based on growth inhibition assays. Growth of most fungi was not affected by the addition of up to 50 μg/mL cereulide, with the exception of *C. albicans*, *Rhodotorula mucilaginosa* and *Rhodotorula glutinis* (Figure S3A). Hence, the growth curves of the latter species were analysed in detail using a logistic model (Sprouffske & Wagner, 2016). The AUC was used as a growth quantification proxy within the investigated 24-h period (Figure 2). The AUC of *C. albicans* was roughly 40% and 15% decreased upon 50 μg/mL and 25 μg/mL cereulide treatment, respectively (Figure 2B). *R. glutinis* growth decreased by more than 80% in response to 25 and 12.5 μg/mL cereulide (Figure 2C). *R. mucilaginosa* growth was inhibited by more than 50% upon cereulide treatment (Figure 2D). Generally, *Rhodotorula* spp. displayed slow growth and did not reach the maximum growth within 24 h in untreated conditions (Figure S3A). Hence, we further monitored growth up to 46 h and still found that *R. glutinis* was more than 80% decreased upon cereulide treatment (25–6.25 μg/mL), while *R. mucilaginosa* growth was reduced by roughly 30% after 46 hours of culture (Figure 2E). Notably, growth of *R. glutinis* and to a lesser extent of *R. mucilaginosa*, was positively affected by the presence of EtOH ≤1.25%, which were the corresponding solvent control for ≤25 μg/mL cereulide treatment, with respect to medium alone (Figure S3A,B). Therefore, lower cereulide concentrations have a seemingly enhanced inhibitory effect.

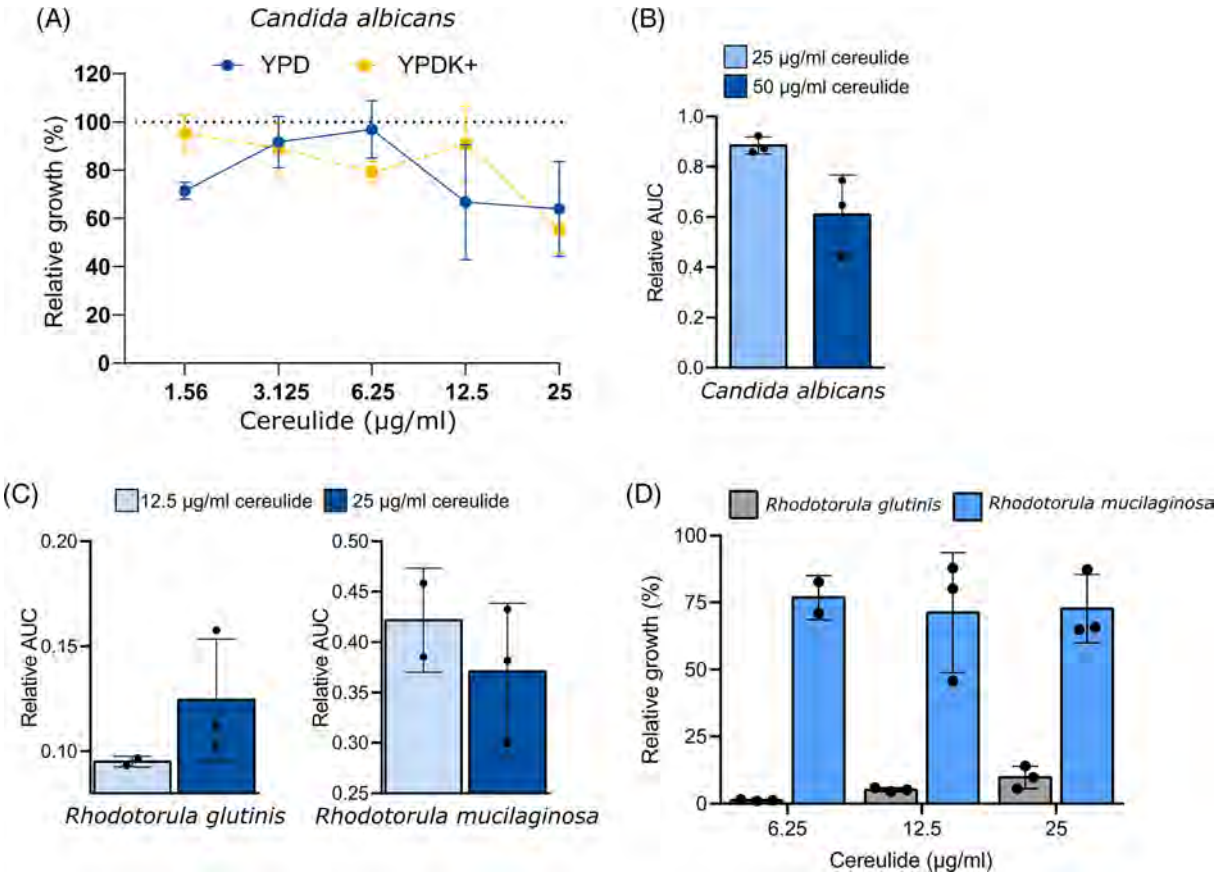

FIGURE 2 Antifungal activity of cereulide. (A) Growth of *Candida albicans* in YPD and YPD with KCl (0.171 M, YPDK+) supplemented with the indicated cereulide concentrations after 20 h of cultivation at 30°C. The relative growth depicts OD_{600} values at the indicated cereulide concentration relative to the corresponding solvent control. (B, C) AUC derived from growth curves shown in Figure S1 relative to the solvent control in response to cereulide. (D) Growth of *Rhodotorula glutinis* and *Rhodotorula mucilaginosa* in YPDK+ supplemented with the indicated cereulide concentrations after 46 h of cultivation at 30°C. Growth relative to the cereulide solvent control is shown. Data represent mean and SD values from 2 to 3 replicates (A–D).

Cereulide-mediated HEp-2 cytotoxicity is modulated by potassium

The cytotoxic effect of cereulide on the human larynx carcinoma cell line HEp-2, is well established (Andersson et al., 2007). We further investigated the effect of various external potassium concentrations on the cytotoxic action of cereulide. In common cell culture medium (MEM; 5 mM KCl), the 50% inhibitory concentration of cereulide was 3×10^{-4} μg/mL (Figure 3). Raising the external KCl concentration augmented cereulide cytotoxicity, indicated by the decreased IC_{50} in a potassium concentration-dependent manner. Supplementation with 25 mM and 50 mM KCl increased cereulide toxicity roughly 3-fold and 10-fold, respectively (Figure 3). These data demonstrate that, in addition to the antibacterial action against *B. casei*, environmental potassium has a major impact on the cytotoxicity of cereulide.

Antinematodal activity

Nematodes inhabit almost all ecosystems on Earth and are key components of terrestrial ecosystems

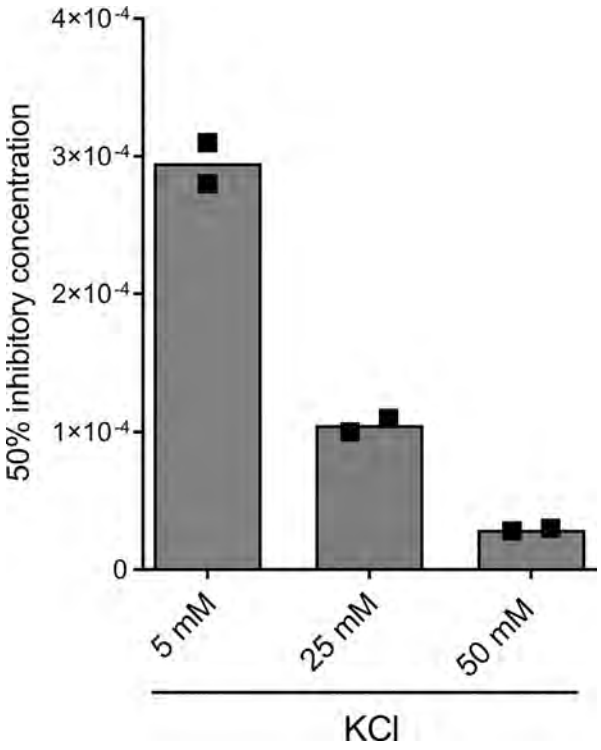

FIGURE 3 Increasing potassium concentrations augment cytotoxicity of cereulide. HEp-2 cells were treated with cereulide and the indicated potassium concentrations for 48 h at 37°C. Viability was measured using a colorimetric assay (WST-1). The 50% inhibitory concentration represents results normalised to cells treated solely with the respective KCl concentration. Data represent mean values from two biological replicates indicated by black squares.

(Kergunteuil et al., 2016). Thus, they may eventually share a common habitat with emetic *B. cereus* and cereulide. To analyse the antinematodal action of cereulide, *Oesophagostomum dentatum* was used due to its good suitability to monitor larval development of nodular worms (Daugschies & Watzel, 1999). Eggs of *O. dentatum* are released into the environment upon completion of its parasitic cycle and develop there into its infective form, the third-stage larvae (L3) (Ondrovics et al., 2013). The moult from L3 to fourth-stage (L4) larvae offers a convenient quantifiable parameter for the investigation of factors influencing the growth and the development of parasitic nematodes. Therefore, the impact of cereulide was first investigated on the development of L3 (Figure S4A) to L4 larvae (Figure S4B). L3 treatment with 2 μg/mL cereulide delayed the onset of larval development compared to untreated larvae, with roughly of 11% L3 larvae developing to L4 by day 5, while all cereulide-treated larvae still remained in the L3 stage. Only by day 7, L3–L4 development was observed upon cereulide treatment with roughly 4% (2 μg/mL) to 1% (10 μg/mL) of larvae displaying the developmental change (Figure 4A). After 13 days, 50% of untreated L3 transited to L4, while 2 μg/mL cereulide treatment reduced L4 development to 21% (Figure 4A). Higher cereulide concentrations exacerbated this effect. Notably, the culture medium in those experiments contained 60 mM KCl. Since increasing the KCl concentration had an effect on the cereulide-exerted cytotoxicity on HEp-2 cells and on the antibacterial effect of *B. casei*, we further tested the impact of external potassium on the antinematodal action of cereulide. Initially, we supplemented the culture medium with 100 mM KCl. The developmental delay of 2 μg/mL cereulide-treated L3 was enhanced in these conditions by roughly two-fold at day 13 (11% at 100 mM KCl vs. 21% at 60 mM KCl; Figure S4C). A similar effect was seen at 10 μg/mL cereulide treatment. However, increasing KCl to 100 mM already negatively affected L3–L4 development of untreated larvae by roughly 1.4-fold (50% vs. 35% at day 13 in 60 mM KCl and 100 mM KCl, respectively; Figure S4C). Hence, we additionally included culture conditions with 5 mM KCl to test whether decreased KCl would ameliorate the cereulide-induced developmental defects of *O. dentatum*. First, lowering the KCl concentration to 5 mM did not negatively affect L3–L4 development of untreated larvae. Further, the delayed transition of L3–L4 of cereulide-treated larvae was not ameliorated by lower KCl concentrations (Figure 4B). These data demonstrate that there is no, or only a minor, cooperative effect of environmental KCl and cereulide on the developmental delay of *O. dentatum*.

Besides the inhibition of larval development, cereulide exhibited a nematocidal effect on L3 larvae from day 7 onwards ranging from 0.6% dead L3 larvae at 2 μg/mL of cereulide to 5.1% at 4 μg/mL (Figure 4C).

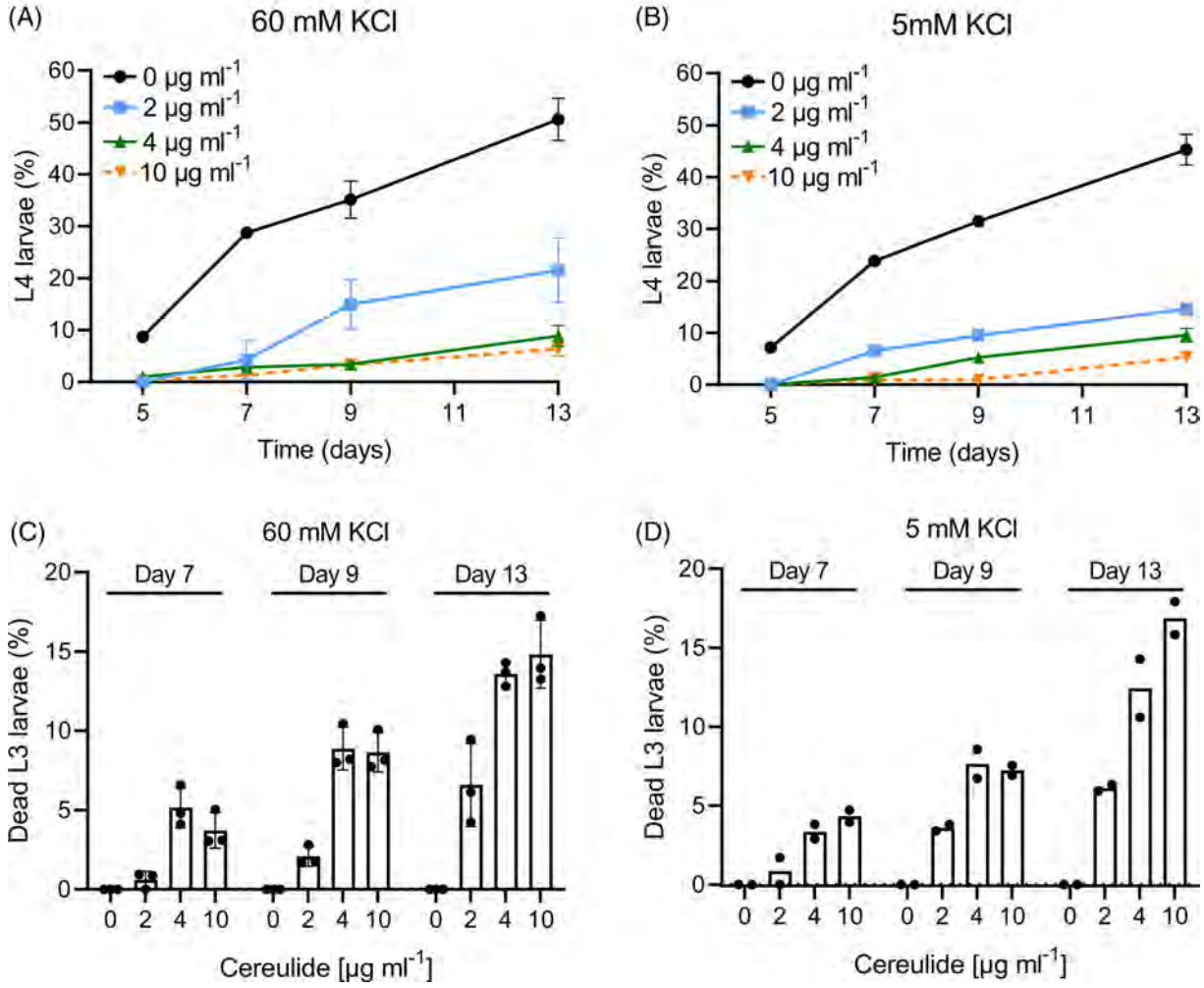

FIGURE 4 Cereulide inhibits development and decreases viability of *Oesophagostomum dentatum*. (A, B) Development from L3 to L4 larvae of *O. dentatum* in response to cereulide in LB supplemented with 60 mM KCl (A) or 5 mM KCl (B). (C, D) Nematocidal effect of cereulide on L3 larvae in LB with 60 mM (C) and 5 mM KCl (D). Data represent mean values from three (A,C) and two (B,D) biological replicates. At least 70 larvae were assessed in each replicate (A–D).

Upon prolonged cereulide treatment, the amount of dead L3 larvae substantially increased with 4 and 10 µg/mL cereulide treatment to roughly 14% (Figure 4C). Importantly, untreated larvae displayed no loss of viability after an incubation time of 13 days in the tested conditions (LB, 60 mM KCl; Figure 4C). In addition, the influence of low KCl concentrations on the nematocidal effect of cereulide on L3 larvae was investigated. However, the nematoxicity of cereulide on L3 larvae was comparable to LB with 60 mM and 5 mM KCl (Figure 4D). Notably, also culture conditions with 100 mM KCl were tested, which increased the nematocidal action of 10 µg/mL cereulide with respect to 60 mM and 5 mM KCl LB supplementation (Figure S4D). However, *O. dentatum* viability without cereulide treatment was already negatively affected (roughly 2% dead L3 larvae at day 13) in response to 100 mM KCl alone, analogously to the delay of L3–L4 development. Taken together, cereulide exerts antinematodal actions on L3 larvae ranging from developmental delays to larval death, which are not substantially modulated by external potassium.

Insecticidal activity

B. cereus is frequently isolated from insects (Feinberg et al., 1999; Swiecicka & Mahillon, 2006), which may thus be exposed to cereulide production. Notably, most larvae of insect host models are highly sensitive to direct injection of *B. cereus* cells (Ehling-Schulz et al., 2019). The larvae *Galleria mellonella* are routinely used as model organisms for in vivo studies of pathogenic bacteria and fungi as well as toxic substances produced by these organisms (Joyce & Gahan, 2010; Mollier et al., 1994). The injection of cereulide led to larval paralysis after a short time and resulted in death at later time points. At concentrations

of 10 μg/g larva, paralysis occurred 1 h after injection, while treatments with lower concentrations led to paralysis symptoms 2–3 h post injection (Figure 5A). At the lowest tested concentration (1.56 μg/g larva), more than 50% of larvae were paralysed after 5 h. Strikingly, cereulide-treated larvae remained in this paralysis state followed by a direct transition into death, as represented by larval melanisation.

Given the observed susceptibilities of *G. mellonella* to cereulide injection, we further analysed whether this toxin is produced in vivo. The genetic modules facilitating cereulide biosynthesis are encoded by the *ces* gene cluster, which is tightly transcriptionally regulated depending on the bacterial growth phase or environmental cues (Dommel et al., 2010; Lücking et al., 2009). To visualize *ces* gene expression in vivo, we employed a transcriptional reporter strain harbouring a plasmid-encoded luciferase cassette regulated by the main *ces* promoter P1 (P1*ces*-lux) (Dommel et al., 2010). Additionally, a strain expressing the luciferase cassette during the vegetative growth at nearly constitutive levels was included as well (P*cspA*-lux). The reporter strains and the unmodified emetic type strain F4810/72 (wild type, WT) were injected directly injected into the hemocoel of *G. mellonella* (see experimental procedures). Notably, *G. mellonella* is highly susceptible to direct injection of *B. cereus* (Ehling-Schulz et al., 2019). Thus, conclusions on bacterial virulence are limited from this experimental model. However, this experiment allowed to assess the temporal resolution of insect host death in our set-up. Larvae were equally sensitive to all *B. cereus* strains with more than 50% of larvae succumbing to the infection after 9 h (Figure S5A). At this time point, bacterial populations have already increased approximately by 3-log units with respect to the infection load (Figure S5B). The peak of bacterial cell numbers was reached 18 h post infection, hence demonstrating a saprophytic lifestyle of *B. cereus* in *G. mellonella* (Figure S5B). This was further reflected in nearly stable P*cspA*-lux signals measured after 16 h post infection (Figure 5B). After 14 h, we measured the first significant P1*ces*-lux

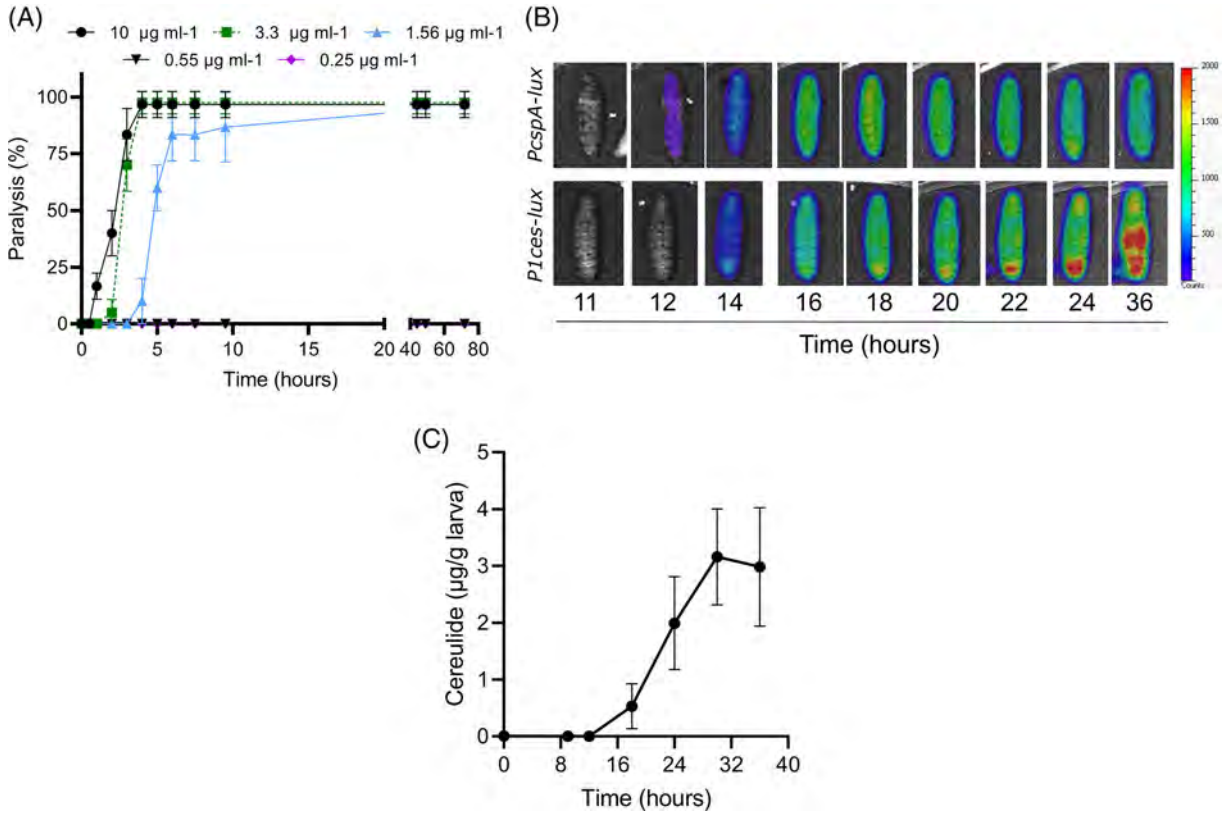

FIGURE 5 Cereulide exerts adverse effects in the insect host *Galleria mellonella*. (A) Paralysis of *G. mellonella* larvae in response to cereulide. Larvae were injected with the indicated cereulide concentration and incubated at room temperature for up to 80 h. Data represent mean and SD values from three replicates encompassing 10 larvae per injection group. (B) Real-time monitoring of *ces* gene cluster transcription. *G. mellonella* larvae were injected with *B. cereus* equipped with a bioluminescence reporter fused to the main *ces* promoter (*P1ces-lux*) or to the promoter of cold shock protein A (*PcspA-lux*) as control. Larvae were kept at 24°C and luminescent signals were recorded using an IVIS imaging system at the indicated time point. Each infection group encompassed eight larvae and one representative larvae is shown. Two independent experiments were performed. (C) Cereulide production in vivo. Larvae were infected with F4810/72 and extracts for cereulide quantification were prepared at the indicated time points. Data represent mean and SD values from a minimum of 30 larvae, except for 30 h, which represents mean and SD from 9 larvae.

signals, indicative of activated *ces* gene cluster transcription (Figure 5B). The luminescent signal increased strongly throughout the remaining observation time (up to 36 h), demonstrating an enhanced *ces* expression during the saprophytic stage of *B. cereus* in *G. mellonella*. Notably, highest cereulide synthase promotor activity was found in the head and abdomen of the larvae, thereby indicating a higher *ces* gene expression in distinct larval regions. In contrast, a nearly even distribution of luciferase activity in the control reporter strain P*cspA*-lux was seen between 18 and 36 h post infection (Figure 5B).To confirm that cereulide is indeed produced in the larval cadaver, cereulide was directly quantified in larvae infected with the WT strain using the SIDA described previously (Bauer et al., 2010). In accordance with luminescent reporter assays, cereulide was first detectable after 18 h post infection and it accumulated continuously up to 3–3.2 μg/g larva in the cadaver after 30–36 h (Figure 5C). In summary, cereulide production is triggered in the dead host and, along with bacterial biomass, increases during further growth in the host tissue. These results highly imply that cereulide functions during the saprophytic lifestyle of *B. cereus* within an insect host.

DISCUSSION

NRPS systems, such as the cereulide biosynthetic machinery, pose a major burden to the producer cell (Süssmuth & Mainz, 2017). Simultaneously, their products often mediate competitive fitness by functioning in nutrient acquisition or directly inhibiting potential competitors or predators (Alonzo & Schmeing, 2020; Brien & Wright, 2011). The benefit of cereulide synthesis for the producer strain in its typical environment is still unclear. Earlier work suggested that cereulide production provides a competitive advantage in environments with limited potassium availability (Ekman et al., 2012). Moreover, extracts from emetic *B. cereus* exert antimicrobial effects on some filamentous fungi (Ladeuze et al., 2011). The present work comprehensively extends our current knowledge on the antagonistic actions of cereulide on other organisms, which potentially share the same ecological niche as emetic *B. cereus*.

Cereulide may mediate niche adaptation against a competitive microflora

Gram-negative bacteria were resistant to cereulide treatment, which is in accordance with earlier work (Tempelaars et al., 2011), while Gram-positive bacteria displayed varying degrees of cereulide susceptibility. Gram-negative bacteria are protected by the OM, which is decorated with lipopolysaccharide molecules, and impedes the toxic actions such as detergents or hydrophilic antimicrobials (Li et al., 2017; Xu et al., 2021). Indeed, enhancing the permeability of the OM via exposure to PMBN increased cereulide susceptibility of *E. coli*, indicating that the OM of Gram-negative bacteria also protects against the action of cereulide. In contrast, cereulide susceptibility of Gram-positive bacteria was species-specific ranging from resistant (*S. aureus* and *L. monocytogenes*) to very sensitive (*A. globiformis* and *Rhodococcus fascians*; Figure S1 and Table 1). The cell envelope of Gram-positive bacteria consists of a thick, relatively porous structure, the peptidoglycan layer, which is permeable for small peptides (Lambert, 2002). However, changes in bacterial cell surface properties, such as the peptidoglycan layer, can modulate antimicrobial susceptibility (Hort et al., 2021). In addition, several other mechanisms determine the intrinsic susceptibility to antimicrobial peptides, such as the presence of efflux pumps that mediate compound export, or enzymes modifying and thus, inactivating the antimicrobial molecule (Blair et al., 2015; Peterson & Kaur, 2018). Similar mechanisms are also employed by the producer strain, thereby conferring so-called self-resistance (Abee, 1995; Riley, 1998). Likewise, the emetic type strain *B. cereus* F4810/72 displayed cereulide resistance, while cereulide susceptibility of the non-emetic *B. cereus* strain ATCC14579 and other *B. cereus* group members (*B. thuringiensis*, *B. weihenstephanensis*) was more pronounced. Yet, the latter species were still able to resume growth after a prolonged lag-phase in response to the highest test concentration. A shift in the growth start indicates a gradual adjustment process mostly by activation of a resistance mechanism that allows similar growth rates comparable to untreated conditions after cellular adaptation. Notably, the fungi *R. mucilaginosa* and *R. glutinis* displayed the highest degree of susceptibility amongst the tested yeast species, while antibacterial actions were most pronounced amongst *Arthrobacter* spp. These species may potentially share an ecological niche with emetic *B. cereus*, which was enriched in potato tubers (Altayar & Sutherland, 2006; Hoornstra et al., 2013) and rice paddy fields (Ueda & Kuwabara, 1988). Likewise, *Rhodotorula* spp and *A. oxydans* have been isolated from similar habitats (Berg et al., 2005; Kot et al., 2017; Lee et al., 2012; Sen et al., 2019; Wongwigkarn et al., 2020). Therefore, the ability to produce cereulide may function in inter-species competition in specialised ecological niches. Further, emetic *B. cereus* may reside as an endophyte, thereby engaging in a symbiotic relationship with certain plants. We found that the plant pathogen *R. fascians*, which persists and infects the aerial parts of herbaceous plants (Dhaouadi et al., 2020), was amongst the most cereulide susceptible Gram-positive bacteria. Additionally, growth of the rice blast fungus *Magnaporthae grisea* was inhibited by

extracts prepared from emetic *B. cereus* (Ladeuze et al., 2011). Generally, the heterogeneity of cereulide susceptibility amongst Gram-positive bacteria and fungi may reflect species-specific properties such as ecological niches and adaptation strategies. Thereby, cereulide production may potentially function in microbial warfare by decreasing the growth of unfavourable competitors. This concept was already postulated in earlier work demonstrating that cereulide can dissipate the bacterial membrane potential at alkaline pH of susceptible bacteria (Tempelaars et al., 2011). Furthermore, *B. subtilis* induces the production of certain antimicrobial NRPs only in the presence of a susceptible species, but not in response to phylogenetically closer and resistant species. Thereby, energy-intensive NRP biosynthesis is restricted (Maan et al., 2022). Hence, species susceptible to cereulide and emetic *B. cereus* may have a common niche. Alternatively, cereulide resistance may as well be a consequence of microbial coevolution and thus, simultaneously might indicate a potential common niche between emetic *B. cereus* and a resistant species. Such interactions may even result in a symbiotic relationship. Indeed, synergistic interactions amongst unrelated species are common (Banerji et al., 2019; D'Souza et al., 2018). For example, the NRP syringafactin is central in the cooperative defence of a *Pseudomonas* and *Paenibacillus* strain against amoebal predators (Zhang et al., 2021). Hence, further studies addressing cereulide expression upon microbial interaction are required to resolve the complex relationship between microbial competition and their molecular mediators.

Cereulide production may enable niche-specialization and flexible lifestyles

In addition to microbial competitors, soil bacteria are often further exposed to or grazed by the resident macroflora such as amoeba or nematodes (Mayrhofer et al., 2021; Rønn et al., 2002). Species from the *Bacillus* genus constitute the major part of the microbiome from free-living amoeba. Recent work further demonstrated that emetic *B. cereus* or bacterial supernatants exerted no adverse effects on amoebal viability and reproduction (Chen et al., 2021). In contrast, cereulide exposure had a major impact on L3 larval development and viability of the nematode *O. dentatum*. These observations suggest that cereulide production potentially enables specialised interactions of *B. cereus* within its ecological niches. Since many nematodes feed on bacteria (Blanc et al., 2006), it is tempting to speculate that cereulide might provide emetic *B. cereus* with an advantage against nematode competitors or predators in certain ecological niches. Indeed, certain habitats are enriched with emetic strains. For example, although *B. cereus* is frequently isolated from soil, the occurrence of emetic isolates is rare (Altayar & Sutherland, 2006; Hoton et al., 2009). In contrast, more than 40% of isolates from paddy rice fields (Ueda & Kuwabara, 1988) and 16% from potato belonged to the emetic lineage (Altayar & Sutherland, 2006). Hence, starch-rich plants may encompass a potential environmental niche of emetic *B. cereus*, which is in line with the frequent contamination of food products derived from the respective plants (Ehling-Schulz et al., 2019). Furthermore, it is suggested that soil constitutes rather a reservoir than a preferred environmental niche (Chen et al., 2021; Jensen et al., 2003). One distinguishable feature is that, unlike true soil bacteria, *B. cereus* is equipped with a limited number of carbohydrate-degrading enzymes, but its genome is enriched with proteo- and lipolytic enzymes (Ivanova et al., 2003), suggesting an adaptation to pathogenic, symbiotic or saprophytic lifestyle within a host such as insects or plants.

Besides plant roots, *B. cereus* has been isolated from a diverse number of insects, such as mosquito larvae and soil-dwelling insects, where it is able to colonise the gut (Swiecicka & Mahillon, 2006). It has been reported that *B. cereus* is able to persist in the gut of mosquito larvae for at least 7 days, while *B. thuringiensis*, a close relative of *B. cereus*, is cleared within 2 days (Luxananil et al., 2001). In addition, a recent study indicates that *B. cereus* is able to replicate in the gut of starved moth larvae (X. Li et al., 2022) while no evidence for replication of *B. thuringiensis* was found (Raymond et al., 2008). Since the referred studies did not specifically distinguish between emetic and non-emetic *B. cereus*, it is so far unknown whether emetic strains are present in insects and if cereulide production plays a role in the infection process. Notably, 64% of detected *B. cereus* group isolates from industrially reared cricket and yellow mealworm belonged to the emetic linage (Vandeweyer et al., 2020). Our results further indicate that cereulide synthesis does not mediate larval killing but rather plays a role in the insect cadaver as activation of cereulide expression and toxin accumulation occurs only in the cadaver of *G. mellonella* larvae. Consequently, the question arises about the biological function of cereulide in the larval cadaver. Dead insects represent a good nutrient source in the soil (Behie & Bidochka, 2013), which can be also exploited by emetic *B. cereus* as exemplified in the present study. Cereulide production may thereby delay or even prevent the growth of other microorganisms enabling persistence in this nutrient-rich environment. Modelling experiments have shown that early access to insect cadavers is key to competitive success in *B. thuringiensis* (van Leeuwen et al., 2015), fostering the hypothesis that cereulide might provide an advantage for a necromenic lifestyle of emetic *B. cereus*. Indeed, emetic strains belong to the *B. cereus* clade I (Ehling-Schulz

et al., 2019), recently suggested to entail necromenic bacteria (White et al., 2022). Interestingly, certain *Streptomyces* species produce valinomycin, a structurally similar potassium-binding NRP, and have a symbiotic relationship with leaf-cutting ants. Thereby, valinomycin functions as an antimicrobial protecting the fungal gardens, which are the major food source of leaf-cutting ants (Ilka et al., 2011). It is therefore tempting to speculate that cereulide-producing *B. cereus* might have a similar relationship with certain insect hosts followed by a saprophytic lifestyle upon host death. Moreover, insects can also contribute to the dispersion of bacteria and spores due to their mobility. Upon insect death or excretion by insectivore animals, bacteria are then released back into the soil or on plant parts. These cycles may also further enable the entry into the food chain of *B. cereus* (Ehling-Schulz et al., 2015; Majed et al., 2016).

Tuning cereulide function by environmental potassium may enable competitive adaptation

While cereulide was not expressed during the infectious phase in *G. mellonella* larvae, the larvae displayed pronounced sensitivity to systemic cereulide application causing rapid larval paralysis followed by larval death. Interestingly, neurological symptoms have also been observed in human food-intoxications related to cereulide (Ichikawa et al., 2010; Pósfay-Barbe et al., 2008) and in pigs orally challenged with cereulide (Bauer et al., 2018). As a potassium ionophore, cereulide interferes with the membrane potential of cellular and mitochondrial membranes with the environmental potassium concentrations affecting potassium fluxes and thus, cereulide toxicity (Andersson et al., 2007; Ekman et al., 2012;Mikkola et al., 1999; Teplova et al., 2006). The cytotoxic action of cereulide on mammalian cells such as HEp-2 (Andersson et al., 2007), Caco-2, HepG-2 (Decleer et al., 2018) and pancreatic beta cells (Vangoitsenhoven et al., 2014) is well established. We further demonstrated that the cytotoxicity on HEp-2 cells was strongly modulated by extracellular potassium concentrations. In contrast to bacterial cells, cereulide may act at two sites in intact eukaryotic cells, the cellular and mitochondrial membrane where it causes potassium efflux on non-energised membranes and potassium influx on energised membranes (Mikkola et al., 1999; Teplova et al., 2006). The synergistic cytotoxic action of potassium and cereulide on HEp-2 cells may stem from increased cellular stress due to hyperkaliemia. However, enhanced toxicity of cereulide in high potassium surroundings does not apply generally to eukaryotic organisms as the antifungal and antinematodal action of cereulide was unaffected upon increased environmental potassium. Regarding the antifungal activity, cell surface properties, which potentially limit cereulide access to cellular and mitochondrial membranes, may further determine cereulide susceptibility. Notably, cereulide has a high affinity for potassium and therefore, exerts its cytotoxic properties towards eukaryotic cells already at low potassium concentration (1–3 mM) (Teplova et al., 2006). Indeed, potassium occurs in the environment in rather low concentrations, typically ranging from 0.1–5 mM in soil (Liebeke et al., 2009; Maathuis, 2009) or extracellular fluids (Palmer & Clegg, 2016). In our study, cereulide was almost non-toxic at low potassium concentrations (5 mM) towards *B. casei*. However, increasing external potassium concentration enhanced the antibacterial action of cereulide as indicated by a prolonged lag phase. Cereulide evokes potassium efflux and an increase in the cellular membrane potential in non-emetic *B. cereus* in potassium-deficient medium, but not when the extracellular potassium levels are in the same order as the intracellular level (120 mM) (Ekman et al., 2012). In the current study, antibacterial assays were performed using a similarly high potassium concentration (171 M KCl). This indicates that cereulide-mediated potassium efflux and increasing membrane potentials played a minor role in the here observed growth inhibition of susceptible bacterial species. Higher potassium concentrations can be found in potash-rich soils, fruits, seeds, beans, humus, animal dung and the insect gut (Leigh & Wyn Jones, 1984; Harvey & Nedergaard, 1964; Tempelaars et al., 2011; Walker et al., 1996; Wang & Wu, 2010), from where *B. cereus* has been isolated (Feinberg et al., 1999; Margulis et al., 1998; Swiecicka & Mahillon, 2006). Furthermore, potato and its peel, which is a potential habitat of emetic *B. cereus*, is known for high potassium content (Altayar & Sutherland, 2006). In nutrient-rich environments, a rivalry amongst different organisms is likely and cereulide-producing *B. cereus* strains may be able to dominate by inhibiting the growth of other organisms. Earlier work already pointed towards a role of cereulide in microbial competition, as well as, a speculated function in signal transduction via potassium flux and cellular differentiation (Tempelaars et al., 2011). In addition, cereulide supports the fitness of emetic *B. cereus* strains in environments with low potassium (Ekman et al., 2012). Thus, it is conceivable that the action of cereulide differs in environments with low and high potassium resources and may further change in a concentration-dependent manner.

CONCLUSION

Taken together, our results demonstrate that cereulide exerts detrimental functions on a variety of organisms and acts in a species-specific manner. This indicates

that emetic *B. cereus* utilizes cereulide in a context-specific manner, thereby enabling an adaptive advantage in environments with fluctuating potassium levels and a competitive microflora. Moreover, cereulide may function as a communication signal thereby mediating adaptive fitness within a population. Hence, the targeted screening for emetic *B. cereus* in distinct environments will aid in unravelling the elusive ecological niche of emetic strains and the biological role of cereulide. A better comprehension of the ecology of cereulide-producing *B. cereus* will further aid in limiting its entry into the food chain and thus, increase food safety.

AUTHOR CONTRIBUTIONS

Sabrina Jenull: Conceptualization (equal); data curation (equal); formal analysis (equal); visualization (equal); writing – original draft (equal); writing – review and editing (equal). **Tobias Bauer:** Conceptualization (equal); data curation (equal); formal analysis (equal); visualization (equal); writing – original draft (equal). **Katja Silbermayr:** Data curation (equal); formal analysis (equal); visualization (equal). **Maximilian Dreer:** Data curation (equal). **Timo D. Stark:** Data curation (equal); formal analysis (equal). **Monika Ehling-Schulz:** Conceptualization (equal); funding acquisition (lead); supervision (equal); writing – original draft (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

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