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Susceptibility of emetic and enterotoxigenic *Bacillus cereus* grown at high temperature to disinfectants

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ABSTRACT

The prevalence of emetic strains in food products is rare; however, infection with these may be fatal to the vulnerable population. Bacterial control of the emetic *Bacillus cereus* strains is still unclear. This study aimed to assess the influence of high temperature on the disinfection of emetic and enterotoxigenic *B. cereus*. Emetic *B. cereus* strains survived up to 50 °C; the lag time and maximum growth rate were higher at 42 °C than those at 30 °C. Compared to enterotoxigenic *B. cereus*, all emetic food strains showed higher minimum inhibitory concentrations and minimum bactericidal concentrations for sodium hypochlorite and citric acid. The disinfectant susceptibility of the emetic *B. cereus* OS-05 strain incubated at a higher temperature did not increase and was maintained at the highest MBC value. In all emetic *B. cereus* strains, enterotoxin gene expression was upregulated at 42 °C and 45 °C. Increased *ces* gene expression was also found in emetic *B. cereus* strains GP-15 and OS-05, with upregulation of 128- and 820-fold at 42 °C. Thus, emetic *B. cereus* grown at high temperatures may resist common disinfectants of the food industry. The findings may help control *B. cereus* in food or the food processing industry.

1. Introduction

Bacillus cereus group belongs to a bacterial complex comprising of the following closely related pathogenic species: *B. anthracis, B. cereus*, *B. thuringiensis, B. mycoides, B. pseudomycoides, B. weihenstephanensis, B. cytotoxicus, B. toyonensis,* and *B. wiedmannii*. *B. cereus* is a gram-positive, spore-forming, rod-shaped pathogen causing diarrheal and emetic illnesses and is present in various foods such as dairy products, soybean products, starchy foods (noodles, rice, and cereals), vegetables, herbs, and spices [1]. Its growth temperature generally ranges between 10 and 45 °C [2]. Some strains (*B. cytotoxicus*) belonging to the *B. cereus* group can grow at temperatures up to 50 °C, whereas psychrotolerant strains (*B. weihenstephanensis* or *B. wiedmannii)* are unable to survive at 35 °C or 42 °C, respectively [3].

The diarrheal foodborne illness is caused by enterotoxins produced by *B. cereus*, and the foodborne illness with vomiting is a result of the preformed heat- and acid-stable toxin, 'cereulide', in food. Toxin genes are responsible for food poisoning by foodborne pathogens, show thermostability or resistance to digestive tract enzymes such as pepsin and trypsin [4]. Although the association between environmental stress and toxin gene expression has not been fully explained, the ability of foodborne pathogens to adapt to stressful environmental conditions may impact the intensity of the infection process [5]. Although antimicrobial agents eliminate

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B. cereus, residual amounts may be present in food and processed materials, making emetic *B. cereus* critical in food microbiology distribution [6]. Vomiting can occur from 15 min to 6 h after food consumption owing to food contaminated with the cereulide toxin produced during bacterial growth. The emetic symptoms are generally mild, and patients can recover within approximately 24 h; however, they can be fatal for vulnerable individuals, such as children or the older population [7]. Toxic levels of cereulide in foodborne diseases reported is between 10^5 and 10^8 colony forming units (CFU)/g in food. However, infective dose for food poisoning can start from 10^3 CFU/g in food due to the variation in emetic *B. cereus* strains [8].

Various antimicrobial agents inhibit the growth of *B. cereus* during food processing, distribution, and storage. Sodium hypochlorite, and organic acid are widely used in the food processing industry. Sodium hypochlorite, is a strong oxidizing agent with fact acting and effectiveness at low concentration [9,10]. Organic acids are popular disinfectants owing to their low cost and ease of manipulation. Acetic acid and citric acid have bacteriostatic and bactericidal effects, with hamper bacterial growth and lead to a loss in viability [11, 12]. Increasing chloride levels and lowering pH generally aid in limiting the growth rate and prolonging the lag phase of *B. cereus* [13]. Temperature is a crucial factor in determining antimicrobial activity of disinfectants such as sodium hypochlorite, and organic acids. The influence of temperature on antimicrobial activity against foodborne pathogens is known. A temperature higher or lower than the optimal for bacterial growth increases bacterial susceptibility to antimicrobial agents [1,14–16]. Psychrotolerant *B. cereus* activated at low temperatures is tolerant to sodium hypochlorite (NaOCl) [17]. Bacteria grown at different temperatures can develop increased resistance or susceptibility to antimicrobial agents. However, such comparisons are common for *Salmonella* spp., *Listeria monocytogenes*, and pathogenic *Escherichia coli*.

In addition to bacterial growth, temperature and environmental conditions can also affect virulence gene expression and the virulence potential of pathogens; however, studies showing the effect of temperature on virulence gene expression in *B. cereus* are limited. The enterotoxin gene expression of foodborne pathogens can differ in a strain-specific manner and is based on environmental conditions [18]. In *Yersinia enterocolitica*, gene expression is temperature-dependent and activates at 20–25 °C or host temperature [19, 20]. However, the presence of virulence-associated genes in microorganisms does not necessarily indicate their expression. Therefore, it is essential to understand the relationship between gene expression and virulence in different temperature conditions.

Standardized conditions such as the optimal temperature for the targeted bacteria were used to compare activities of the antimicrobial agents. However, changes in environmental conditions, such as temperature fluctuations during processing, cooking, storage, and serving, which could favor environment for bacterial growth. The incidence of emetic strain in food products is rare but their infection may be fatal to newborns and elders [7]. The emetic *B. cereus* should be inactivated before the production of cereulide toxin in food. However, there is a lack of reports on how to effectively inhibit or control the growth of these bacteria. Our study aimed to investigate the antimicrobial activity of disinfectant agents against *B. cereus* with a focus on emetic *B. cereus* strains under different temperature condition considering bacterial growth temperature range. Sodium hypochlorite, citric acid, and acetic acid, which are widely used in the food processing industry, were selected as the chemical or organic disinfectant agents, In addition, we investigated the expression of toxin genes such as non-hemolytic enterotoxin (NHE) complex (*nheA, nheB, nheC*), enterotoxin FM (*entFM),* cytotoxin K (*cytK*), and *ces*, with varying temperature.

2. Materials and methods

2.1. Bacterial strains

For this study, the following four reference enterotoxigenic *B. cereus* were used: *B. cereus* ATCC 14579, *B. cereus* ATCC 10876, *B. cereus* ATCC 21772, and *B. cereus* ATCC 11778. Additionally, the following six emetic *B. cereus* strains, *B. cereus* NCCP 14796 (emetic reference strain), GP-6, GP-15, GP-16 (isolated from RTE grain powder product) [21], MC-05, and OS-05 (isolated from fermented soybean products) [22], were used. Five emetic *B. cereus* isolates (GP-6, GP-15, GP-16, MC-05, and OS-05) obtained from food products harbored enterotoxin genes, such as *nheA, nheB, nheC, cytK*, and *entFM*.

2.2. Growth properties of emetic and enterotoxigenic B. cereus

All the *B. cereus* strains were screened for growth ability at 7, 10, 40, 42, 45, 47, 50, and 52 °C. *B. cereus* strains were first inoculated on tryptic soy agar (TSA) and incubated at 30 °C for 18 h. One colony of overnight culture was inoculated on a fresh TSA plate and incubated at 7 °C for 30 days, 10 °C for 20 days, and 40, 42, 45, 47, 50, and 52 °C for 18 h. After incubation, bacterial growth on the plates was examined. To analyze the growth curve and parameters, all strains were inoculated in 10 mL of tryptic soy broth (TSB) and the bacterial cultures were incubated for 24 h at 30 °C. This culture (50 μL) was inoculated into 5 mL of fresh TSB at an initial concentration of 10^3 CFU/mL and incubated at 30, 42, 45, and 50 °C for 18 h. The samples from all tested temperatures were analyzed every 2 h, and *B. cereus* cells were enumerated on mannitol-egg-yolk-polymyxin B agar (Merck, Darmstadt, Germany) and incubated at 30 °C for 18 h. Growth curves were obtained by plotting log CFU/mL against the exposure time. The ComBase software tool, DMfit 2.0 program (*Baranyi and Roberts* model, https://www.combase.cc, accessed on: 10 September 2022) was used to determine the lag/shoulder (h), and maximum growth rate (LogCFU/ml/h) for all tested strains at varying temperatures. Triplicate assays were performed using *B. cereus* isolates at different temperatures.

2.3. Antibiotic susceptibility

The antibiotic susceptibility profile was determined using Muller–Hinton agar. Two temperatures, 30 °C and 45 °C, were used for

incubation. The antimicrobial agents were tested and their concentrations were as follows: cefotaxime (30 μg), ceftriaxone (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), clindamycin (2 μg), erythromycin (15 μg), gentamicin (10 μg), imipenem (10 μg), penicillin (10 μg), rifampin (5 μg), tetracycline (30 μg), and vancomycin (10 μg) [23]. Inhibition zones were measured using a ruler, and antibiotic activity was determined based on the diameter of the inhibition zone. The results were classified as resistant, intermediate, and susceptible in accordance with the criteria provided by the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2022) guidelines.

2.4. Minimum inhibitory concentration and minimum bactericidal concentration to determine disinfectant susceptibility

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of NaOCl (10–15%; Sigma-Aldrich, St. Louis, MO, USA), acetic acid (≥99.8%; Sigma-Aldrich), and citric acid (purity ≥99%, Sigma-Aldrich) were determined using the broth dilution method in a 96-well plate [17]. The plates were prepared using a two-fold dilution of NaOCl (concentration ranging from 4 to 0.03125%), citric acid (concentration ranging from 3 to 0.0625%), and acetic acid (concentration ranging from 3 to 0.0625%) with a total volume of 200 μL. The final concentration of all bacterial suspensions was adjusted to 10^5–10^6 log CFU/mL and the plates were incubated at 30, 42, and 45 °C for 18 h. The MIC of each disinfectant was defined as the lowest concentration at which no bacterial growth was observed. To define the MBC value, 0.1 mL was removed from the wells where no growth was observed and inoculated onto the TSA plates. The value of that the lowest concentration with no perceivable growth on the plate was identified as the MBC.

2.5. Virulence gene expression using quantitative real time reverse transcription-polymerase chain reaction

Total RNA from *B. cereus* strains was extracted using the RNeasy Mini Kit (Qiagen, Valencia, CA), and the extracted RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA), according to the manufacturer's instructions. The primer sequences used for quantitative real-time polymerase chain reaction (PCR) are illustrated in Table 1. Quantitative reverse transcription-PCR was performed in a 20 μL reaction volume with 50 ng of cDNA, SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 10 pmol of each primer, using the QuantSudio 3 Real Time PCR System (Applied Biosystems, USA). Triplicate assays were performed using cDNA samples isolated from the *B. cereus* strains at three different temperatures. For negative controls, distilled water was used. Negative results or no amplification curves were considered when the cycle threshold (Ct) values were more than 40. The data confirmed that the transcript levels of the *rpoA* gene for data normalization were not significantly different at 42 °C and 45 °C, compared to those at 30 °C. The fold change was calculated using the comparative Ct method ($2^{-\Delta\Delta CT}$ method).

2.6. Statistical analyses

Mean values were obtained from three replicate experiments, and all data were statistically analyzed using analysis of variance and Tukey's test using SPSS software (Statistical Package for the Social Sciences, version 22.0, IBM, NY, USA). For all tests, a *p*-value of 0.05 or less was considered statistically significant.

3. Results

3.1. Growth parameter of emetic and enterotoxigenic B. cereus

The growth of emetic and enterotoxigenic *B. cereus* strains in different temperature conditions was analyzed on TSA plates. Emetic *B cereus* strains, including GP-6, GP-16, MC-05, and NCCP 14796, grew at temperatures ranging from 10 to 50 °C and the maximum

Table 1
Sequences of primers for reverse transcription and quantitative Real-time PCR.

Target gene	Primer sequence (5′-3′)	Length (bp)	Reference
rpoA	*rpoA*-F: GTATACGCCAGCTGATGCAA *rpoA*-R: ATGCTTCCATCCGTCCATAC	171	Gao et al., 2021
nheA	*nheA*-F: TTCAAATTCAAAAGAATGTTGAAGAAGG *nheA-R:* GATTTGTTTGCTTATTCATTTCATCAC	111	Wehrle et al., 2010
nheB	*nheB*-F: TGCGAAGCAATGGTTAGATG *nheB*-R: AACTGATCCACTTGCGCTTT	198	Melnick et al., 2012
nheC	*nheC*-F: CAGCACCAAAGAGATGCAAA *nheC*-R: CGCGAAAAGCTTTCAAATTC	250	Melnick et al., 2012
entFM	*entFM*-F: ATTGCAGGTTTAGCAGCAGCTT *entFM*-R: GCGCTTCATTTGAAACTTGTGC	85	Li et al., 2016
cytK	*cytK*-F: GCGCTGATAAACAGATTGCCGT *cytK*-R: TAGCGCCAGGGATTGGGTAGTT	105	Li et al., 2016
ces	*ces*-F: GCTTTGTATAAGCAACTTGGATAG *ces*-R: AGCCTCTGTAACACCAAGC	389	Wehrle et al., 2010

growth temperature of two strains (GP-15 and OS-05) was 52 °C (Table 2). In contrast, all enterotoxigenic ATCC *B. cereus* strains exhibited growth in the temperature range of 10–45 °C. Thus, we selected 30, 42, 45, 47, 50, and 52 °C for the growth curve analysis. The growth curve and parameters, including lag time and maximum growth rate at each temperature, were calculated using the Baranyi model (Fig. 1(a–d) and Table 3). All emetic *B. cereus* strains maintained bacterial viability at temperatures ranging from 42 to 47 °C, while enterotoxigenic ATCC *B. cereus* strains exhibited a bactericidal curve after 6 h at 45 °C and 5 h at 47 °C (Fig. 1(a–c)). All *B. cereus* strains, including the emetic and enterotoxigenic strains, showed a bactericidal curve during incubation at 50 °C and bacteria were not detected after 12 h (Fig. 1(d)). In addition, none of the tested *B. cereus* strains were detected after incubation for 1 h at 52 °C (data not shown). Growth temperature affected the lag time and maximum growth rate of *B. cereus* strains. In emetic *B. cereus* strains, a higher growth temperature (42 °C) resulted in a shorter lag time and a higher maximum growth rate than those at the optimal temperature (30 °C). GP-16 incubated at 42 °C showed the shortest lag time (0.5 ± 0.45 h), while GP-15 exhibited the shortest lag time (0.6 h) at 45 °C. Unlike enterotoxigenic *B. cereus* strains that survived up to 45 °C, emetic strains maintained their growth ability until 47 °C in TSB and were detected for up to 52 °C in TSA plates, indicating that emetic *B. cereus* strains were more tolerant to high-temperature conditions than were the enterotoxigenic strains.

3.2. Antibiotic susceptibility at different growth temperatures

Table 4 shows the resistance, intermediate susceptibility, and susceptibility patterns of *B. cereus* strains grown at 30 °C and 45 °C. All emetic and enterotoxigenic *B. cereus* strains were resistant to penicillin, ceftriaxone, rifampicin, and cefotaxime, regardless of their growth temperature, and all strains were susceptible to imipenem, ciprofloxacin, chloramphenicol, and gentamicin at both 30 and 45 °C. At 30 °C, most *B. cereus* strains were susceptible to clindamycin; however, four emetic food strains (GP-6, GP-15, MC-05, and OS-05) exhibited intermediate susceptibility. Furthermore, five emetic *B. cereus* strains were susceptible to tetracycline antibiotics; in contrast, enterotoxigenic ATCC strains were moderately susceptible or resistant to tetracycline. The antibiotic susceptibility of emetic and enterotoxigenic *B. cereus* strains decreased or remained unchanged at 45 °C, compared to that for strains grown at 30 °C. For instance, MC-05 and OS-05 were moderately susceptible to rifampicin at 30 °C, but were susceptible to it at 45 °C. In addition, OS-05 was resistant to vancomycin at 30 °C, whereas the strain grown at 45 °C exhibited enhanced sensitivity.

3.3. Minimum inhibitory concentration of NaOCl, acetic acid, and citric acid at different growth temperatures

The MICs of NaOCl, acetic acid, and citric acid for emetic *B. cereus* strains were investigated at 30 °C and compared to those at 42 °C and 45 °C. The emetic and enterotoxigenic *B. cereus* strains were more susceptible to the tested disinfectants at higher temperatures of 42 °C or 45 °C than that at 30 °C (Table 5). MIC values between emetic and enterotoxigenic *B. cereus* strains showed considerable differences, regardless of the growth temperature. At 30 °C, the MICs of NaOCl for five emetic *B. cereus* food strains, including NCCP 14796, were 0.5%, and these values were at least two- (0.25% of *B. cereus* ATCC 11778 and *B. cereus* ATCC 21772) or four- (0.125% of *B. cereus* ATCC 14579 and *B. cereus* 10876) times higher than those of enterotoxigenic ATCC *B. cereus* strains. When the temperature increased to 42 °C, the MIC values decreased to 0.125% (for *B. cereus* ATCC 21772), 0.0625% (for *B. cereus* ATCC 11778), and 0.25% (for all emetic strains except for OS-05). The MICs at 45 °C were similar to the antimicrobial activity to NaOCl at 42 °C. Furthermore, at 30 °C, the MICs of citric acid were 0.5% against all emetic *B. cereus* strains and 0.3125% against all the tested enterotoxigenic *B. cereus* strains (Table 4). On increasing the temperature to 42 °C and 45 °C, the MICs of citric acid decreased to 0.25% in all emetic and enterotoxigenic *B. cereus* strains, except for *B. cereus* ATCC 14579 and OS-05. Conversely, these two strains retained a 0.5% MIC value for citric acid, regardless of the growth temperature. Incubation temperature did not affect the MIC for acetic acid and exhibited the same value (0.3125%) at 30 °C.

3.4. Minimum bactericidal concentration of NaOCl, acetic acid, and citric acid at different growth temperatures

The MBCs of NaOCl, acetic acid, and citric acid were greater than or equal to those of the MICs in the emetic and enterotoxigenic

Table 2
Evaluation of the growth ability of emetic and enterotoxigenic *B. cereus sensu stricto* strains in TSA agar plate.

	7 °C	10 °C	40 °C	42 °C	45 °C	47 °C	50 °C	52 °C
Emetic *Bacillus cereus sensu stricto*								
B. cereus NCCP 14796	-*	+	+	+	+	+	+	–
GP-16	–	+	+	+	+	+	+	–
GP-15	–	+	+	+	+	+	+	+
GP-6	–	+	+	+	+	+	+	–
MC-05	–	+	+	+	+	+	+	+
OS-05	–	+	+	+	+	+	+	–
Enterotoxigenic *Bacillus cereus sensu stricto*								
B. cereus ATCC 14579	–	+	+	+	+	–	–	–
B. cereus ATCC 21772	–	+	+	+	+	–	–	–
B. cereus ATCC 10876	–	+	+	+	+	–	–	–
B. cereus ATCC 11778	–	+	+	+	+	–	–	–

*-: non-growth of *B. cereus s.s.* strain; +: growth of *B. cereus s.s.* strain.

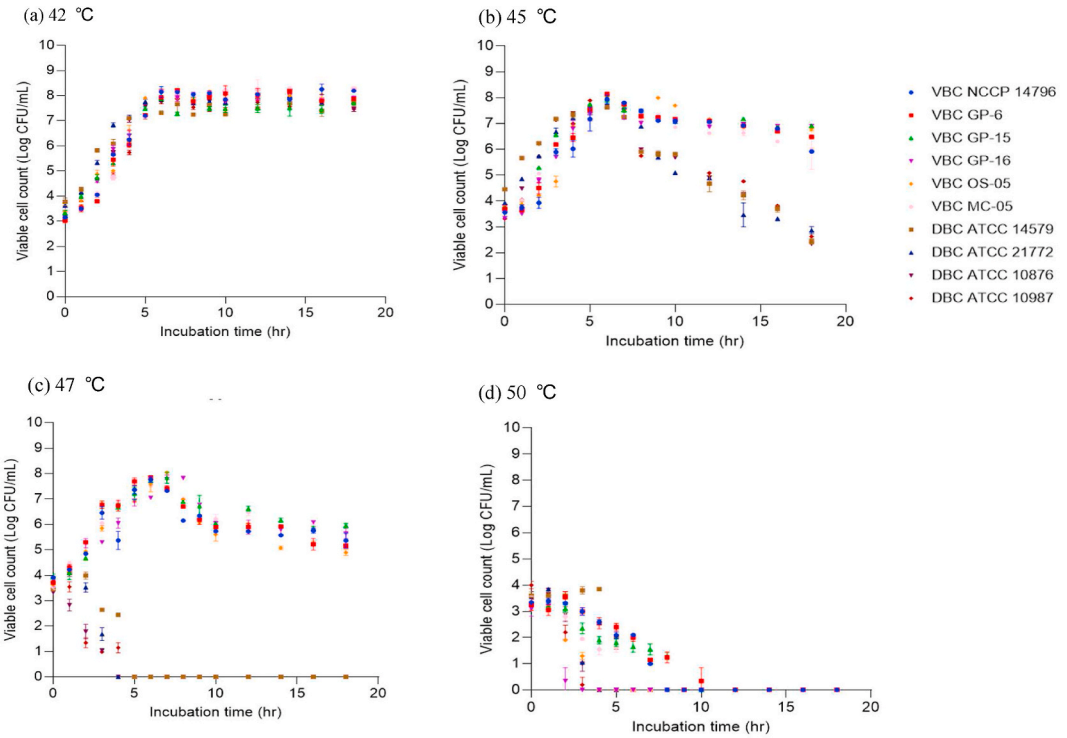

Fig. 1. Growth curve for emetic and enterotoxigenic *Bacillus cereus sensu stricto* strains obtained at 42 °C (a), 45 °C (b), 47 °C (c), and 50 °C. All data were presented as mean ± SD (standard deviation).

B. cereus strains tested (Table 6). The MBC of NaOCl at 30 °C was the highest (2.0%) in GP-6, GP-15, and OS-05, including the emetic reference strain NCCP 14796, followed by 1.0% in GP-6 and MC-05. Enterotoxigenic *B. cereus* ATCC 10876 and ATCC 11778 exhibited the lowest MBC (0.25%) at 30 °C. Considerable differences in MBC were observed between emetic and enterotoxigenic *B. cereus* strains. For instance, the MBCs of emetic *B. cereus* strains were four- (0.5%) to eight- (0.25%) times higher than that of enterotoxigenic ATCC *B. cereus*. The MBCs of NaOCl against emetic *B. cereus* strains at 42 °C were 2.0% for *B. cereus* NCCP 14796, GP-16, GP-15, and OS-15; and 0.5% for GP-6 and MC-05. *B. cereus* ATCC 14579, ATCC 10876, and ATCC 21772 showed MBCs of 0.25%, and *B. cereus* ATCC 11778 showed an MBC of 0.125% to NaOCl. The MBCs at 45 °C were lower or equal to those at 42 °C. However, two emetic *B. cereus* strains, GP-15 and OS-05, maintained their MBC values and were detected in 2% NaOCl at all tested temperatures.

The MBCs of acetic acid were 0.3125% against most of the tested *B. cereus* strains, regardless of the incubation temperature (Table 6). However, among emetic *B. cereus* strains, GP-15 grown at 30 °C exhibited the highest MBC (3%), which was approximately ten times higher than the lowest MBC value (0.3125%) obtained from all enterotoxigenic *B. cereus* strains and two emetic strains (GP-6 and OS-05). Increasing the growth temperature from 30 °C to 42 °C or 45 °C decreased MBC values against GP-16 (0.75% at 30 °C vs. 0.3125% at 42 and 45 °C), MC-05 (0.75% at 30 °C vs. 0.3125% at 42 and 45 °C), and GP-15 (3% at 30 °C vs. 2.5% at 42 and 45 °C). Three emetic *B. cereus* strains (NCCP 14796, GP-6, and OS-05) exhibited similar MBCs at 42 °C and 45 °C, and all enterotoxigenic *B. cereus* strains showed 0.3125% MBC for acetic acid in comparison to those at 30 °C. Among the tested *B. cereus* strains, only GP-15 was detected at acetic acid concentrations of up to 2% and maintained survival regardless of the incubation temperature. The MBCs for citric acid exhibited significant differences between emetic and enterotoxigenic *B. cereus* strains. At 30 °C, MBCs of emetic *B. cereus* strains were more remarkable, with two- (2.0%) or three- (3.0%) fold increase in comparison to 0.5% (*B. cereus* ATCC 14579) and 1.0% (*B. cereus* ATCC 21772, *B. cereus* ATCC 10876, and *B. cereus* ATCC 11778) against enterotoxigenic *B. cereus* strains. The MBCs for GP-15, GP-6, MC-05, and OS-05 among the emetic *B. cereus* strains at 42 °C were similar to those at 30 °C. Most of the tested *B. cereus* strains showed decreased MBC values at 45 °C; OS-05 was detected at a citric acid concentration of 3% at 45 °C.

3.5. Virulence gene expression

Genes involved in bacterial virulence were differentially expressed in emetic and enterotoxigenic *B. cereus* strains at different incubation temperatures (Fig. 2(a–f)). Although gene expression varied with the strain type, the most emetic *B. cereus* strains exhibited upregulated gene expression for *nheA, nheB, nheC, cytK,* and *entFM* at 42 °C or 45 °C as compared to those at 30 °C (Fig. 2(a–e)). Interestingly, the *nheA* and *cytK* genes were more overexpressed in GP-16 at 45 °C than that at 30 °C, and the relative gene expression at 42 °C was similar to that at 45 °C. The expression profiles of *ces* differed in emetic *B. cereus* strains. GP-15 and OS-05 overexpressed *ces* at 42 and 45 °C, where the upregulation at 42 °C was significantly higher than that at 45 °C (Fig. 2 (f)).

Table 3
Mean values of growth parameters at different temperatures calculated for the emetic and enterotoxigenic *Bacillus cereus sensu stricto* strains.

	30 °C		42 °C		45 °C		47 °C	
	Lag phage (h)	Maximum specific growth Rate (μmax, h^{-1})	Lag phage (h)	Maximum specific growth Rate (μmax, h^{-1})	Lag phage (h)	Maximum specific growth Rate (μmax, h^{-1})	Lag phage (h)	Maximum specific growth Rate (μmax, h^{-1})
Emetic *Bacillus cereus sensu stricto*								
B. cereus NCCP 14796	1.3 ± 0.60^{ab}*	0.7 ± 0.05^{a}	0.9 ± 0.31^{b}	1.1 ± 0.09^{ab}	1.1 ± 0.67^{b}	1.1 ± 0.28^{b}	0.9 ± 2.11^{a}	0.8 ± 0.6^{a}
GP-6	1.3 ± 0.32^{ab}	1.2 ± 0.13^{c}	1.1 ± 0.26^{bc}	1.1 ± 0.07 ^{ab}	1.1 ± 0.63^{b}	1.2 ± 0.28^{b}	1.3 ± 0.89^{ab}	1.8 ± 1.40^{bc}
GP-15	1.1 ± 0.43^{a}	0.7 ± 0.04^{a}	0.7 ± 0.48 ^{ab}	0.9 ± 0.13^{a}	0.6 ± 0.72^{a}	1.1 ± 0.29^{b}	1.9 ± 0.45^{b}	2.7 ± 1.66^{c}
GP-16	2.1 ± 0.60^{c}	0.9 ± 0.12^{b}	0.5 ± 0.45^{a}	1.0 ± 0.11 ^{ab}	0.9 ± 0.53^{ab}	1.2 ± 0.24^{b}	0.9 ± 1.41^{a}	0.7 ± 0.27^{a}
OS-05	1.8 ± 0.85^{bc}	0.7 ± 0.10^{a}	1.2 ± 0.42^{bc}	1.1 ± 0.16 ^{ab}	1.6 ± 0.23^{c}	2.1 ± 0.43^{c}	1.1 ± 1.43^{ab}	0.9 ± 0.57^{ab}
MC-05	1.9 ± 0.68^{bc}	0.8 ± 0.10^{ab}	1.6 ± 0.29^{c}	1.2 ± 0.12^{b}	0.7 ± 0.91^{a}	0.8 ± 0.22^{a}	1.1 ± 1.11^{ab}	1.1 ± 0.57^{b}
Enterotoxigenic *Bacillus cereus sensu stricto*								
B. cereus ATCC 14579	1.7 ± 0.38^{bc}	0.8 ± 0.05^{ab}	0.8 ± 0.50^{b}	0.9 ± 0.13^{a}	NS**	NS	NS	NS
B. cereus ATCC 21772	1.8 ± 0.27^{bc}	0.8 ± 0.03^{ab}	0.7 ± 0.27^{ab}	1.3 ± 0.15^{b}	NS	NS	NS	NS
B. cereus ATCC 10876	1.7 ± 0.27^{b}	0.6 ± 0.01^{a}	0.7 ± 0.15 ^{ab}	1.1 ± 0.05^{ab}	NS	NS	NS	NS
B. cereus ATCC 11778	2.1 ± 0.60^{c}	0.6 ± 0.06^{a}	1.4 ± 0.27^{c}	1.1 ± 0.09^{ab}	NS	NS	NS	NS

*Value with different superscripts of lowercase letter in the same column for each strain and growth parameter are significantly different ($P < 0.05$).
**no significant lag-phase and maximum specific growth rate detected.

Table 4
Antibiotic susceptibility pattern of emetic and enterotoxigenic *Bacillus cereus sensu stricto* strains at different temperature condition.

		P*	IPM	CRO	DA	RD	CIP	CTX	TE	C	E	VA	CN
Emetic *Bacillus cereus sensu stricto*													
B. cereus	30 °C	R**	S	R	S	R	S	R	S	S	S	S	S
NCCP 14796	45 °C	R	S	R	S	R	S	R	S	S	S	S	S
GP-16	30 °C	R	S	R	S	R	S	R	S	S	S	S	S
	45 °C	R	S	R	S	R	S	R	S	S	S	S	S
GP-15	30 °C	R	S	R	I	R	S	R	S	S	S	S	S
	45 °C	R	S	R	I	R	S	R	S	S	S	S	S
GP-6	30 °C	R	S	R	I	R	S	R	S	S	S	S	S
	45 °C	R	S	R	I	R	S	R	S	S	S	S	S
MC-05	30 °C	R	S	R	I	R	S	R	S	S	S	S	S
	45 °C	R	S	R	S	R	S	R	S	S	S	S	S
OS-05	30 °C	R	S	R	I	R	S	R	S	S	S	R	S
	45 °C	R	S	R	S	R	S	R	S	S	S	S	S
Enterotoxigenic *Bacillus cereus sensu stricto*													
B. cereus	30 °C	R	S	R	S	R	S	R	I	S	S	S	S
ATCC 14579	45 °C	R	S	R	S	R	S	R	I	S	S	S	S
B. cereus	30 °C	R	S	R	S	R	S	R	I	S	I	S	S
ATCC 21772	45 °C	R	S	R	S	R	S	R	I	S	I	S	S
B. cereus	30 °C	R	S	R	S	R	S	R	R	S	S	S	S
ATCC 10876	45 °C	R	S	R	S	R	S	R	R	S	S	S	S
B. cereus	30 °C	R	S	R	S	R	S	R	I	S	S	S	S
ATCC 11778	45 °C	R	S	R	S	R	S	R	I	S	S	S	S

*P: Penicillin; IPM: Imipenem; CRO: Chloramphenicol; DA: Clindamycin; RD: Rifampin; CIP: Ciprofloxacin; CTX: Ceftriaxone TE: Tetracycline; C: Cefotaxime E: Erythromycin; VA: Vancomycin; CN: Gentamicin.
**R: Resistant; S: Susceptible; I: Intermediate.

Table 5
Minimum inhibitory concentrations (% *w/v*) of three disinfectant agents against emetic and enterotoxigenic *Bacillus cereus sensu stricto* strains at different temperature.

MIC	Sodium hypochlorite			Citric acid			Acetic acid		
	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C
Emetic *Bacillus cereus sensu stricto*									
B. cereus NCCP 14796	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
GP-16	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
GP-15	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
GP-6	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
MC-05	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
OS-05	0.5	0.5	0.25	0.5	0.5	0.5	0.3125	0.3125	0.3125
Enterotoxigenic *Bacillus cereus sensu stricto*									
B. cereus ATCC 14579	0.125	0.125	0.125	0.5	0.25	0.25	0.3125	0.3125	0.3125
B. cereus ATCC 21772	0.25	0.125	0.125	0.5	0.25	0.25	0.3125	0.3125	0.3125
B. cereus ATCC 10876	0.125	0.125	0.0625	0.5	0.25	0.25	0.3125	0.3125	0.3125
B. cereus ATCC 11778	0.25	0.0625	0.0625	0.5	0.25	0.25	0.3125	0.3125	0.3125

Table 6
Minimum bactericidal concentrations (% *w/v*) of three disinfectant agents against emetic and enterotoxigenic *Bacillus cereus sensu stricto* strains at different temperature.

MBC	Sodium hypochlorite			Citric acid			Acetic acid		
	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C	30 °C	42 °C	45 °C
Emetic *Bacillus cereus sensu stricto*									
B. cereus NCCP 14796	2	2	0.5	3	2	0.5	0.3125	0.3125	0.3125
GP-16	2	2	0.5	3	2	1	0.75	0.3125	0.3125
GP-15	2	2	2	3	3	2	3	2.5	2.5
GP-6	1	0.5	0.5	2	2	0.5	0.3125	0.3125	0.3125
MC-05	1	0.5	0.5	2	2	0.5	0.75	0.3125	0.3125
OS-05	2	2	2	3	3	3	0.3125	0.3125	0.3125
Enterotoxigenic *Bacillus cereus sensu stricto*									
B. cereus ATCC 14579	0.5	0.25	0.25	0.5	0.25	0.25	0.3125	0.3125	0.3125
B. cereus ATCC 21772	0.5	0.25	0.25	1	0.5	0.5	0.3125	0.3125	0.3125
B. cereus ATCC 10876	0.25	0.25	0.0625	1	0.5	0.25	0.3125	0.3125	0.3125
B. cereus ATCC 11778	0.25	0.125	0.125	1	0.25	0.25	0.3125	0.3125	0.3125

4. Discussion

Emetic *B. cereus* lacks the *hbl* complex genes encoding hemolysin BL [2] but possesses enterotoxin genes for NHE complexes, CytK, and EntFM, including the synthetase gene cluster linked to cereulide production [21,22,24]. This finding also suggests its involvement in diarrheal syndromes. The five emetic *B. cereus* strains collected in the present study also harbored enterotoxin genes, including *nheA, nheB, nheC, cytK, entFM*, and emetic toxin encoded by the *ces* gene [21,22]. All emetic *B. cereus* strains and the *ces* positive-NCCP 14796 strain could survive at temperatures between 10 and 50 °C, whereas enterotoxigenic ATCC *B. cereus* strains showed maximum growth at 45 °C. Compared to other *B. cereus* strains, the emetic strains display strain-specific characteristics, including no or weak hemolysis and the inability to hydrolyze starch and salicin [25]. Most cereulide-producing *B. cereus* strains are mesophilic, but some can grow at 8 °C [26,27]. Furthermore, emetic *B. cereus* strains do not grow below 10 °C and can grow at 48 °C [28]. In this study, emetic *B. cereus* strains exhibited a lower lag time and a higher maximum growth rate at 42 °C than that at 30 °C. Dobrić et al. also found that the maximum growth of *B. cereus* was much faster at 30 °C than that at 10 °C [29]. The metabolic rate of *Bacillus megaterium* also increases with increasing temperature.

Temperature is critical for regulating antibiotic susceptibility. Increased susceptibility to amikacin has been observed in *E. coli, S. enterica,* and *Staphylococcus aureus* after incubation at high temperatures (45 °C) [30]. Elevated temperatures (41 °C) increase the antibiotic sensitivity of the resistant strain [31]. These results are concordant with our study, where the antibiotic susceptibility of most emetic and enterotoxigenic *B. cereus* strains remained unchanged with increasing temperature; however, some emetic *B. cereus* strains

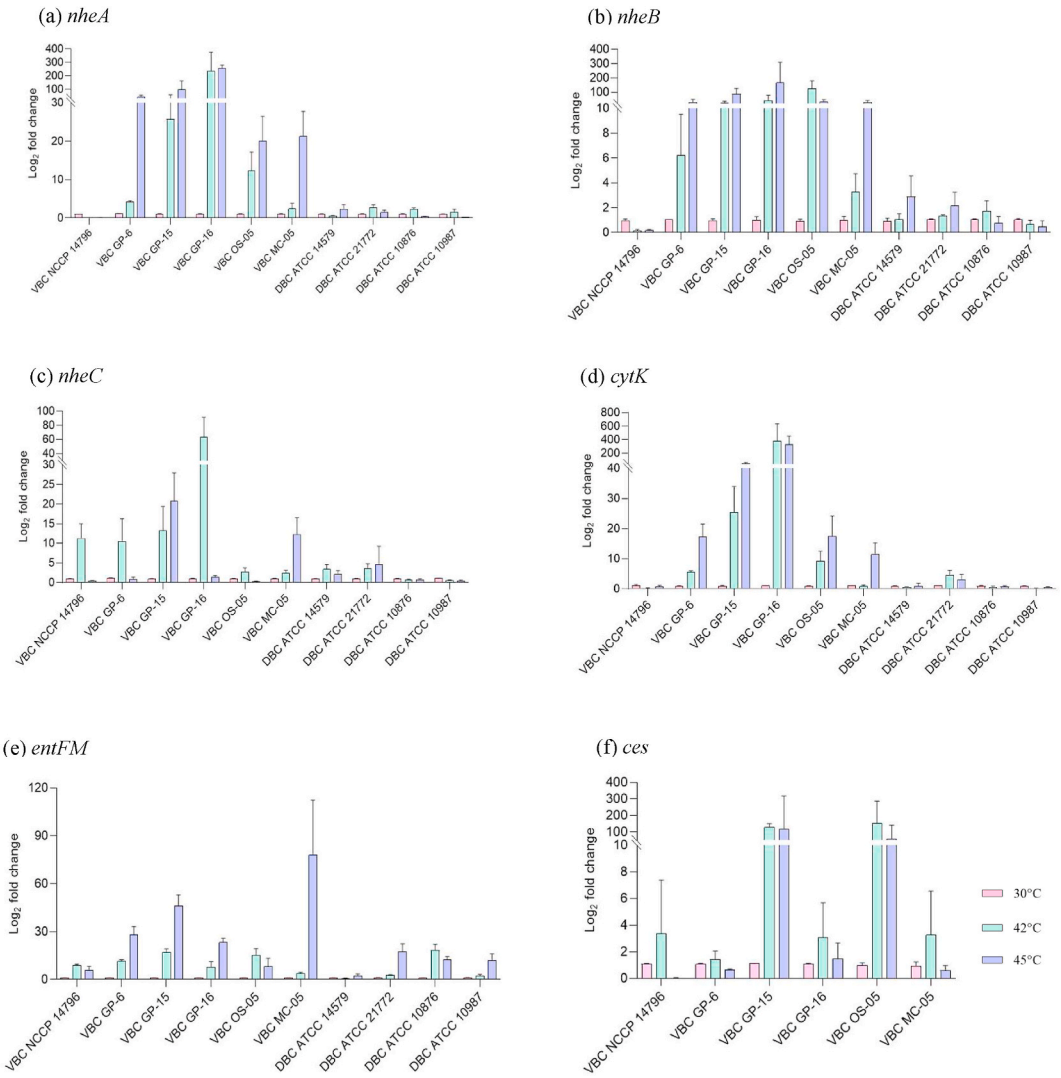

Fig. 2. Expression of *nheA* (a), *nhe*B (b), *nheC* (c), *cytK* (d), *entFM* (e), and *ces* (f) genes in emetic (VBC) and enterotoxigenic (DBC) *Bacillus cereus sensu stricto* strains at 42 and 45 °C. Gene expression level in the control (30 °C) was set as 1.0. The data were expressed as the mean fold change (n = 3). All data were presented as mean ± SD (standard deviation).

that were intermediate susceptible to clindamycin exhibited increased sensitivity to the antibiotic. Additionally, the OS-05 strain, resistant to vancomycin at 30 °C, developed sensitivity at 45 °C. In contrast, *Acinetobacter baumannii* treated at a high temperature (45 °C) displayed an increased survival rate after exposure to streptomycin and aminoglycosides [14].

Susceptibility testing is generally performed under standardized conditions, while antimicrobial activity is highly dependent on the bacterial species or environmental conditions. Therefore, the present study evaluated the influence of temperature on the disinfectant susceptibility of emetic *B. cereus* strains, in comparison with enterotoxigenic strains. Our results indicate that the antimicrobial susceptibility of disinfectants varies with the type of *B. cereus* strains and their temperature conditions. The antimicrobial activity of NaOCl against five emetic *B. cereus* strains, including emetic *B. cereus* NCCP 14796, exhibited two- or four-fold higher MICs and four- or eight-fold higher MBCs than those of enterotoxigenic *B. cereus* strains at standard temperatures. The antimicrobial susceptibility of most emetic and enterotoxigenic *B. cereus* strains to NaOCl increased with temperature. However, two emetic *B. cereus* strains, GP-15 and OS-05, grown at 42 °C and 45 °C, maintained 2% MBC at 30 °C, indicating that emetic *B. cereus* may exhibit a greater tolerance to NaOCl than that by enterotoxigenic *B. cereus* strains, regardless of increased temperature. NaOCl is extensively utilized for disinfection despite its decreased efficacy in the presence of organic material [32]. The effect of NaOCl depends on various factors, such as concentration, contact time, and temperature, and presents a wide spectrum of antimicrobial activity over a broad temperature range [15, 33]. The bactericidal action of NaOCl solutions can be altered at high temperatures [15]. A 2.6% NaOCl concentration effectively reduces the vegetative cells of *S. aureus*, *E. coli,* and *Bacillus subtilis* [15]. The present study reported that the tested bacteria showed higher inhibitory activity of NaOCl at 37 °C than that at 21 °C. The bactericidal effect of NaOCl against *Enterococcus faecalis* also

exhibits a 100-fold increase at 45 °C compared to that at 20 °C [34]. Furthermore, the highest antibacterial activity against *Enterococcus faecalis* has been reported at 45 °C compared to that at 20 °C [16]. Temperature can influence the bactericidal action of NaOCl as well as affect the viscosity and surface tension of NaOCl-based irrigants [9,10]. Heating the NaOCl solution increases the kinetic energy and movement of the molecules. Because this movement has enough energy to overcome the force responsible for binding the molecules together, the solution becomes more fluid, and its viscosity decreases. This motion may be explained by thermal agitation of the molecules with rapid movements at a higher temperature (37 °C) compared to that at 22 °C [9]. Similarly, the current study showed that the antimicrobial activity of NaOCl increased when the growth temperature increased from 30 °C to 42 °C or 45 °C, and the antimicrobial effectiveness was greater in enterotoxigenic strains than that in emetic *B. cereus* strains. However, emetic *B. cereus* strains had higher MBC values than those in enterotoxigenic strains, even with an increase in incubation temperature. Interestingly, GP-15 and OS-05, among emetic *B. cereus* strains, maintained the highest MBC value of 2% at all the tested temperatures.

Organic acids are popular disinfectants owing to their low cost and ease of manipulation [11]. Many reports on antimicrobial activity of organic acids against foodborne pathogens such as *E. coli* O157:H7, *Salmonella* Enteritidis and *L. monocytogenes*, which may occur due to altered bacterial membrane permeability and unfavorable environment due to low extracellular pH [12]. Organic acids have both bacteriostatic and bactericidal effects, which hamper bacterial growth and lead to a loss in viability [11]. Undissociated organic acid molecules that permeate microbial cell membranes show inhibitory activity by lowering intracellular pH and dissociating within the cell [35]. However, research on *B. cereus* strains is lacking. The MICs of citric acid and acetic acid showed similar antimicrobial activity at all tested temperatures. However, the bacteriostatic efficacy according to the MBC of citric acid was stronger against enterotoxigenic ATCC *B. cereus* strains than that against emetic *B. cereus* strains at all temperatures. Unlike emetic *B. cereus* strains that exhibit high MBC for citric acid at 42 and 45 °C as well as at optimal temperature, acetic acid eliminated the vegetative cells of most emetic and enterotoxigenic *B. cereus* strains at the same concentration, regardless of their incubation temperature. Acetic acid is relatively non-toxic, inexpensive, and readily available for killing foodborne pathogens. Our previous study [17] reported strong antibacterial activity of acetic acid against psychrotolerant *B. cereus*. Acetic acid is the most effective acid against *B. subtilis* [36]. Furthermore, Bell et al. found that dipping beef in 1.2% acetic acid reduced the population of *Salmonella* ser Typhimurium by 73.3% [37]. Doores has also reported that acetic acid inhibits the growth of *Bacillus* spp., *Pseudomonas aeruginosa, E. coli,* and *S. aureus* [38]. Citric acid also inhibits microbial growth by altering the cytoplasmic membrane permeability and chelating divalent metal ions [39]. The MIC of citric acid is $>$ 5 mg/mL against *E. coli* [40], and 2–10 mg/mL against *Candida albicans* [41]. Citric acid also exhibits antimicrobial activity against *E. coli* and *S. aureus*, with an MIC of 0.03–0.06 g/mL [42]. Low pH may alter the role of the cytoplasmic membrane, which controls the permeability between the intracellular and extracellular environments, and therefore eliminates bacterial growth during food processing [43]. found that citric acid treatment at 40 °C eliminates the vegetative cells of *B. cereus* by more than 2 log CFU/g and the antimicrobial effectiveness against *B. cereus* increases with temperature [44]. However, our results also indicate that, compared to citric acid, acetic acid may be a more effective disinfectant for simultaneously eliminating the cells of both emetic and enterotoxigenic *B. cereus* strains. This may be attributed to the presence of a higher concentration of the undissociated form of acetic acid than that of citric acid. Mortimore and Wallace [45] reported that the proportion of undissociated citric acid is smaller than that of acetic acid in the pH range of 3.0–7.0, which may enhance acetic acid dissociation in the intracellular environment, thereby increasing antimicrobial activity. Although the relationship between organic acids and temperature against foodborne pathogens is unexplored, acetic acid enhances the inhibition of bacterial growth at higher temperatures than that at optimal temperatures with increased reactive oxygen species levels [46]. These levels may increase with cultivation temperature and induce a reduced specific growth rate or cease cell growth. Hence, microorganisms grown at high temperatures are more susceptible to organic acids, such as acetic acid. The antimicrobial activity of the tested disinfectants may differ depending on the type and growth temperature of *B. cereus*. This is an unexpected result owing to the tolerance response to NaOCl and citric acid while maintaining the MBC against emetic *B. cereus* strains at increased temperatures. Further studies are necessary to understand the bacterial stress response linked to decreased disinfectant activity against emetic *B. cereus* with increasing temperature.

The gene expression of virulence factors can be modulated by various environmental factors including temperature, pH, salt, and water activity. In our study, *B. cereus* strains displayed increased expression of the enterotoxin gene when incubated at 42 °C or 45 °C compared to those grown at 30 °C. These characteristics were more evident in the emetic *B. cereus* strains. In particular, the *nheA* and *cytK* genes in GP-16 showed the highest upregulation, with a mean fold change of 256 and 381 at 45 °C when compared to that in other strains. These results demonstrated transcriptional upregulation of virulence genes in most emetic *B. cereus* strains at higher temperatures, with most enterotoxin genes being expressed at 42 °C and 45 °C. The regulation of *nhe* and *hbl* gene expression is subject to a combination of oxygen and substrate limitations [47]. Enterotoxin gene expression and production are enhanced under simulated growth conditions [48]. Cereulide synthesis is initiated by the *ces* gene transcription for cereulide toxin production. Four emetic *B. cereus* strains, NCCP 14796, GP-16, GP-6, and MC-05, showed marginally increased *ces* gene expression at 42 °C, whereas *ces* gene expression at 45 °C was lower than that at 30 °C. These results are in accordance with the findings of Kranzler et al., that *ces* gene expression decreases at higher temperatures in a strain-specific manner [18]. The influence of higher temperatures on the transcription of botulinum toxin complex, L-TC, and its stability was lower than those at the optimal temperature [49]. In comparison, a significant increase in the gene expression of *ces* in two emetic *B. cereus* strains, GP-15 and OS-05, was observed at higher temperatures (42 °C and 45 °C) than that at the optimal temperature (30 °C). Various studies have analyzed the increase in virulence gene expression under high-temperature conditions. Fujikawa et al. [20] demonstrated that temperature is one of the main factors affecting the toxin gene of *S. aureus* in dairy products, and a temperature above 46 °C against *S. aureus* increases toxin production [50]. Bavoc et al. [51] also found that the gene expression levels and production of enterotoxin are associated with storage temperature and time. Gene expression of *Yersinia enterocolitica* is temperature-dependent and is activated at room temperature or host temperature [19]. Thus, at higher growth temperatures, *ces* gene expression may increase in some emetic *B. cereus* strains isolated from various food products. However,

further studies are required to elucidate the association between toxin gene expression and toxin production. Furthermore, the exact role of temperature on cereulide toxin synthesis should be elucidated, as toxin production is independent of temperature changes, even when the *ces* gene is expressed [18].

5. Conclusion

This study demonstrated that emetic *B. cereus* strains exposed to temperatures higher than the optimal temperature (30 °C) might be more tolerant to disinfectants, such as NaOCl and citric acid, and become more virulent. Most of the enterotoxin genes tested in this study were highly expressed in emetic *B. cereus* under high-temperature conditions, and the *ces* gene was overexpressed in two emetic strains (GP-15 and OS-05) at 42 °C. Further genomic and functional analyses are needed to demonstrate the bacterial tolerance response to disinfectants, which is an unexpected finding for emetic *B. cereus* strains. Thus, the study findings may aid in understanding and controlling *B. cereus* in food or the food processing industry.

Author contribution statement

Kyung Min Park: Conceived and designed the experiments; Performed the experiments; Wrote the paper.
Hyun Jung Kim: Performed the experiments; Analyzed and interpreted the data.
Kee Jae Park: Contributed reagents, materials, analysis tools or data.
Minseon Koo: Conceived and designed the experiments; Wrote the paper.

Data availability

Data will be made available on request.

Declaration of competing interest

The authors have no interests to declare.

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Abbreviations

CFU: colony forming units
MBC: minimum bactericidal concentration
MIC: minimum inhibitory concentration
NHE: non-haemolytic enterotoxin
PCR: polymerase chain reaction
NaOCl: sodium hypochlorite

TSA: tryptic soy agar
TSB: tryptic soy broth