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OPTIMIZATION OF RAPID CYTOTOXICITY ASSAYS FOR SCREENING *BACILLUS CEREUS* GROUP ISOLATES

A Thesis in

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ABSTRACT

The Centers for Disease Control and Prevention estimates 63,400 cases of foodborne illness occurring annually in the U.S. due to *Bacillus cereus*. Additionally, the number of cases is likely underreported, as the disease usually resolves within 24 hours, although deadly cases have been reported. The *B. cereus* group of microorgansms is composed of 18 phylogenetically related species. Some representatives of the *B. cereus* group species can cause one of the two types of foodborne illnesses – emetic and diarrheal. Foodborne pathogenic strains are capable of secreting several known and putative diarrheal toxins, including hemolysin BL (encoded by *hbl*), non-hemolytic enterotoxin (encoded by *nhe*), and cytotoxin K (encoded by *cytK*). However, the mechanism of the diarrheal syndrome caused by *B. cereus* is still not well understood. One of the approaches to studying *B. cereus* group isolates' pathogenic potential is through immunoassays and cytotoxicity assessment using human cell lines. With an objective of developing a rapid and reliable screening technique, we evaluated and optimized colorimetry-based cytotoxicity assays to screen *B. cereus* group isolates and compared the results with those obtained using immunoassays.

We found that the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was not appropriate for assessment of the cytotoxic effects of *B. cereus* supernatants on HeLa human cervical cancer cells due to cell detachment that created error in the readout. We alternatively optimized the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay and used it to characterize 9 novel *B. cereus* group species validated in 2017, and the 2016 foodborne outbreak-associated isolates. We showed that *B. cereus sensu stricto* (*s.s.*) type strain ATCC 14579 was highly cytotoxic towards HeLa cells, in agreement with previous studies. We further conducted comparative analyses of 15 clade IV isolates (13 dairy-associated isolates, and the type strains *B. cereus s.s.* ATCC 14579 and *B. thuringiensis* ATCC 10792) in terms of their cytotoxicities towards HeLa, undifferentiated and differentiated Caco-2 cells using

both WST-1 and lactate dehydrogenase (LDH) assays. We applied 15% volume/volume (v/v) supernatants on Caco-2 cells and 5% v/v supernatants on HeLa cells to optimally differentiate among isolates with high and low cytotoxicity. We found the LDH assay results from undifferentiated Caco-2 cells to be concordant ($P < 0.05$) with those from HeLa and differentiated Caco-2 cells, which may indicate a similar mode of action against all tested cells. Further, results of the LDH and WST-1 assay were found to be concordant with each other for HeLa and undifferentiated Caco-2 cells ($P < 0.05$). This may indicate that for HeLa and undifferentiated Caco-2 cells, the pore-forming nature of the toxins could be the main contributor to the reduced viability of the cells. We additionally saw that the immune-based detection of non-hemolytic enterotoxin B positively correlated with the LDH assay results on differentiated Caco-2 cells ($P < 0.05$).

The assays optimized in the present study will be used for high-throughput screening of larger sets of *B. cereus* group isolates to elucidate the underlying mechanisms of isolates' virulence. Furthermore, the growth and cell harvesting conditions determined through this study will be used for the secretome characterization of the different *B. cereus* isolates through proteomics.

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LIST OF ABBREVIATIONS

s.l.	*sensu lato*
s.s.	*sensu stricto*
MLST	Multilocus sequence typing
Hbl	Hemolysin BL
Nhe	Non-hemolytic enterotoxin
Cyt K	Cytotoxin K
HlyII	Hemolysin II
HEp-2	Human epithelial type-2
EntFM	Enterotoxin FM
BceT	*B. cereus* enterotoxin
PI-PLC	phosphatidylinositol specific phospholipase C
SMase	Sphingomyelinase
HlyI	Hemolysin I
InhA1	Immune inhibitor A1
Npr	Neutral protease
LC	Liquid chromatography
MS	Mass spectrometry
UPLC-TOF	Ultra-performance liquid chromatography – time of flight
PCR	Polymerase chain reaction
GS	Genetic signature
ClyA	Cytolysin A
CHO	Chinese Hamster Ovary

LDH	Lactate dehydrogenase
Clo	Cereolysin O
CwpFM	Cell wall peptidase FM
PC-PLC	phosphatidylcholine-specific phospholipase C
TB	Trypan blue
PI	Propidium iodide
DAPI	4′,6-diamidino-2-phenylindole
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride; iodonitrotetrazolium
NAD	Nicotinamide-adenine dinucleotide
CV	Crystal violet
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide
WST-1	4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate; water soluble tetrazolium salt-1
NR	Neutral red
SRB	Sulforhodamine B
ORP	Oxidation-reduction potential
EntD	Enterobactin synthase component D
GI	Gastrointestinal
IMF	Infant milk formula
BHI	Brain heart infusion
KCTC	Korean Collection for Type Cultures
ATCC	American Type Culture Collection
PBS	Phosphate buffered saline

MOD	Minimal medium
OD_{600}	Optical density at 600 nm
cfu	Colony-forming units
EMEM	Eagle's Minimum Essential Medium
FBS	Fetal bovine serum
v/v	Volume/volume
DMSO	Dimethyl sulfoxide
$A_{550\ nm}$	Absorbance at 550 nm
$A_{450\ nm}$	Absorbance at 450 nm
$A_{690\ nm}$	Absorbance at 690 nm
$A_{490\ nm}$	Absorbance at 490 nm
$A_{595\ nm}$	Absorbance at 595 nm
PMS	Phenazine methosulfate
MRS	De Man, Rogosa and Sharpe
ATP	Adenosine triphosphate
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt
iTRAQ	Isobaric tags for relative and absolute quantitation
PMSF	Phenylmethylsulfonyl fluoride
HPLC	High performance liquid chromatography

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Chapter 1.

Literature Review

1.1. Introduction

Bacillus cereus group [also known as *sensu lato* (*s.l.*)] encompasses eighteen taxonomically valid species namely *B. cereus sensu stricto* (*s.s.*) (Carroll et al., 2019; Frankland et al., 1887; Miller et al., 2018), *B. thuringiensis* (Nakamura, 1994), *B. anthracis* (Logan et al., 1985), *B. wiedmannii* (Miller et al., 2016), *B. weihenstephanensis* (Lechner et al., 1998), *B. toyonensis* (Jiménez et al., 2013), *B. mycoides* (Lechner et al., 1998), *B. pseudomycoides* (Nakamura, 1998), *B. cytotoxicus* (Guinebretière et al., 2013), *B. paranthracis* (Liu et al., 2017b), *B. pacificus* (Liu et al., 2017b), *B. tropicus* (Liu et al., 2017b), *B. albus* (Liu et al., 2017b), *B. mobilis* (Liu et al., 2017b), *B. luti* (Liu et al., 2017b), *B. proteolyticus* (Liu et al., 2017b), *B. nitratireducens* (Liu et al., 2017b) and *B. paramycoides* (Nakamura, 1998), and three effectively published (published outside of the International Journal of Systematic and Evolutionary Microbiology; Tindall, 2015) species namely *B. bingmayongensis* (Liu et al., 2014), *B. gaemokensis* (Jung et al., 2010), and *B. manliponensis* (Jung et al., 2011).

This group of bacteria has been implicated in causing serious maladies like anthrax (Mock and Fouet, 2001) and meningitis (Gaur et al., 2001), as well as emetic and diarrheal foodborne illnesses (Carroll et al., 2019; Glasset et al., 2016). Some strains of *B. cereus* group are known to cause infections in humans and animals (Duport et al., 2016; Tewari and Abdullah, 2015), fish (Orozova et al., 2018), and insects (Bravo, 2018), while some strains are used as probiotics in humans and livestock (Kamar et al., 2013; Zhu et al., 2016b). A few endophytic and rhizosphere

inhabiting strains belonging to species *B. thuringiensis* and *B. cereus* have also been reported to offer protection against phytopathogenic microorganisms, insects, and nematodes, and to promote plant (e.g., soybean, wheat and Chinese cabbage) growth (Ku et al., 2018; Lopes et al., 2018).

A great amount of variability exists in the population of the *B. cereus* group, and several methods have evolved over time to classify the population into phylogenetic groups. The most accepted and recent method is by Carroll et al. (2017) who developed and used BTyper, which is based on *in silico* virulence gene detection, multilocus sequence typing (MLST), *panC* (encodes pantothenate sythetase) clade typing, and *rpoB* (encodes the β subunit of bacterial RNA polymerase) allelic typing, for nucleotide sequence-based phylogenetic grouping. They grouped 31 clusters into 7 *panC* clades (Carroll et al., 2017).

1.2. *Bacillus cereus* group and associated types of foodborne illness

Foodborne pathogenic *B. cereus* strains are broadly classified as emetic (i.e., carrying genes encoding cereulide synthetase) and diarrheal (i.e., encoding diarrheal enterotoxins). Enterotoxins produced during growth in the intestine are implicated in the diarrheal syndrome, while emesis/vomiting is caused by the dodecadepsipeptide cereulide pre-formed in food (Bauer et al., 2018; R et al., 2018). While cereulide as a cause of emesis is well established (Marxen et al., 2015), the exact causes of diarrhea are not yet fully understood, although a number of virulence factors including hemolysin BL (Hbl; encoded by *hbl*), non-hemolytic enterotoxin (Nhe; encoded by *nhe*), cytotoxin K (CytK; encoded by *cytK*) have been identified (outlined in Section 1.3.). That the *B. cereus* diarrheal disease causes are not well understood may be due to the existence of high phylogenetic diversity and frequent horizontal gene transfer that affects virulence gene dissemination, expression and activity and/or implication of a large number of putative virulence factors (Clair et al., 2010).

There have been a few gene deletion experiments conducted to establish the role of virulence factors in toxicity of *B. cereus*. A few examples are illustrated here. Deletion of *resE* encoding a sensor histidine kinase, which is a part of the redox signal system of *B. cereus*, resulted in the ablation of enterotoxin production (Duport et al., 2006). Expression of Hbl and Nhe, and their biosynthesis were strongly downregulated in *B. cereus* mutants lacking the anaerobic regulatory protein Fnr in a study looking at the transcription of genes and toxin detection (Messaoudi et al., 2010). Fnr is a fumarate and nitrate reductase that serves as a regulator of adaptation to low oxygen tension. Deletion of the gene encoding hemolysin II (HlyII) strongly reduced the virulence of the *B. thuringiensis* strain 407 Cry^- (Bt 407) that was used as a model for *B. cereus* in insect model and mice (Tran et al., 2011). Similarly, deletion of *B. cereus* Hbl-encoding genes significantly reduced cytotoxicity (Sastalla et al., 2013). Deletion of a gene encoding global transcriptional regulator in *B. cereus* (*codY*) resulted in significant reduction of virulence (Lindbäck et al., 2012).

Although some earlier studies reported use of animal models for pathogenicity studies as described in Section 1.7. (Hoštacká et al., 1992; Shinagawa et al., 1991; Wong et al., 1988), cell culture-based assays have been established as proxies for *in vivo* pathogenicity assessment. Use of cell culture proxies may be one of the reasons why pathogenicity is poorly understood for these bacteria. Researchers have extensively used various cell line-based assays for assessment of cytotoxicity and then extrapolated cytotoxicity results to virulence in humans (Section 1.6.).

Unlike the case of emetic strains, where a single virulence factor is considered to be implicated in pathogenesis, several virulence factors ranging from enzymes to lytic toxins have been proposed to be associated with diarrheal symptoms. Moreover, the cytotoxicity reaction may be specific to the cell lines used for the assays, as well as the state of the cells (Jeßberger et al., 2014; Zhang et al., 2015). Co-existence of the emetic toxin and enterotoxins in the same strain complicates the assessment of isolates' pathogenic capacity (Carroll et al., 2019; Glasset et al.,

2016; Heini et al., 2018; Oh et al., 2012). In recent years, whole genome sequencing, coupled with other omics tools such as transcriptomics and proteomics, has been used to better understand the association between virulence factors and pathogenicity (Carroll et al., 2019; Clair et al., 2010; Clair et al., 2013; Gilois et al., 2007; Jeßberger et al., 2015; Miller et al., 2018; Omer et al., 2015).

1.3. *Bacillus cereus* toxins associated with foodborne illness

Cereulide is a non-ribosomally synthesized peptide synthesized by non-ribosomal peptide synthetases. It is produced by certain strains of *B. cereus* group and is well-established as a cause of emesis (Ehling-Schulz et al., 2015; Messelhäußer and Ehling-Schulz, 2018). On the other hand, a series of *B. cereus* group enterotoxins and enzymes has been inconsistently associated with foodborne diarrheal illness (Griffiths and Schraft, 2017; McKillip, 2000; Schoeni and Wong, 2005). The pore-forming enterotoxins Hbl, Nhe and CytK are the most well-studied (Ramarao and Sanchis, 2013). Hbl is comprised of three antigenically distinct proteins (B, L_1, and L_2) and is a membrane-lytic system rarely present in emetic strains (Biesta-Peters et al., 2016; Glasset et al., 2016). Nhe, also a three-component enterotoxin, is present in most strains of *B. cereus* (Biesta-Peters et al., 2016; Cui et al., 2016a; Heini et al., 2018; Owusu-Kwarteng et al., 2017). CytK can occur in two different forms, CytK-1 that is specific to *B. cytotoxicus*, and CytK-2 that has been found in a number of other *B. cereus* group species (Castiaux et al., 2015; Miller et al., 2018). Other putative diarrheal toxicoinfection virulence factors include enterotoxin FM (EntFM), *B. cereus* enterotoxin (BceT), phosphatidylinositol-specific phospholipase C (PI-PLC), sphingomyelinase (SMase), hemolysin I (HlyI), immune inhibitor A1 (InhA1, a metalloprotease), neutral protease (NprA alternatively designated as NprB, a metalloprotease bacillolysin; Npr599 found in *B. anthracis*) and HlyII, all of them being single proteins (López et al., 2015; Stenfors Arnesen et al., 2008).

1.3.1. Emetic toxin

Emetic strains of the *B. cereus* group harbor a plasmid pCER270 that carries the cereulide biosynthesis gene cluster *ces* (Ehling-Schulz et al., 2006). The *ces* operon is comprised of 7 coding DNA sequences (*cesH*, *cesP*, *cesT*, *cesA*, *cesB*, *cesC* and *cesD*) and is located on the pCER270 megaplasmid that has similarity with the *B. anthracis* plasmid pXO1 (Ehling-Schulz et al., 2015). Cereulide is a dodecadepsipeptide that resembles valinomycin, a non-ribosomally synthesized antibiotic that simulates K^+ uptake and H^+ efflux from mitochondria. Cereulide is highly stable and is not sensitive to heat, extremes of pH, and proteolytic enzymes (Guérin et al., 2017; Rajkovic et al., 2008). Thermal treatment of food contaminated with cereulide does not reduce the risk for human intoxication upon food ingestion.

Cereulide has high affinity for monovalent cations, preferably K^+, and is highly hydrophobic, a characteristic that allows it to diffuse through cell membranes and cross the blood-brain barrier (Bauer et al., 2018; Mikkola et al., 1999). Cereulide is an optimal ionophore (a lipid-soluble entity that localizes in cell membrane and transports ions across the cell membrane) that can collapse electrochemical gradient of human cell membranes (Bauer et al., 2018; Mikkola et al., 1999). It is also an uncoupler of oxidative phosphorylation in mitochondria and causes mitochondrial swelling in human epithelial type-2 (HEp-2) laryngeal carcinoma cells (Agata et al., 1994; Sakurai et al., 1994; Shinagawa et al., 1992).

PlcR is a transcription factor known as a master regulator of most known virulence factors in *B. cereus* (Agaisse et al., 1999; Grenha et al., 2013). In *B. cereus s.s.* ATCC 14579 (a non-emetic strain), PlcR regulates the expression of as many as 45 genes that code for phospholipases, proteases, toxins, bacteriocins, transporters, cell wall biogenesis proteins, two-component sensors, chemotaxis proteins, diguanylate cyclase family regulators, and cytoplasmic regulators (Agaisse et al., 1999). In emetic strains, however, PlcR appears to be a negative regulator of *ces* genes. Deletion

of CodY (a nutrient-responsive regulator) results in the downregulation of PlcR regulon and a concomitant upregulation of *ces* genes (Frenzel et al. 2012). The transition state regulator AbrB, not PlcR, directly affects the biosynthesis of cereulide (Lücking et al., 2009). Furthermore, Kranzler et al. (2016) showed that temperature impacts cereulide synthesis on post-transcriptional levels, thereby altering the composition of cereulide isoforms (Kranzler et al., 2016) that have been shown to have different emetic potency (Ehling-Schulz et al., 2015).

Several quantitative methods such as LC (liquid chromatography) – MS (mass spectrometry) and LC-MS/MS have been developed to measure cereulide (Ueda et al., 2012; Yamaguchi et al., 2013). Using UPLC-TOF (Ultra-performance liquid chromatography – time of flight) MS and ion trap MS(n) sequencing, ^{13}C-labeling, and post-hydrolytic dipeptide and enantioselective amino acid analysis, Stark et al. (2013) observed 18 cereulide variants including the new variants isocereulides A-G. Isocereulide A is approximately 10-fold more cytotoxic than cereulide (Stark et al., 2013). The highest level of isocereulide A was detected at 33 ºC and 37 ºC, while that of isocereulide B was detected at 18 ºC and 24 ºC (Kranzler et al., 2016).

Apart from *B. cereus*, some strains of the psychrotolerant *B. weihenstephanensis* (phylogenetic group VI) have also been reported to produce cereulide (Guérin et al., 2017). Ehling-Schulz et al. (2005) used polymerase chain reaction (PCR) using M13 primers, random amplification of polymorphic DNA, MLST, Fourier transform infrared spectroscopy, protein profiling, and biochemical assays to type 90 *B. cereus* isolates; the analysis revealed a single, distinct cluster for emetic strains (Ehling-Schulz et al., 2005). Using whole genome-based classification, *cesABCD* genes were found to be associated with cluster 12 (*k-mer* based analysis) belonging to *panC* clade III, and cluster 24 belonging to *panC* clade VI of *B. cereus* (Carroll et al., 2017). Overall, the emetic toxin cereulide is among the most well characterized *B. cereus* toxins with well-defined genetics, phylogenetic distribution, biosynthesis, and mode of action.

1.3.2. Diarrheal toxins

Unlike the emetic toxin cereulide, the putative diarrheal toxins (e.g. Hbl, Nhe, CytK, HlyII) are ribosomally synthesized proteins. The expression of diarrheal toxins (CytK, Nhe and Hbl) is regulated by the PlcR-PapR quorum-sensing system (Grenha et al., 2013; Yehuda et al., 2018). PlcR is activated upon binding of its cognate signaling peptide PapR on a tetratricopeptide repeat-type regulatory domain (Grenha et al., 2013). In *B. thuringiensis*, activity of the virulence quorum sensor PlcR is regulated by CodY by controlling the import of the signaling peptide PapR (Slamti et al., 2016).

While emetic and diarrheal toxins are regulated by different primary regulators, their regulatory networks are interconnected through CodY (Frenzel et al., 2012). Furthermore, it is not uncommon for strains to carry both *ces* genes and *nhe* genes (Carroll et al., 2017; Glasset et al., 2016). The co-occurrence of emetic and diarrheal toxins in the same strain can complicate investigation and identification of virulence determinants responsible for foodborne illness. Among emetic *B. cereus* strains that carry *ces* genes, cluster 24 (*panC* clade 6), but not cluster 12 (*panC* clade 3) harbored *hbl* genes, but both these clusters harbored *nhe* genes (Carroll et al., 2017). Based on the common functional and structural properties, Fagerlund et al. (2008) proposed that Hbl, as well as Nhe families of toxins constitute a superfamily of pore-forming cytotoxins (Fagerlund et al., 2008). In a survey by Biesta-Peters et al. (2016), none of the *hbl*-containing isolates possessed the *ces* gene, whereas all strains contained the *nhe* genes, indicating common co-occurrence of genes encoding Hbl and Nhe (Biesta-Peters et al., 2016). A similar observation has been recorded in the studies conducted by Miller et al. (2018), where 15 of 44 isolates investigated carried both *hbl* and *nhe* genes (Miller et al., 2018). Of 662 isolates analyzed that fell into 31 clusters, only 8 isolates [belonging to *panC* clades 1 (only *nheC* present), 3 (only *nheA* and *nheB* present) and an

undefined clade (all three absent)] were negative for genes for Nhe, whereas 18 clusters representing 379 isolates were positive for Hbl genes (Carroll et al., 2017).

Glasset et al. (2016) studied 564 strains associated with 140 foodborne outbreaks that had occurred in France between 2007 and 2014. They investigated the presence of genes encoding cereulide biosynthetic pathway, diarrheal enterotoxins (Nhe, Hbl, CytK-1 and CytK-2), and HlyII in a sub-panel of 149 strains (Glasset et al., 2016). Twelve genetic virulence signatures (GSs) were obtained, of which GS1 (*panC* group II, III, IV; only *nhe* genes present) and GS2 (*panC* group IV; *nhe*, *hbl* and *cytK-2* present) were the most prevalent GSs and were found in 28% and 31% of foodborne outbreaks, respectively (Glasset et al., 2016).

Enterotoxicity of *B. cereus* group of pathogens is a complex phenomenon and attempts to define the role of individual components is a challenge. We reviewed individual diarrheal enterotoxins and other virulence factors that had been associated with diarrheal illness caused by *B. cereus* group microorganisms in following subsections.

1.3.2.1. Hemolysin BL (Hbl)

The two tripartite enterotoxins, Hbl and Nhe, are most commonly linked to the *B. cereus* diarrheal syndrome (Schraft and Griffiths, 2006). Hbl is a pore-forming toxin (Fagerlund et al., 2008). Hbl requires the combined action of the three proteins, namely a binding component Hbl-B (37.5 kDa) along with lytic components Hbl-L_1 (38.2 kDa*)* and Hbl-L_2 (43.5 kDa) (Sastalla et al., 2013).

Hbl-B has a high structural similarity to *Escherichia coli* Cytolysin A (ClyA), and it was predicted that Hbl-B alone might be able to oligomerize on the cell surface and form a pore, and that L_2 and L_1 either stabilize B, induce conformational changes to B, or enter the mammalian cell (Madegowda et al., 2008). Sastalla et al. (2013) reported that *B. cereus* strain ATCC 10876, which is highly cytotoxic to HT1080 human fibroblasts, hPMNs human neutrophils, Chinese hamster ovary (CHO) cells, and RAW264.7 mouse macrophages, harbors two copies of the *hbl* operon/loci

(*hbl-I* and *hbl-II*, both encoding the Hbl proteins L_2, L_1, and B). They identified Hbl as the major secreted toxin responsible for inducing rapid lysis of RAW264.7 macrophages *in vitro* and in an intraperitoneal mouse toxicity model (Sastalla et al., 2013). By using specific monoclonal antibodies in dot blots, indirect and hybrid sandwich enzyme immunoassays, complex formation between Hbl L_1 and B, as well as L_1 and L_2 in solution (separately) was shown (Tausch et al., 2017). Recently, Mathur et al. (2019) demonstrated that Hbl-induced pore results in the efflux of K^+, leading to activation of the NLRP3 inflammasome and secretion of interleukin-1β and interleukin-18, and pyroptosis. Furthermore, Hbl-producing *B. cereus* was shown to cause rapid inflammasome-mediated mortality (Mathur et al., 2019). Based on the existing literature, the three-component toxin Hbl appears to be an important virulence determinant in diarrheal strains of *B. cereus*. However, the fact that all Hbl positive strains are not cytotoxic/virulent, indicates that its toxicity may be dependent on some other extra-/intracellular toxins/enzymes. Investigating Hbl activity in *B. cereus* culture supernatants is complicated by the fact that almost all known enteropathogenic strains that bear *hbl* also have the *nhe* operon (Tausch et al., 2017) and their interactions and potential synergistic effects are not well characterized.

1.3.2.2. Non-hemolytic enterotoxin (Nhe)

B. cereus produces a pore-forming enterotoxin Nhe, that is similar in structure and function to ClyA (also known as hemolysin E and silent hemolysin locus A) produced by certain strains of *E. coli*, *Salmonella enterica*, and *Shigella flexneri*. Nhe forms large conductance pores in planar lipid bilayers and can induce osmotic lysis in epithelial cells (Fagerlund et al. 2008). Nhe has been characterized as a beta-barrel toxin that elicits symptoms of diarrhea due to the cellular damage caused by pore formation in gut epithelial cells (Ramarao and Sanchis, 2013). This has been supported by molecular study wherein mutants lacking NheB and NheC lost the capacity to cause cytotoxic effects on Caco-2 human epithelial colorectal adenocarcinoma cells (Fagerlund et al.,

2008). The *nhe* genes were found in all enteropathogenic *B. cereus* strains examined by Tausch et al. 2017 (Tausch et al., 2017). The *B. cereus nheA* and *nheC* genes are capable of being expressed in *E. coli* and *nheB* in *B. subtilis* (Lindbäck et al., 2004). All the Nhe components, i.e. NheA, NheB and NheC were required for biological activity, with the highest toxicity against Vero African green monkey kidney cells having been observed at a molar ratio of 10:10:1 (Lindbäck et al. 2004). In the same study, NheB was shown to function as a binding component (Lindbäck et al., 2004). Of 100 *B. cereus* strains examined, 42% and 99% of the strains harbored the genes for Hbl and Nhe respectively, and the level of Nhe seems to explain most of the *in vitro* cytotoxic activity of *B. cereus* isolates and may indicate a high diarrheic potential (Moravek et al., 2006). The concentrations of the Hbl-L_2 ranged from 0.02 to 5.6 μg/mL and of NheB component ranged from 0.03 to 14.2 μg/ml (Moravek et al., 2006). Using PCR, genes for NheA, B, and C were detected in all strains except one CytK-producing strain.

In order to elucidate the functional role of the various components of Nhe, Haug et al. (2010) used strains of *B. cereus* lacking either NheA or –C. In both Vero and GH4 rodent pituitary cells, NheA+B+C induced release of lactate dehydrogenase (LDH) and K^+ as well as influx of Ca^{2+}, which are indicators of pore formation in membranes. NheA + B caused a similar K^+ efflux and elevation of Ca^{2+}, while NheB + C had no effect. Based on these observations, they concluded that NheA and NheB are necessary and sufficient for formation of large-conductance channels in GH4 cells, whereas in Vero cells, such large-conductance channels are in addition, dependent on NheC (Haug et al., 2010). Lindbäck et al. (2010) demonstrated that both NheB and NheC were able to bind to Vero cells directly while NheA could not. In addition, the authors found that Nhe-induced cytotoxicity required a specific binding order (NheC-B-A) (Lindbäck et al., 2010).

Based on interaction studies with the non-ionic detergent dodecyl maltoside, Phung et al. (2012) proposed that conformational changes and oligomerization of NheB are prerequisite events in the process of pore formation (Phung et al., 2012). Amino acids 122-150 of NheB are involved

in interaction between NheB and NheC, both on the epithelium cell surface, and in solution, and amino acids 321 to 341 are involved in association between NheA and cell-bound NheB in a competitive assay where solutions of different molar ratios of the antibody and NheA were added simultaneously to cells that have been primed with NheB and NheC (Didier et al., 2012). Heilkenbrinker et al. (2013) provided evidence that a defined level of NheB-NheC complexes as well as a sufficient amount of free NheB is necessary for efficient cell binding and cytotoxicity against Vero cells (Heilkenbrinker et al., 2013). Further, Zhu et al. (2016a) recently studied the properties of the NheB/C complex and the fate of the target cell upon binding. High molecular mass hetero-oligomers (620 kDa) probably consisting of one NheC and up to 15 NheB were detected by size-exclusion chromatography and on native polyacrylamide gel electrophoresis immunoblots. They concluded that the NheB/C complex tends to increase the membrane permeability prior to the emergence of full pores which also contain NheA (Zhu et al., 2016a). In addition to pore-forming properties, Nhe also induces cell cycle arrest at G0/G1 phase provoking apoptosis in Vero cells, which is most likely associated with mitogen-activated protein kinase and death receptor pathways (Liu et al., 2017a). From these studies, it appears that Nhe has a role in cytotoxicity, which is contradictory to the fact that Nhe is present in nearly all strains of *B. cereus* examined to date, including pathogenic and non-pathogenic ones. Nhe may thus have a role of an assistor or act in conjunction with other virulence factors, either imparting additive or synergistic effects.

1.3.2.3. Cytotoxin K (CytK)

CytK is a relatively recently discovered virulence factor of *B. cereus* having been reported in 2000 and incriminated in an outbreak of severe gastroenteritis causing the death of three patients in France in 1998 (Lund et al., 2000). CytK, a protein of molecular mass 34 kDa, was necrotic and hemolytic in guinea pigs, and no other known *B. cereus* enterotoxin was detected in this strain, indicating an important role of CytK in virulence in the studied case. Based on a cytotoxicity assay

in Caco-2 cells, CytK, a beta-barrel pore-forming toxin was found to be linked with human cases of necrotic enteritis (Hardy et al., 2001). Among the two forms of CytK, CytK-1 (associated with *B. cytotoxicus*) is more toxic than CytK-2 to Caco-2 and Vero cells (Fagerlund et al., 2004). Fagerlund et al. (2007) described a strain of *B. cereus* (NVH 391/98) that lacked Hbl- and Nhe-encoding genes, but harbored CytK-1 (Fagerlund et al., 2007); this strain was later on described as a type strain of species *B. cytotoxicus* (Guinebretière et al., 2013). The genome of strain NVH 391/98 harbored a variant of Nhe (80% protein sequence identity to known Nhe) and the culture supernatants of strains NVH 391/98 and INRA AF2 (both *B. cytotoxicus*) contained CytK-1 and Nhe, and were highly cytotoxic to Vero cells. Based on the distribution of CytK genes among 390 *B. cereus* strains studied (Castiaux et al., 2015), the authors questioned the relevance of CytK as a virulence factor for the diarrheal pathotype, although they did not completely rule out the involvement of CytK in the diarrheal syndrome.

1.3.2.4. Hemolysin I (HlyI) and hemolysin II (HlyII)

HlyI belongs to the cholesterol-dependent cytolysin family, while HlyII and CytK are oligomeric ß-barrel pore-forming toxins (Ramarao and Sanchis, 2013). HlyI produced by *B. cereus* is called cereolysin O (Clo); that produced by *B. thuringiensis* is called thuringiolysin O, and by *B. anthracis* is called anthrolycin O. *B. cereus* Clo is hemolytic at 1 ng/ml concentration, and is lethal to mice when injected intravenously at 1-2 µg dose (Ramarao and Sanchis, 2013).

HlyII has been shown to be responsible for host cell death, has hemolytic properties against a broad range of erythrocytes, induces pore formation in the membranes of various eukaryotic cells, and induces apoptosis of host monocytes and macrophages *in vivo* (Ramarao and Sanchis, 2013). HlyII transcription is regulated by *HlyIIR* and the ferric uptake regulator Fur, but not by PlcR (Ramarao and Sanchis, 2013). Andreeva et al. (2006) expressed HlyII from *B. cereus* in *E. coli* and showed that two types of human cells (Paju neuroblastoma and Caco-2) were sensitive to this toxin.

They inferred, based on its broad cytotoxic activity and the ability to interact with artificial membranes, that HlyII binds to cell membranes in a non-specific manner (Andreeva et al., 2006). Recently, Kaplan et al. (2017) solved the structure of HlyII by NMR-based studies and found a novel fold, consisting of two subdomains αA-β1-β2 and β3-β4-αB-β5 that come together in a barrel-like structure (Kaplan et al., 2017) that is typical for pore-forming enterotoxins. Cadot et al. (2010) used PCR to detect presence of the genes *inhA1*, *nprA,* and *hlyII*, quantitative reverse transcription PCR to study the transcription, and virulence *in vivo* by injecting the bacteria into the hemolymph of the model insect *Galleria mellonella* larvae. They found all strains carrying *hlyII* were strongly toxic to *G. mellonella*. Based on this, Cadot et al. (2010) suggested that *hlyII* may be a good indicator of pathogenicity, even though only 31% of strains involved in food poisoning outbreaks carried *hlyII*. Further, *inhA1* and *nprA* were expressed in higher levels in pathogenic compared to the non-pathogenic group of strains studied. They hypothesized based on the high production of virulence factors InhA1, NprA, and HlyII in the two most virulent strains studied (F2081A/98 and AH1293) that *B. cereus* may use InhA1, NprA, and HlyII (when present) simultaneously rather than separately in causing an infection (Cadot et al., 2010).

1.3.2.5. *Bacillus cereus* enterotoxin T (BceT) and Enterotoxin FM (EntFM)

BceT is a protein of molecular mass 46 kDa and is encoded by the *bceT* gene. Agata et al. (1995) cloned and expressed *bceT* gene from *B. cereus* in *E. coli* and demonstrated that the recombinant toxin exhibited cytotoxicity on Vero cells. Based on this finding, it was concluded that *bceT* codes for one of the enterotoxic proteins that contributes to foodborne diarrhea (Agata et al., 1995). Choma and Graum (2002) attempted to express BceT in *E. coli*, and using the supernatant and cell extract, found no cytotoxicity on Vero cells., which could be due to lack of expression or due to a low level of homology (50–60% identity) to the original BceT, indicating a different gene studied (Choma and Granum, 2002).

EntFM was first reported from *B. cereus* strain FM1 as a protein of molecular mass 45 kDa (Asano et al., 1997). The *entFM* gene coding for the *B. cereus* cell wall peptidase EntFM is present as a single copy, is chromosomal, and common to *B. thuringiensis* and *B. cereus* strains (Hsieh et al., 1999; Ngamwongsatit et al., 2008; Tran et al., 2010). All thirty-five *B. cereus* strains isolated from 100 commercial Sunsik (a ready-to-eat food in Korea, comprised of various agricultural and marine products) samples were positive for *entFM* gene (Chon et al., 2012). Of 29 *B. cereus* isolates from India examined, the *entFM* gene was amplified from 93% samples (Tewari et al., 2015). In another study, 100% of 87 *B. cereus* isolates examined from Korean fermented soybean products were positive for the *entFM* gene (Yim et al., 2015). Such observations are also supported by Miller et al. (2018) who reported presence of *entFM* gene in all phylogenetic groups, indicating widespread occurrence of this gene in *B. cereus* (Miller et al., 2018). Since EntFM is related to cell-wall peptidases, Tran et al. (2010) suggested renaming it as cell wall peptidase FM (CwpFM; Tran et al., 2010).

To elucidate the biological function of CwpFM, a *cwpFM*-deficient mutant of *B. cereus* strain *B. thuringiensis* 407 (Cry^- strain) was constructed and used as a model for *B. cereus*. Additionally, the recombinant *B. cereus* CwpFM protein was produced in *E. coli* to study its activity (Tran et al., 2010). Comparison of the wild-type and *cwpFM* mutant strains revealed that the mutant displayed a "peanut shape" throughout bacterial growth and seemed to agglutinate and form filaments. The *cwpFM* deletion reduced motility by 73.5%, and adhesion of the *cwpFM* mutant to HeLa human epithelial cervix adenocarcinoma cells was reduced by 10-fold compared to the wild-type strain. The *cwpFM* mutant was also severely compromised in its ability to form biofilm. Recombinant CwpFM induced lengthening and strong vacuolization of macrophages. However, the *cwpFM* mutant was as cytotoxic as the wild-type strain (both 100%), hence CwpFM was not shown to be involved in *B. cereus* toxicity toward either HeLa cells or J774 macrophages; however, it still may have played a role in pathogenesis through motility and adhesion (Tran et al.,

2010). Tran et al. (2010) assessed cell viability using a dye exclusion method and reported cytotoxicity as the percentage of cells that were permeable to the dye. An assay with insect model *G. mellonella* showed that bacterial virulence in insects was strongly reduced in the *cwpFM* mutant compared to the wild-type strain, revealing a clear role of CwpFM in *B. cereus* virulence (Tran et al., 2010).

1.3.2.6. Sphingomyelinase (SMase)

B. cereus SMase is an extracellular hemolysin classified as a Mg^{2+}-dependent neutral SMase, and has a molecular mass of 34 kDa (Obama et al., 2003). This enzyme was first purified from *B. cereus* broth. It is encoded by the gene *sph*. Purified SMase from *B. cereus* specifically hydrolyzed sphingomyelin to ceramide and phosphorylcholine (Hiroh et al., 1978). Subsequently, it was found to be specifically adsorbed onto mammalian erythrocyte membranes in the presence of either Ca^{2+} or Ca^{2+} plus Mg^{2+} (Tomita et al., 1983). Beecher and Wong (2000) proved that SMase alone could lyse ruminant erythrocytes, however, the cooperative action of PC-PLC (phosphatidylcholine-specific phospholipase C) and SMase was needed to lyse swine and human erythrocytes. SMase synergistically enhanced hemolysis caused by Hbl for all erythrocytes tested (Beecher and Wong, 2000). *B. cereus* SMase-induced hemolysis of sheep erythrocytes is related to the formation of interface between ceramide-rich locations and ceramide-poor locations through production of ceramide from sphingomyelin (Oda et al., 2010). *B. cereus* SMase is a virulence factor for septicemia, and clinical isolates that produced large amounts of SMase caused death in mice (Oda et al., 2012). *B. cereus* SMase is essential for the hydrolysis of sphingomyelin in membranes, leading to a reduction in phagocytosis (Oda et al., 2012). *B. cereus ΔnheBC* mutant strain lacking Nhe was cytotoxic to intestinal epithelial cells. Using single and double deletion mutants of *sph* and *nheBC* in *B. cereus*, it was demonstrated that SMase is an important factor for *B. cereus* cytotoxicity *in vitro* and pathogenicity *in vivo* (Doll et al., 2013).

It should be emphasized that various toxin genes often co-exist in the same *B. cereus* strains and may act synergistically, hence, the relative contribution of individual toxins to the overall cytotoxicity is challenging to study and is not fully understood. Attempts have been made in the past to study the association between cytotoxicity, toxin gene presence, toxin expression and species identity, which have been hindered due to inaccurate taxonomic identification of species based on phenotypic characteristics (Fox et al., 1992; Liu et al., 2015; Prüß et al., 1999). Similarly, comparisons of cytotoxicity and toxin expression among different studies are hindered by the lack of reported experimental conditions (e.g., temperature at which strains were grown). Finally, different cytotoxicity cell models have been used in different studies that may affect the outcomes and predicted pathogenic potential. We critically review and compare existing literature to assess the effects of different cell lines on the observed level of isolates' cytotoxicity in Section 1.6.

1.4. Cytotoxicity assessment techniques

Cytotoxicity assays are widely used as indicators of biological effects of toxins and drugs on cells *in vitro* (Aslantürk, 2017; Fotakis and Timbrell, 2006). Different biological indicators such as damage to cell membranes, activity of mitochondrial enzymes, and cell proliferation can be used to assess cell damage and death via these assays (Aslantürk 2017). In this section, we briefly describe some of these assays that have been widely used for assessing effects of bacterial toxins on mammalian cell lines.

1.4.1. Trypan blue dye exclusion assay

The dye exclusion assay is based on the principle that live cells exclude dye, but dead cells, due to compromised membrane integrity, uptake the stain (Aslantürk, 2017). Trypan blue (TB) is the most commonly used dye, but others including eosin, Congo red and erythrosine B have also been used

(Aslantürk, 2017; Strober, 1997). Cells after treatment with toxins are washed, trypsinised, mixed with TB, which is a large negatively charged molecule impermeable to membranes of live cells, and examined under a light microscope. The dead cells stain blue, and the proportion of live to dead cells is recorded using a hemocytometer (Aslantürk 2017). This method is simple, inexpensive, and a good indicator of membrane integrity (Aslantürk, 2017; Ruben, 1988).

1.4.2. Propidium iodide fluoresence assay

Propidium iodide (PI) penetrates only damaged cellular membranes and intercalates with double-stranded DNA, resulting in an amplification of the fluorescence (Dengler et al., 1995; Wlodkowic et al., 2011). In the first measurement, cells are incubated with PI and the fluorescence detection allows assessment of the number of non-viable cells. The cells are then frozen at – 20 °C for 24 hours; this allows PI to stain all the cells. The number of viable cells is then calculated by the difference between these two measurements (Dengler et al., 1995). Instead of the freezing step, Miller et al. (2016) permeabilized cells with Triton X-100 and incubated permeabilized cells with 4′,6-diamidino-2-phenylindole (DAPI; 1 $\mu g\ ml^{-1}$) at room temperature for 1–5 min to facilitate the total cell counts using fluorescence microscopy. This assay is simple, does not involve any washing steps, and requires a small number of cells for drug testing (Dengler et al., 1995). However, it is laborious, as it requires microscopic imaging and calculation of live-to-dead cells ratios post-imaging. Assays that allow for higher throughput are therefore desired for rapid screening of larger sets of isolates.

1.4.3. Lactate dehydrogenase assay

The LDH assay is a widely used colorimetric method for assaying cytotoxicity (Korzeniewski and Callewaert, 1983). LDH is a stable enzyme that is found in the cytoplasm of cells (Kumar et al., 2018). This assay measures the LDH that is released from cells when the cell membrane is

damaged, using a coupled enzymatic reaction that converts a tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride [also known as iodonitrotetrazolium (INT)] into formazan. The reaction takes place in two steps. The first step involves conversion of lactate to pyruvate by LDH, resulting in reduction of nicotinamide-adenine dinucleotide (NAD) to $NADH/H^+$. Subsequently, an electron mediator such as diaphorase or phenazine methosulfate transfers H/H^+ from $NADH/H^+$ to INT, reducing it to red formazan. The red formazan can be measured quantitatively at 490 nm (Aslantürk, 2017; Decker and Lohmann-Matthes, 1988; Fotakis and Timbrell, 2006; Hisada and Yagi, 1977). This assay is fast, simple and reliable, and is an indicator of cell membrane damage (Aslantürk, 2017). The ability to carry out LDH assay in a microtiter plate format and obtain the readout using a microplate reader in a matter of minutes make it suitable for high-throughput screenings.

1.4.4. Crystal violet staining assay

Adherent cells detach from cell culture plates during cell death, and the crystal violet (CV) staining assay is used to detect the maintained adherence of cells (Feoktistova et al., 2016). The CV dye binds to DNA and proteins of viable cells, and thus, attached cells are stained with this dye. Dead, non-adhering cells are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. CV assay is a quick and reliable screening method that is suitable for the examination of the impact of compounds on cell survival and growth inhibition, but cannot measure cell proliferation rate (Feoktisova et al., 2016).

1.4.5. MTT assay

The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay is one of the most popular colorimetric assays, where the MTT dye gets reduced to formazan crystals by living cells (van Meerloo et al., 2011). This assay determines cell viability by measuring activity of

mitochondrial enzymes such as succinate dehydrogenase (Supino, 1995). The purple formazan formed can be quantified by measuring the absorbance at 450 nm. However, the MTT formazan is insoluble in water, forming needle-shaped crystals in the cells. Hence, prior to measuring the absorbance, an organic solvent such as dimethyl sulfoxide or isopropanol is required to solubilize the crystals (Aslantürk, 2017; Morgan, 1998). The MTT assay protocol requires cell washing and replacement of reagents in test wells, which increases the risk of unintentional removal of cells from test wells in cases where cells are detached due to the activity of enzymes or toxins present in tested bacterial supernatants.

1.4.6. WST-1 assay

4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (water-soluble tetrazolium salt-1; WST-1) is a simple, colorimetric assay for determining the metabolic activity of cells (Ishiyama et al., 1997; Tominaga et al., 1999). In this assay, WST-1, in the presence of an intermediate electron acceptor such as 1-methoxy-5-methyl-phenazinium methyl sulfate, is converted to a highly water-soluble yellow colored formazan by mitochondrial dehydrogenase enzymes (Aslantürk, 2017; Ishiyama et al., 1997). The formazan is released into the cell culture medium resulting in a color change which is read at 450 nm with a reference reading at 690 nm (to account for interference from cell debris in the wells of the plate; Fisichella et al., 2009; Ma et al., 2010). Since the colored dye which is produced at the end of experiment is water-soluble, addition of solvent is not required (Aslantürk, 2017). WST-1 assay is a rapid assay suitable for high-throughput screening. Given that the protocol does not include any cell washing steps or replacement of test reagents in test wells, this assay does not pose risk of unintentional removal of cells from test wells.

1.4.7. Neutral red uptake assay

The neutral red (NR) uptake assay is another widely used cytotoxicity/cell viability assay (Borenfreund and Puerner, 1985; Repetto et al., 2008). At physiological pH, net charge of the NR dye is close to zero, which allows the dye to penetrate the cell membranes through non-ionic passive diffusion and concentrate in the lysosomes (where, due to a lower pH than the cytoplasmic pH, the dye becomes charged and is retained; Repetto et al., 2008). After washing the excess dye away, the dye bound by viable cells is extracted with an acidified ethanol solution and the absorbance is measured at 540 nm using a spectrophotometer (Aslantürk, 2017; Ates et al., 2017). When the cell dies or pH gradient is reduced, the dye cannot be retained (Repetto et al., 2008). The uptake of NR by viable cells can also be modified by alterations in cell surface or lysosomal membranes. Using NR assay, it is thus possible to distinguish between viable and damaged/dead cells (Aslantürk, 2017).

1.4.8. Sulforhodamine B assay

The sulforhodamine B (SRB) assay is a rapid and sensitive colorimetric method used to measure the drug-induced cytotoxicity in both adherent and suspension cell cultures (Aslantürk, 2017; Skehan et al., 1990). SRB is a bright pink aminoxanthene dye with two sulfonic groups, which, under mildly acidic conditions, binds electrostatically to protein basic amino acid residues in trichloroacetic acid-fixed cells to provide a sensitive index of cellular protein used to investigate cell proliferation (Aslantürk, 2017; Orellana and Kasinski, 2016). The absorbance is measured at 510 nm (Orellana and Kasinski, 2016; Vichai and Kirtikara, 2006).

1.5. Proteome-based studies investigating the regulation of *B. cereus* toxicity

Advancements in modern genomics and proteomics tools, especially next generation sequencing and high throughput MS-based quantitative protein detection have helped resolve many complex biological problems and have also been applied to understanding the biology and toxicity of *B. cereus*. In this section we discuss some such applications.

Gilois et al. (2007) did a time-course study of secretome of *B. cereus* ATCC 14579 and found that corresponding to PlcR activation, cytotoxicity against HeLa cells (TB uptake by dead cells) also peaked in the early stationary phase, and the cytotoxicity was directly related to the concentration of CytK (a PlcR-controlled toxin) (Gilois et al., 2007). The same group, using a PlcR deletion mutant of *B. cereus* ATCC 14579, inferred that most of the proteins secreted at the onset of the stationary phase are putative virulence factors, all of which are regulated, directly or indirectly, by PlcR (Gohar et al., 2008). This finding is in agreement with Gilois et al. (2007). Clair et al. (2010) used a combination of transcriptomics and proteomics-based approaches, and concluded that an early growth (37 ºC and 300 rpm, pH 7.2 in a 2-L bioreactor) phase-dependent pathway could regulate the expression of several virulence factors, allowing *B. cereus* to infect a host irrespective of the redox conditions, and this early growth phase-dependent pathway may function, at least in part, independently of the pleiotropic virulence gene regulator PlcR. In their study, growth inhibition of Caco-2 cells (measured using SRB method) was more when exposed to bacterial aerobic and anaerobic high redox potential, also known as oxidation-reduction potential (ORP) filtrate supernatants than when exposed to bacterial anaerobic low ORP filtrate supernatant. They draw this conclusion based on observation of less than 25% cells surviving at 48 and 96 h at high ORP compared to more than 50% cells surviving at 48 and 96 h at low ORP (Clair et al., 2010). Réjasse et al. (2012), using two-dimensional gel electrophoresis, showed that the production of the Hbl enterotoxin and of two proteases, NprB and NprP2 by *B. weihenstephanensis* was greater

at a growth temperature of 15 ºC than at 30 ºC (Réjasse et al., 2012). It should be noted that *B. weihenstephanensis* is a psychrotolerant bacterium that can grow at a temperature as low as 4 ºC (Lechner et al., 1998). OhrA (organic hydroperoxide resistance protein A) and OhrR (organic hydroperoxide resistance transcriptional regulator) belong to a thiol-dependent peroxidase-like protein family, and MarR (a multiple antibiotic resistance-controlled toxin) family of transcriptional regulators, respectively (Clair et al., 2013). Clair et al. (2013) compared exoproteomes from Δ*ohrA,* Δ*ohrR* and wild-type cells and found significant changes in the abundance levels of toxin-related proteins that were dependent on the extracellular redox potential. These results indicated that OhrRA is crucial for adaptation of *B. cereus* in the lumen of the small intestine that has an extreme redox environment (Clair et al., 2013). Omer et al. (2015) investigated another potential virulence gene regulator, the exoprotein EntD (enterobactin synthase component D; encoded by *entD*) that was found to play an important role in *B. cereus* virulence. EntD has been previously shown to play an important role in *E. coli* and *Salmonella typhi* virulence. Using a *ΔentD* mutant, Omer et al. (2015) identified 308 and 79 proteins that were regulated by EntD in the cellular proteome and the exoproteome, respectively. Caco-2 cells were more sensitive (as assessed by the MTT assay) to wild-type than to Δ*entD* filtrate supernatant, indicating that EntD could be an important player in *B. cereus* virulence. Among the cellular proteome, 154 proteins were upregulated in the Δ*entD* mutant compared with the wild-type, while the same number of proteins were downregulated. Motility- and toxin-related proteins (CytK, Nhe and the 3 Hbl components) were mainly downregulated during growth of Δ*entD* mutants indicating a role in the regulation of virulence factors and motility. They proposed that EntD regulates the cytotoxic potential of *B. cereus* cells by modulating its exoproteome (Omer et al., 2015). In order to study the role of *B. cereus* flagellar biosynthesis protein FlhF on protein export, Mazzantini et al. (2016) constructed a knockout mutant. In the extracellular proteome of the *ΔflhF* mutant, there was an increase in abundance of certain proteins like the B component of Nhe, Clo and enolase, while the abundance

of proteins like flagellin, L_2 component of Hbl, bacillolysin, SMase, PC-PLC, PI-PLC and CytK were reduced. Deletion of *flhF* also resulted in significant attenuation in the pathogenicity to *G. mellonella* larvae, highlighting the multifunctional role of FlhF in *B. cereus* toxicity, as this protein is involved in bacterial flagellation, swarming, protein secretion, and pathogenicity (Mazzantini et al., 2016). All these studies have identified some targets for future research but failed to clearly establish role of a particular toxin in cytotoxicity/virulence. More research investigating the correlations between genomes and proteomes (especially secretomes) of closely and distantly related strains is needed to better understand *B. cereus* group pathogenicity.

1.6. Using *B. cereus* cytotoxicity assays as a model for assessment of isolates' pathogenic potential

As discussed earlier, *B. cereus* group strains can cause serious maladies in animals and humans. In earlier research reports, animal models (see Section 1.7.) were used to study toxicity of these pathogens. However, expenses and ethical issues demanded a simple *in vitro* tool for assessing toxicity of *B. cereus* strains. This was especially true in experiments involving hundreds of isolates, as in population studies. Several cell lines have been used for such studies, with the most common being HEp-2, Vero, Caco-2, and HeLa.

1.6.1. Assessment of cytotoxicity due to emetic toxin

HEp-2 cells originating from a human laryngeal carcinoma have been used to study the response of cells to cereulide (Agata et al., 1994; Kamata et al., 2012; Mikami et al., 1994; Nishikawa et al., 1996; Sakurai et al., 1994; Shinagawa et al., 1992; Stark et al., 2013). Finlay et al. (1999) described a specific, sensitive, semi-automated, and quantitative HEp-2 cell culture-based MTT assay for assessment of cytotoxic effects of *B. cereus* emetic toxin. Since cereulide affects mitochondrial

function, which in turn affects cell viability, MTT was used as an indicator of cell viability and hence cytotoxicity (Finlay et al., 1999). Cell-free supernatant fluids from 11 *Bacillus* isolates from veterinary samples associated with bovine mastitis, bovine abortion, ovine abortion and caprine mastitis exhibited cytotoxicity in HEp-2 cells, as assessed by the MTT assay (Rowan et al., 2003). From Sunsik (a ready-to-eat Korean food) samples, 35 *B. cereus* strains were isolated, and all the strains harbored at least 1 enterotoxin gene (Chon et al., 2012). The occurrence of *nheABC*, *hblCDA*, *cytK*, and *entFM* enterotoxin genes among all isolates were 97%, 86%, 77%, and 100%, respectively. The diversity within *B. cereus* was studied by DiversiLab^{TM}Bacillus Kit (BioMérieux), a PCR-based tool. One strain (2.9%) carried the emetic toxin genes, including *ces* and *em1*, and was positive for HEp-2 cell vacuolation. Stark et al. (2013) et al. classified 78 *B. cereus* strains into no/low, medium and high producers of the emetic toxin cereulide, based on UPLC-TOF MS profiling, and compared with cytotoxicity (WST-1 assay) against HEp-2 cells; HEp-2 bioassay data and MS data were correlated (Stark et al., 2013).

The boar sperm motility assay is also used for assessment of cereulide activity. Within five minutes of exposure of boar sperm to heat-treated (100 ºC) extract of a cereulide producing *B. cereus* strain at 37 ºC, cessation of motility could be observed (Andersson et al., 2004). In a study by Biesta-Peters et al. (2010), boar sperm treated with synthetic cereulide concentration above 6.25 ng/ml lost their motility within 10 min of exposure. Treatment with synthetic cereulide resulted in toxicity/malformation of HEp-2 cells as examined under a microscope. When HEp-2 cells were incubated with cereulide concentrations above 7.81 ng/ml for 48 h, less than 10% of the cells survived (Biesta-Peters et al., 2010). Boar sperm assay was compared with the PI uptake-based cytotoxicity assay on four human cell lines (Calu-3 human lung adenocarcinoma, HeLa, Caco-2, and Paju), and comparable toxicity across all assays was recorded (Jääskeläinen et al., 2003) - human cells and boar sperm were equally sensitive to cereulide. In this study, the threshold concentration of cereulide provoking visible mitochondrial damage in boar sperm exposed *in vitro*

was 2 ng of cereulide ml^{-1} of extended boar sperm. Hoton et al. (2009) studied by PCR, two thousand *B. cereus* group isolates from food and environmental matrices for the presence of cereulide-encoding gene (*cesB*, cereulide synthetase), resulting in the identification of 73 potential emetic strains (belonging to *B. cereus* and *B. weihenstephanensis*), as determined by MLST (including *cesB* and *repX* from pXO1-like plasmids), large plasmid gel electrophoresis and Southern blot hybridization. None of the 450 well-characterized *B. thuringiensis*, *B. mycoides* and *B. pseudomycoides* strains was PCR-positive for the cereulide genetic determinants. This study, which also included boar sperm toxicity and cereulide production assays, led to the identification of two distinct clusters of cereulide-producing strains, with members of the second group (cluster II) identified as psychrotolerant *B. weihenstephanensis* capable of growing at 8°C (Hoton et al., 2009). Carroll et al. (2017) detected emetic toxin genes *cesABCD* in 24 assemblies belonging to *panC* clades III (encompasses *B. anthracis*, *B. thuringiensis* and *B. cereus s.s.*) and VI (encompasses *B. weihenstephanensis*) isolated from food, clinical, and environmental settings (Carroll et al., 2017).

Using MTT and SRB-based assays, Rajkovic et al. (2014) showed that Caco-2 cells were sensitive to sub-emetic doses of cereulide of less than 4 ng/g or 4 ng/ml (Rajkovic et al., 2014). In an earlier study, an analysis of samples originating from patients suffering from diagnosed emetic food poisoning revealed cereulide in high concentrations in gastric fluid (4 ng/mL), blood serum (4 ng/mL), urine (8 ng/mL) and, especially, stool (160–800 ng/g) (Shiota et al., 2010). In a recent study on sub-optimal dose of cereulide, both Caco-2 and HepG2 human liver cancer cells experienced measurable mitochondrial impairment after prolonged exposure of 10 days to 0.25 nM of cereulide (assessed using the MTT assay; Decleer et al., 2018). Carroll et al. (2019) studied 33 isolates of *B. cereus* group associated with a foodborne outbreak. These strains had only mild toxicity on HeLa cells compared to reference diarrheal strain *B. cereus s.s.* ATCC 14579, as found using the WST-1 assay. It was proposed that the outbreak was caused by emetic group III *B. cereus*

belonging to the *B. paranthracis* species. However, food samples were not tested for presence of cereulide (Carroll et al., 2019).

1.6.2. Assessment of cytotoxicity due to diarrheal enterotoxins

Here we review the role of diarrheal enterotoxins in cytotoxicity that has been assayed using three common cell lines namely, Vero, Caco-2 and HeLa cells. Vero cells, derived from kidney epithelial cells of an African green monkey, have been widely used in cytotoxicity assays assessing diarrheal disease potential of *B. cereus* strains (Cui et al., 2016a; Heini et al., 2018; Tausch et al., 2017). The human intestinal Caco-2 cell line, originally obtained from a human colon adenocarcinoma, has been frequently used in cytotoxicity assays of *B. cereus* enterotoxins, because it mimics the epithelial cells of the small intestine (Andersson et al., 1998; Castiaux et al., 2016; Decleer et al., 2018; López et al., 2015; Rajkovic et al., 2014; Wijnands et al., 2007). The HeLa cell line is derived from cervical cancer cells. Being fast growing, this cell line is widely used in research, but less commonly as a model system for cytotoxicity assay for *B. cereus*, which is primarily a pathogen of the gastrointestinal (GI) system. Nevertheless, this cell line is amenable to microscopy-based assays due to clear cell morphology and has hence been used for *B. cereus* cytotoxicity assays that require cell counting, such as the PI assay (Jääskeläinen et al., 2003; Miller et al., 2016, 2018; Simon et al., 2013). Below we summarize and synthesize current knowledge related to the mechanisms of action and the role of individual *B. cereus* group GI toxins in the development of cytotoxic effects on human cells.

As noted above, one of the cell culture lines used in assessment of *B. cereus* group cytotoxicity has been Vero cells (Agata et al., 1995; Cherif et al., 2003; Choma and Granum, 2002; Christiansson et al., 1989; Einar Granum and Nissen, 1993; Granum et al., 1996; Guaycurus et al., 1993; Hoštacká et al., 1992; Hoult and Tuxford, 1991; Lindbäck et al., 2004; Lund et al., 2000; Lund and Granum, 1996; Moravek et al., 2006; Stenfors et al., 2002; Stenfors Arnesen et al., 2007;

Stenfors and Granum, 2001; Te Giffel et al., 1997; Wilde et al., 2003; Wong et al., 1988). Lund and Granum (1997) compared cytotoxicity (by measuring the inhibition of protein synthesis) of the two different enterotoxin complexes Hbl and Nhe of *B. cereus* after isolation from three different strains using the Vero cell cytotoxicity assay. Inhibition of protein synthesis was measured as the percentage inhibition of C^{14}-leucine incorporation in cells due to the cells being treated with *B. cereus* supernatants, calculated relative to untreated cells (Fagerlund et al., 2010; Sandvig and Olsnes, 1982). While all the three strains produced 39 kDa, 45 kDa and 105 kDa components of Nhe, only two strains (F837-76 and 1230-88) produced B, L_1 and L_2 components of Hbl as detected in the supernatants of the strains. The Hbl complex from strain F837-76 was highly toxic against Vero cells. They concluded that when measuring cytotoxic enterotoxins, at least two different toxins (Nhe and Hbl) and six different proteins comprising these two toxins should be considered (Lund and Granum, 1997). Of the fifty strains of *B. weihenstephanensis* tested for cytotoxicity against Vero cells, 72% were not cytotoxic (in terms of inhibition of protein synthesis), even though all strains had at least one of the *B. cereus* enterotoxin-encoding genes Hbl, Nhe or CytK (Stenfors et al., 2002). Of 321 strains of *B. cereus* isolated from various sources from 4 countries, as many as 239 exhibited cytotoxicity on (inhibition of protein synthesis of) Vero cells, while only 53% of these cytotoxic strains were positive for the B-component gene of Hbl as assessed by PCR (Granum et al., 1996). Rowan et al. (2001) examined 47 isolates belonging to 14 species, including 21 *B. cereus s.s.*, for expression of virulence factors in reconstituted infant milk formula (IMF); eight isolates belonging to the species *B. cereus*, *B. licheniformis*, *B. circulans*, and *B. megaterium* were found to produce Hbl (detected by reversed passive latex agglutination kit) after growth in reconstituted IMF. Three isolates of *B. cereus* and 1 isolate of *B. subtilis* produced the Hbl enterotoxin after 18 h of growth (at 37 ºC) in brain heart infusion broth (Rowan et al., 2001). It is important to note here that most studies before 2010 relied on phenotypic characteristics for identification of species and the classification may not have been reliable. In one of the studies, of

the 26 strains of *B. cereus* grown at 42 ºC that were tested for cytotoxicity against Vero cells (in terms of inhibition of protein synthesis), only 19 strains were cytotoxic even though all strains had at least one of genes encoding the *B. cereus* enterotoxins Hbl, Nhe or CytK (Stenfors and Granum, 2001). Treatment of Vero cells with the supernatants of 14 out of the 19 cytotoxic strains resulted in a > 90% inhibition of protein synthesis of Vero cells (Stenfors and Granum, 2001). Wehrle et al. (2009) compared results of PCR (for detection of *hblC*, *hblD*, *hblA*, *nheA*, *nheB*, *nheC*, *ces* and *cytK-1* genes), immunoassay detection of three components of Hbl and Nhe, and cytotoxicity (assayed using WST-1 dye) in Vero and HEp-2 cells. The Vero cell assay results showed a positive correlation with immunoassay detection and PCR. Only three strains, one each from group II, III and V (grouping was done based on sequence of PCR-amplified enterotoxin genes), expressing all the Nhe components (determined by ELISA), albeit in very low amounts, were negative in the Vero cell assay. One strain lacking NheB (pattern VII), and a second strain producing only NheA (pattern VIII), did not exhibit cytotoxic effects. For emetic strains, PCR results were confirmed with a test based on HEp-2 cells. They proposed that multiplex PCR systems could be an easy way to identify enterotoxinogenic *B. cereus* isolates at that point of time when genome-based approach was not very common (Wehrle et al., 2009). Ninety-two *B. cereus*-like bacteria were isolated from 306 milk and environmental samples, of which 91.3% showed cytotoxicity in Vero cells, as assessed by the WST-1 assay (Cui et al., 2016a). Three major toxin genes *nhe*, *hbl* and *ces* were detected with rates of 100.0%, 78.3% and 1.1%, but no strain harbored gene for CytK-1, indicating that CytK-1 is not essential for cytotoxicity in Vero cells.

Minnaard et al. (2007) undertook a systematic study on the relationship between presence of virulence genes (*entS*, *entFM*, *nheA, nheB, nheC*, *sph*, *hblA, hblB, hblC, hblD*, *piplC* and *bceT*) and cytotoxicity. In 21 strains of *B. cereus* group, cytotoxicity indicators like decreased mitochondrial dehydrogenase activity (MTT assay), necrosis (PI uptake assay), cell detachment (CV assay), and hemolytic activity were detected in a strain-dependent manner in the Caco-2 cell

line. For the cell detachment assay, differentiated Caco-2 monolayers were co-incubated with isolates' supernatants at 37 ºC for 2 h. Of 21 strains examined, filtrate from all but one strain was positive in the detachment assay. Significant positive correlation was observed between presence of *entS*, *nheC* and *sph* sequences and detachment. There were also trends of positive correlations with sequences of genes *piplC* and *bceT* and detachment. High correlation was found between sequences of the Hbl complex, but there was a lack of significant correlation between sequences of the *nhe* operon and detachment (Minnaard et al., 2007). It may be noted that all the strains under study were positive for NheA when tested by the ELISA assay.

Guinebretière et al. (2008) classified *B. cereus* group into seven phylogenetic groups based on fluorescent amplified fragment length polymorphism patterns, ribosomal gene sequences, partial *panC* gene sequences, 'psychrotolerant' DNA sequence signatures, and phenotypic and descriptive data from a range of growth temperatures, psychrotolerance, and thermal niches (Guinebretière et al., 2008). They further determined the presence of known toxin genes (*hbl*, *nhe*, *cytK-1*, *cytK-2*, *ces*) in 391 isolates of the *B. cereus* group using PCR (Guinebretière et al., 2010). Cytotoxicity of culture filtrates of a subset of 97 strains, belonging to the different phylogenetic groups (II to VII), was assayed against Caco-2 cells using CV staining. According to their classification, which was not based on whole genome analysis, phylogenetic group I composed of *B. pseudomycoides*, II, IV and V composed of *B. cereus* and *B. thuringiensis*, group III of *B. cereus, B. thuringiensis and B. anthracis*, VI of *B. weihenstephanensis, B. mycoides,* and *B. thuringiensis* and group VII of *B. cytotoxicus*. Genes (*ces*) for cereulide were found only in group III, in subgroup III-2 containing the emetic strain F4810/72, and subgroup III-3, while *nhe* genes were present in 100% of the tested isolates. The frequency of *hbl* gene varied from 40% to 97% among phylogenetic groups I, II, IV, V, and VI. The gene *cytK-1* was present only in group VII "*B. cytotoxicus*", while *cytK-2* was frequently detected in mesophilic groups III and IV. Further, *cytK-2* was absent or rarely present in the psychrotolerant or moderately psychrotolerant groups VI, II and V. The *cytK* gene was also

absent in group I strains (*B. pseudomycoides*). A relation between phylogenetic affiliation (Groups I to VII) of *B. cereus* group isolates and cytotoxicity was thus seen and it was proposed that phylogenetic classification is more reliable than species-based classification for assessing food-poisoning risk (Guinebretière et al., 2010). The authors inferred that food poisoning risk is highest for group III (containing *B. cereus*, *B. thuringiensis*, and *B. anthracis*). Böhm et al. (2015) sequenced 25 strains of *B. cereus*, analyzed, and compared with 142 existing whole genome sequences. They proposed subdivision of *B. cereus group* into seven phylogenetic groups. Based on a comparison of phylogeny of enterotoxin genes, Böhm et al. (2015) provided evidence in favor of horizontal transfer of *hbl*, *cytK* and *plcR* genes, frequent deletion of both toxins, as well as duplication of *hbl*. Contrary to this, evidence in favor of horizontal transfer of *nhe* was rare, and deletion of this gene was not found in their analysis (Böhm et al., 2015). In a study encompassing 70 *B. cereus* strains, enterotoxicity could not be fully explained by the presence of any of the virulence genes screened (*ces, cytK, entFM, entS, hbl, nhe, nprA, piplC* and *sph*) by PCR (Castiaux et al., 2016). Similarly, there was no correlation between clade, presence of toxin genes and cytotoxicity (against HeLa cells) among 8 type strains and 44 dairy isolates of *B. cereus* group (Miller et al., 2018). In this study, one of the *B. weihenstephanensis*/*B. mycoides* isolates was cytotoxic (by PI uptake method) when grown at 37 ºC and all of the *B. pseudomycoides* isolates exhibited cytotoxicity.

Miller et al. (2016) identified a psychrotolerant, new species *B. wiedmannii* that produced Hbl and Nhe (determined using immunoassay kit Duopath after growing for 20 h at 32 °C), and exhibited cytotoxicity in HeLa cells (Miller et al., 2016), as measured using PI/DAPI staining. Miller et al. (2018) systematically investigated the association between Hbl and Nhe proteins, genes and cytotoxicity. No strict correlation was seen between the detection of toxin genes and pathogenic potential of *B. cereus* group isolates, as the presence of toxin genes was not always consistent with cytotoxicity phenotype. Hbl-L_2 was detected in phylogenetic groups II, III, IV and V, while NheB

was detected in phylogenetic groups II, III, IV, V and VI; hemolytic activity was detected in groups I to VII (Miller et al., 2018). Carroll et al. (2019) characterized *B. cereus* strains associated with 2016 foodborne outbreak from New York. Outbreak associated three diarrheal strains produced Hbl and Nhe while six representatives of the emetic isolates tested produced Nhe, but not Hbl (Carroll et al., 2019).

Jeßberger et al. (2014) did a comparative analysis on the relevance of Nhe, Hbl and CytK with respect to their toxic activity towards different target cell lines using the WST-1 assay; Nhe and Hbl complexes accounted for over 90% of the total toxicity. Vero and HUVEC primary vascular endothelial cells were highly susceptible to Nhe, whereas Hep-G2 human hepatocellular carcinoma cells, Vero and A549 human non-small cell lung cancer cells were most sensitive to Nhe plus Hbl. Of all cell lines studied, the Caco-2 cells were found to be the most sensitive to CytK. Since the tested Hbl positive strains always produced Nhe in parallel, the individual contribution was determined by consecutively removing their single components. The contribution of Nhe and Hbl to the cytotoxicity against most cell lines was between 40 and 60%; the relative activity of Nhe being 90%, and of Hbl being 75% against HUVEC and A549 cells, respectively. Moreover, Nhe/Hbl producing strains generally caused rapid pore formation (as indicated by faster influx of PI into the target cells) compared to solely Nhe-producing strains. This data highlighted the importance of Hbl in pore formation in the cell membranes. A correlation between cytotoxicity and expression of Hbl-L_1, NheB and HblB could be seen for Nhe/Hbl producing isolates. A correlation was also observed between cytotoxicity of solely Nhe producing strains and NheB. Since cell lines of different histological origin were susceptible to *B. cereus* toxins, it was concluded that the toxicity might not be restricted to GI infections (Jeßberger et al., 2014). This thorough study established for the first time the differential toxicity of enterotoxins to different cell lines.

The Jeßberger et al. (2014) study is significant as there often exists a weak correlation between the genome-based taxonomy and phenotypes, especially with respect to the toxicity pattern

and virulence in *B. cereus* (Castiaux et al., 2016; Miller et al., 2018), which might be due to use of different cell lines in the assays. This also could be due to the presence/absence of mobilizable megaplasmids resulting from massive horizontal gene transfer across this species group under strong selection pressure, as suggested by Böhm et al. (2015) (Böhm et al., 2015). This may explain high intraclade variability in cytotoxicity but does not explain the poor correlation between presence of toxin genes and production of toxins/cytotoxicity (Castiaux et al., 2016; Miller et al., 2018). For example, *B. cereus s.s.* and *B. thuringiensis* are similar except for the presence of the megaplasmids carrying the *cry* genes in the latter. Similarly, emetic strains of *B. cereus* also may harbor *nhe*, *cytK-2* and *hbl* genes (Guinebretière et al., 2010). Another example is the presence of "pXO1", the megaplasmid coding for virulence determinants from *B. anthracis* that has been found to be present in *B. cereus* biovar *anthracis* (Antonation et al., 2016). Cytotoxicity assays have been a valuable tool for assessing the virulence potential of *B. cereus* strains, especially for characterizing the large number of samples required in population genetics studies. However, as discussed above, there seems to be an interaction between strains (and toxin profile) and cell types, which needs to be taken into account while interpreting such results.

1.7. Relation between *in vitro* cytotoxicity and virulence in animal models

A few initial studies used animal models to assess the toxicity of *B. cereus* group strains or purified toxins (Agata et al., 1995; Duncan and Strong, 1969; Guaycurus et al., 1993; Hoštacká et al., 1992; Shinagawa et al., 1991; 1995; 1996; Wong et al., 1988). However, *in vitro* cell culture-based assays became preferred methods due to the ease of performing experiments, low cost, and ethical issues. However, a weakness of *in vitro* experiments is that results from *in vitro* experiments may not always correlate with outcomes of animal experimental infections (for example Rolny et al., 2014). Here we report studies where a good correlation between *in vitro* and *in vivo* experiments was

demonstrated, and where more animal-based studies are needed in order to better understand *B. cereus* group virulence and capacity to cause emetic and/or diarrheal foodborne illness.

As stated earlier, cereulide causes vacuolation in cells due to uncoupling of mitochondrial phosphorylation (Agata et al., 1994; Sakurai et al., 1994; Shinagawa et al., 1992). Similar to symptoms in humans, partially purified emetic toxin cereulide was shown to induce vomiting (no lethality) in rhesus monkeys when administered orally (30000-36000 units per monkey of 6-8 kg body weight; 1 unit was defined as 5 ng), and also resulted in vacuolation in HEp-2 cells (Shinagawa et al., 1995). Intravenous and intraperitoneal injections of cereulide were lethal to mouse and *Suncus* (a genus of shrews in the family Soricidae) (Shinagawa et al., 1996). In the study by Cui et al. (2016b), rabbits were administered single dose of 5 μg cereulide and concentration in plasma was monitored by MS. Maximum concentration of cereulide in plasma peaked after 2.6 ± 3.4 hours of intravenous injection in rabbits (Cui et al., 2016b). No clinical symptom or mortality at this dose was reported, even though there was transient liver damage (Cui et al., 2016b). In human intoxication cases, vomiting is induced within 1-5 hours of ingestion (Häggblom et al., 2002).

Bauer et al. (2018) recently did a systematic study on bioaccumulation of cereulide in pigs. Animals were orally administered cereulide, either 10–150 μg cereulide kg^{-1} body weight, single dose, or daily doses of 10 μg cereulide kg^{-1} body weight for a period of 7 days. Symptoms like vomiting, depressive behavior, recurrent seizures, shivering, lethargy were observed in the experimental pigs at 150 μg/kg. From pigs orally challenged with 150 μg cereulide kg^{-1} part of the cereulide ingested with food was rapidly excreted with feces and urine (up to 5 μg cereulide g^{-1} feces and up to 2 ng cereulide mL^{-1} urine); up to 45 ng cereulide mL^{-1} was detected in blood, while part of the toxin was absorbed, passed through membranes and got distributed within the body. Bioaccumulation of cereulide in tissues and organs (i.e., kidney, liver, muscles and fat tissues) was shown. In animals challenged with 150 μg cereulide kg^{-1}, the toxin was found in the content of the

large intestine (up to 140 ng g^{-1}) and in contents of small intestine (max. 1 ng g^{-1}). Cereulide was also detectable in stomach, spleen, liver and brain and fat tissues of all animals. Cereulide crossed the blood–brain barrier (Bauer et al., 2018), explaining, in part, the cerebral effects reported in humans. Considering these findings, pigs seems to be a good model for studying pathogenicity of emetic strains of *B. cereus*. Being heat stable, it has been fairly easy to establish the role of cereulide in pathogenicity of *B. cereus*. Simply by boiling the culture filtrate, it is possible to exclude the role of other virulence factors if produced by the same strain. Also, the clinical symptoms are clear and there is establish correlation between reaction of HEp-2 cells (vacuolation) and animal symptoms in animal models.

Wong et al. (1988) studied toxicity of 183 dairy isolates of *B. cereus* through hemolytic activity on rabbit erythrocytes, disruption of monolayer of Vero cells, and alteration of shape of CHO cells to an abnormal shape. All the tested isolates obtained from these samples lysed rabbit erythrocytes, 98% showed toxicity to Vero cells and 68% to CHO cells. Based on strong hemolytic activity, 11 isolates were selected for animal studies and half ml of cell-free filtrates of cultures grown at 35 ºC for 10 h at 280 rpm were injected to caudal vein of adult mice. The filtrates of only three isolates killed adult mice at the administrated dose, while the remaining filtrates significantly lowered physical activities of the mice (Wong et al., 1988).

In another study, a native, purified enterotoxin of molecular mass 45 kDa (probably Nhe) from *B. cereus* showed toxicity against Vero cells, but no hemolytic or phospholipase C activity and vascular permeability in rabbits (0.02-0.04 μg/rabbit), caused fluid accumulation in mouse ligated intestinal loops (8-16 μg/loop), and was lethal to mice (10 μg with i.v.), indicating a role of Nhe as a virulence factor (Shinagawa et al., 1991). In another study with culture filtrate of 89 strains of *B. cereus* (isolated from foodstuffs), only three were found to cause fluid accumulation in the ligated rabbit ileal loop (Hoštacká et al., 1992). This model was commonly used since fluid

accumulation in ligated rabbit ileal loop is a method for assessing diarrheal toxins (Duncan and Strong, 1969).

Of the 89 isolates tested, 71 caused vascular permeability increase in rabbit skin (injected intradermally with 0.05 ml of culture filtrates; rabbit skin assay is an indicator of permeability), and 59 were toxic (by examination under the microscope; criterion for cytotoxicity unspecified) against Vero cells (Hoštacká et al., 1992). In the same study, filtrates (grown at 32 ºC) from 31 strains were lethal, 81 induced hemolysis and 55 showed lecithinase activity (zone of turbidity on egg yolk agar). They inferred that there exists a relationship between extent of permeability reaction and cytotoxicity, lecithinase and lethality. There was no relationship between the permeability reaction of culture filtrates and the reaction in the ligated rabbit ileal loop, and only a low correlation was found between the permeability reaction and hemolysin.

Guaycurus et al. (1993) purified three secreted proteins of molecular mass > 30 kDa from *B. cereus* AL-42 and AL-15 strains. The major peak of 66 kDa resulted in cytotoxicity (disruption of cell monolayer) to Vero- and HeLa cells and lethality to mice (Guaycurus et al., 1993). Agata et al. (1995) expressed the BceT enterotoxin in *E. coli*; this protein showed toxicity against Vero cells (disruption of cell monolayer) and vascular permeability and fluid accumulation in ligated mice illeal loop and was lethal to mice upon injection (Agata et al., 1995). Rolny et al. (2014) administered 6-8-week-old female mice with 10^8-10^9 *B. cereus* strain B10502 cells by intragastric gavage using a blunt end needle. Infection led to significant modification in relevant immune cells in the spleen, Peyer's patches and mesenteric lymph nodes (Rolny et al., 2014). Significantly, this strain was unable to invade enterocytes *in vitro* (Minnaard et al., 2004). More animal model studies are required to establish the role of individual or combination cytotoxic components in virulence of diarrheal strains of *B. cereus*. The same is true for differentiating between environmental strains and true pathogens. As is seen from discussion in this section, the animal-model based studies are very old and pre-date the omics-based studies. There is a need for re-visiting animal-based studies

and combining with cytotoxicity and whole-genome based assays to better understand the virulence system in *B. cereus*.

1.8. References

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Chapter 2.

Statement of the Problem

The *Bacillus cereus* group of bacteria encompasses as many as 18 taxonomically valid species. Some of these species are responsible for serious maladies of humans and animals, e.g., anthrax caused by *B. anthracis*. Foodborne pathogenic isolates belonging to *B. cereus* group may be classified into two groups - those that cause emesis (vomiting), and those that cause diarrhea. The pathogenicity of emetic strains is well understood, as the virulence factor is well-defined and characterized.

In contrast to the emetic strains, the pathogenicity of the diarrheal strains is poorly understood. Part of the problem is that many putative virulence factors, ranging from enzymes to lytic toxins, have been implicated in pathogenicity, primarily based on *in vitro* cytotoxicity assays. *B. cereus* strains are ubiquitous and have been isolated from different environments including human excreta, milk, processed and raw food, soil, and the plant rhizosphere. However, there is no reliable model for testing whether a specific strain of *B. cereus* can cause diarrhea. With recent advancements in genomics, researchers have attempted to study the associations between whole genome sequence, proteome and cytotoxicity (Carroll et al., 2019; Clair et al., 2010; Clair et al., 2013; Gilois et al., 2007; Jeßberger et al., 2015; Miller et al., 2018; Omer et al., 2015). While these analyses have resulted in robust phylogenetic classification and groupings, assigning a virulence phenotype to a phylogenetic group has remained elusive. Among the *B. cereus* toxins that have been implicated in diarrhea, the pore forming toxins non-hemolytic enterotoxin (Nhe) and hemolysin BL (Hbl) are best studied (Schraft and Griffiths, 2006). Nhe, but not Hbl, seems to be

present across strains of *B. cereus*, indicating the specific role of Hbl in pathogenicity (Carroll et al., 2017). However, since all Hbl positive strains are not cytotoxic/virulent, its sole role as a pathogenicity factor is questionable, and there is a possibility that its toxicity may be dependent on some other extra-/intracellular toxins/enzymes. Cytotoxin K, hemolysin I, hemolysin II, sphingomyelinase, enterotoxin T and enterotoxin FM are other components that have been perceived as putative virulence factors/toxins.

The effects of *B. cereus* group virulence factors have been studied using various cell lines and several assays. HEp-2 human laryngeal carcinoma cells have been used for the emetic strains, while for the non-emetic strains, Vero African green monkey kidney cells, HeLa human epithelial cervix adenocarcinoma cells and Caco-2 human epithelial colorectal adenocarcinoma cells have been commonly used. However, studies involving the use of combination of different cytotoxicity assays and different cell lines for the systematic screening of large number of *B. cereus* isolates are lacking. Comparative analyses and optimization of cytotoxicity assays are therefore needed in order to support high-throughput screening of *B. cereus* group isolates to better understand their pathogenic potential and distribution of pathogenic isolates throughout the food supply chains. The overall goal of the thesis was the optimization of high throughput cytotoxicity assays for screening of *B. cereus* isolates, and their application to characterize *B. cereus* group species type strains, 2016 foodborne outbreak isolates, and *B. cereus* clade IV isolates.

The dairy environment is a major source of *B. cereus* contamination introduced to milk and transmitted through the food supply chain. Recently, Miller et al. (2018) studied 52 isolates, including 9 type strains and 44 isolates from dairy-associated sources for detailed phylogenetic analysis, presence of toxin genes, presence of NheB and Hbl-L_2, and cytotoxicity against HeLa cells, assayed by propidium iodide (PI) staining. Being fast-growing, HeLa cells are amenable to be used for screening a large number of isolates. However, PI staining is a laborious and time-consuming cytotoxicity assay that does not allow for rapid screening of larger sets of isolates. There

was no report in literature on a rapid cytotoxicity assay for screening *B. cereus* isolates using HeLa cells. Hence, the first objective of our study was to optimize a rapid cytotoxicity assay using HeLa cells and apply it on type strains of *B. cereus* group (Liu et al., 2017; Miller et al., 2018) and isolates from a 2016 foodborne outbreak (Carroll et al., 2019).

Miller et al. (2018) had reported a wide range of cytotoxicities based on the PI staining for 15 isolates classified as *B. cereus* clade IV, which includes *B. cereus s.s.*, a known foodborne pathogen (Glasset et al., 2016), and *B. thuringiensis*. The cytotoxicity on HeLa cells determined using the PI assay did not correlate well with the presence of different known and putative toxin genes. Moreover, the PI assay they had used was laborious. One of the long term goals of our project was to characterize the proteome profile of the *B. cereus* secretome in order to understand the associations of cytotoxicity and proteins for a large number of *B. cereus* isolates. For this purpose, the bacteria had to be cultured in a minimal (MOD) medium. Rapid cytotoxicity assays for screening large number of *B. cereus* isolates grown in MOD medium have not been developed so far. Hence, the second objective of our study was to conduct comparative cytotoxicity analyses of clade IV isolates (grown in MOD medium) using two different rapid cytotoxicity assays, on two different cell lines (HeLa and Caco-2 cells). While HeLa cells are fast-growing and easier to maintain, the Caco-2 cells, when differentiated, make for a more relevant cell model to study gastrointestinal foodborne illness due to *B. cereus*. This is because they resemble the enterocytes lining the small intestine once differentiated. However, differentiation takes 21 days of time, which makes it more challenging to conduct on a routine basis, hence we assayed undifferentiated Caco-2 cells to see if the effects were similar for both states of Caco-2 cells. We also compared the results of the Caco-2 cells with those of HeLa cells using the optimized assays.

Lastly, we looked for associations between the putative virulence gene presence/absence, detection of Hbl and Nhe components secreted by the bacteria, and cytotoxicity data from all three cell lines obtained for the 15 clade IV isolates. This was the final objective of our study.

To summarize, the main objectives of this study were:

1. To optimize a rapid cytotoxicity assay with HeLa cells and apply it on *B. cereus* group species type strains and foodborne outbreak-associated isolates

2. To characterize the cytotoxic potential of *B. cereus* clade IV isolates in HeLa and Caco-2 cell lines using two different high-throughput cytotoxicity assays

3. To test for associations between presence of putative virulence genes, detection of hemolysin BL and non-hemolytic enterotoxin, and cytotoxicity in HeLa and Caco-2 cell lines

Chapter 3.

Materials and Methods

3.1. Source of isolates

3.1.1. Bacillus cereus group species type strains

The *Bacillus cereus sensu lato* (*s.l.*) group species type strains *B. cereus sensu stricto* (*s.s.*) ATCC 14579, *B. pseudomycoides* DSM 12442, *B. wiedmannii* FSL W8-0169, *B. toyonensis* BCT-7112, *B. cytotoxicus* NVH 391-98, *B. thuringiensis* ATCC 10792, *B. mycoides* DSM 2048, and *B. weihenstephanensis* WSBC 10204 were obtained from the Food Microbe Tracker culture collection (Vangay et al., 2013). These were further streaked for isolation and stored in the form of glycerol stocks in brain heart infusion (BHI) medium containing 25% glycerol at – 80 °C. The 9 novel *B. cereus* group type strains which were added into the *B. cereus* group (Liu et al., 2017), namely *B. albus* N35-10-2, *B. luti* TD41, *B. mobilis* 0711P9-1, *B. nitratireducens* 4049, *B. pacificus* EB422, *B. paramycoides* NH24A2, *B. paranthracis* Mn5, *B. proteolyticus* TD42, and *B. tropicus* N24 were purchased from the Korean Collection for Type Cultures (KCTC; South Korea) and received in lyophilized form. *B. mycoides* ATCC 21929 was purchased from the American Type Culture Collection (ATCC; U.S.A) and received in a lyophilized form. The strains that arrived in lyophilized form were suspended in 100 µl of phosphate buffered saline (PBS), gently mixed, and streaked onto BHI agar plates for isolation. They were made into glycerol cryostocks by growing single isolated colonies of the revived cultures in BHI broth overnight at 32 °C, supplementing them with 25% sterile glycerol and storing them at – 80 °C. The *B. anthracis* type strain ATCC

14578 was not obtained or used in this study due to its classification as a Biosafety Level 3 microorganism. Being a threat to both human and animal health, *B. anthracis* has been used as a biological weapon in the past. Hence, *B. anthracis* (as well as *B. cereus* biovar *anthracis*) was classified as a Tier 1 agent on the Select Agents and Toxins Lists, as regulated by the U. S. Department of Health and Human Services, and the U.S. Department of Agriculture.

3.1.2. Foodborne outbreak isolates

The 33 *B. cereus* group isolates from the 2016 Mighty Taco outbreak were received in the cryostock form Cornell University, Ithaca, New York, and were originally sourced from the New York State Department of Health (Carroll et al., 2019). These were comprised of 3 diarrheal outbreak-associated isolates and 30 emetic outbreak-associated isolates. While the *B. cereus s.s.* type strain ATCC 14579 (Section 3.1.1.) served as a reference for the diarrheal isolates, the *B. cereus* strain DSM 4312 was used as the reference strain for comparison of the emetic isolates. Here, emetic isolates refer to isolates that carry *ces* genes, while diarrheal isolates have been denoted as those carrying either *hbl* or *cytK-2* genes but lacking *ces* genes. DSM 4312 was received in a cryostock form from Cornell University, Ithaca, New York, was streaked for isolation, and stored in the form of glycerol stocks at – 80 °C.

3.1.3. Clade IV isolates

The 15 *B. cereus* clade IV isolates included 13 dairy-associated *B. cereus s.s.* isolates that had previously been classified into phylogenetic group (i.e., clade) IV (Miller et al., 2018) which were obtained from the culture collection of Cornell University, Ithaca, New York. Fresh glycerol stocks were made from the isolates and frozen at – 80 °C as described in Section 3.1.1. The clade IV group also included the type strains of *B. cereus s.s.* and *B. thuringiensis*. A summary of the sources of isolates used and characteristics of tests performed using the isolates is presented in Table 3-1.

Table **3-1**: Sources of *B. cereus* group isolates and tests performed

Study	Isolates	Source	Growth medium	Cytotoxicity assay on	
				HeLa cells	Caco-2 cells
Characterization of *B. cereus* group type strains	*B. cereus s.s.* ATCC 14579 [(a)], *B. pseudomycoides* DSM 12442, *B. wiedmannii* FSL W8-0169, *B. toyonensis* BCT-7112, *B. cytotoxicus* NVH 391-98, *B. thuringiensis* ATCC 10792, *B. mycoides* DSM 2048, *B. weihenstephanensis* WSBC 10204	Food Microbe Tracker culture collection	BHI	WST-1	N.D. [(b)]
	B. albus N35-10-2 [(a)], *B. luti* TD41 [(a)], *B. mobilis* 0711P9-1 [(a)], *B. nitratireducens* 4049 [(a)], *B. pacificus* EB422 [(a)], *B. paramycoides* NH24A2 [(a)], *B. paranthracis* Mn5 [(a)], *B. proteolyticus* TD42 [(a)], *B. tropicus* N24 [(a)]	Korean Collection for Type Cultures			
Characterization of 2016 Mighty Taco foodborne	FSL R9-6381 [(a)], FSL R9-6382 [(a)], FSL R9-6383, FSL R9-6384 [(a)], FSL R9-6385, FSL R9-6386, FSL R9-6387, FSL R9-6388, FSL R9-6389 [(a)], FSL R9-6390, FSL R9-6391, FSL R9-6392, FSL R9-6393, FSL R9-6394, FSL R9-6395	Food Microbe Tracker	BHI	WST-1	N.D. [(b)]

associated isolates	[a], FSL R9-6396, FSL R9-6397, FSL R9-6398, FSL R9-6399 [a], FSL R9-6400, FSL R9-6401, FSL R9-6402, FSL R9-6403, FSL R9-6404, FSL R9-6405, FSL R9-6406, FSL R9-6407, FSL R9-6408, FSL R9-6409, FSL R9-6410, FSL R9-6411, FSL R9-6412, *B. cereus* DSM 4312, FSL R9-6387, *B. cereus s.s.* ATCC 14579 [a]	culture collection			
Characterization of *B. cereus* group clade IV isolates	FSL K6-0040 [a], FSL K6-0043 [a], FSL K6-0073 [a], FSL K6-0268 [a], FSL K6-1030 [a], FSL M8-0139 [a], FSL R5-0184 [a], FSL R5-0585 [a], FSL R5-0859 [a], FSL W7-1101 [a], FSL W7-1334 [a], FSL W8-0640 [a], FSL W8-0824 [a], *B. thuringiensis* ATCC 10792 [a], *B. cereus s.s.* ATCC 14579 [a]	Food Microbe Tracker culture collection	MOD	WST-1, LDH	WST-1, LDH

[a] Isolate was also characterized for production of NheB and Hbl-L_2 using lateral flow immunoassay.

[b] N.D. means cytotoxicity assay was not done.

3.1.4. Other B. cereus group isolates

Dairy-associated isolates FSL K6-0069, FSL M8-0214, FSL W8-0003, FSL J3-0113, and FSL M7-0109 were obtained from the Food Microbe Tracker culture collection of the Cornell University (Vangay et al., 2013). The environmental isolate UBC1 (= ML-A2C4) was obtained in a stab culture form from the University of British Columbia. All isolates were streaked for isolation and stored in the form of glycerol stocks at – 80 °C as described in Section 3.1.1. These isolates were only used for optimization of the MTT assay (Section 3.12.), and were not intended to be compared and characterized using cytotoxicity and immunoassays. Hence, they have not been listed in Table 3-1 for simplicity.

3.2. Source of cell lines

HeLa human cervical cancer cells were received from Cornell University, Ithaca, New York in the cryostock form. Caco-2 human colon adenocarcinoma cells (ATCC HTB-37) were purchased from ATCC, U.S.A.

3.3. Preparation of the MOD medium

MOD medium is a minimal medium that was used for growing *B. cereus* group isolates in experiments in which bacterial supernatants were intended to be used for further proteomics-based characterization. Defined minimal medium was selected over BHI broth to minimize the potential interference of medium protiens with subsequent proteomics analyses. Sterile MOD medium having the composition shown in Table 3-2 (Rosenfeld et al., 2005) was prepared. The pH was adjusted to 7.2 using 0.5 M KOH solution.

Table **3-2**: Composition of MOD medium

Component	Quantity per liter [(a)]
L-arginine	0.46 g
L-aspartic acid	0.91 g
L-cysteine	0.04 g
L-glutamic acid	2 g
L-histidine	0.36 g
L-isoleucine	0.7 g
L-leucine	1.37 g
L-methionine	0.4 g
L-phenylalanine	0.28 g
L-threonine	0.71 g
L-valine	0.91 g
L-glycine	0.39 g
L-lysine	1.18 g
L-serine	0.66 g
L-tyrosine	0.71 g
K_2HPO_4	1 g
$(NH_4)_2SO_4$	6 g
$MgSO_4 \cdot 7H_2O$	0.04 g
$CaCl_2$	275 µg
$FeCl_2 \cdot 6H_2O$	675 µg
$MnCl_2 \cdot 4H_2O$	50 µg
$ZnCl_2$	85 µg

$CuSO_4$	40 µg
$CoCl_2 \cdot 6H_2O$	30 µg
$Na_2MoO_4 \cdot 2H_2O$	30 µg
Na_2SeO_4	24 µg
Glucose	5.4 g

[(a)] Quantities taken from Rosenfeld et al., 2005.

3.4. Bacterial growth conditions

All *B. cereus* isolates were streaked onto BHI agar from their cryostocks and incubated for 18-24 h at 37 °C.

3.4.1. Outbreak isolates and type strains

Isolated colonies of *B. cereus* group species type strains, outbreak isolates, as well as the emetic reference strain DSM 4312 were inoculated in 5 ml of sterile BHI and incubated at 37 °C under non-shaking conditions overnight (16-19 h). 5 µl of the overnight cultures obtained were used to inoculate fresh 5 ml BHI broth, and the cultures were allowed to grow up to an optical density at 600 nm (OD_{600}) of approximately 1.5. This value of OD_{600} was chosen, as it corresponded to ~ 10^8 colony-forming units (cfu)/ml based on colony counts of a few representative isolates (FSL R9-6407, FSL R9-6381, FSL R9-6413), indicative of the early stationary phase.

3.4.2. Clade IV isolates

The Clade IV isolates were cultured in the MOD medium, a protein-free minimal medium (essential for subsequent proteome characterization of supernatants; Appendix A). Briefly, isolated colonies were inoculated in 5 ml of sterile MOD medium in tubes and incubated at 37 °C with shaking at

150 rpm. 5 µl of the overnight cultures were used to inoculate fresh tubes containing 5 ml MOD medium, which were again incubated at 37 °C and 150 rpm to enhance bacterial growth and ensure homogenous environment. Overnight cultures were spun down at 16,000 g for 10 minutes (Beckman Coulter Avanti® J-26 XPI), and the spent medium was discarded. The pellet was resuspended in 5 ml MOD medium, and the washing step was repeated again by spinning down and resuspending in MOD medium. This medium containing bacteria was used to inoculate fresh 250 ml flasks containing 125 ml of MOD medium such that the calculated OD_{600} at the beginning of the incubation was 0.02 (Rosenfeld et al., 2005). Culturing was carried out at 37 °C under 150 rpm shaking conditions.

3.5. Standard curve describing a linear relationship between optical density and culture concentration

As described in 3.4.2, two isolated colonies obtained from a streaked plate of the *B. cereus s.s.* type strain ATCC 14579 were grown up independently in the MOD medium, following which the harvested overnight culture was used to inoculate fresh MOD medium contained in two flasks such that the starting OD_{600} was 0.02. The cultures contained in the flasks were incubated at 37 °C at shaking speed of 150 rpm. The growth was stopped as OD_{600} reached between 1.20 – 1.22 by placing the flasks on ice. The cultures were adjusted to OD_{600} of 0.9, 0.7, 0.5, 0.3, and 0.1 by diluting the cultures in MOD medium. Serial dilutions of the cultures of different adjusted OD_{600} values were performed in Butterfield's PBS in triplicate, with 100 µl of the appropriate dilution spread plated onto BHI agar plates. The plates were incubated overnight at 37 °C, and the colonies on the plates counted. The colony counts of the cultures of OD_{600} 0.9, 0.7, 0.5, 0.3, and 0.1 were calculated. The average counts of 3 technical replicates corresponding to each biological replicate were calculated. A standard curve was plotted using mean counts of the two biological replicates,

with the standard error of mean shown with error bars. A linear line of best fit was generated, and the corresponding equation was found.

3.6. Growth curves for clade IV isolates

The OD_{600} of *B. cereus* clade IV isolates cultured at 37 °C and 150 rpm in flasks (described in Section 3.4.2.) was measured over time (cultures were diluted to OD_{600} in the range of 0.1 to 0.9 with MOD medium, and the resultant OD_{600} values were multiplied by the dilution factors), and were recorded for all the clade IV isolates in two independent biological replicates. The growth curves based on OD_{600} as a function of time were constructed.

The equation describing the relationship between OD_{600} and culture concentration determined using methods described in Section 3.5. was used to calculate the approximate cfu/ml values of the isolates at each time point from the OD_{600} values in the range of 0.1 to 0.9. The cfu/ml values were hence calculated, and estimated $\log_{10}$ cfu/ml vs. time curves were constructed. The early stationary phase was identified as the time point when the log cfu/ml vs. time curves flattened, which was when the culture reached ~ 8 log cfu/ml.

3.7. Collection of bacterial supernatants

3.7.1. Outbreak isolates and type strains

After incubation, the bacterial cultures were placed on ice to stop their growth. Cultures were then spun down at 16,000 g for 2 min, following which the supernatants were collected. They were preserved in 1 ml aliquots by storing at – 80 °C until further use (Carroll et al., 2019).

3.7.2. Clade IV isolates

The bacteria were grown in MOD medium as described in Section 3.4.2. up to the early stationary phase based on the time of harvest obtained in Section 3.6. OD_{600} values were measured, recorded, and converted to cfu/ml values from the standard curve obtained as per Section 3.5. to confirm that approximately 8 logs of cfu/ml were reached. Growth was quenched by placing the cultures on ice. Cultures were spun down at 11,900 g for 5 min at 4 °C. The decanted liquid was filtered using polyethersulfone 0.2 µm filters, following which the cell-free supernatants were collected and aliquoted. The aliquots were preserved by storing at – 80 °C until further use.

3.8. Qualitative detection of hemolysin BL and non-hemolytic enterotoxin using a lateral flow immunoassay

3.8.1. Outbreak isolates and type strains

The nine novel *B. cereus* type strains *B. albus* N35-10-2, *B. luti* TD41, *B. mobilis* 0711P9-1, *B. nitratireducens* 4049, *B. pacificus* EB422, *B. paramycoides* NH24A2, *B. paranthracis* Mn5, *B. proteolyticus* TD42, and *B. tropicus* N24, as well as of *B. mycoides* ATCC 21929 were tested for production of enterotoxins hemolysin BL (Hbl) and non-hemolytic enterotoxin (Nhe) by way of lateral flow immunoassay. The results for the rest of the type strains were previously published (Miller et al., 2018), and hence these isolates were not tested. Briefly, the cultures and immunoassay kits (Duopath® Cereus Enterotoxins, Millipore Sigma) were brought to room temperature. As per the manufacturer's instructions, 150 µl of the overnight cultures (Section 3.4.1.) were added to the immunoassay port. The results were read after incubation of the kits at room temperature for 30 min. A red line corresponding to Hbl-L_2 and NheB was read as positive ('+') for production of toxins Hbl-L_2 and NheB respectively, provided there was a red line corresponding to the control.

The latter ensured that the results from the kits were valid. The same procedure was followed for the detection of Hbl-L_2 and NheB for a subset of outbreak-associated isolates. Of the 33 outbreak isolates, the diarrheal isolates (FSL R9-6406, FSL R9-6413, and FSL R9-6410) and representatives of the emetic isolates (FSL R9-6381, FSL R9-6382, FSL R9-6384, FSL R9-6389, FSL R9-6395, and FSL R9-6399) were tested as well.

3.8.2. Clade IV isolates

The supernatants of the 15 *B. cereus* clade IV isolates were tested for production of enterotoxins Hbl and Nhe using immunoassay to ensure that the two enterotoxins were not lost during the filtration and subsequent freezing process. Briefly, the supernatants were thawed from – 80 °C, and they, along with the immunoassay kits (Duopath® Cereus Enterotoxins, Millipore Sigma), were brought to room temperature. 150 µl of the supernatants (cell-free supernatants that were obtained in Section 3.7.2.) was added to the sample port of the immunoassay kit, and the kits were incubated for 30 min at room temperature. Red bands corresponding to Hbl-L_2 and NheB were taken as positive for the production of toxins Hbl-L_2 and NheB respectively, when a red band corresponding to the control (C) was confirmed.

3.9. Maintenance and propagation of cell lines

HeLa and Caco-2 cells were grown in a cell culture medium [Eagle's Minimum Essential Medium (EMEM) containing 1.5 g/L sodium bicarbonate, non-essential amino acids, L-glutamine, and sodium pyruvate (Corning) supplemented with fetal bovine serum (FBS; VWR)] in T-75 vented flasks (Corning) at 37 °C and 5% CO_2. At 80% confluency, the cells were washed with PBS (Invitrogen) and passaged using 0.25% trypsin (Corning). After resuspending in fresh cell culture medium, the cells were spun down at 4000 rpm for 10 min. The medium was aspirated, fresh

medium was added, and the cell suspension after mixing was used to seed fresh T-75 flasks for propagating the cell line further and 96 well plates (Corning) for the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (water-soluble tetrazolium salt-1; WST-1) assay, and the lactate dehydrogenase (LDH) assays. For the HeLa cells, the percentage of FBS used was 10%. For the studies involving the clade IV isolates, additionally, the cell culture medium was supplemented with antibiotics streptomycin and penicillin at 100 µg/ml and 100 U/ml respectively (1 U equals 1667 mg sodium penicillin G; Alicino, 1946). For the Caco-2 cells, the cell culture medium composed of EMEM supplemented with 20% FBS along with 0.1 100 µg/ml streptomycin and 100 U/ml penicillin.

3.10. Preparation of cells for treatment with supernatant

3.10.1. HeLa cells

HeLa cells were seeded in 96-well plates at a density of 8 x 10^4 cells/cm^2 (Fisichella et al., 2009) in 100 µl of EMEM supplemented with 10% FBS. They were incubated at 37 °C and 5% CO_2 for 18-24 h to allow for attachment and growth.

3.10.2. Caco-2 cells

Caco-2 cells were seeded in 96 well plates at a density of 6.25 x 10^4 cells/cm^2 (Jeßberger et al., 2017) in 100 µl of EMEM supplemented with 20% FBS. For undifferentiated Caco-2 cells, the plates were incubated for 48 h before treatment. For assays using differentiated Caco-2 cells, the seeded Caco-2 cells were allowed to differentiate under incubation conditions of 37 °C and 5% CO_2 for 21 days. The medium was changed every 3-4 days.

3.11. Treatment of cells with supernatant

3.11.1. Outbreak isolates and type strains

The medium from each well of the 96 well plate containing HeLa cells was aspirated and replaced with fresh medium (EMEM with 10% FBS) containing 5% volume of volume (v/v) of the bacterial supernatants. The supernatants were thawed and pre-warmed to 37 °C before mixing with the cell culture medium. The medium with supernatants was added to the wells containing HeLa cells using a multichannel pipettor. Cell culture medium containing 5% BHI broth was used as negative control, while cell culture medium containing Triton X-100 (final concentration of 0.05%) was used as a positive (cytotoxicity) control. Being a detergent, Triton X-100 was expected to lyse the cells and reduce their viability. The plates with the cells treated with bacterial supernatants were incubated for 15 min under 37 °C, 5% CO_2 (Miller et al., 2018).

3.11.2. Clade IV isolates

The WST-1 assay was used to determine the optimal dose of supernatant for treating Caco-2 and HeLa cells with supernatants of clade IV isolates. Medium from each well of the 96 well plate containing undifferentiated Caco-2 cells and HeLa cells was aspirated and replaced with fresh medium (EMEM with FBS) containing 5%, 15%, 25%, and 50% v/v of the bacterial supernatants. The supernatants were thawed and pre-warmed to 37 °C before mixing with the cell culture medium. The medium with supernatants was added to the test wells containing cells using a multichannel pipettor. Cell culture medium containing EMEM and FBS was used as negative control, while cell culture medium containing Triton X-100 (final concentration of 0.05%) was used as positive control. Triton X-100 was expected to lyse the cells and reduce their viability. The effect of MOD medium was also observed at the different concentrations to control for the effect of the MOD medium on the viability of cells when treated with the bacterial supernatants. The

plates with the cells treated with bacterial supernatants were incubated for 15 min under 37 °C, 5% CO_2 (Miller et al., 2018) before the WST-1 dye was added.

The optimized conditions of 5% (v/v) supernatant for HeLa cells, and 15% (v/v) supernatant for Caco-2 cells were used further for characterizing the clade IV isolates on HeLa, and undifferentiated as well as differentiated cell lines respectively using WST-1 and LDH assays.

3.12. MTT assay

A single colony of a given *B. cereus* isolate was inoculated in 5 ml BHI broth and incubated overnight (18-20 h). The culture was centrifuged at 16,000 g for 2 min and supernatants were collected, aliquoted and stored in – 80 °C freezer until further use. HeLa cells seeded into 96 well plates were allowed to grow up to 80% confluency. One hour before intoxication of the HeLa cells using supernatants, the cell culture medium was replaced using 95 µl fresh EMEM containing 10% FBS. Next, 5 µl of the supernatants was added, and the plates gently shaken before placing back in the incubator set to 5% CO_2, 37 °C. After intoxication (variable times of intoxication; described in subsections below), the cells were washed with 100 µl of PBS, following which, 100 µl of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) dye working solution (0.01 g MTT dissolved in 8 ml EMEM with 10% FBS, and 2 ml PBS) was added to each well, i.e. each well was treated with 100 µg of MTT (Bruggisser et al., 2002). After incubation with the dye (variable times of incubation; described in subsections below), the dye solution was removed from each well, and dimethyl sulfoxide (DMSO; variable volumes; described in subsections below) was added to solubilize the formazan formed. The plates were shaken in a microplate shaker for 10 min at 600 rpm (VWR; catalog number 12620-926), followed by reading of the absorbances at 550 nm ($A_{550\ nm}$) (Bruggisser et al., 2002) using a microplate reader (Thermo Scientific Multiskan GO, Thermo Fisher Scientific).

3.12.1. Optimization of cell-free medium supernatant concentration and incubation time of HeLa cells with supernatant

Supernatants obtained from growing *B. cereus s.s.* ATCC 14579 were thawed from – 80 °C (without bringing the supernatants to 37 °C) and either used undiluted or diluted 2 x, 4 x, 8 x, and 16 x in BHI to treat HeLa cells at 5% v/v concentration of the cell culture medium. After incubation times of 15 min, 20 min, and 30 min with the toxin, the medium was removed, the cells were washed with PBS, and treated with MTT dye solution. After 25 min, 50 µl DMSO was added, the plate was shaken, and $A_{550\ nm}$ values were measured. Percent viability was calculated as ratio of $A_{550\ nm}$ values corresponding to the isolates (Bruggisser et al., 2002) to those of 5% v/v BHI (Miller et al., 2018).

Use of undiluted supernatants at 5% v/v of the cell culture medium, and a treatment time of 15 min of HeLa cells with *B. cereus* supernatants was found to be appropriate for further use. Using these conditions, eight isolates of *B. cereus s.l.* group (FSL R5-0184, *B. cereus s.s.* ATCC 14579, FSL K6-0069, FSL M8-0214, FSL W8-0003, FSL K6-0073, FSL J3-0113, and FSL M7-0109) were tested on two separate 96 well plates. Four test wells were used per isolate per plate.

3.12.2. Optimization of incubation time with the dye and volume of dimethyl sulfoxide for solubilization of the dye

B. cereus s.s. ATCC 14579 was previously shown to be highly cytotoxic (Miller et al., 2018). Using supernatant of *B. cereus s.s.* ATCC 14579, we attempted to identify optimal incubation time with the dye (15 min, 20 min, 25 min, 30 min, 35 min, and 40 min) and DMSO volume (50 µl, 60 µl, and 70 µl) so as to get the lowest viability of HeLa cells relative to 5% v/v BHI. This time, the supernatants were brought to 37 °C before use.

Using the optimized conditions (5% v/v undiluted supernatant, 15 min incubation with supernatant, 15 min incubation with dye, and 60 µl DMSO used for solubilizing the dye), isolates FSL R9-6381, FSL R9-6382, FSL R9-6384, FSL R9-6389, FSL R9-6395, FSL R9-6399, FSL R9-6406, FSL R9-6410, FSL R9-6413, and *B. cereus s.s.* ATCC 14579, as well as a cytotoxic environmental isolate UBC1 were tested in two independent replicates (two different plates).

3.13. WST-1 assay

According to the manufacturer's instructions, 10 µl of water-soluble tetrazolium salt-1 (WST-1; Roche) solution was added to each well of the 96 well plate with cells that had been incubated with supernatants as described in Section 3.11. After 30 s of shaking at 600 rpm in a microplate shaker (VWR), the absorbances were read by a microplate reader (Thermo Scientific Multiskan GO, Thermo Fisher Scientific). The absorbances were read in the precision mode at 450 nm ($A_{450\ nm}$) and 690 nm ($A_{690\ nm}$) (Fisichella et al., 2009). $A_{690\ nm}$ corresponding to the background signal was subtracted from $A_{450\ nm}$ corresponding to the signal from the formed colored product (i.e. formazan in the solution).

3.13.1. Optimization of dye incubation time

HeLa cells were treated with the supernatant from the type strain *B. cereus s.s.* ATCC 14579 at 5% v/v, after which the WST-1 dye was added with or without a 15 min incubation with the supernatant. After addition of the WST-1 dye, the plate was incubated for 15 min, 25 min, 35 min, 1 h, or 2 h at 37 °C, 5% CO_2. $A_{450\ nm}$ and $A_{690\ nm}$ values were recorded. Viability of the HeLa cells when treated with the *B. cereus* supernatant was calculated as the ratio of the corrected absorbances of the solution when HeLa cells were treated with the bacterial supernatant to that of cells treated with 5% v/v BHI medium and expressed as a percentage.

3.13.2. Outbreak isolates and type strains

After addition of the WST-1 dye, the plates were incubated for 25 min at 37 °C, 5% CO_2. Hence, the total exposure time of the HeLa cells to bacterial supernatants was 40 min (inclusive of 15 min pre-incubation of the HeLa cells with *B. cereus* supernatants; Section 3.11.1.). The percentage of viability of HeLa cells was calculated with respect to the negative control (5% BHI). Each test, including that using 0.05% Triton X-100, was conducted with 6 technical replicates per test on two different HeLa passages, resulting in 12 technical replicates per test. The supernatants from the isolates were from one biological replicate. The mean viabilities of 12 technical replicates were calculated for all tested isolates and the results were compared with those of 0.05% Triton X-100, supernatant of *B. cereus* type strain ATCC 14579, which served as the reference for diarrheal isolates, and supernatant of *B. cereus* strain DSM 4312, which served as the reference for emetic isolates.

3.13.3. Clade IV isolates

To assess whether 5% v/v of supernatants could be used for treating Caco-2 cells, as was done with HeLa cells, the undifferentiated Caco-2 cells were treated with 5% v/v supernatant, incubated for 15 min, and the WST-1 dye was added as described in Section 3.13.1. The incubation with the dye was done with the supernatant and the absorbance measured at 2.5 h (150 min), 3.25 h (195 min), and 3.5 h (210 min) total incubation times with the supernatant. Higher doses of supernatants were tested next. For studying the dose-response of supernatants of *B. cereus* clade IV isolates involving Caco-2 cells at higher doses (15%, 25%, and 50% v/v), after treatment and incubation with supernatants for 15 min as previously described, plates were incubated for 25 min, 1 h, 2 h, 2.5 h, 3 h, and 3.25 h more at 37 °C, 5% CO_2. Hence, the total exposure time of the Caco-2 cells with the bacterial supernatants were 40 min, 75 min, 135 min, 165 min, 195 min, and 210 min respectively.

The effect of the MOD medium on the cells was tested by calculating the viability of cells treated with MOD medium to that of untreated cells (cells incubated with cell culture medium). The viability of the isolates with respect to 15%, 25%, and 50% v/v MOD medium was calculated and the mean viabilities of 3 technical replicates per isolate was plotted. The supernatants from the isolates were from one biological replicate. The calculation of viability of cells with respect to the MOD medium was done to account for the effect of the MOD medium against cells. The optimal dose-time combination for undifferentiated Caco-2 cells was found to be 15% (v/v) of supernatant, with a total incubation time of 135 min with the bacterial supernatant (inclusive of 15 min pre-incubation of the Caco-2 cells with *B. cereus* supernatants; Section 3.11.1.). These conditions were used for testing a second plate of undifferentiated Caco-2 cells with 3 technical replicates of each isolate in the plate, resulting in a total of 6 technical replicates for undifferentiated Caco-2 cells at exposure to 15% (v/v) supernatant and total incubation time of 135 min.

The optimized conditions obtained for undifferentiated Caco-2 cells were used for differentiated Caco-2 cells as well, to enable effective comparison between the results of the two states of Caco-2 cells. Hence, for the differentiated Caco-2 cells, 10 µl of WST-1 reagent was added to each well after treatment with 15% (v/v) supernatant for 15 min at 37 °C, 5% CO_2, and incubated for 2 h, resulting in a total incubation time of 135 min of the cells with the bacterial supernatant. This was done for all clade IV isolates on two independent plates.

For the clade IV isolates, the optimal dose for HeLa cells was found to be 5% (v/v) of supernatant, with a total incubation time of 40 min with the bacterial supernatant (inclusive of 15 min pre-incubation of the HeLa cells with *B. cereus* supernatants). These conditions were used for running a second plate of HeLa cells with 3 technical replicates of each isolate in the plate, resulting in a total of 6 technical replicates for HeLa cells at incubation conditions of 5% (v/v) supernatant and total incubation time of 40 min. The dose-response graph for HeLa cells was also plotted using the mean viability of cells calculated with respect to MOD medium in order to account for the effect

of the MOD medium towards cells. The percentage viability of HeLa cells was calculated with respect to the MOD medium. Each test, including that using 0.05% Triton X-100, was conducted with 3 technical replicates per test on two different passages of cells, resulting in 6 technical replicates per test. The supernatants from the isolates were from one biological replicate. The mean viabilities of 6 technical replicates were calculated for all tested isolates and the results were compared with those of 0.05% Triton X-100 and MOD medium.

3.14. LDH assay

HeLa cells and Caco-2 cells were treated with supernatants according to the optimized conditions as previously described for the WST-1 assay. Hence, the conditions for HeLa cells were a 40 min total incubation time of the cells with 5% v/v supernatants of *B. cereus* clade IV isolates. For Caco-2 (both, undifferentiated and differentiated) cells, the conditions were a 135 min total incubation time of the cells with 15% v/v supernatants. At the end of the incubation period, 50 µl of the contents of the sample wells (spent medium of the cells) was carefully transferred to wells of an empty 96 well plate (Corning). 100 µl of LDH assay reagent [prepared by adding equal volumes of 204 mM lithium lactate, 200 mM Tris pH 8.0, and iodonitrotetrazolium (INT)/phenazine methosulfate (PMS)/nicotinamide-adenine dinucleotide (NAD) (solution containing 100 µl of 33 mg/ml INT solution, 100 µl of 9 mg/ml PMS, and 2.3 ml of 86 mg of 3.74 mg/ml NAD)] was added to each of the wells containing the spent medium of the cells. The reaction was incubated in the dark for 15 min (Kumar et al., 2018), and the absorbances corresponding to the colored product (signal) at 490 nm ($A_{490\ nm}$) and that of the background ($A_{690\ nm}$) were measured. While literature typically does not use the background subtraction for LDH assay, it was necessary to carry this out in our case, as the cell debris carried over from detached cells could alter the $A_{490\ nm}$ values (Jan and Lashuel, 2012; Kumar et al., 2018). The ratio of corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) with

respect to the MOD medium was calculated for each isolate. The supernatants from the isolates were from one biological replicate. The mean ratios obtained for 6 technical replicates were calculated for all tested isolates. The higher the ratio, the higher the LDH release from membrane-damaged cells, and hence, higher is the degree of pore formation of the isolate's supernatant in the cell membrane of the cells. To test the effect of the MOD medium on the cells, the ratio of corrected absorbances ($A_{490\,nm} - A_{690\,nm}$) of solution obtained from treating MOD medium to that of untreated cells (cells incubated with cell culture medium) was calculated.

Table 3-1 shows a summary of the isolates characterized using cytotoxicity assays and lateral flow immunoassay.

3.15. Finding the virulence gene profiles

BTyper version 2.3.0, an open-source *B. cereus* group subtyping tool for the detection of virulence genes using whole genome sequencing data (Carroll et al., 2017), was used to identify the virulence gene profile of the clade IV isolates.

3.16. Protein estimation by Bradford assay using standard curve

Solutions of bovine serum albumin (BSA) with the concentrations 1.25, 2.5, 5, 10, and 20 µg/ml were prepared by diluting a BSA solution of concentration 1 mg/ml in water. To 1 ml of the solutions, 1 ml of Bradford reagent (Sigma) was added, and the absorbance values recorded at 595 nm ($A_{595\ nm}$) after subtracting from the blank after an incubation period of 5 min, as per the manufacturer's instructions. The average absorbances were used to plot $A_{595\ nm}$ versus the concentration of protein with respect to BSA solution (µg/ml). A line of best fit was constructed for the linear graph, and the equation was used to calculate protein content of the *B. cereus* supernatants according to the $A_{595\,nm}$ values obtained.

3.17. Statistical analyses

The statistical analyses were performed in R version 3.5.2 (R Core Team, 2018). The R codes for all the analyses are provided in Appendix B. During the optimization of the MTT and WST-1 assays, for testing whether percentage viability of isolates obtained in two independent plates were statistically different or not, paired Student's t-tests were performed. For this, the function "t.test" under the package {stats} was utilized. In order to check for the non-homogeneity of variances of percentage viabilities (WST-1 assay) or absorbance ratios (LDH assay) across the different isolates/treatments, the Bartlett's test (function "bartlett.test" under the package {stats}) was utilized.

When the data had non-homogenous variances across the isolates and more than 6 number of replicates per isolate, the Welch's test and the Games-Howell post-hoc test (with Bonferroni correction to account for error due to multiple comparisons) were employed. The analyses were carried out on the results of all technical replicates of the isolates tested. The Welch test was performed using the function "welch.test" under the package {onewaytests}, while the Games-Howell test was performed using the function "posthocTGH" under the package {userfriendlyscience} in R. When the data had ≤ 6 number of replicates, even if the variances were non-homogenous variances across the isolates as assessed by the Bartlett's test, one-way ANOVA (using "lm" and "aov" functions in {stats} package) with Tukey post-hoc test (using "HSD.test" under {agricolae}) was used. This is because the Games-Howell test is inappropriate for small sample sizes and can be too liberal (Lee and Lee, 2018; Shingala and Rajyaguru, 2015). We found that the Games-Howell test, when used with a samples size of 6 replicates per isolate, gave illogical trends (for example, a cytotoxicity of 14.5 ± 2.1 was shown to be statistically different ($P < 0.05$)

from a cytotoxicity of 106.1 ± 10.4, but not statistically different from a cytotoxicity of 108.8 ± 20.6 when Games-Howell method was used).

For testing correlations between samples, Pearson's product moment correlation coefficients were calculated using the function "cor.test" under {stats}. To plot the correlation matrix, the functions "cor" and "corrplot" from packages {stats} and {corrplot} respectively, were utilized.

3.18. References

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Chapter 4.

Results and Discussion

4.1. MTT assay optimization for HeLa cells

Due to rapid growth rate and ease of handling, HeLa human cervical cancer cells were used in cytotoxicity assays of *B. cereus sensu lato* (*s.l.*) toxins by Miller et al. (2018). In these studies, cytotoxicity was determined by measuring the influx of propidium iodide (PI) relative to 4′,6-diamidino-2-phenylindole (DAPI) into HeLa cells exposed to cell-free medium supernatants [5 % volume/volume (v/v)]. The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay is widely used for assessing cell viability, and is based on the conversion of the MTT dye to insoluble formazan crystals by living cells, a phenomenon that is correlated to mitochondrial activity (van Meerloo et al., 2011). The MTT assay has not been used to assess toxicity of *Bacillus cereus* supernatants on the HeLa cell line. We therefore attempted to find the appropriate MTT assay conditions with HeLa cells in order to get reproducible results between two plates of cells.

4.1.1. Optimization of the cell-free medium supernatant concentration and incubation time of HeLa cells with supernatant

We evaluated the supernatant concentration (% v/v) and incubation time-dependence of *B. cereus sensu stricto* (*s.s.*) type strain ATCC 14579 cytotoxicity against HeLa cells using the MTT assay. A 15-min treatment of HeLa cells with 5% v/v *B. cereus s.s.* ATCC 14579 was found to be sufficient for reducing the metabolic activity of the HeLa cells (Fig. 4-1). At this supernatant concentration, the HeLa cells appeared to be ruptured, as observed under the microscope.

Additionally, we selected the undiluted supernatant over diluted supernatants because the trend observed at different incubation times was as expected (i.e., shortest incubation resulted in least inhibition in viability of HeLa cells). Based on these results and the published literature for treatment of HeLa cells with *B. cereus* supernatants (Miller et al., 2018), we used the conditions of 5% v/v undiluted supernatant of *B. cereus* isolates with 15 min treatment time of HeLa cells with supernatant for further assays. It is interesting to note here that, at 4 x and 8 x dilution of supernatants with brain heart infusion medium (BHI), the viability was less at 20 minutes incubation, compared to 30 minutes incubation with the supernatant. The possible explanation for this could be cell detachment, addressed later in the section.

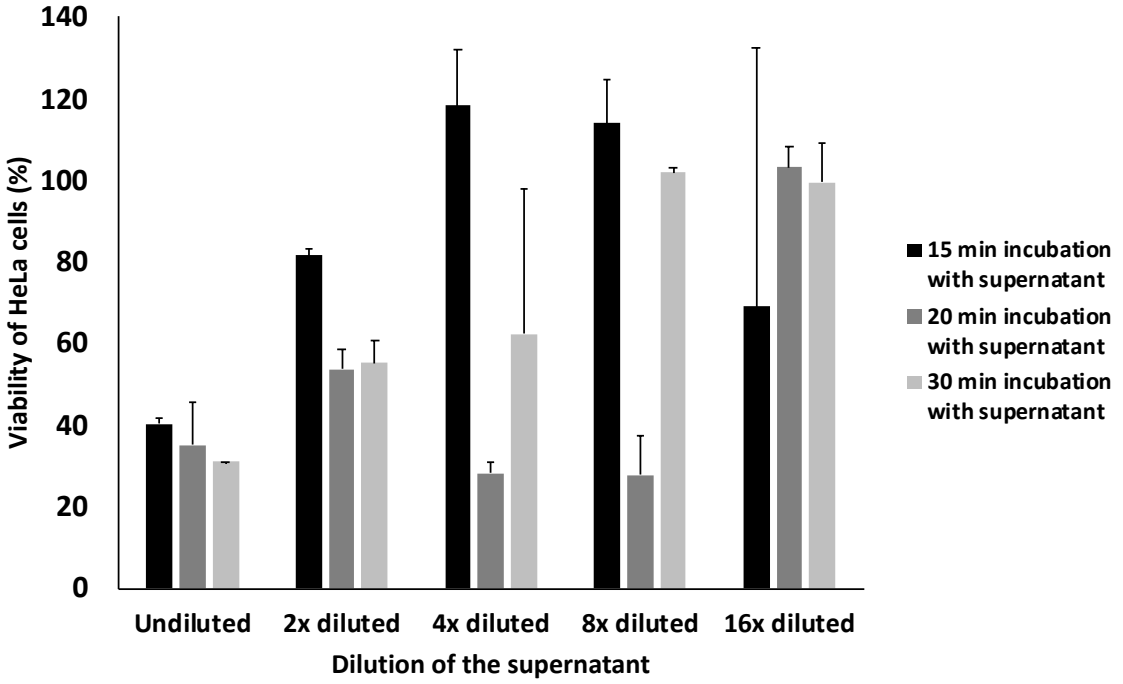

Figure **4-1**: Effect of dilution of 5% v/v *B. cereus s.s.* ATCC 14579 supernatant on the viability of HeLa cells with respect to 5% v/v BHI medium, as determined using MTT assay. Viability of cells was calculated by taking the ratio of absorbance at 550 nm obtained for HeLa cells treated with *B. cereus s.s.* ATCC 14579 supernatants of varying dilutions (undiluted, 2 x diluted, 4 x diluted, 8 x diluted, and 16 x diluted) at 5% v/v level to that of 5% v/v BHI medium and converting

to percentages. Columns show the mean of two technical replicates and the bars show the standard deviation.

To assess if the assay worked using a broader set of *B. cereus* isolates, we repeated the MTT assay on two separate 96 well microtiter plates (Fig. 4-2) using undiluted supernatants of 8 *B. cereus s.l.* group isolates at 5% v/v level and 15-minute incubation with HeLa cells. The results (Fig. 4-2) indicated that for 5 out of 8 of isolates tested, there was a statistically-significant difference between the results from the two plates ($P < 0.05$). Moreover, the differences between the means of the viabilities from the two plates for these 5 isolates was large (49-72% difference in mean viabilites between plates). Further investigation of the cause of these differences led to observations of assay wells under the microscope, which revealed a variable and considerably large detachment of cells in plate 2 for most of the isolates, compared to plate 1, where cell detachment was observed in fewer wells.

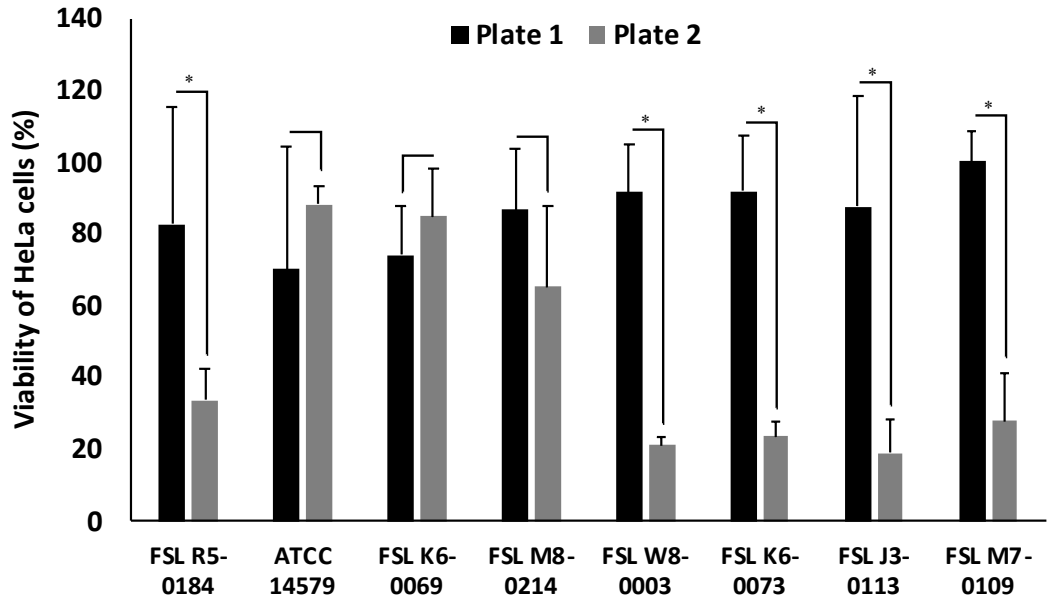

Figure **4-2**: Comparison of viabilities determined using MTT assay of HeLa cells with respect to 5% v/v BHI medium from two different plates when treated with 5% v/v supernatants of 7 *B. cereus*

group isolates and *B. cereus s.s.* ATCC 14579. Viability of cells was calculated by taking the ratio of absorbance at 550 nm obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. Columns show the mean of 4 replicates for a particular plate, while error bars show the standard deviation. Results are shown from two separate plates. A '*' indicates that there is significant ($P < 0.05$) difference between the results of the two plates for a particular isolate, as determined using paired t tests.

We hypothesized that the observed differences in cell detachment across wells and plates were due to temperature differences in the supernatants applied to the HeLa cells due to the supernatants not being applied at a uniform temperature for both plates, which could be causing stress to the HeLa cells even at 5% v/v levels. Hence, in the next experiments, we thawed and pre-warmed the supernatants to 37 °C before treating the cells. The hypothesis was in congruence with the Repetto et al. (2008) recommendation to pre-warm the neutral red reagent to cell culture temperature before use on the cells.

4.1.2. Optimization of the incubation time with dye and volume of dimethyl sulfoxide for solubilization of the dye

Further, we tested the effect of incubation time with the dye (15-40 min) and volume of dimethyl sulfoxide (DMSO) used to solubilize the dye (50, 60, and 70 µl) on the results of the MTT assay using *B. cereus s.s.* ATCC 14579 as the test strain (Fig. 4-3). This was done to arrive at a condition that would give the lowest viability of HeLa cells when treated with supernatant of *B. cereus s.s.* ATCC 14579 (highly cytotoxic isolate), so that isolates with lower cytotoxicity relative to *B. cereus s.s.* ATCC 14579 would be better differentiated. As shown in Fig. 4-3, we chose an incubation time of 15 min with the dye to be used further, with 60 µl of DMSO for solubilization of the formazan formed, because this set of combined conditions gave the lowest absorbance of solution, and hence lowest calculated viability of HeLa cells.

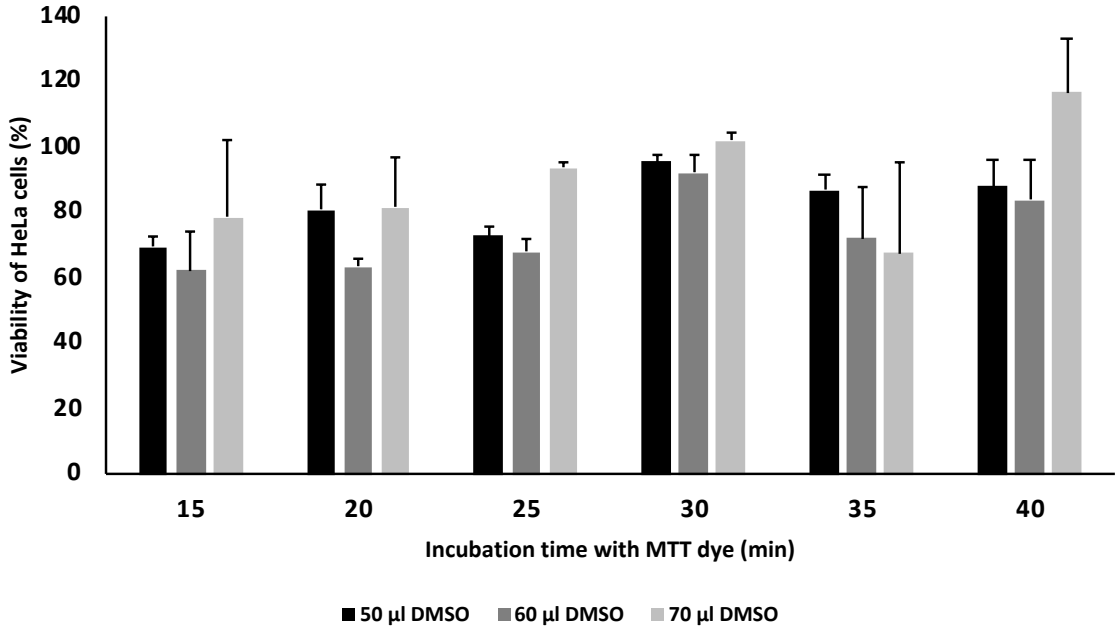

Figure **4-3**: Effect of incubation time of MTT dye with HeLa cells, and the volume of DMSO used to solubilize the formed dye on the viability of HeLa cells with respect to 5% v/v BHI medium when treated with 5% v/v supernatants of *B. cereus s.s.* ATCC 14579, as determined using MTT assay. Viability of cells was calculated by taking the ratio of absorbance at 550 nm obtained for HeLa cells treated with *B. cereus s.s.* ATCC 14579 supernatant at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. Columns show the mean of 2 technical replicates (single plate), while error bars show the standard deviation.

Under these conditions (15 min incubation with 5% v/v supernatant, 15 min incubation with the dye, 60 µl DMSO used for solubilization), we tested 9 outbreak-associated isolates, and an environmental isolate UBC1, as well as the *B. cereus* type strain ATCC 14579 by the MTT assay on two 96 well plates (Fig. 4-4) in order to assess whether the assay produced reproducible results. From Fig. 4-4, it was observed that the two plates yielded statistically-significantly different results for replicate tests for 8 out of 11 isolates tested ($P < 0.05$). Out of these 8 isolates, however, only 2 isolates (FSL R9-6381 and FSL R9-6389) gave a large difference in mean viabilities (71-85%) between two plates (Fig. 4-4), indicating that warming bacterial supernatants to 37 °C before treating cells resulted in lesser degree of detachment of cells compared to when bacterial supernatants were not warmed to 37 °C (Fig. 4-2). This was also confirmed from microscopic

examination of cells. Hence, in all subsequent experiments, we pre-warmed bacterial supernatants to 37 °C before treating cells.

However, the variability in results from two plates of cells (Fig. 4-4) could not be explained solely by temperature of supernatant used. Under the microscope, we noticed that for UBC1 isolate, which is highly toxic to the cells, there were differences in detachment of cells among wells and across plates after performing the MTT assay even when the supernatant was pre-warmed to 37 °C before use. The same was observed for the *B. cereus s.s.* ATCC 14579. According to a review by Cueppens et al. (2013), in addition to pore-forming toxins such as hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin K (CytK) which may contribute to cytotoxicity, *B. cereus* also produces other factors like enterotoxin EntFM, hemolysins, phospholipases C, and other degradative enzymes that are not directly cytotoxic, but they may contribute to the cytotoxicity mechanism through detachment of the host's epithelial cells, damage to microvilli, a decrease in metabolic activity of cells and possibly other mechanisms (Ceuppens et al., 2013). These observations suggested that the detached cells were being removed during the washing step before application of the dye solution, and during replacement of MTT dye solution with DMSO (used for solubilization of formed formazan dye). This was hypothesized to be a probable issue when attempting to measure the viability based on the metabolic activity of the cells. The MTT assay measures the amount of the dye converted by viable cells, hence the detachment and loss of cells leads to reduction of the signal that may be incorrectly interpreted as reduced cell viability.

This led us to the conclusion that the MTT assay is not an appropriate method for assessing the cytotoxic effects of *B. cereus* supernatants on HeLa cells. Additionally, to the best of our knowledge, there is no report in the literature on the use of MTT assay for cytotoxicity assessment of *B. cereus* supernatants on HeLa cells, although researchers have used the MTT assay to assess cytotoxicity of *B. cereus* supernatants in other cell lines such as Chinese Hamster Ovary (CHO;

Ngamwongsatit et al., 2008) and HEp-2 human laryngeal carcinoma cells (for assessing toxicity of cereulide; Finlay et al., 1999).

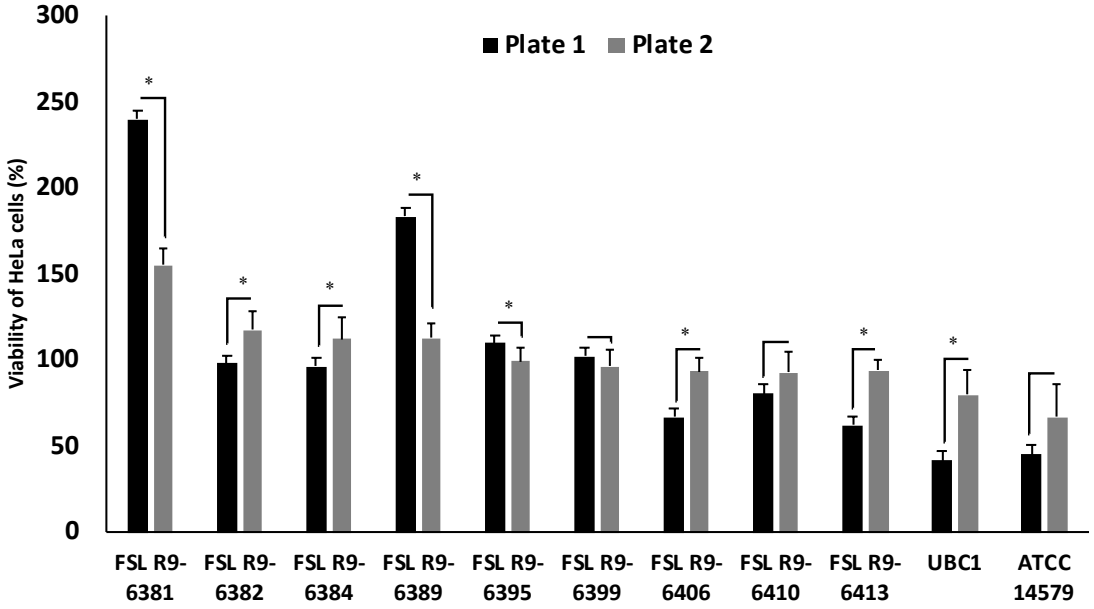

Figure **4-4**: Comparison of viabilities determined using MTT assay of HeLa cells with respect to 5% v/v BHI medium from two different plates when treated with 5% v/v supernatants of 9 outbreak-associated isolates, an environmental isolate UBC1, and the *B. cereus s.s.* type strain ATCC 14579 of the *B. cereus* group. Viability of cells was calculated by taking the ratio of absorbance at 550 nm obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. Columns show the mean of 8 replicates for a particular plate, while error bars show the standard deviation. Results are shown from two separate plates. A '*' indicates that there is significant ($P < 0.05$) difference between the results of the two plates for a particular isolate, as determined using paired t tests.

4.2. WST-1 assay optimization for HeLa cells

Since the MTT assay was found to be unsuitable for assessing the cytotoxicity of *B. cereus* cell-free medium supernatant in HeLa cells, we next tried to optimize the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (water-soluble tetrazolium salt-1; WST-1) assay which has been suggested as a substitute for MTT assay for rapid cytotoxicity detection of

Bacillus spp. using CHO cell lines (Ngamwongsatit et al. 2008). One of the main benefits mentioned was that the WST-1 dye is more rapidly metabolized (incubation time of cells with dye being 2 h) compared to the MTT dye (incubation time of cells with dye being 4 h). In addition, the solubilization step of end product with DMSO required of the MTT assay is eliminated with the WST-1 assay, as the latter assay yields a water-soluble colored formazan as the end product (Ngamwongsatit et al., 2008). As shown in our MTT assay optimization experiments in Section 4.1., a workflow that does not neccessitate reagent removal would be less likely to lead to cell detachment. This supposition is supported by the work of Ngamwongsatit et al. (2008), who found the WST-1 assay to have higher reproducibility as compared to the MTT assay. Based on these findings and the fact that a few other studies have used this assay for cytotoxicity of *Bacillus* spp. (Blanch et al., 2014; Jeßberger et al., 2014), we proceeded to optimize the WST-1 assay with HeLa cells.

4.2.1. Optimization of the dye incubation time

In order to optimize the incubation time using a single isolate, we first treated HeLa cells with 5% v/v *B. cereus s.s.* ATCC 14579 supernatant and incubated for 15 min, before adding the WST-1 dye (black columns in Fig. 4-5). In an additional set of wells, we added the WST-1 dye simultaneously with the 5% v/v *B. cereus s.s.* ATCC 14579 supernatant and incubated them together (gray columns in Fig 4-5). We chose 5% v/v as the concentration of supernatant to be used for treating HeLa cells, as it was sufficient to rupture HeLa cells when treated with supernatant of *B. cereus s.s.* ATCC 14579, as observed under the microscope. We measured the absorbance values at 450 nm ($A_{450\,nm}$) and at 690 nm ($A_{690\,nm}$) at 15, 25, 35, 60, and 120 min post addition of the WST-1 dye. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\,nm} - A_{690\,nm}$) obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. $A_{450\,nm}$ measures the signal from the colored product formed, while

$A_{690\ nm}$ is measured to account for the background signal from cell debris in the wells of the plate (Fisichella et al., 2009).

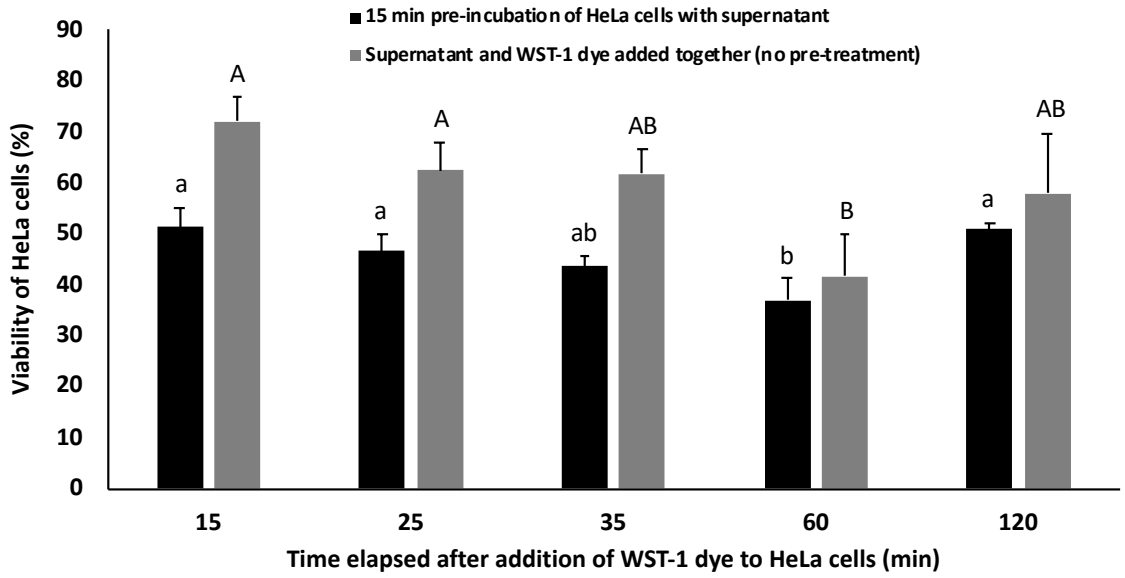

Figure **4-5**: Effect of pre-incubation of HeLa cells with 5% v/v *B. cereus s.s.* ATCC 14579 supernatant and incubation time with WST-1 dye on the viability of HeLa cells with respect to 5% v/v BHI medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. Columns show the mean of 3 replicates of one plate. Any two columns that do not share a common lowercase alphabetic character had significantly different percentage viability values ($P < 0.05$) for HeLa cells pre-incubated with *B. cereus* supernatant, as determined by Tukey HSD method. Any two columns that do not share a common uppercase alphabetic character had significantly different percentage viability values ($P < 0.05$) for HeLa cells without *B. cereus* supernatant pre-treatment, as determined by Tukey HSD method.

Fig. 4-6 shows the increase in absorbance ($A_{450\ nm} - A_{690\ nm}$) values over time for the HeLa cells treated with supernatants in comparison with cells incubated with BHI medium. When cells were incubated with the dye for 60 min and 120 min, the resultant absorbance values for the negative control (5% v/v BHI) exceeded 2, which exceeded the linear range of the spectrophotometer. Hence, we considered the 60 min and 120 min time points as inappropriate incubation times for further use. The rate of dye development slowed down when cells were

incubated with supernatants compared to that of BHI, which meant the supernatant decreased the ability of the cells to convert the dye. As time progressed, the gap in the absorbances corresponding to BHI-treated HeLa cells and supernatant-treated cells increased, with the gap larger when cells were pre-incubated with supernatant for 15 min (Fig. 4-6).

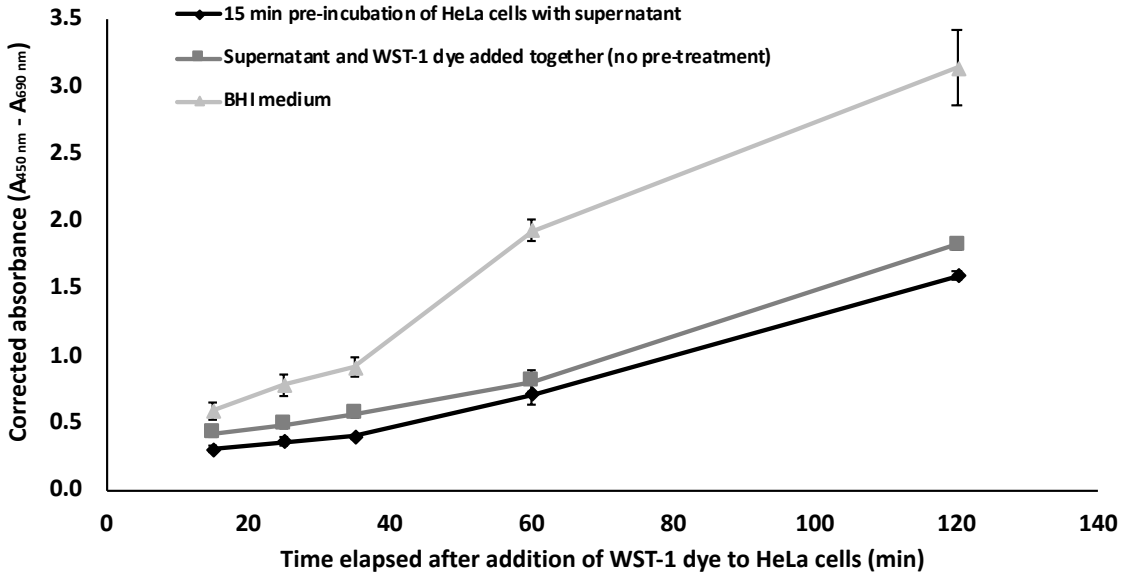

Figure **4-6**: Corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) of solution obtained for HeLa cells treated with supernatants and BHI medium at 5% v/v concentration as a function of incubation time with dye, as determined by the WST-1 assay. Data points show the mean absorbance of 3 replicates from a single plate, while error bars show the standard deviation.

Co-incubating the HeLa cells with the dye without pre-treatment with *B. cereus* cell-free medium supernatant resulted in around 62% viability (Fig. 4-5). We thought that this decrease was too small to facilitate comparison with less cytotoxic *B. cereus*. For this reason, we pre-incubated the cells with the supernatants or the Triton X-100 (positive control) for 15 min prior to the addition of the dye in subsequent experiments. The mean viabilities of HeLa cells pre-incubated with supernatants for 15 min, and then incubated with the WST-1 dye for an additional 15 min, 25 min, and 35 min were 51%, 46%, and 43%, respectively. Because the mean viability of cells became

near constant after 25 min of incubation with the dye, we chose 25 min as the time for incubation with WST-1 dye for further experiments. This meant, including the 15 min pre-incubation with the supernatant, the total incubation time with cell-free medium supernatant was 40 min.

4.2.2. Application of the assay on foodborne outbreak isolates

In order to apply and further assess the performance of the assay described above, the cytotoxicity of supernatants from 33 outbreak associated isolates along with the emetic reference isolate DSM 4312 and the *B. cereus s.s.* type strain ATCC 14579 was assessed using HeLa cells and WST-1 assay. When we tested the supernatants of isolates associated with the 2016 foodborne outbreak of *B. cereus* on HeLa cells in two different plates, we found that the results for 10 isolates from both plates statistically different, and for the rest 23 isolates, the results were not statistically different between plates (Fig. 4-7; $P < 0.05$). Hence, a lesser proportion of isolates gave significantly different viabilities between plates (Fig. 4-7) compared to the MTT assay previously used (Fig. 4-4). The results obtained here also corroborated the fact that these outbreak-associated isolates were genetically clonal (Carroll et al., 2019). Hence, this method was considered suitable for further use. The conditions used in the WST-1 assay for HeLa cells are summarized in Table 4-1. The seeding density of cells (8×10^4 cells/cm^2) is as per Fisichella et al. (2009). Supernatants are to be applied at to HeLa cells at a concentration of 5% v/v of the cell culture medium (Eagle's minimum essential medium (EMEM) supplemented with 10% fetal bovine serum (FBS)), and the HeLa cells are to be incubated for 15 min, followed by the addition of 10 µl of the WST-1 dye. The cells are to be incubated for an additional 25 min for the dye development process, following which the $A_{450\ nm}$ and $A_{690\ nm}$ values (Fisichella et al., 2009) are to be recorded.

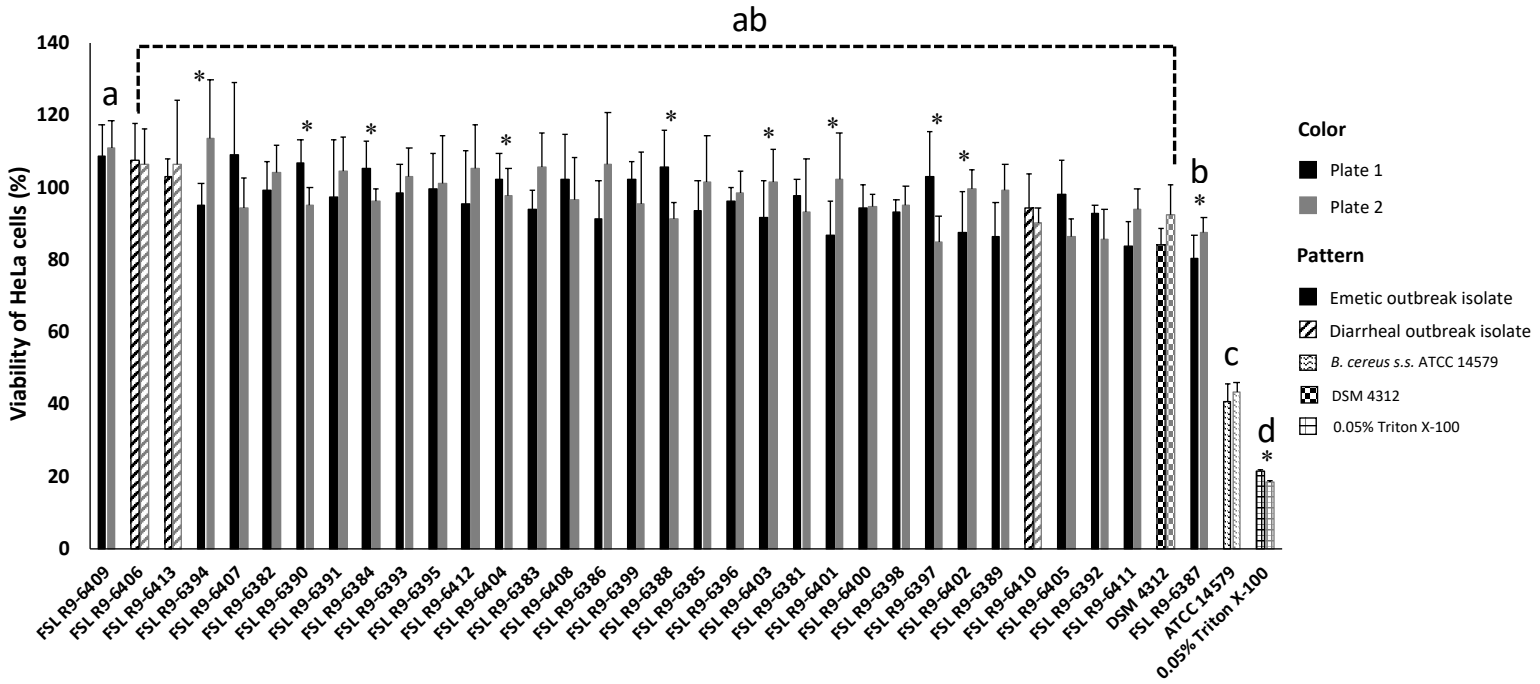

Figure **4-7**: Effect of 5% v/v supernatants of 33 outbreak associated isolates, the emetic reference isolate DSM 4312, and the *B. cereus s.s.* type strain ATCC 14579 on the mean viability of HeLa cells with respect to 5% BHI medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean of 6 replicates for a particular plate, while error bars show the standard deviation. Results are shown from two separate plates. A '*' indicates that there is significant ($P < 0.05$) difference between the results of the two plates for a particular isolate, as determined using paired t tests. Any two treatments that do not share a common alphabetic character had significantly different mean (of 12 replicates, 6 each from 2 plates for each isolate) percentage viability values ($P < 0.05$), as determined by Games-Howell method. Figure adapted from Carroll et al., 2019.

Table **4-1**: Conditions used in the WST-1 assay for HeLa cells

Parameter	Value
Seeding density of cells [(a)]	8 x 10^4 cells/cm^2
Concentration of cell-free *B. cereus* medium supernatant	5% v/v in cell culture medium [(b)]
Pre-incubation time of cells with only *B. cereus* supernatant	15 min
Volume of WST-1 dye added [(c)]	10 µl
Time of incubation of cells with WST-1 dye with the supernatant also present	25 min
Wavelength for measurement of signal [(c)]	450 nm
Wavelength for measurement of background [(c)]	690 nm

[(a)] Conditions taken from Fisichella et al., 2009.
[(b)] EMEM with 10% FBS.
[(c)] Conditions taken from manufacturer's (Roche) instructions.

In the study of outbreak-associated isolates, any isolate that carried *ces* genes encoding the cereulide biosynthetic pathway were designated as emetic isolates, while isolates that lacked *ces* genes but carried either *hbl* or *cytK-2* genes that encode diarrheal enterotoxins were classified as diarrheal isolates (Carroll et al., 2019). The mean viability of HeLa cells when treated with supernatants of the three diarrheal isolates (FSL R9-6406, FSL R9-6410, and FSL R9-6413) was found to be 101.4 ± 2.3% relative to the BHI control, while that of the remaining 33 emetic isolates (clonal, based on the whole genome sequence analyses; Carroll et al., 2019) was found to be 97.5 ± 1.5% with respect to the bacterial growth medium, BHI. We found that the data had non-homogenous variances, as evaluated by the Bartlett test ($P > 0.05$), and hence resorted to using the Welch's test and Games-Howell posthoc test (appropriate for use on data with non-homogenous variances) for statistical grouping of data (to assess differences between isolates). Among all pairs of the emetic outbreak-associated isolates, only the mean viabilities of HeLa cells exposed to the supernatants of emetic isolates FSL R9-6409 and FSL R9- 6387 were found to be different (Games-Howell $P < 0.05$; Fig. 4-7), which could be due to well-to-well variability in the metabolic activity of cells. The rationale behind this conclusion was that all the emetic isolates were in fact, clonal (Carroll et al., 2019). Amongst all the strains tested, the *B. cereus s.s.* type strain ATCC 14579 was found to be most cytotoxic towards HeLa cells, with a 42.1 ± 0.8% mean viability of HeLa cells post treatment. This was an effect of approximately two times less compared to the maximum kill achieved by the treatment with 0.05% Triton X-100, which resulted in a mean viability of 20.2 ± 0.9% (Fig. 4-7).

After the completion of this study, we realized that the workflow we used for treating HeLa cells with *B. cereus* supernatants may not be appropriate for assessment of the cytotoxic effects of supernatants of emetic strains, since the emetic toxin cereulide is hydrophobic and likely binds with the cell membranes of bacteria that were separated from supernatants during centrifugation (Ramarao et al., 2015). This could explain why we did not observe cytotoxic effects of emetic

strains' supernatants on HeLa cells. In the future work, we therefore recommend assessing the cytotoxic effects using both bacterial supernatants as well as crude lysates of boiled cells, which is expected to facilitate the release of the membrane-bound heat-stable cereulide without compromising its activity. Alternatively, methanolic extraction of cereulide can also be performed (Andersson et al., 2007; Rajkovic et al., 2008) before testing on cells.

4.3. Detection of NheB and Hbl-L_2 in supernatants of outbreak-associated isolates by immunoassay

Since all the emetic outbreak-associated isolates were found to be clonal as determined by the whole genome sequence analyses (Carroll et al., 2019), we chose only six representatives (FSL R9-6381, FSL R9-6382, FSL R9-6384, FSL R9-6389, FSL R9-6395, and FSL R9-6399) for carrying out the immunoassay detection of Hbl-L_2 and NheB toxins. In addition, we tested all three diarrheal outbreak-associated isolates (FSL R9-6406, FSL R9-6410, and FSL R9-6413), as well as the *B. cereus s.s.* type strain ATCC 14579 for the production of NheB and Hbl-L_2 components (Table 4.2.). We detected the toxin components in the culture broth of bacteria grown overnight in BHI medium using Duopath® Cereus Enterotoxins (Merck) kits. The results, summarized in Table 4-2, revealed that the six representatives of the emetic isolates tested were found to produce NheB, but not Hbl-L_2. All three diarrheal strains that had been isolated during the investigation of the outbreak tested positive for the production of both Hbl-L_2 and NheB. This was also true for the *B. cereus s.s.* type strain ATCC 14579. It should be noted that this test is qualitative in nature, with detection limits of 6 ng/ml for NheB, and 20 ng/ml for Hbl-L_2 (Krause et al., 2010).

The HeLa cells showed varied viabilities on treatment with the outbreak-associated diarrheal isolates in comparison with that of the *B. cereus s.s.* type strain ATCC 14579 despite all four producing both Hbl and Nhe. This could be because of differences in the quantities of Nhe and

Hbl toxins produced by the bacteria, which the immunoassay assay-based detection method was unable to distinguish. The observation that qualitative detection of Hbl (Christiansson et al., 1989; Miller et al., 2018) and Nhe (Miller et al., 2018) is not necessarily correlated with HeLa cell cytotoxicity has been previously reported. For example, Hbl-L_2 was not detected in *B. pseudomycoides*, *B. mycoides*/*B. weihenstephanensis*, and *B. cytotoxicus* isolates, while the distribution of NheB detection was more abundant (38 isolates positive of 52 tested by Miller et al., 2018). However, *B. pseudomycoides* strains were found to be highly toxic against HeLa cells as assayed by PI/DAPI staining. Similarly, *B. cytotoxicus* NVH 391-98 was negative for both Hbl-L_2 and NheB, but showed cytotoxicity against HeLa cells (Miller et al., 2018).

Table **4-2**: Detection of NheB and Hbl-L_2 in cultures of outbreak-associated isolates and *B. cereus s.s.* type strain ATCC 14579 grown in BHI broth at 37 °C using the Duopath immunoassay kit

Isolate name	Type of isolate	NheB detection	Hbl-L_2 detection
FSL R9-6381	Emetic	+	-
FSL R9-6382	Emetic	+	-
FSL R9-6384	Emetic	+	-
FSL R9-6389	Emetic	+	-
FSL R9-6395	Emetic	+	-
FSL R9-6399	Emetic	+	-
FSL R9-6406	Diarrheal	+	+
FSL R9-6410	Diarrheal	+	+
FSL R9-6413	Diarrheal	+	+
B. cereus s.s. ATCC 14579	Type strain	+	+

'+' denotes that the *B. cereus* isolate was positive for a given virulence factor, while '-' denotes that the virulence factor was not detected.

4.4. Application of the WST-1 assay to test toxicity of *B. cereus* group type strains

We further tested supernatants from 17 type strains of the *B. cereus* group on HeLa cells using the WST-1 assay. This list also includes 8 type strains tested earlier (*B. cereus s.s.* ATCC 14579, *B. pseudomycoides* DSM 12442, *B. wiedmannii* FSL W8-0169, *B. toyonensis* BCT-7112, *B. cytotoxicus* NVH 391-98, *B. thuringiensis* ATCC 10792, *B. mycoides* DSM 2048, and *B. weihenstephanensis* WSBC 10204) by Miller et al. (2018). The objective was to compare the toxicity spectrum obtained here with the earlier study using HeLa cells and PI assay (Miller et al., 2018). The results of the WST-1 assay (Fig. 4-8) indicated that the *B. pseudomycoides* DSM 12442 type strain is the most cytotoxic towards HeLa cells, followed by the *B. cereus s.s.* type strain ATCC 14579 (Games-Howell $P < 0.05$), which is in agreement with Miller et al. (2018). Toxicity of the other strains was low and there were no significant differences in cell viability amongst the low toxic strains (letter 'a', Fig. 4-8). These results indicate that even though PI-based assay (detects membrane-compromised cells under microscope) and WST-1 assay (detects viable cells based on the amount of formazan dye formed due to activity of the mitochondrial dehydrogenases) are based on two different biological effects, in HeLa cells, both can be used for distinguishing highly cytotoxic *B. cereus* isolates from isolates of low cytotoxicity. However, the WST-1 assay may be advantageous to use as it is faster and easier to do with less chance for user error. Hence, it allows screening of more isolates in a shorter period of time.

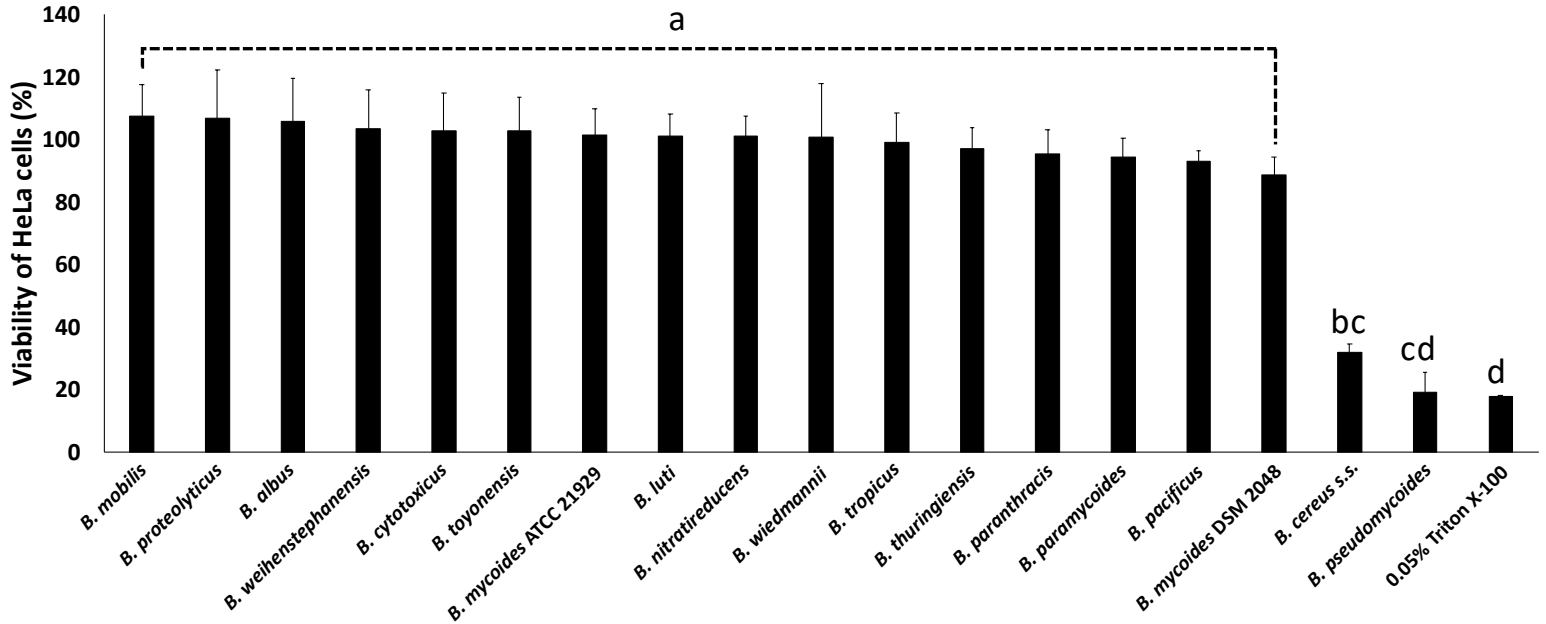

Figure **4-8**: Effect of 5% v/v supernatants of 17 type strains of *B. cereus* group and *B. mycoides* ATCC 21929 on the mean viability of HeLa cells with respect to BHI medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\,nm} - A_{690\,nm}$) obtained for HeLa cells treated with supernatants at 5% v/v level to that of 5% v/v BHI medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean of 12 replicates (6 replicates each from 2 plates for each strain), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different percentage viability values ($P < 0.05$), as determined by Games-Howell method. *B. mycoides* ATCC 21929 strain, based on our studies, has been putatively identified as a new species, and hence included here in the list of type strains.

4.5. Detection of NheB and Hbl-L_2 in supernatants of novel *B. cereus* group type strains by immunoassay

The type strains of 9 novel species of the *B. cereus* group were recently described (Liu et al., 2017). These were isolated from marine environments, and include *B. albus* N35-10-2, *B. luti* TD41, *B. mobilis* 0711P9-1, *B. nitratireducens* 4049, *B. pacificus* EB422, *B. paramycoides* NH24A2, *B. paranthracis* Mn5, *B. proteolyticus* TD42, and *B. tropicus* N24. These strains had not been tested for the presence of Hbl-L_2 and NheB toxins. Hence, we analyzed the strains to detect the presence of Hbl-L_2 and NheB using a lateral flow immunoassay and see if the results correlate with the observed cytotoxicity (or the absence of it). We compared the results of both cytotoxicity and immunological tests conducted using type strains with the results from the *B. cereus s.s.* type strain ATCC 14579, a known cytotoxic strain (Section 4.2.2.). We detected Hbl-L_2 in the supernatant of only *B. albus* N35-10-2 when grown in BHI medium (Table 4-3). Despite producing detectable amounts of the Hbl-L_2, *B. albus* N35-10-2 did not show detrimental effects on HeLa cells when applied in 5% v/v concentration.

Table **4-3**: Detection of NheB and Hbl-L_2 in cultures of *B. cereus* group type strains grown in BHI broth at 37 °C using the Duopath immunoassay kit

B. cereus type strain	NheB detection	Hbl-L_2 detection
B. albus N35-10-2	+	+
B. luti TD41	+	-
B. mobilis 0711P9-1	+	-
B. nitratireducens 4049	+	-
B. pacificus EB422	+	-
B. paramycoides NH24A2	+	-
B. paranthracis Mn5	+	-
B. proteolyticus TD42	+	-
B. tropicus N24	+	-
B. cereus s.s. ATCC 14579	+	+
B. pseudomycoides DSM 12442 [(a)]	-	-
B. wiedmannii FSL W8-0169 [(a)]	+	+
B. toyonensis BCT-7112 [(a)]	+	+
B. cytotoxicus NVH 391-98 [(a)]	-	-
B. thuringiensis ATCC 10792 [(a)]	+	+
B. mycoides DSM 2048 [(a)]	+	-
B. weihenstephanensis WSBC 10204 [(a)]	+	-
B. mycoides ATCC 21929 [(b)]	-	-

[(a)] Results taken from Miller et al., 2018.

[(b)] This strain, based on our studies, has been putatively identified as a new species, and hence included here in the list of type strains.

'+' denotes that the *B. cereus* isolate was positive for a given virulence factor, while '-' denotes that the virulence factor was not detected.

4.6. Study of *B. cereus* clade IV isolates

B. cereus s.s. is associated with foodborne illness in many parts of the world (Glasset et al., 2016). Miller et al. (2018) has previously studied at the cytotoxicity (HeLa cells – PI/DAPI staining) of *B. cereus* clade IV isolates which comprised majorly of *B. cereus s.s.* isolates (13 dairy-associated *B. cereus s.s.* isolates, the *B. cereus s.s.* type strain ATCC 14579, and the *B. thuringiensis* type strain ATCC 10792). They found that the clade IV isolates exhibited a wide range of cytotoxicity in HeLa cells based on PI staining. A probable reason could be the differential production of virulence factors between isolates, which we wanted to investigate by characterizing the proteome profile of the supernatants as a long term goal of the study. For this purpose, the bacteria were cultured in a minimal medium (MOD medium) (Clair et al., 2010; Omer et al., 2015; Rosenfeld et al., 2005) for cytotoxicity studies. Initial steps towards obtaining purified protein for the purpose of the secretome proteomic characterization are described in the Appendix A.

Miller et al. (2018) had cultured *B. cereus* clade IV isolates in nutrient-rich BHI medium for cytotoxicity studies of supernatants on HeLa cells. Hence, we began by assaying cytotoxicity of the supernatants of clade IV isolates grown in MOD medium on HeLa cells, to see if they exhibited a range of cytotoxicities as was seen by Miller et al., 2018. Next, we proceeded to assay cytotoxicity of the clade IV isolates against Caco-2 human epithelial colorectal adenocarcinoma cells, a more relevant cell line for studying diarrheal strains (Jeßberger et al., 2017). We assayed both undifferentiated and differentiated Caco-2 cells. We judged the cytotoxicity of the isolates on the cells using two different methods – the WST-1 assay, which gives a measure of the overall metabolic activity of the cells (Aslantürk, 2017), and the LDH (lactate dehydrogenase) assay, which gives a measure of the membrane integrity of the cells, and is indicative of the pore forming toxins present in the supernatants (Riss and Moravec, 2004). Although the differentiated Caco-2 cell line resembles the intestinal mucosal epithelium, it takes 2 – 3 weeks to fully differentiate the cells. For

this reason, we compared the results obtained for differentiated Caco-2 cells with results obtained for undifferentiated Caco-2 to see if the effects were similar for both states of Caco-2 cells. Finally, we tested for associations between presence of putative virulence genes, detection of Hbl and Nhe components, and the observed cytotoxicity in HeLa and Caco-2 cell lines.

4.6.1. Growth curves for clade IV isolates

Since the growth of the clade IV isolates in the MOD medium was not studied earlier, we started with the preparation of growth curves in order to obtain supernatants at the appropriate growth stage (late exponential or early stationary phase). It is known from literature that the enterotoxin activity of *B. cereus* peaks at the late exponential or early stationary phase of its growth (Gilois et al., 2007; Granum and Lund, 1997; Minnaard et al., 2001). Hence, for effective comparison of the clade IV isolates, we aimed to harvest the supernatants at the early stationary phase for each of the isolates. Growth curves of these 13 dairy isolates along with *B. cereus s.s.* type strain ATCC 14579, and the *B. thuringiensis* type strain ATCC 10792 were constructed based on the optical density measured at 600 nm (OD_{600}) over a particular time range (0-14 h) after inoculation of MOD medium with an overnight culture. The inoculated MOD medium had an approximate initial (time = 0) bacterial concentration of 6 log colony-forming units (cfu)/ml. The curves (solid curves in Fig. 4-9, 4-10, and 4-11) revealed differences between the OD_{600} reached at a particular time point, both between two biological replicates of the same isolate, as well as between different isolates. Moreover, in some cases (e.g., one of the replicates of FSL R5-0184 (Fig. 4-10) and FSL W8-0640 (Fig. 4-11)), the OD_{600} values became constant and then rose again. Hence, we developed a standard curve to correlate the OD_{600} values with the concentration of the cultures (Fig. 4-12) and assess the magnitude of these variations in cfu/ml.

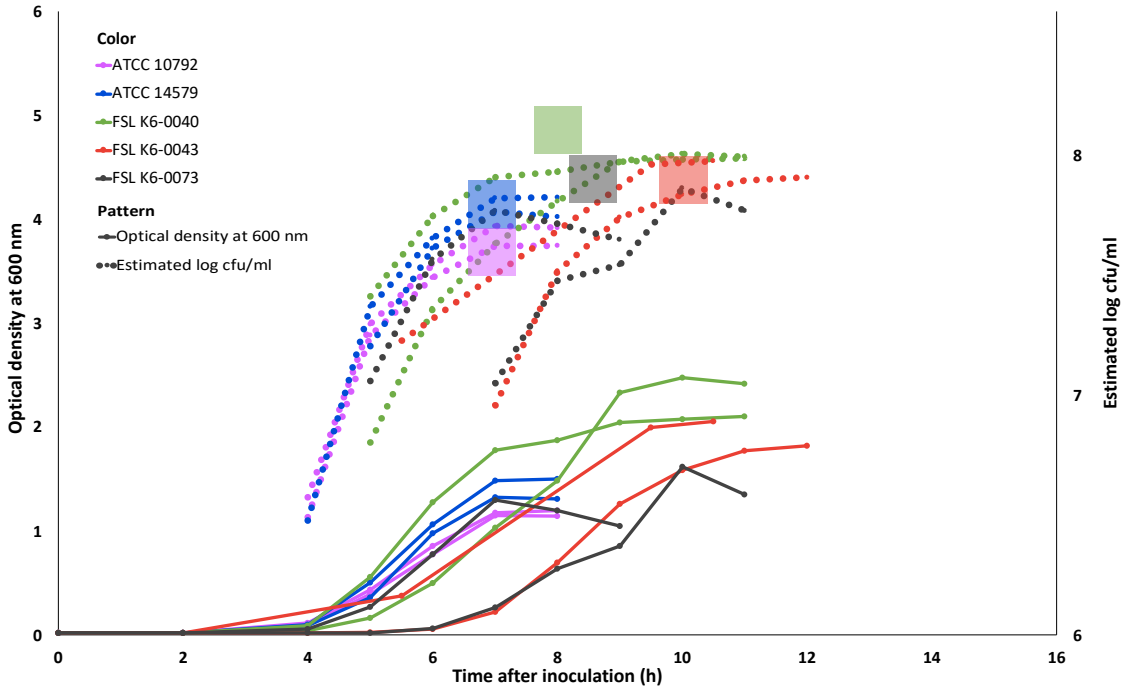

Figure **4-9**: Growth curves showing the optical density measured at 600 nm (solid curves), and the corresponding estimated log cfu/ml (dotted curves) as a function of time for *B. cereus* clade IV isolates *B. thuringiensis* ATCC 10792, *B. cereus s.s.* ATCC 14579, FSL K6-0040, FSL K6-0043, and FSL K6-0073 grown in MOD medium at 37 °C. The curves of same color are from two biological replicates of the same isolate. The colored boxes represent the time points and estimated log cfu/ml values of the cultures when the supernatants were harvested for further experiments.

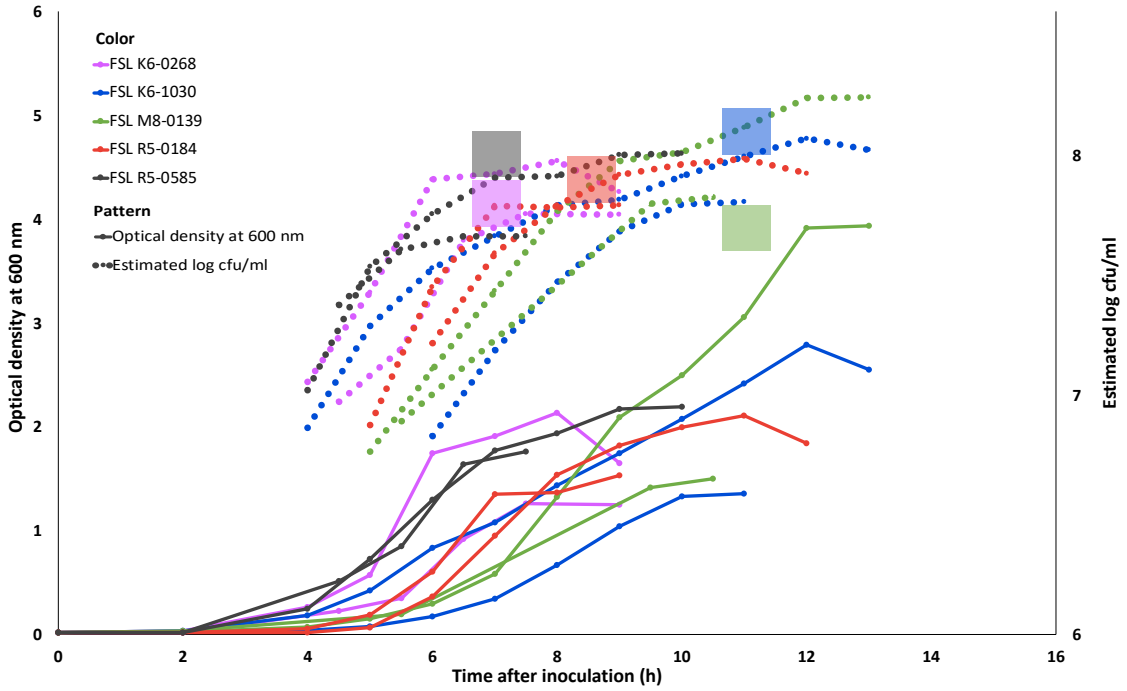

Figure **4-10**: Growth curves showing the optical density measured at 600 nm (solid curves), and the corresponding estimated log cfu/ml (dotted curves) as a function of time for *B. cereus* clade IV isolates FSL K6-0268, FSL K6-1030, FSL M8-0139, FSL R5-0184, and FSL R5-0585 grown in MOD medium at 37 °C. The curves of same color are from two biological replicates of the same isolate. The colored boxes represent the time points and estimated log cfu/ml values of the cultures when the supernatants were harvested for further experiments.

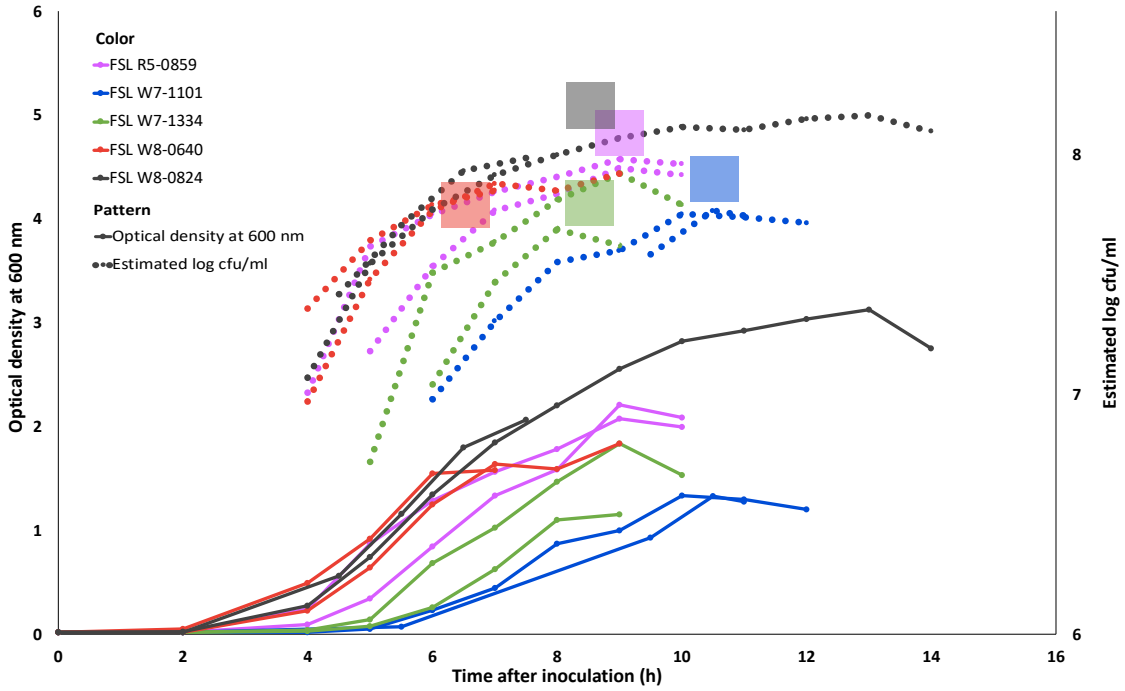

Figure **4-11**: Growth curves showing the optical density measured at 600 nm (solid curves), and the corresponding estimated log cfu/ml (dotted curves) as a function of time for *B. cereus* clade IV isolates FSL R5-0859, FSL W7-1101, FSL W7-1334, FSL W8-0640, and FSL W8-0824 grown in MOD medium at 37 °C. The curves of same color are from two biological replicates of the same isolate. The colored boxes represent the time points and estimated log cfu/ml values of the cultures when the supernatants were harvested for further experiments.

A standard curve from growing the *B. cereus s.s.* type strain ATCC 14579 in MOD medium under the same conditions as used for the growth curves (150 rpm, 37 °C, 125 ml MOD medium) showed a linear relationship between OD_{600} in the range of 0.1 to 0.9, and the corresponding culture concentration (Fig. 4-12). The R^2 value was found to be 0.9755 for the linear equation obtained [cfu/ml = $(5 \times 10^7 \times (OD_{600})) - (2 \times 10^6)$] for OD_{600} values ranging from 0.1 to 0.9. The OD_{600} values for growth curves represented in solid curves in Figures 4-9, 4-10, and 4-11 were converted to their estimated colony counts using this linear equation.

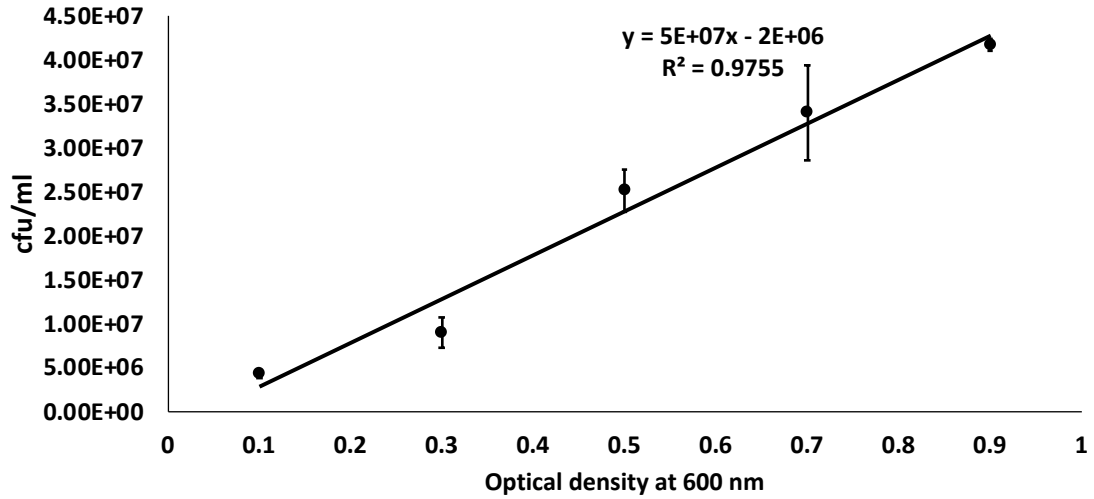

Figure **4-12**: Standard curve describing a linear relationship between optical density and culture concentration for *B. cereus s.s.* ATCC 14579 grown in MOD medium. The culture concentration (cfu/ml) was obtained by performing plate counts serial dilutions of *B. cereus s.s.* ATCC 14579 culture in triplicate for two biological replicates. Error bars represent the standard error of means of the two biological replicates.

We drew the growth curves using the predicted log cfu/ml values with respect to time (dotted curves in Figures 4-9, 4-10, and 4-11). From the predicted log cfu/ml values, we determined the time point when the log cfu/ml vs. time curve flattened, and marked it as the stationary phase, which was between 6.5 to 11 hours, depending on the isolate in question. The time periods of harvest for each isolate are shown in colored boxes in Figures 4-9, 4-10, and 4-11. The time points of early stationary phase corresponded with approximately 8 logs of bacterial counts from the log cfu/ml vs. time curves constructed. As per Aguirre et al. (2015), *B. cereus* ATCC 10876 reached a maximum of approximately 8 logs of bacterial counts at its stationary phase (Aguirre et al., 2015). At these time points, we measured the optical density again at the time of harvest and converted to the log cfu/ml values to confirm that the 8 log cfu/ml bacterial concentration was reached. We found that the bacteria had reached 8 log cfu/ml based on the estimate from the standard curve at

the time of the harvest of the supernatants of each isolate (colored boxes in Figures 4-9, 4-10, and 4-11).

4.6.2. Dose-dependent response in HeLa cells to B. cereus clade IV supernatants as measured by the WST-1 assay

We used the WST-1 assay for testing the effect of *B. cereus* clade IV isolates' supernatants at different doses (5% v/v, 15% v/v, 25% v/v, and 50% v/v) on HeLa cells, to confirm that the 5% v/v concentration of supernatant that had been used earlier to test outbreak isolates and *B. cereus* group type strains (Sections 4.2.2. and 4.4.) was optimal for differentiation among clade IV *B. cereus* isolates based on their cytotoxicity, and to extend our results to higher concentrations. We treated HeLa with *B. cereus* clade IV supernatants (13 dairy isolates along with *B. cereus s.s.* type strain ATCC 14579, and the *B. thuringiensis* type strain ATCC 10792) for 15 min at 5% v/v, 15% v/v, 25% v/v, and 50% v/v concentrations, which was followed by the addition of the WST-1 dye, and incubation for 25 additional minutes. We also monitored the effect of treatment with fresh MOD medium at 5% v/v, 15% v/v, 25% v/v, and 50% v/v levels on HeLa cells. Additionally, we measured the absorbance of solutions in wells (once the WST-1 dye was added) containing cells incubated with only cell culture medium. This was done to see if MOD medium had any effect in itself on the cells in comparison with untreated cells. Hence, we had three internal controls per assay – cells incubated with cell culture medium, cells incubated with MOD medium (at the same concentration as supernatants used to treat the cells), and 0.05% Triton X-100 (the positive cytotoxicity control). Fig. 4-13 shows the percentage viability of HeLa cells normalized to the cell culture medium when treated with the MOD medium and 0.05% Triton X-100. The MOD medium had a detrimental effect on viability of HeLa cells at 15% v/v, 25% v/v, and 50% v/v concentrations (Fig. 4-13). However, MOD medium at 5% concentration had a negligible effect on the viability of HeLa cells (Fig. 4-13). The decrease in cell viability after exposure to higher concentrations of the MOD was

most likely due to the medium components rather than a reduction in nutrient concentration. Christiansson et al. (1989) had tested the toxin production by dairy isolates of *B. cereus* at low temperatures using a HeLa cell model, with supernatant concentrations ranging from 0.15% to 20%, with the use of sterile BHI broth and skim milk as controls (Christiansson et al., 1989). Moreover, Nouri et al. (2018) conducted a study to see the effect of *Lactobacillus* spp. supernatants on the HeLa cell line using MTT assay at 5-25% v/v levels. They saw a negligible effect on the cell viability when using De Man, Rogosa and Sharpe (MRS) broth (negative control) at 5-25% v/v concentrations (Nouri et al., 2018). It should be noted that BHI and MRS broths are more nutrient rich than MOD medium and could supplement some of the nutrients of the cell culture medium that is being replaced.

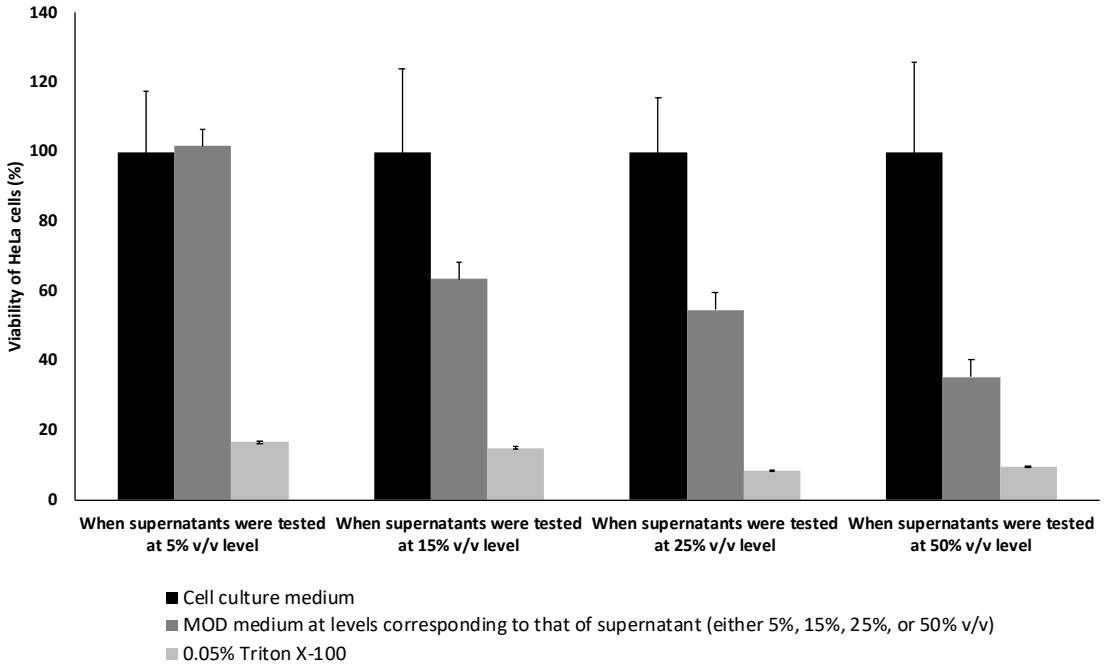

Figure **4-13**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 5%, 15%, 25%, and 50% v/v MOD medium) on the viability of HeLa cells with respect to cell culture medium, as determined using WST-1 assay. Viability of cells treated with MOD medium has been calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with MOD medium at 5%, 15%, 25%, and 50% v/v levels to that of the cell culture medium and

converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates from a single plate, while error bars show the standard deviation.

We calculated the viabilities of HeLa cells treated with 5% v/v, 15% v/v, 25% v/v, and 50% v/v *B. cereus* clade IV isolates' supernatants with respect to 5% v/v, 15% v/v, 25% v/v, and 50% v/v MOD medium, respectively (Fig. 4-14). We chose to use MOD medium as the basis for calculating viabilities, as this would take into consideration the effects of the medium in which the bacteria were grown and supernatants harvested, while also taking into consideration the plate-to-plate differences in biological activity of the cells. Overall, use of the supernatants at 5% v/v concentration gave a good range of viabilities (49 ± 9% for *B. cereus s.s.* ATCC 14579 to 119% ± 14% for FSL K6-0268) that allowed for differentiation among isolates (Fig. 4-14). Regardless of whether it was the effect of nutrient depletion or the effect of MOD medium components themselves, or a combination of both, the fact remained that the HeLa cells were sensitive to cell culture medium replacement with MOD medium at concentrations higher than 5% v/v. Miller et al. (2018) had previously used the 5% v/v concentration for their testing of *B. cereus* supernatants on HeLa cells (Miller et al., 2018) and we continued using this level as the MOD at this concentration showed least detrimental effects on HeLa cell viability.

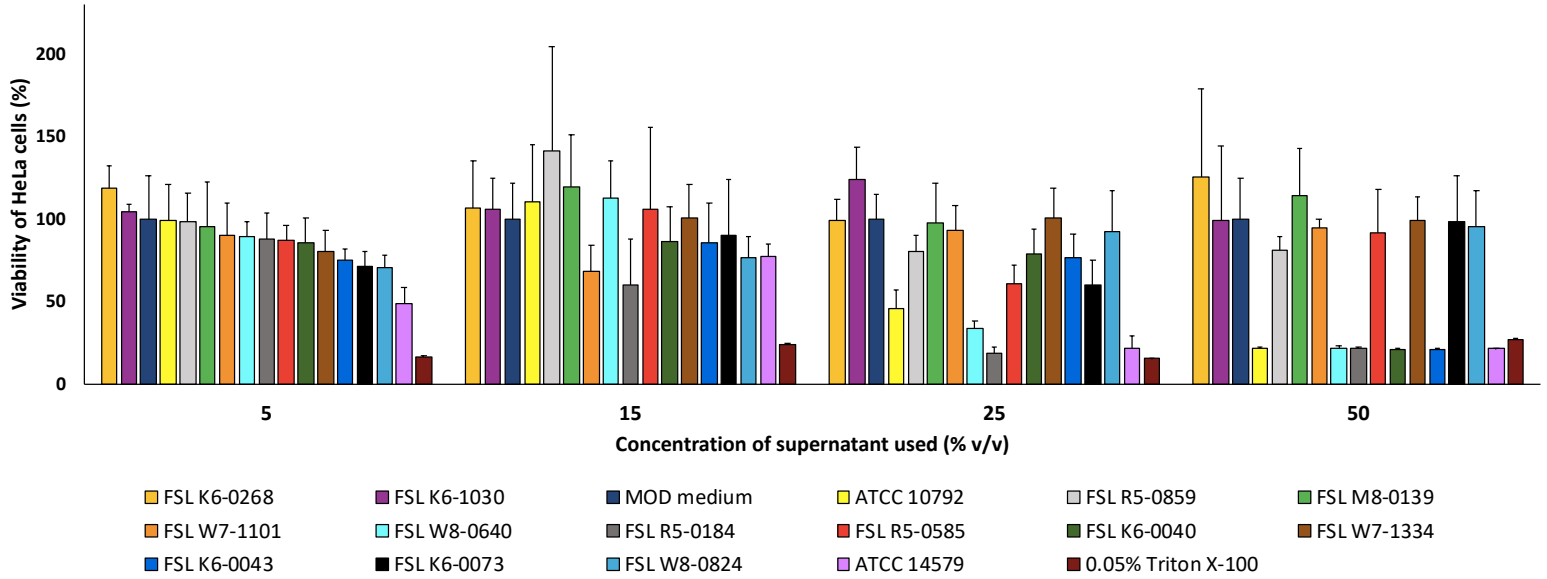

Figure **4-14**: Effect of concentration of supernatants (5%, 15%, 25%, and 50% v/v) of *B. cereus* clade IV isolates on the viability of HeLa cells with respect to MOD medium at levels corresponding to that of the supernatant (5%, 15%, 25%, and 50% v/v respectively), as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with supernatants at 5, 15, 25, and 50% v/v levels to that of 5, 15, 25, and 50% v/v MOD medium respectively and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean of 3 replicates from a single plate, while error bars show the standard deviation.

Researchers who have worked with HeLa cells and *B. cereus* supernatants in the past used 5% v/v concentrations of supernatants (Christiansson et al., 1989; Miller et al., 2018). Hence, the conditions for testing the effect for *B. cereus* supernatants via the WST-1 assay remained the same as used in Sections 4.2.2. and 4.4. The conditions are summarized in Table 4-1.

4.6.3. Toxicity of B. cereus clade IV supernatants in HeLa cells

4.6.3.1. Effect on the viability of HeLa cells, studied using the WST-1 assay

After deciding the concentration of supernatant to be used on HeLa cells (5% v/v), we assessed the effect of supernatants of the 15 isolates of *B. cereus* clade IV (13 dairy isolates, *B. cereus s.s.* ATCC 14579, and *B. thuringiensis* ATCC 10792) on HeLa cells on two separate plates. There were 3 replicates per isolate per plate. We recorded the effect of the MOD medium (negative control) and 0.05% Triton X-100 (positive control) on cells with respect to untreated cells (cells incubated with only cell culture medium) in each plate. For the two plates of HeLa cells that we conducted the WST-1 assay on, the effect of the controls is presented in Fig. 4-15. Cells incubated with the cell culture medium were considered to be 100% viable.

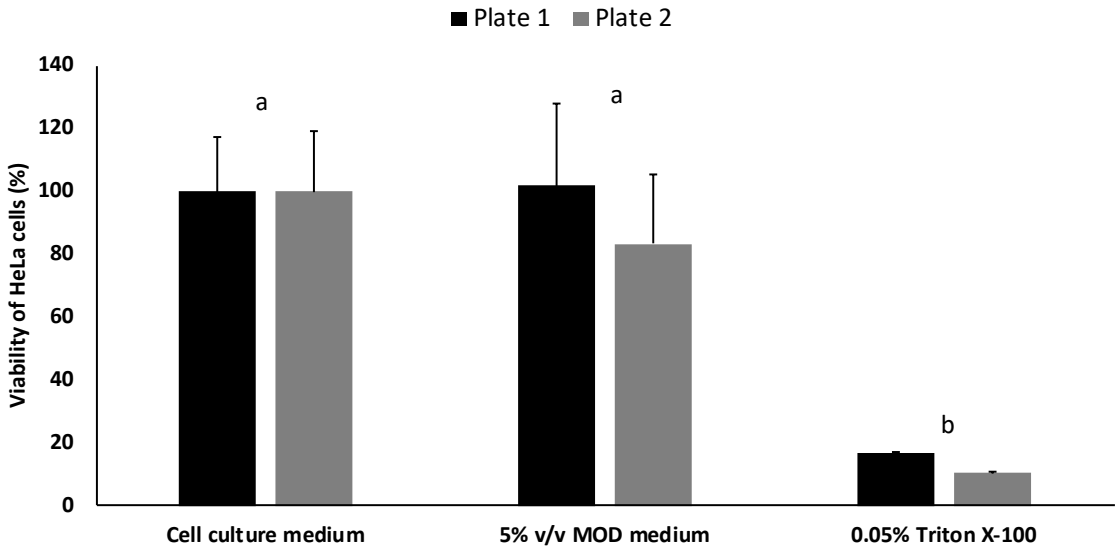

Figure **4-15**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 5% v/v MOD medium) on the viability of HeLa cells from two plates with respect to cell culture medium, as determined using WST-1 assay. Viability of cells treated with MOD medium has been calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with 5% v/v MOD medium to that of the cell culture medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had significantly different mean percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

After establishing that 5% v/v MOD medium had a minimal effect on HeLa cells ($P < 0.05$; Fig. 4-15), we assessed the viability of HeLa cells exposed to 5% v/v culture filtrate from *B. cereus* clade IV isolates. In terms of cell viability, statistically, we found 3 isolates (FSL W8-0824, FSL W7-1101, and FSL K6-0043) to be as toxic as the highly cytotoxic *B. cereus s.s.* type strain ATCC 14579 by the WST-1 assay ($P < 0.05$; Fig. 4-16). The mean viabilities ranged from 42% for *B. cereus s.s.* ATCC 14579 to 124% for FSL K6-0268 (Fig. 4-16).

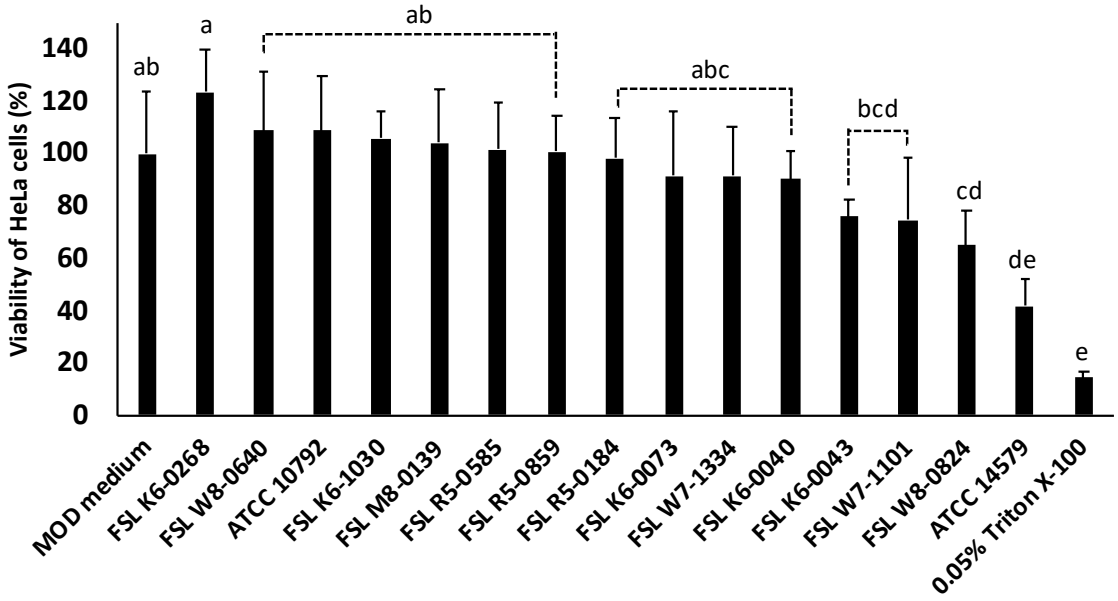

Figure **4-16**: Effect of 5% v/v supernatants of *B. cereus* clade IV isolates on the viability of HeLa cells respect to 5% v/v MOD medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for HeLa cells treated with *B. cereus* supernatants to that of the MOD medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

4.6.3.2. Effect on the cell membrane integrity of HeLa cells, studied using the LDH assay

In addition to the WST-1 assay, we used the LDH assay (which gives a measure of the membrane integrity of the cells) to test for the effect of the *B. cereus* supernatants on the disruption of the membrane integrity of the HeLa cells. While in the past, Miller et al. (2018) have tested for the degree of pore formation using the *B. cereus* clade IV supernatants on HeLa cells, they have used a time-consuming microscopy-based PI/DAPI method (Miller et al., 2018). We wanted to use a more rapid dye-based assay for this purpose, and also compare the results with the literature (Miller et al., 2018). We kept the total treatment time of HeLa cells with the supernatants the same as what

we had used in the WST-1 assay. A summary of the conditions of the LDH assay used has been provided in Table 4-4.

Table **4-4**: Conditions used in the LDH assay for HeLa cells

Parameter	Value
Seeding density of cells [(a)]	8 x 10^4 cells/cm^2
Concentration of cell-free *B. cereus* medium supernatant	5% v/v in cell culture medium [(b)]
Incubation time of cells with *B. cereus* supernatant	40 min
Volume of HeLa cell supernatant (spent medium) used for assay [(c, d)]	50 µl
Volume of LDH assay reagent added [(c, d)]	100 µl
Time of incubation of LDH assay reagent with the HeLa spent medium [(c)]	15 min
Wavelength for measurement of signal [(c, d)]	490 nm
Wavelength for measurement of background [(c, d)]	690 nm

[(a)] Conditions taken from Fisichella et al., 2009.
[(b)] EMEM with 10% FBS.
[(c)] Conditions taken from Kumar et al., 2018.
[(d)] Conditions taken from Jan and Lashuel et al., 2012.

In order to control for the potential effect of the MOD medium on the cell membrane integrity of HeLa cells, we calculated the ratio of corrected absorbances of solutions obtained by treating HeLa cells with 5% v/v MOD medium relative to that of the cell culture medium (Fig. 4-17). We saw that the use of 5% v/v MOD medium did not affect the HeLa cells in terms of the pore-forming ability ($P < 0.05$; Fig. 4-17). Hence, 5% v/v MOD medium was found to be non-cytotoxic in HeLa cells as judged by both the WST-1 (Fig. 4-15) and the LDH (Fig. 4-17) assays.

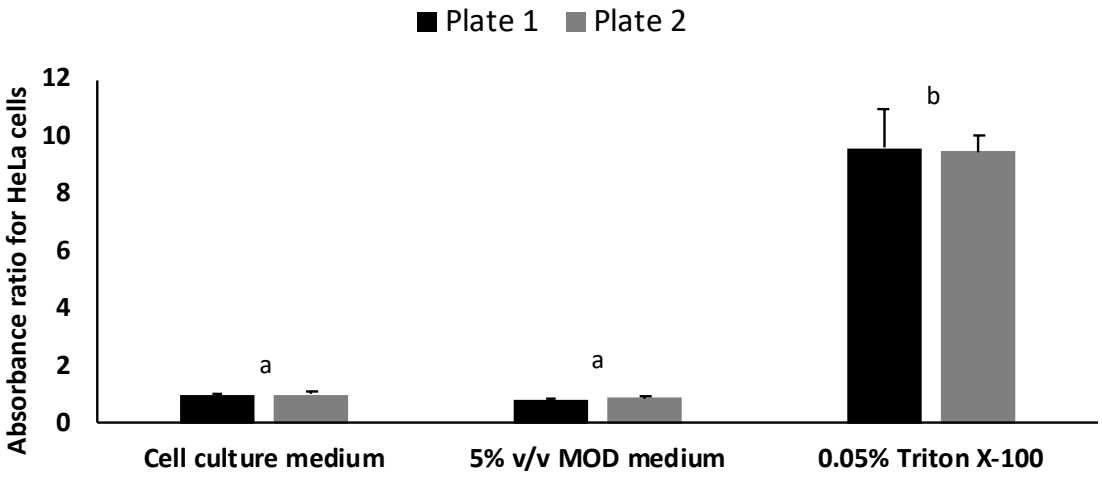

Figure **4-17**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 5% v/v MOD medium) on the absorbance ratio of solution relative to that of 5% v/v MOD medium for HeLa cells, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had significantly different mean absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method.

We proceeded to calculate the absorbance ratios of solutions obtained from HeLa cells treated with 5% v/v supernatants of *B. cereus* clade IV isolates to that of 5% v/v MOD medium. The results have been presented in Figure 4-18. Statistically, *B. cereus s.s.* ATCC 14579 was found to be the isolate of highest cytotoxicity ($P < 0.05$), with a mean LDH assay ratio of 1.86. All other

isolates were found to be of low cytotoxicity (mean LDH ratios of 1.01 to 1.18). This is in contrast with the results obtained by Miller et al. (2018) which looked at the assessment of degree of pore formation via PI/DAPI staining. While they did not conduct posthoc statistics to group their clade IV isolates based on the data of PI-positive HeLa cells, they considered isolates that showed an average of greater than or equal to 5% PI-positive HeLa cells as cytotoxic (Miller et al., 2018). Based on this arbitrary cutoff, they found 7 out of the 15 clade IV isolates to be cytotoxic towards HeLa cells (Miller et al., 2018). The differences in cytotoxicity results could be because of different assays employed or differences in culture conditions (e.g. different growth media, shaking conditions, and time of harvest). Miller et al. (2018) cultured the bacteria in BHI medium. That medium composition might affect toxicity was reported by Christiansson et al. (1989). They observed differences in cytotoxicity when dairy-sourced *B. cereus* isolates were cultured in milk versus in BHI medium. More than 50% of 136 isolates were found to produce detectable cytotoxicity when cultured in milk for 24 h versus around 37% of the isolates that were cytotoxic when cultured under identical conditions in BHI broth. Hence, a higher number of isolates were found to produce cytotoxins in milk than in BHI broth (Christiansson et al., 1989). These differences may be explained by the fact that milk is more of a natural substrate for the *B. cereus* isolates studied compared to BHI medium.

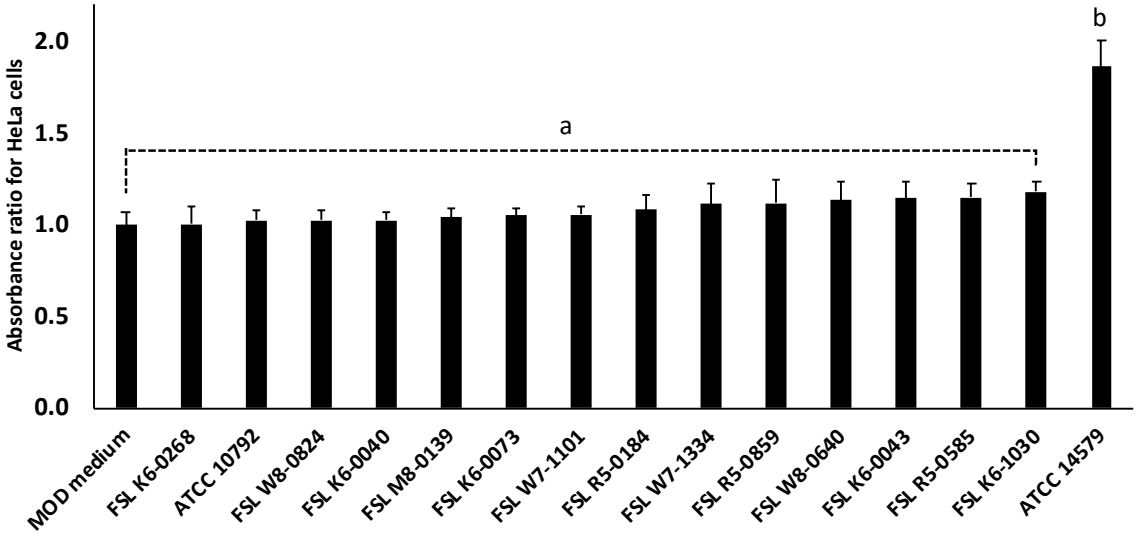

Figure **4-18**: Effect of 5% v/v supernatants of *B. cereus* clade IV isolates on the absorbance ratio of solution relative to that of 5% v/v MOD medium for HeLa cells, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of 6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method. 0.05% Triton X-100 is not shown on the graph because of its large value (11.42 ± 1.24; received the letter 'c' during statistical grouping based on the Tukey HSD method).

4.6.4. Dose-dependent response in undifferentiated Caco-2 cells to B. cereus clade IV supernatants as measured by the WST-1 assay

The goal of this experiment was to optimize the WST-1 assay conditions of *B. cereus* supernatant doses and dye development times for use with Caco-2 cells, so as to get a wide range of cell viabilities in order to differentiate the cytotoxic potential of the clade IV isolates. We used undifferentiated, rather than differentiated Caco-2 cells for this purpose as they could be made ready faster, and were easier to maintain compared to differentiated Caco-2 cells. We first tested the effect of 5% v/v *B. cereus* supernatants (which was the same concentration of supernatants used

earlier with HeLa cells) on Caco-2 cells to see if this dose could elicit a similar response in Caco-2 cells. For this, undifferentiated Caco-2 cells were treated with 5% v/v *B. cereus* clade IV supernatants (13 dairy isolates along with *B. cereus s.s.* type strain ATCC 14579, and the *B. thuringiensis* type strain ATCC 10792) for 15 min, followed by addition of the WST-1 dye. The absorbance was monitored over time. We saw that even after 3.5 h (210 min) of total incubation with the *B. cereus* supernatants, the effects of the supernatants of the different isolates on the cells (barring *B. cereus s.s.* type strain ATCC 14579) was negligible (Fig. 4-19), with a mean ranging from 92% to 111% (Fig. 4-19). For comparison, the *B. cereus s.s.* type strain ATCC 14579, and the positive control 0.05% Triton X-100 yielded mean viabilities of 53% and 21%, respectively. Hence, we decided to test higher doses of the *B. cereus* supernatants on the undifferentiated Caco-2 cells in order to obtain a wide range of viabilities of Caco-2 cells which would enable us to better differentiate between the isolates based on their cytotoxic potentials.

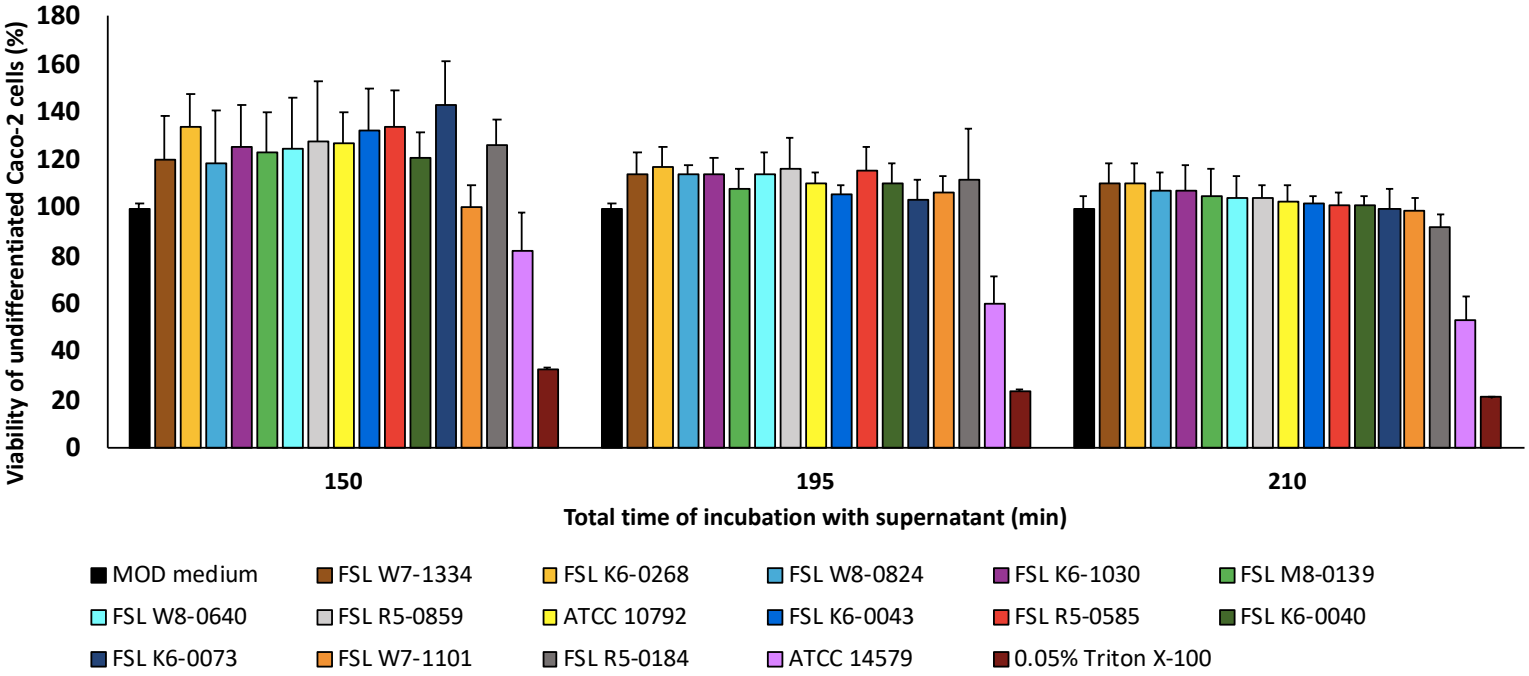

Figure **4-19**: Effect of incubation time with 5% v/v supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells with respect to 5% v/v MOD medium, as determined using WST-1 assay. The viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with supernatants at 5% v/v level to that of 5% v/v MOD medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean of 6 replicates from a single plate, while error bars show the standard deviation.

We treated undifferentiated Caco-2 cells with *B. cereus* supernatants for 15 min at 15% v/v, 25% v/v, or 50% v/v concentrations, following which we added the WST-1 dye. As the incubation progressed, we monitored the absorbance ($A_{450\ nm} - A_{690\ nm}$) of the solutions in the wells over time. We also monitored the effect of MOD medium at 15% v/v, 25% v/v, and 50% v/v levels side-by-side. Additionally, cells incubated with only cell culture medium were also monitored via absorbance measurements once the WST-1 dye was added to see if MOD medium affected the cells in any way, as was done with HeLa cells. Fig. 4-20 shows the percentage viability of the undifferentiated Caco-2 cells normalized to the cell culture medium when treated with the MOD medium and 0.05% Triton X-100 over time. There was a decrease in the cell viability as the concentration was increased to 50% v/v MOD medium from 25% v/v MOD medium. This data indicates that either the MOD medium at higher concentrations is toxic in itself to undifferentiated Caco-2 cells, or that removal of 50% cell culture medium is affecting the viability of cells. This should be taken into account in any cytotoxicity assays with the bacterial supernatants.

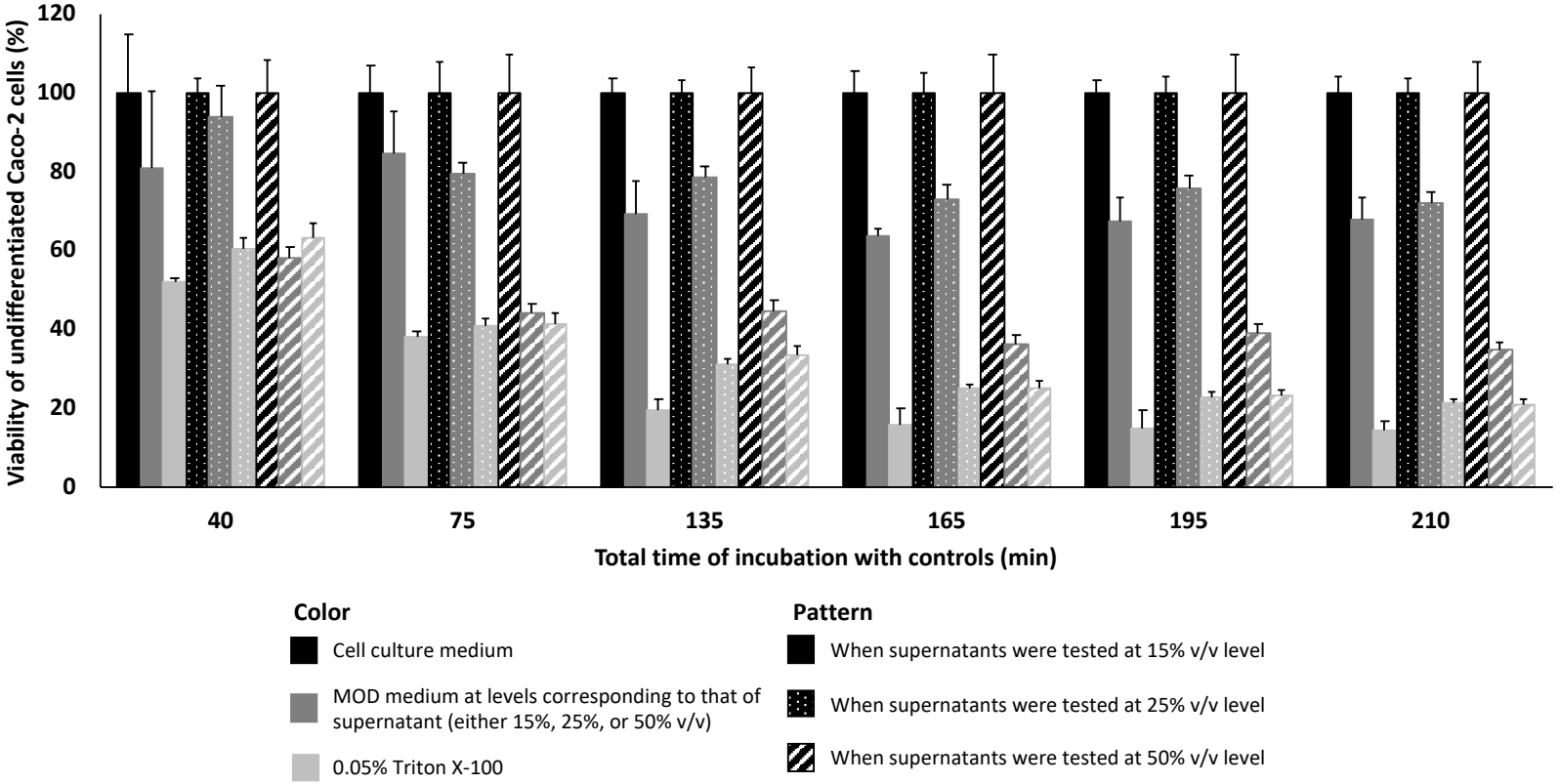

Figure **4-20**: Effect of incubation time with controls (cell culture medium, 0.05% Triton X-100, and 15%, 25%, and 50% v/v MOD medium) on the viability of undifferentiated Caco-2 cells with respect to cell culture medium, as determined using WST-1 assay. Viability of cells treated with MOD medium has been calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with MOD medium at 15%, 25%, and 50% v/v levels to that of the cell culture medium and converting to percentages. 0.05% Triton X-100 was used as a positive cytotoxicity control. Columns show the mean viability of 3 replicates from a single plate, while error bars show the standard deviation.

Figures 4-21, 4-22, and 4-23 show the corrected absorbances ($A_{450\,nm} - A_{690\,nm}$) as a function of time for undifferentiated Caco-2 cells treated with controls or 15%, 25%, and 50% v/v supernatants of clade IV species' type strains. It is useful to take a look at the raw data of absorbances vs. time before taking a look at the cell viability, as the effect of the treatment of supernatants on the dye development can be seen with the progression of time and compared with the progression of dye development when cells are treated with controls. For cells treated with *B. cereus s.s.* ATCC 14579 supernatant, the resultant absorbances remained near constant (and equivalent to that of Triton X-100) at all supernatant concentrations tested, indicating that the cytotoxicity is high even at low concentrations (Fig. 4-21, 4-22, and 4-23). The cytotoxicity of *B. thuringiensis* ATCC 10792 supernatant, however, is least at 15% v/v (Fig. 4-21), and highest at 50% v/v concentration (Fig. 4-23) for the same incubation time, based solely on the absorbance values for cells treated with the supernatant. Moreover, the effect of the B. *thuringiensis* ATCC 10792 does not elicit a linear absorbance response at 15% v/v (Fig. 4-21) with respect to time, as the cell culture medium does (Fig. 4-21), indicating that the ability of the cells to convert dye is affected in an irregular (non-linear with respect to time) manner when treated with supernatants (Fig. 4-21).

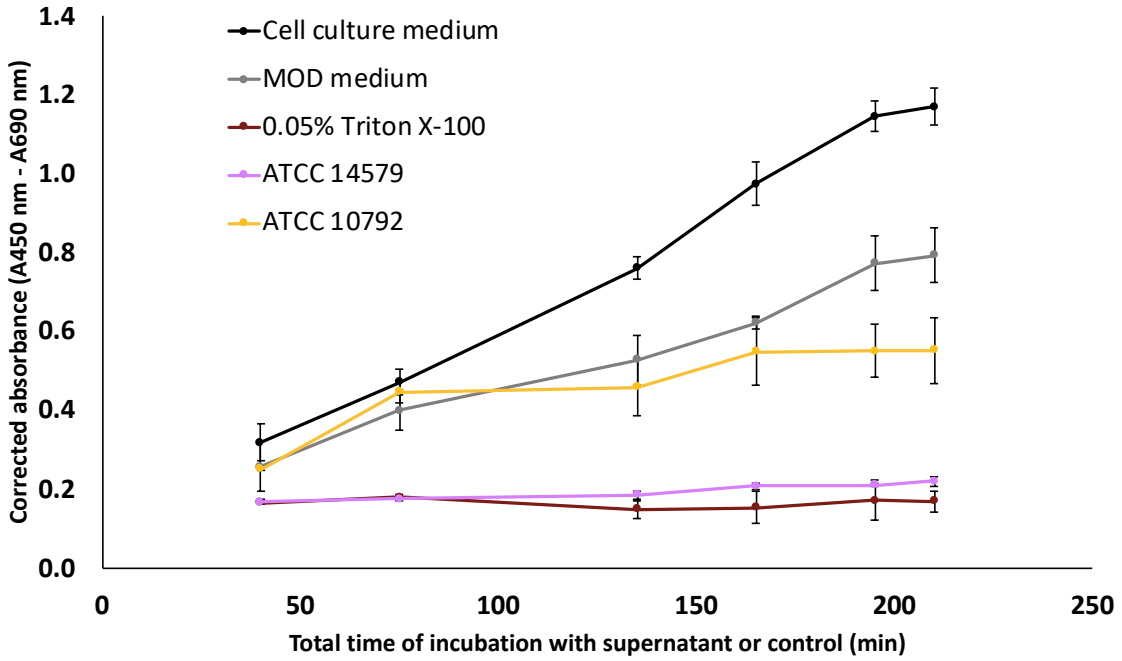

Figure **4-21**: Corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) of solution obtained for undifferentiated Caco-2 cells treated with MOD medium and supernatants of *B. cereus s.s.* ATCC 14579 and *B. thuringiensis* ATCC 10792 at 15% v/v concentration as a function of time, as determined by the WST-1 assay. 0.05% Triton X-100 was used as a positive control. Data points show the mean absorbance of 3 replicates from a single plate, while error bars show the standard deviation.

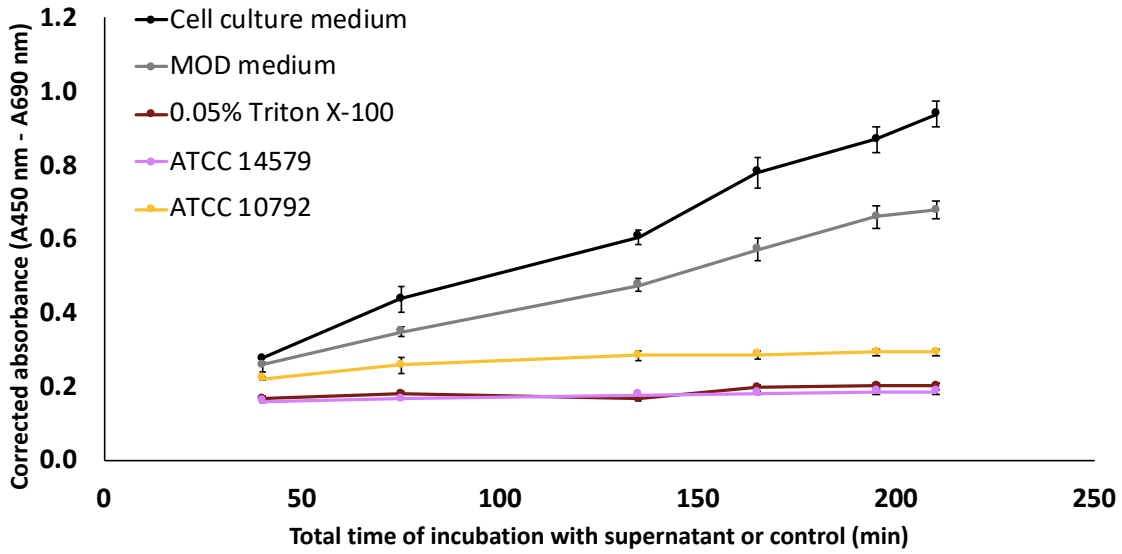

Figure **4-22**: Corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) of solution obtained for undifferentiated Caco-2 cells treated with MOD medium and supernatants of *B. cereus s.s.* ATCC 14579 and *B. thuringiensis* ATCC 10792 at 25% v/v concentration as a function of time, as determined by the

WST-1 assay. 0.05% Triton X-100 was used as a positive control. Data points show the mean absorbance of 3 replicates from a single plate, while error bars show the standard deviation.

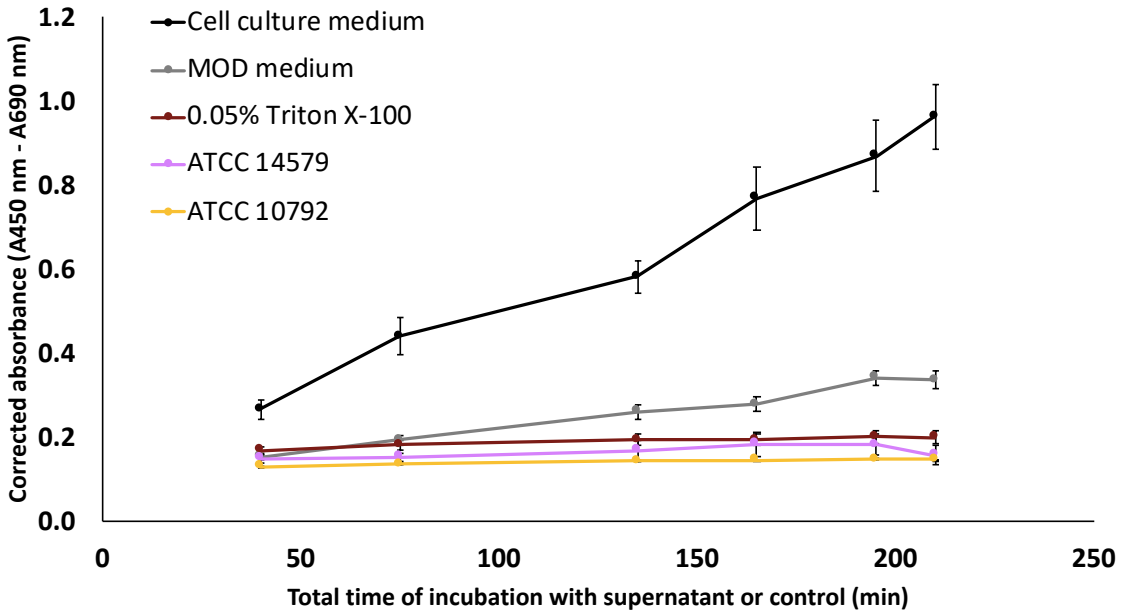

Figure **4-23**: Corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) of solution obtained for undifferentiated Caco-2 cells treated with MOD medium and supernatants of *B. cereus s.s.* ATCC 14579 and *B. thuringiensis* ATCC 10792 at 50% v/v concentration as a function of time, as determined by the WST-1 assay. 0.05% Triton X-100 was used as a positive control. Data points show the mean absorbance of 3 replicates from a single plate, while error bars show the standard deviation.

The viabilities of undifferentiated Caco-2 cells treated with 15%, 25%, and 50% v/v supernatant of the clade IV isolates (15 isolates, inclusive of the *B. cereus s.s.* and *B. thuringiensis* type strains) for different incubation times are presented in Figures 4-24, 4-25, and 4-26 respectively. Here, the viabilities have been normalized to 15%, 25%, and 50% v/v MOD medium respectively.

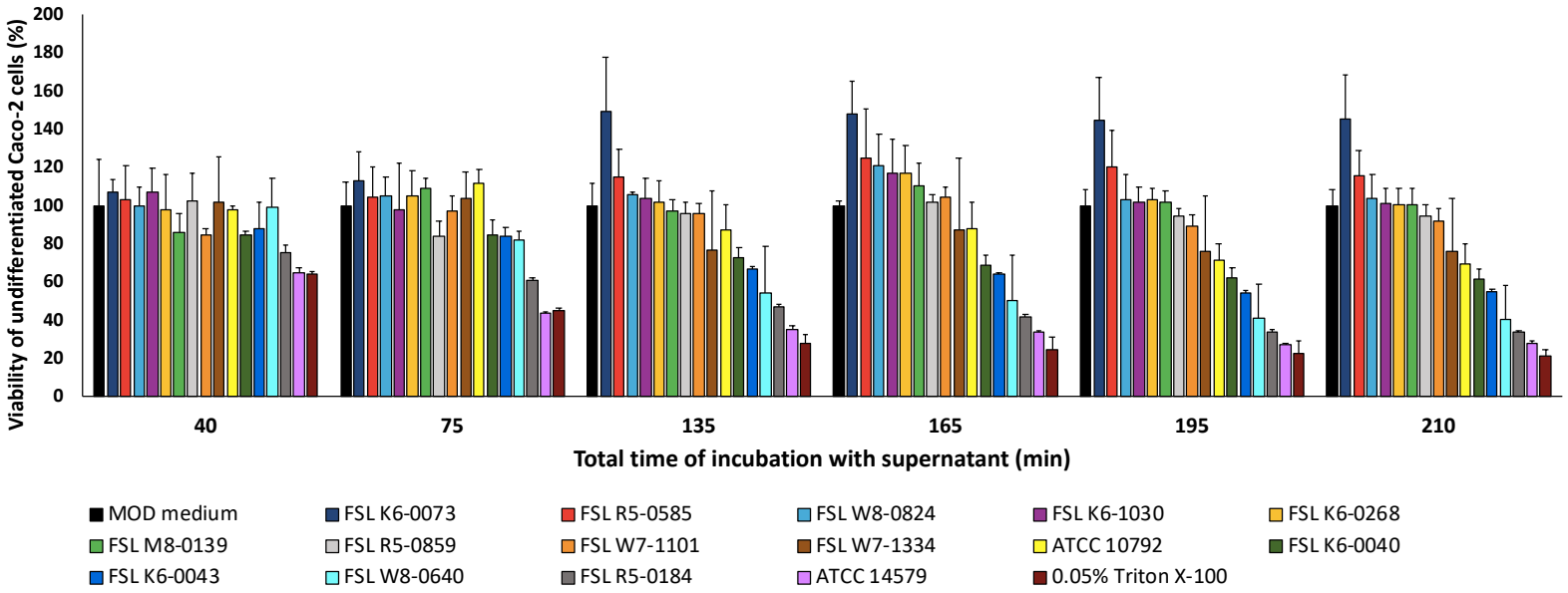

Figure **4-24**: Effect of incubation time of 15% v/v supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells with respect to 15% v/v MOD medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with 15% v/v *B. cereus* supernatants to that of the MOD medium at 15% v/v concentration and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates from a single plate, while error bars show the standard deviation.

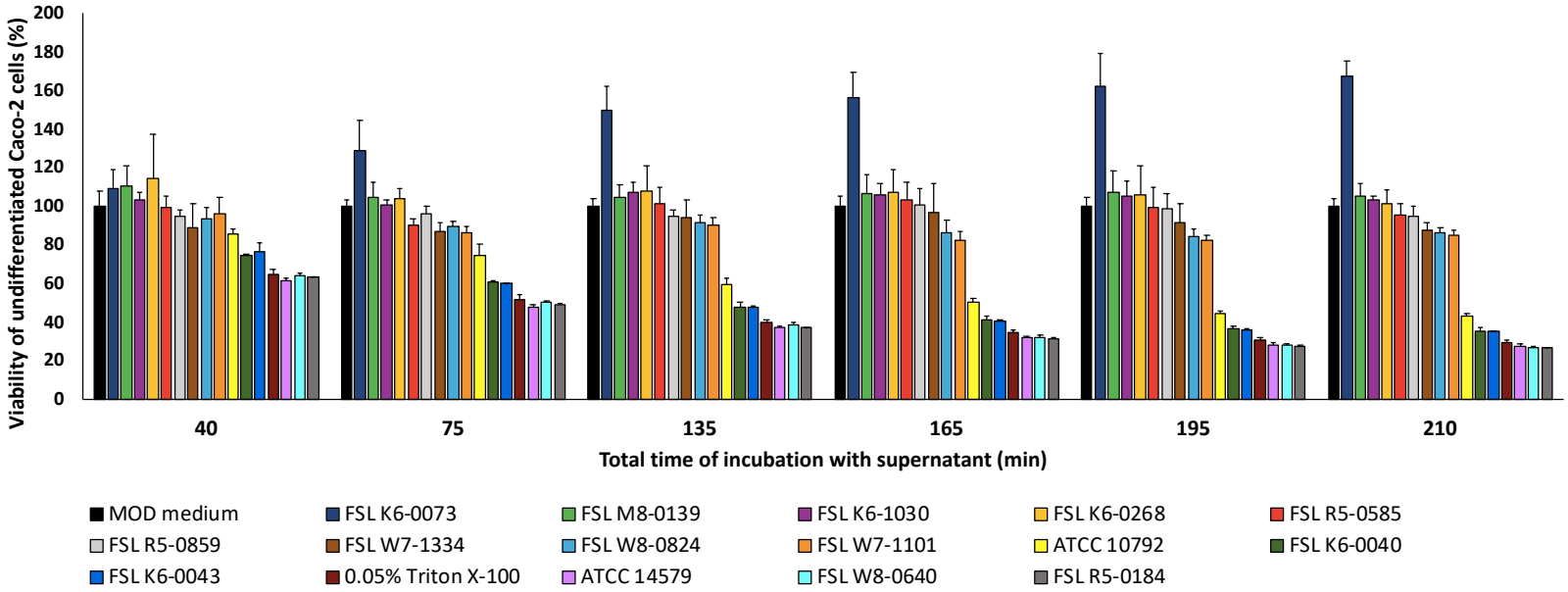

Figure **4-25**: Effect of incubation time of 25% v/v supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells with respect to 25% v/v MOD medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with 25% v/v *B. cereus* supernatants to that of the MOD medium at 25% v/v concentration and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates from a single plate, while error bars show the standard deviation.

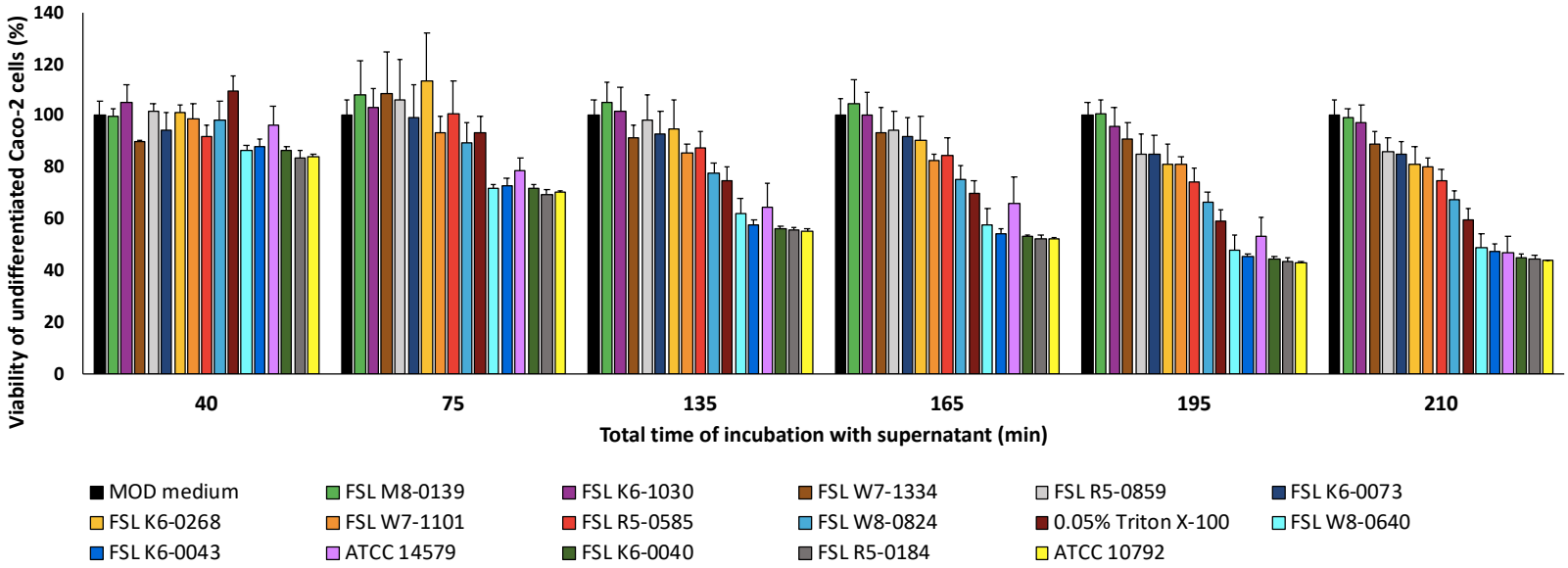

Figure **4-26**: Effect of incubation time of 50% v/v supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells with respect to 50% v/v MOD medium, as determined using the WST-1 assay. The viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with 50% v/v *B. cereus* supernatants to that of the MOD medium at 50% v/v concentration and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates from a single plate, while error bars show the standard deviation.

Overall, the trends shown by the viabilities of Caco-2 cells when treated with 15% and 25% v/v supernatants were similar in terms of relative placement of isolates in the order of their increasing cytotoxic potentials (decreasing viabilities) for incubation times 135 min (2 h 15 min) and longer (Figures 4-24 and 4-25), but different from that of the 50% v/v supernatants (Fig. 4-26). It may be recalled from Fig. 4-20 that the effect of the use of 50% v/v MOD medium was such that it had resulted in a 60% loss of viability of cells (striped dark gray bars in Fig. 4-20). Hence, it is possible that even small differences in absorbances of cells treated with supernatants (Fig. 4-23) relative to the cells treated with the MOD medium resulted in a representation of data in such a way that the resultant viabilities do not show the actual cytotoxic potential of the isolates.

Our observations indicate that replacing a part of the cell culture medium with MOD medium is having an effect on the outcome of the assay. Minnaard et al. (2001) employed the MTT assay as well as a cell detachment assay for testing effect of *B. cereus* supernatants on Caco-2 cells at concentrations up to 100%. They presented their data with respect to the negative control (BHI medium) that was used to grow the bacteria. It was reported that the substitution of cell culture medium with BHI medium did not have a significant effect on the cell viability (Minnaard et al., 2001). Hence, this leads to the conclusion that it was not the effect of removal of 15, 25, and 50% cell culture medium that was causing the effects, but it was the components in the MOD medium themselves. However, it is also possible that the cells were capable of utilizing some of the nutrients from BHI medium, which would compensate for the effect of depleting the cell culture medium, while they were unable to do so from MOD medium.

Nevertheless, to minimize the effect of the MOD medium, we chose the lowest possible incubation time with 15% v/v supernatant required to effectively differentiate the isolates based on the obtained viabilities. From Fig. 4-24, we selected the optimized condition as 135 min (2 h 15 min) incubation with 15% v/v supernatant for undifferentiated Caco-2 cells. In order to compare

the effects of the supernatants between undifferentiated and differentiated Caco-2 cells, we used the same conditions for differentiated Caco-2 cells as well. These conditions are summarized in Table 4-5.

Table **4-5**: Conditions used in the WST-1 assay for Caco-2 cells

Parameter	Value
Seeding density of cells [(a)]	6.25×10^4 cells/cm^2
Concentration of cell-free *B. cereus* medium supernatant	15% v/v in cell culture medium [(b)]
Pre-incubation time of cells with only *B. cereus* supernatant	15 min
Volume of WST-1 dye added [(c)]	10 µl
Time of incubation of cells with WST-1 dye with the supernatant also present	120 min (2 h)
Wavelength for measurement of signal [(c)]	450 nm
Wavelength for measurement of background [(c)]	690 nm

[(a)] Conditions taken from Jeßberger et al., 2017.
[(b)] EMEM with 20% FBS.
[(c)] Conditions taken from manufacturer's (Roche) instructions.

4.6.5. Toxicity of B. cereus clade IV supernatants in undifferentiated Caco-2 cells

4.6.5.1. Effect on the viability of undifferentiated Caco-2 cells, studied using the WST-1 assay

After optimizing the concentration of supernatant and time for dye development, we proceeded by assessing the effect of the supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells using the WST-1 assay. We treated undifferentiated Caco-2 cells with 15% v/v *B. cereus* supernatants and conducted the WST-1 assay as per conditions listed in Table 4-5 on two separate plates. We also monitored the effect of appropriate controls (15% v/v MOD medium and 0.05% Triton X-100) with respect to the cell culture medium, as we had done with HeLa cells. Fig. 4-27 shows the effects of 15% v/v MOD medium and 0.05% Triton X-100 on Caco-2 cells as judged by percentage viabilities of the cells with respect to the cell culture medium (WST-1 assay) from two different plates. Relative to the cell culture medium, 0.05% Triton X-100 reduced the viability to 21%, while treatment with 15% v/v MOD medium gave a cell viability of about 68% (Fig. 4-27).

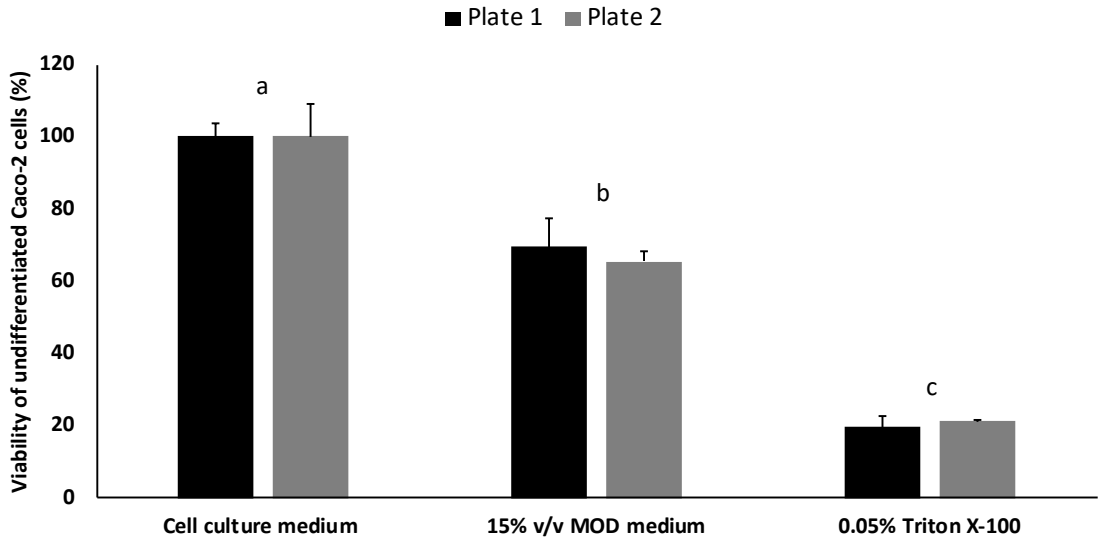

Figure **4-27**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 15% v/v MOD medium) on the viability of undifferentiated Caco-2 cells from two plates with respect to cell culture medium, as determined using WST-1 assay. Viability of cells treated with MOD medium has been calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with 15% v/v MOD medium to that of the cell culture medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had significantly different mean percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

The results of the WST-1 assay for undifferentiated Caco-2 cells treated with clade IV *B. cereus* supernatants indicates that the isolates can be segregated into statistically different groups based on the Tukey HSD method ($P < 0.05$; Fig. 4-28). Within the same grouping as the positive control (0.05% Triton X-100) were the *B. cereus* type strain ATCC 14579 (mean viability of 56%) and the isolate FSL R5-0184 (mean viability of 57%). These were hence considered to be the two of the most highly cytotoxic isolates (Fig. 4-28). A considerable number of isolates (FSL W8-0640, FSL K6-0043, FSL K6-0040, FSL W7-1334, and *B. thuringiensis* ATCC 10792) grouped in the same category as *B. cereus s.s.* ATCC 14579, but were not as cytotoxic as 0.05% Triton X-100 (Fig. 4-28).

Clair et al. (2010) exposed cultured Caco-2 cells to supernatants of *B. cereus s.s.* ATCC 14579 (cultured aerobically in triplicate) after diluting to a final protein concentration of 1.6 µg/ml in cell culture medium for 48 and 96 h. Using Sulforhodamine B assay, they saw a less than 25% survival of Caco-2 cells, even after a very long incubation time, which was an effect comparable to that observed in our study. Like our results here (Fig. 4-28), Jeßberger et al. (2015) also found using the WST-1 assay that the cytotoxicity of *B. cereus* supernatants towards Caco-2 cells was strain-specific (Jeßberger et al., 2015).

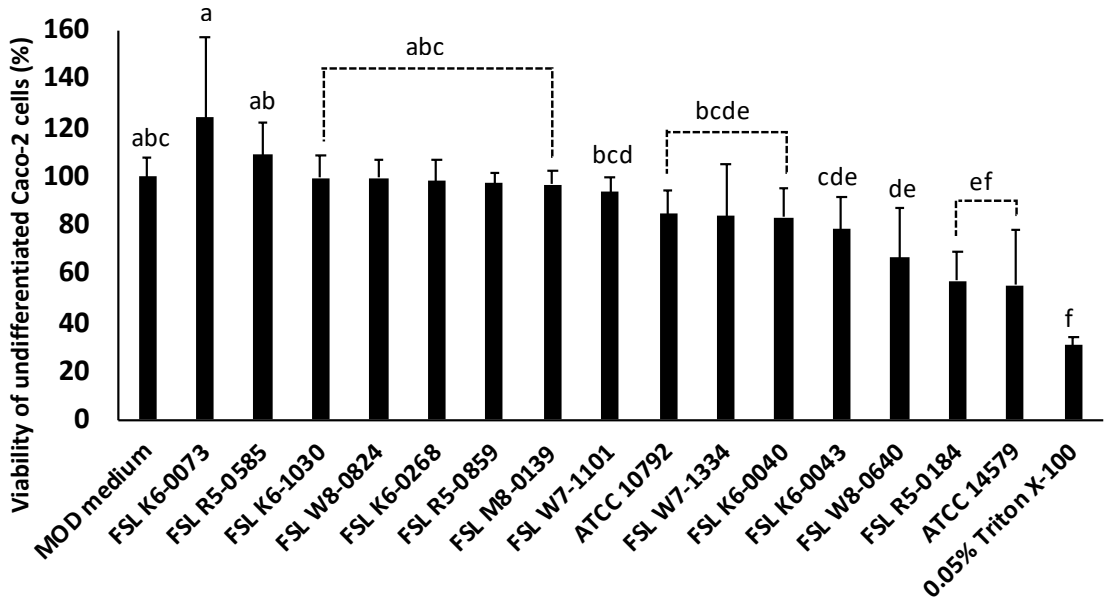

Figure **4-28**: Effect of 15% v/v supernatants of *B. cereus* clade IV isolates on the viability of undifferentiated Caco-2 cells respect to 15% v/v MOD medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for undifferentiated Caco-2 cells treated with *B. cereus* supernatants to that of the MOD medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

4.6.5.2. Effect on the cell membrane integrity of undifferentiated Caco-2 cells, studied using the LDH assay

Similar to studies with HeLa cells, we also wanted to test the cytotoxicity of supernatants *of B. cereus* clade IV isolates towards undifferentiated Caco-2 cells by LDH assay with appropriate controls to test for the effect of the MOD medium. The LDH assay would permit us to test for the effect of the *B. cereus* supernatants towards pore formation in the cell membranes of undifferentiated Caco-2 cells. The treatment conditions such as the total treatment time of Caco-2 cells with supernatant, and the supernatant concentration, were kept the same as that of the WST-1 assay. A summary of the conditions of the assay used has been summarized in Table 4-6.

Table **4-6**: Conditions used in the LDH assay for Caco-2 cells

Parameter	Value
Seeding density of cells [(a)]	6.25×10^4 cells/cm^2
Concentration of cell-free *B. cereus* medium supernatant	15% v/v in cell culture medium [(b)]
Incubation time of cells with *B. cereus* supernatant	135 min (2 h 15 m)
Volume of Caco-2 cell supernatant (spent medium) used for assay [(c, d)]	50 µl
Volume of LDH assay reagent added [(c, d)]	100 µl
Time of incubation of LDH assay reagent with the Caco-2 spent medium [(c)]	15 min
Wavelength for measurement of signal [(c, d)]	490 nm
Wavelength for measurement of background [(c, d)]	690 nm

[(a)] Conditions taken from Jeßberger et al., 2017.
[(b)] EMEM with 20% FBS.
[(c)] Conditions taken from Kumar et al., 2018.
[(d)] Conditions taken from Jan and Lashuel et al., 2012.

Fig. 4-29 shows the effects of the controls used in this assay (15% MOD medium and 0.05% Triton X-100) on the undifferentiated Caco-2 cell viability. The MOD medium did not seem to affect the Caco-2 cells relative to the cell culture medium in terms of compromising the membrane integrity of the cells ($P < 0.05$), as shown in Fig. 4-29. Hence, based on the WST-1 and LDH assay results, the MOD medium was hypothesized to impact the overall metabolic activity of the undifferentiated Caco-2 cells without causing pore formation in the cell membranes of the cells.

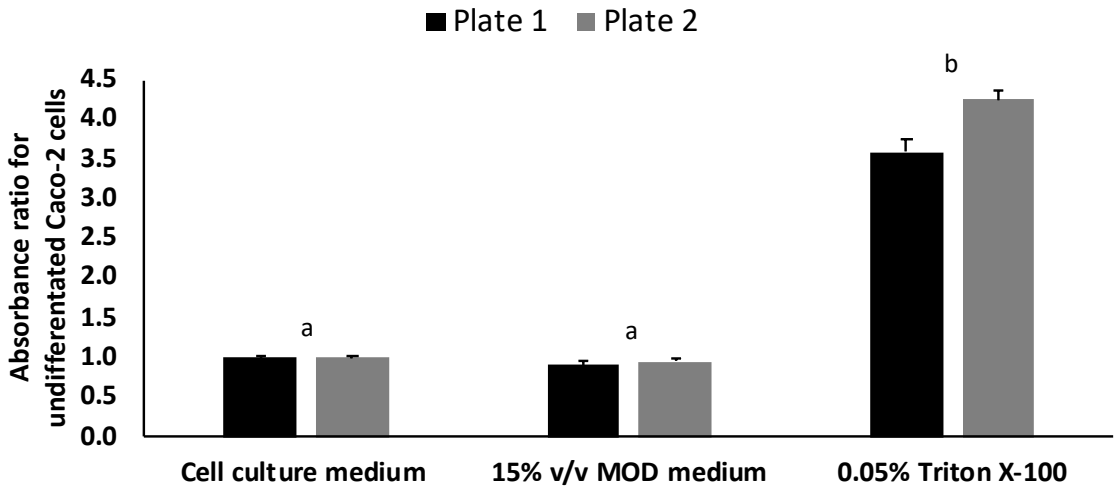

Figure **4-29**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 15% v/v MOD medium) on the absorbance ratio of solution relative to that of cell culture medium for undifferentiated Caco-2 cells from two plates, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had significantly different mean absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method.

The results of the effect of the *B. cereus* supernatants on the undifferentiated Caco-2 cells, as assessed by the LDH assay show that the isolates can be segregated into statistically different groups based on the Tukey HSD method ($P < 0.05$; Fig. 4-30). Triton X-100 (0.05%), being the positive control, showed the highest LDH release based on the highest absorbance ratio with respect

to MOD medium, and was placed in a statistical category by itself (Tukey $P < 0.05$), as expected. Among the clade IV isolates, FSL R5-0184 and *B. cereus s.s.* ATCC 14579 were placed in the highly cytotoxic category 'c' (Tukey $P < 0.05$), with mean absorbance ratios of 1.49 and 1.48 respectively (Fig. 4-30). Comparing with the WST-1 results, the isolates FSL R5-0184 and *B. cereus s.s.* ATCC 14579 were placed in the highly toxic category by both assays (Figures 4-28 and 4-30).

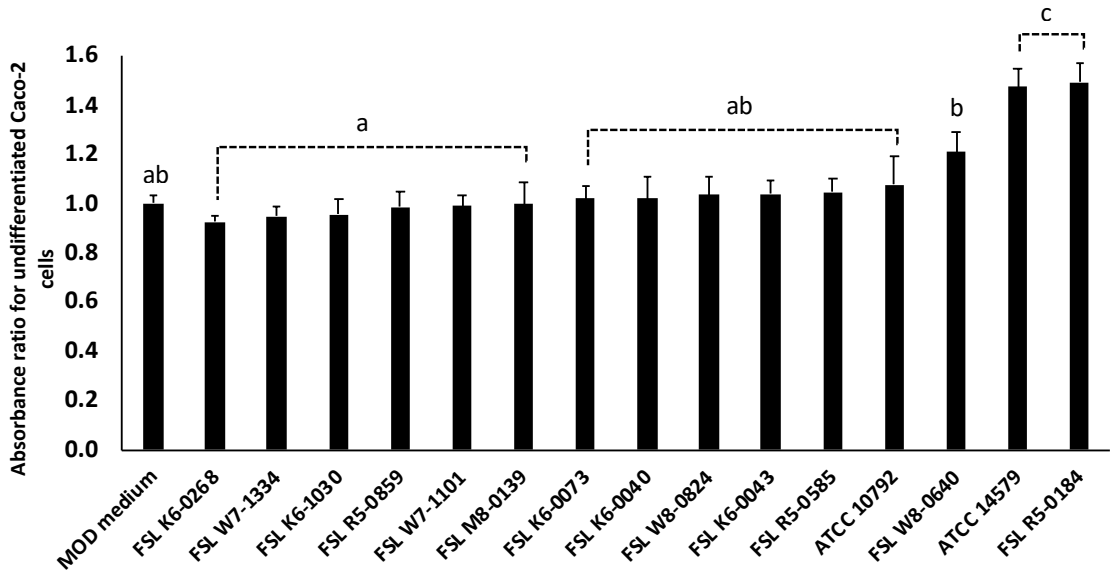

Figure **4-30**: Effect of 15% v/v supernatants of *B. cereus* clade IV isolates on the absorbance ratio of solution relative to that of 15% v/v MOD medium for undifferentiated Caco-2 cells, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of 6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method. 0.05% Triton X-100 is not shown on the graph because of its large value (4.22 ± 0.32; received the letter 'd' during statistical grouping based on the Tukey HSD method).

In order to assess strain-specific toxicity, Jeßberger et al. (2015) studied the effect of supernatants of different *B. cereus* isolates on Caco-2 cells via the PI influx assay and the WST-1 assay. It was seen that the ability to differentiate between isolates based on cytotoxicity was reduced when PI

assay was employed, compared to when the WST-1 assay was used (Jeßberger et al., 2015). We saw this in our study; while LDH assay placed only 2 out of 15 isolates in the highly toxic category, the WST-1 assay was able to place 7 isolates in the high toxicity category (based on the categorization into the same statistical group as the highly cytotoxic *B. cereus s.s.* ATCC 14579). Overall, for undifferentiated Caco-2 cells, we saw that the supernatants of the isolates which gave high ratios with the LDH assay i.e. FSL R5-0184 and *B. cereus s.s.* ATCC 14579, were also highly toxic by the WST-1 assay. However, the opposite was not found to be true, i.e. isolates that were placed in the highly toxic category by WST-1 assay did not necessarily (FSL W8-0640, FSL K6-0043, FSL K6-0040, FSL W7-1334, and *B. thuringiensis* ATCC 10792) cause a high degree of pore formation as judged by the LDH assay.

4.6.6. Toxicity of B. cereus clade IV supernatants in differentiated Caco-2 cells

4.6.6.1. Effect on the viability of differentiated Caco-2 cells, studied using the WST-1 assay

Caco-2 cells seeded and grown for 21 days in cell culture medium become differentiated, and they mimic the small intestinal epithelial barrier (Liévin-Le Moal and Servin, 2013; Natoli et al., 2012). Due to this, differentiated Caco-2 cells are often used as a model for studying the virulence/toxicity of human enteric pathogens (Liévin-Le Moal and Servin, 2013). Therefore, the WST-1 assay was performed on differentiated Caco-2 cells treated with supernatants of *B. cereus* clade IV isolates as per conditions enlisted in Table 4-5. The effects of 15% MOD medium and 0.05% Triton X-100 on the viability of differentiated Caco-2 cells are as shown in Fig. 4-31. As seen with undifferentiated Caco-2 cells in Section 4.6.5.1., 15% v/v MOD medium was found to decrease the

viability of cells when compared with the cell culture medium also in the case of differentiated Caco-2 cells ($P < 0.05$; Fig. 4-31).

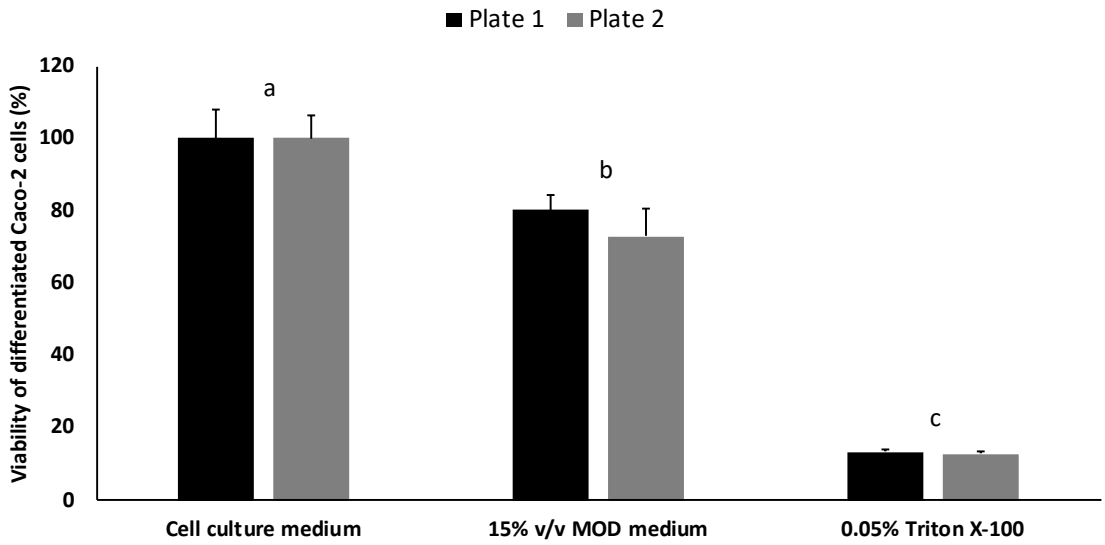

Figure **4-31**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 15% v/v MOD medium) on the viability of differentiated Caco-2 cells from two plates with respect to cell culture medium, as determined using WST-1 assay. Viability of cells treated with MOD medium has been calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for differentiated Caco-2 cells treated with 15% v/v MOD medium to that of the cell culture medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had significantly different mean percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

The viability of the differentiated Caco-2 cells exposed to *B. cereus* supernatants was expressed as a percentage viability of MOD-treated cells (Fig. 4-32). We calculated this based on the ratio of the corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) of the cells treated with the *B. cereus* supernatants relative to that of 15% v/v MOD medium. Statistical segregation of isolates could not be done based on their viabilities (Tukey $P < 0.05$), which could result from the high standard deviations observed during this assay (Fig. 4-32). The lowest mean viabilities were observed for *B. cereus s.s.* ATCC 14579 (78%) and FSL R5-0184 (79%), which were consistent with the findings

of the WST-1 assay results for undifferentiated Caco-2 cells (Fig. 4-28). We attempt to compare our results obtained from the WST-1 with those reported in literature. Minnaard et al. (2001) coincubated differentiated Caco-2 cells with supernatants of *B. cereus* cultures for 1 h, and reported a wide range of viabilities, ranging from 16.3% to 76.8% for strains 2 and 273, respectively. While they reported that these activities were calculated relative to the control (BHI medium), the authors did not report the concentration of supernatants used for this purpose. It may be assumed, considering they used 24-well plates and 0.5 ml filter sterilized supernatants per well (Minnaard et al., 2001), that the percentage was 100% v/v. This demonstrates that it is important to know the treatment times of cells with cell-free bacterial medium supernatants and concentrations of supernatants used, in order to compare results.

Castiaux et al. (2016) monitored the cytotoxic effects of *B. cereus* supernatants on differentiated Caco-2 cells via the use of the adenosine triphosphate (ATP) assay, which assesses the viability of cells through the luminescent quantification of the cytoplasmic ATP. They also monitored the viability of cells via the neutral red (NR) assay, which is based on the measurement of uptake of NR dye by lysosomes of metabolically active cells. They diluted the supernatants two times in Hank's Balanced Salt Solution (HBSS) buffer and incubated the cells with the supernatants for 3 h (for comparison, we had incubated cells with supernatants diluted to 15% v/v in cell culture medium for 2 h 15 min). Viabilities were calculated on the basis of 100% v/v HBSS buffer. For NR assay, viabilities ranged from 0.84% (strain FM1) to 103.99% (strain AH621), with *B. cereus s.s.* ATCC 14579 showing 1.59% viability (which was very low compared to 78% viability we reported with *B. cereus s.s.* ATCC 14579). In the ATP assay, only a few isolates were tested, with the range being 0.11% viability (strain 45) to 53.76% (NVH883-00). In the ATP assay, the cytotoxicity of *B. cereus s.s.* ATCC 14579 was found to be 0.37% (which was again low compared to 78% viability we reported with *B. cereus s.s.* ATCC 14579). However, while they tested the cytotoxicity of the bacterial growth medium used (Luria-Bertani broth) on Caco-2 cells via the NR

assay, they did not use it to calculate percentage viabilities; we had used 15% v/v MOD medium as the basis for calculating the viability of cells. Moreover, they replaced the cell culture medium (EMEM with 10% FBS) completely with HBSS buffer and used this as a negative control as well as for diluting the supernatants in without keeping part of the cell culture medium with the cells. This may have drastically affected the viability of cells as a result, and the meaningfulness of the data due to a lack of appropriate controls for the experiment.

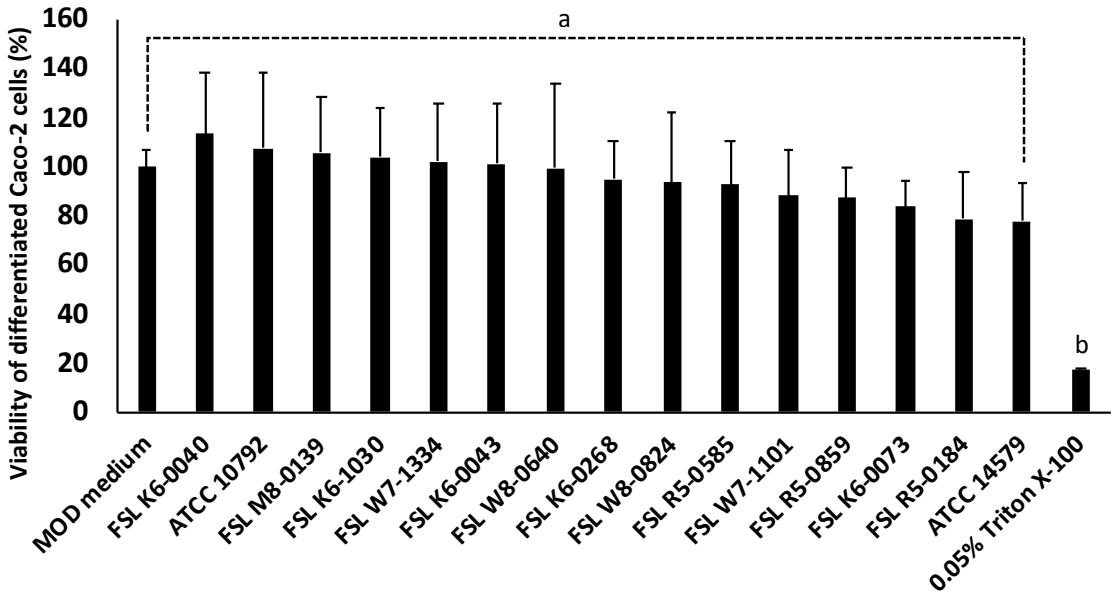

Figure **4-32**: Effect of 15% v/v supernatants of *B. cereus* clade IV isolates on the viability of differentiated Caco-2 cells respect to 15% v/v MOD medium, as determined using WST-1 assay. Viability of cells was calculated by taking the ratio of corrected absorbances ($A_{450\ nm} - A_{690\ nm}$) obtained for differentiated Caco-2 cells treated with *B. cereus* supernatants to that of the MOD medium and converting to percentages. 0.05% Triton X-100 was used as a positive control. Columns show the mean viability of 6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different percentage viability values ($P < 0.05$), as determined by Tukey HSD method.

4.6.6.2. Effect on the cell membrane integrity of differentiated Caco-2 cells, studied using the LDH assay

As we had done for undifferentiated Caco-2 cells, in order to assess the pore-forming effect of supernatants of the *B. cereus* clade IV isolates, we performed the LDH assay on the differentiated Caco-2 cells using as per conditions listed in Table 4-6. We found MOD medium at 15% v/v concentration to have no significant effect on the cell membrane integrity of Caco-2 cells based on the ratio of corrected absorbances of solutions obtained by treating differentiated Caco-2 cells with 15% v/v MOD medium relative to that of the cell culture medium ($P < 0.05$; Fig. 4-33). The average ratio obtained for 15% MOD medium relative to that of the cell culture medium was found to be 1.25. Hence, 15% v/v MOD medium decreased the overall metabolic activity of the cells (Section 4.6.6.1.), without causing pore formation in the cell membranes of differentiated Caco-2 cells ($P < 0.05$), a result also shared by undifferentiated Caco-2 cells as previously discussed (Section 4.6.5.).

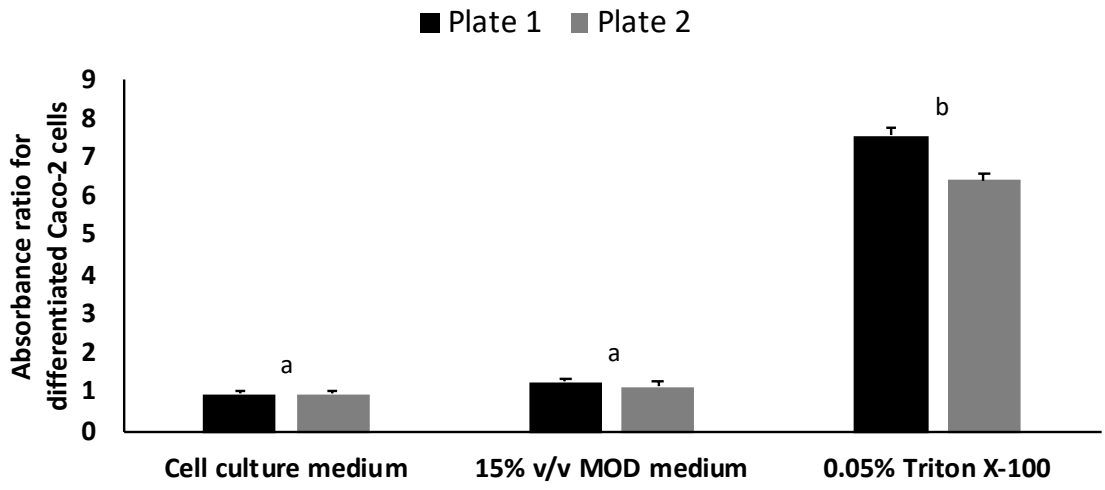

Figure **4-33**: Effect of controls (cell culture medium, 0.05% Triton X-100, and 15% v/v MOD medium) on the absorbance ratio of solution relative to that of cell culture medium for differentiated Caco-2 cells from two plates, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\ nm} - A_{690\ nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of 3 replicates for a given plate, while error bars show the standard deviation. Controls that do not share a common alphabetic character had

significantly different mean absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method.

The effect of the cell-free medium supernatants of *B. cereus* clade IV isolates on the differentiated Caco-2 cells as assessed by the LDH assay yielded better segregation of isolates (Fig. 4-34) compared to the WST-1 assay (Fig. 4-32). Based on the Tukey post-hoc test, we categorized the isolates as highly toxic (indicated with letter 'e'; Tukey $P < 0.05$; FSL R5-0184, *B. cereus s.s.* ATCC 14579, and FSL R5-0585); medium toxic (indicated by letter 'd'; Tukey $P < 0.05$; FSL K6-0043, FSL K6-0040, FSL K6-0073, FSL R5-0859, *B. thuringiensis* ATCC 10792, FSL W8-0824, and FSL W8-0640); and low or non-toxic (those not indicated by letter 'd', nor the letter 'e'; Tukey $P < 0.05$) towards differentiated Caco-2 cells (Fig. 4-34). The two isolates that gave highest mean LDH ratios (FSL R5-0184 and *B. cereus s.s.* ATCC 14579; Fig. 4-34) were the sames ones that resulted in the lowest mean viabilities of differentiated Caco-2 cells as judged by the WST-1 assay (Fig. 4-32).

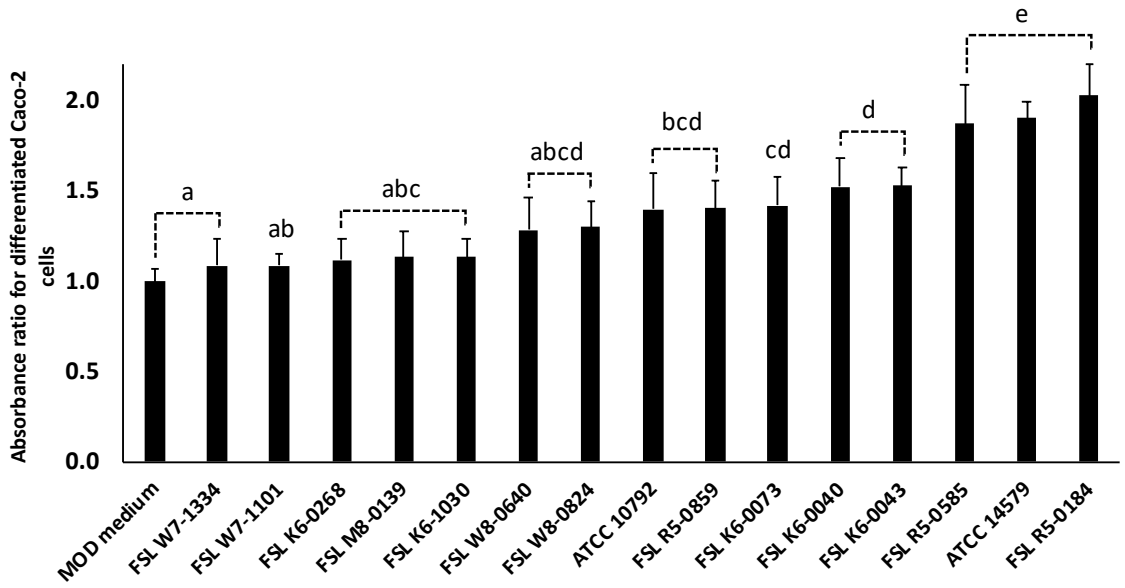

Figure **4-34**: Effect of 15% v/v supernatants of *B. cereus* clade IV isolates on the absorbance ratio of solution relative to that of 15% v/v MOD medium for differentiated Caco-2 cells, as determined using LDH assay. For the ratio calculation, the corrected absorbances ($A_{490\,nm} - A_{690\,nm}$) were used. 0.05% Triton X-100 was used as a positive control. Columns show the mean absorbance ratios of

6 replicates (3 replicates from each of two plates), while error bars show the standard deviation. Any two columns that do not share a common alphabetic character had significantly different absorbance ratio values ($P < 0.05$), as determined by Tukey HSD method. 0.05% Triton X-100 is not shown on the graph because of its large value (5.62 ± 0.24; received the letter 'f' during statistical grouping based on the Tukey HSD method).

The conclusions with reference to the LDH assay with differentiated Caco-2 cells are that all but one isolate (FSL W7-1334) caused loss of cell membrane integrity, and this activity varied across isolates (Fig. 4-34), and isolates could be grouped based on this ability as judged by the LDH assay. However, the differences in the viability of the cells was less apparent between supernatants of different isolates (as judged by the WST-1 assay; Fig. 4-32). Castiaux et al. (2016) observed that with strains that were classified to be low toxic, the NR assay (which measures viability of cells) was more sensitive in detecting the harmful effects of these strains compared to the LDH assay. On the other hand, strains that were classified by the LDH assay as highly toxic were found to be of medium toxicity via the NR assay (Castiaux et al., 2016).

In our study, differentiated Caco-2 cells were found to be more resistant to the *B. cereus* supernatants, as indicated by the lowest recorded viability of 78% for *B. cereus s.s.* ATCC 14579, compared to the lowest viability of 56% again for *B. cereus s.s.* ATCC 14579 obtained for the undifferentiated Caco-2 cells using the WST-1 assay and the same dosing conditions. However, the LDH assay showed an opposite trend, i.e., differentiated Caco-2 cells gave higher absorbance ratios with respect to MOD medium on treatment with *B. cereus* supernatants (highest ratio of 2.03, for FSL R5-0184) than undifferentiated Caco-2 cells (highest ratio of 1.49, again for FSL R5-0184). This may be because of the higher density and state of cells in a plate of differentiated Caco-2 cells compared with that of undifferentiated Caco-2 cells, and hence a higher concentration of the LDH end product being produced in the solution in the former than the latter when treated with *B. cereus* supernatants. There seems to be no literature on the absolute levels of LDH in differentiated Caco-

2 cells compared to undifferentiated Caco-2 cells to suggest that differentiated cells have higher levels of LDH.

To the best of our knowledge, there appears to be no report on a comparative assessment of the effects of bacterial supernatants on the differentiated and the undifferentiated Caco-2 cell lines. Gerloff et al. (2013) studied the toxic potential of SiO_2 and ZnO nanoparticles on differentiated and undifferentiated Caco-2 cells using the WST-1 assay. They found that the observed cytotoxicity depended on the Caco-2 cells' differentiation status. Further, it was seen that while SiO_2 decreased viability of only undifferentiated cells, ZnO was cytotoxic to both, undifferentiated and differentiated Caco-2 cells after 24 h treatment (Gerloff et al., 2013). The observation that the sensitivity of Caco-2 cells in their differentiated and undifferentiated forms depends on the toxins themselves is also demonstrated by Puerto et al. (2009), who compared the toxicity of pure cyanobacterial toxins microcystin-RR and microcystin-YR on differentiated and undifferentiated Caco-2 cells using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt (MTS) assay (used for studying cell viability). The MTS assay results showed that the sensitivity of the cell lines to these toxins was affected by the exposure time to the toxin (24 h vs. 48 h) to a larger extent than it was by the differentiation status of the cells, with microcystin-YR found to be more toxic than microcystin-RR (Puerto et al., 2009). In our results, we show that the supernatants of different isolates gave different cytotoxicities in the undifferentiated Caco-2 cell line, which, taken together with the findings of Puerto et al., 2009, suggests that there might be different levels of toxins or different toxins being secreted by the *B. cereus* isolates that is driving the differences in observed cytotoxicities.

Zhang et al. (2015) found after a 72 h exposure time that the mycotoxin aflatoxin B1 was more toxic compared to aflatoxin M1 on both undifferentiated and differentiated Caco-2 cells based on the MTT and LDH assays, with the differentiated cells more sensitive to the aflatoxin B1 compared to the undifferentiated cells by both assays (Zhang et al., 2015) at the same dosing

conditions (1 µg/ml). It may be worthwhile to note that when a 24 h exposure time with aflatoxin B1 (1 µg/ml) was studied, the differentiated cells were as sensitive to the toxin as the undifferentiated cells by the LDH assay, but less sensitive to the toxin compared to undifferentiated Caco-2 cells based on the MTT assay (Zhang et al., 2015), again shedding light on the importance of exposure time of cells to toxins. While we found differentiated Caco-2 cells to be more sensitive to 15% v/v *B. cereus* supernatants compared to the undifferentiated Caco-2 cells by the LDH assay, the opposite trend was observed for the WST-1 assay for a 2 h 15 min exposure.

The aforementioned studies highlight the need for the study of longer exposure times of differentiated Caco-2 cells with *B. cereus* supernatants at different dosing conditions to be able to statistically distinguish the isolates based on their WST-1 assay responses better.

Overall, we saw that the two most cytotoxic *B. cereus* clade IV isolates shown by WST-1 assay (FSL R5-0184 and *B. cereus s.s.* ATCC 14579) were also the two most cytotoxic isolates by LDH assay in Caco-2 cells, regardless of the differentiation status (Sections 4.6.5. and 4.6.6.). While supernatant of *B. cereus s.s.* ATCC 14579 was cytotoxic even to HeLa cells, the isolate FSL R5-0184 was not found to be among the highly cytotoxic isolates in HeLa cells (Section 4.6.3.) as judged by both WST-1 and LDH assays.

4.6.7. Estimation of protein content and immunoassay detection of Hbl and Nhe components in supernatants of B. cereus clade IV isolates

In order to correlate cytotoxicity results with protein content, and also prepare proteins extracted from bacterial supernatants for proteomics analysis (Appendix A), we measured the protein content of the harvested supernatants of *B. cereus* clade IV isolates (grown in MOD medium) via the Bradford assay. First, we prepared a standard curve (Fig. 4-35) using 1.25 - 20 µg/ml of bovine serum albumin, and the corresponding absorbance values measured at 595 nm. The resulting equation was further used for measuring concentration of proteins in the *B. cereus* clade IV isolates'

supernatants, which are summarized in Table 4-7. The concentration of secreted proteins in MOD medium ranged from about 9.6 to 18.6 µg/ml. Jeßberger et al. (2015) had previously reported a secreted protein concentration of 20 to 60 µg/ml when *B. cereus* strains were grown in casitone glycerol yeast autolysate medium at 30 °C at 125 rpm, which is in the same order of magnitude as what we obtained.

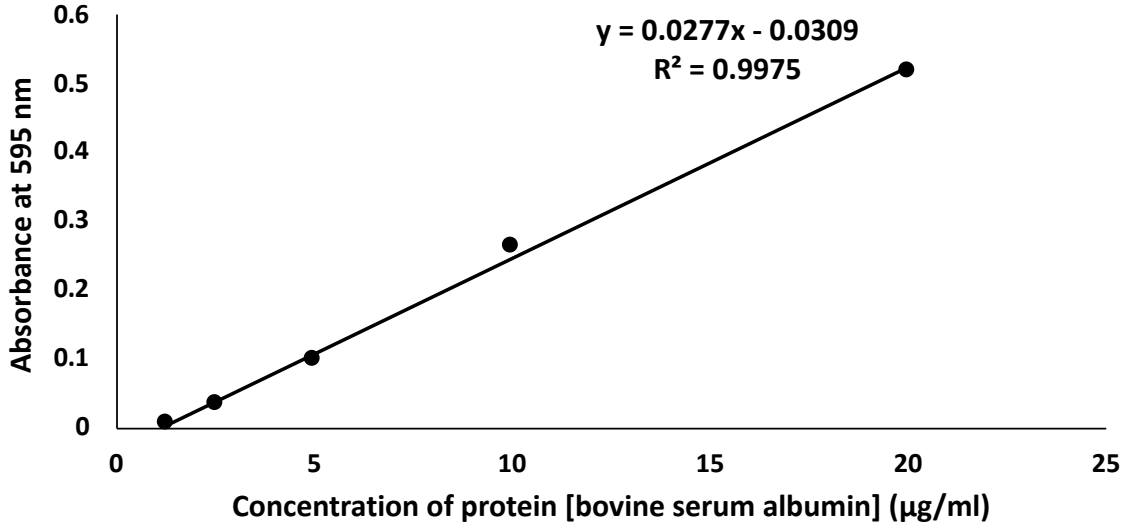

Figure **4-35**: Standard curve describing the linear relationship between absorbance measured at 595 nm as a function of the concentration of protein (bovine serum albumin), obtained via the Bradford assay.

We also tested the supernatants of *B. cereus* clade IV isolates grown in MOD medium for the presence of NheB and Hbl-L_2 components via qualitative immunoassay detection using Duopath kits. Results are shown in Table 4-7. For comparison, we have shown the results from the same clade IV isolates grown in BHI broth as previously published (Miller et al., 2018) in the same table (Table 4-7). NheB toxin component could not be detected in supernatants of isolates FSL K6-1030, FSL M8-0139, and FSL K6-0268 when they were grown in MOD medium (Table 4-7), while they could be detected when they were grown in BHI medium (Miller et al., 2018), however, some

of these differences may be due to different nutrient profiles of the growth media, and culture incubation times used. It may also be possible that the toxin components were produced in MOD medium in quantities that were below the detection limit of the immunoassay (6 ng/ml for NheB and 20 ng/ml for the Hbl-L_2; Krause et al., 2010). Another difference in the detection of the toxins that was noticed was that while in BHI medium, the Hbl-L_2 toxin was not detected when isolate FSL R5-0585 was cultured (Miller et al., 2018), this toxin was detected as part of the supernatant when the isolate was grown in MOD medium (Table 4-7). The qualitative detection results of all the other isolates were the same when grown in BHI medium (Miller et al., 2018), and in MOD medium (Table 4-7).

Table **4-7**: Detection of NheB and Hbl-L_2 using the Duopath immunoassay kit, and estimated protein content in *B. cereus* supernatants of clade IV isolates

B. cereus clade IV isolate	NheB detection in supernatant of *B. cereus* grown in		Hbl-L_2 detection in supernatant of *B. cereus* grown in		Total protein content in supernatant (µg/ml)
	MOD	BHI [(a)]	MOD	BHI [(a)]	
B. cereus s.s. ATCC 14579	+	+	+	+	14.05
FSL K6-1030	-	+	-	-	13.36
FSL M8-0139	-	+	-	-	9.61
FSL K6-0268	-	+	-	-	9.75
B. thuringiensis ATCC 10792	+	+	+	+	17.94
FSL W8-0640	+	+	+	+	13.97
FSL W7-1334	-	-	+	+	9.72

FSL W8-0824	+	+	+	+	17.90
FSL W7-1101	+	+	+	+	18.01
FSL K6-0043	+	+	+	+	18.59
FSL K6-0040	+	+	+	+	13.79
FSL R5-0859	+	+	+	+	17.69
FSL R5-0184	+	+	+	+	16.75
FSL R5-0585	+	+	+	-	12.71
FSL K6-0073	+	+	+	+	15.56

[(a)] Results taken from Miller et al., 2018.

'+' denotes that the *B. cereus* isolate was positive for a given virulence factor, while '-' denotes that the virulence factor was not detected.

4.6.8. Toxin gene profiles of the B. cereus clade IV isolates

The virulence gene profile of the clade IV isolates that were being tested was found using the whole genome sequences of the isolates and the software BTyper (version 2.3.0), an open-source *B. cereus* group subtyping tool for the detection of virulence genes (Carroll et al., 2017). A total of 27 putative virulence genes were identified through BTyper. These were *entFM* (encoding enterotoxin FM), *bceT* (*B. cereus* enteroxin T), *clo* (cereolysin O), *hblC* (Hbl lytic component L_2), *plcA* (1-phosphatidylinositol phosphodiesterase precursor), *entA* (enterotoxin/cell wall binding protein), *hblD* (Hbl lytic component L_1), *inhA2* (an immune inhibitor A precursor), *bpsE* (*B. cereus* exopolysaccharide operon gene E; UTP-glucose-1-phosphate uridylyltransferase), *cerA* (cereolysin A), *nheC* (Nhe component C), *cerB* (cereolysin B), *hblB* (Hbl binding component precursor), *bpsH* (*B. cereus* exo-polysaccharide operon gene H LytR family transcriptional regulator), *inhA1* (an immune inhibitor A precursor), *nheA* (Nhe component A), *nheB* (Nhe component B), *hblA* (Hbl binding component), *plcB* (phospholipase C), *sph* (sphingomyelinase C), *plcR* (transcriptional

activator), *cytK-2* (cytotoxin K), *bpsF* (*B. cereus* exopolysaccharide operon gene F; tyrosine protein kinase), *bpsD* (*B. cereus* exopolysaccharide operon gene D; glycosyl transferase), *cesC* (ABC transporter ATP-binding protein), *hlyII* (hemolysin II), and *hlyR* (hemolysin II regulatory protein). Of these genes, only the genes which showed differences in terms of presence/absence across the 15 isolates are represented in Fig. 4-36. The rest of the genes, i.e. *entFM*, *clo*, *plcA*, *entA*, *inhA2*, *bpsE*, *nheC*, *cerB*, *bpsH*, *inhA1*, *nheA*, *nheB*, *plcR*, and *bpsF* were present in the sequences of all of the isolates. The *B. anthracis* hyaluronic acid capsule *hasA* gene was not detected in any of the isolates.

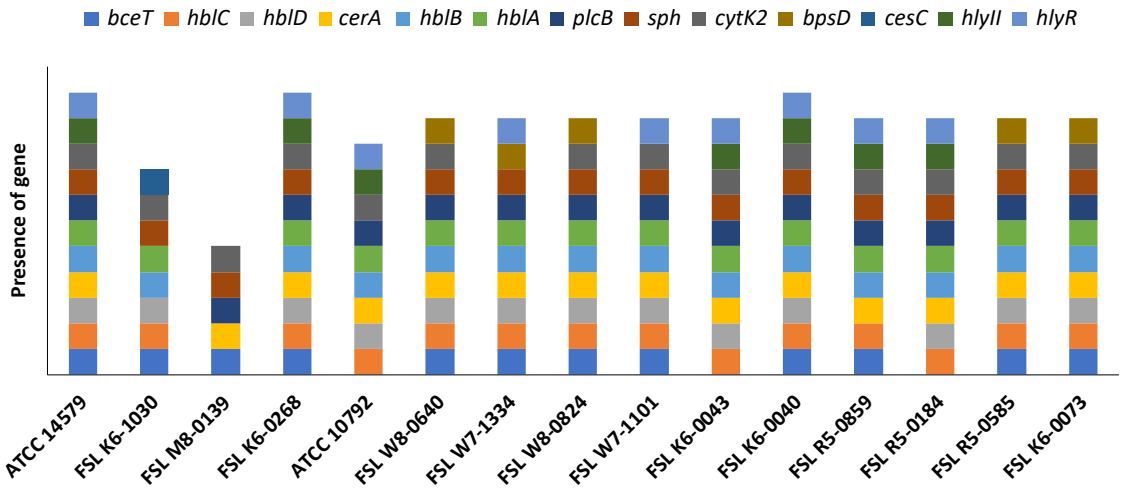

Figure **4-36**: Virulence gene profile of *B. cereus* clade IV isolates. Only genes which showed differences in terms of presence/absence across the 15 isolates have been represented.

4.6.9. Associations between presence of virulence genes, detection of enterotoxins, and cytotoxicity

The possible associations between the WST-1 and LDH assay results for different cell lines with the virulence genes, protein content in supernatant, immunoassay results, and the time of harvest of supernatants (time taken for isolates to reach stationary phase) were assessed via the

Pearson's correlation test using results from all 15 isolates of the *B. cereus* clade IV (Fig. 4-37). For the ease of interpretation of results, the cell viabilities obtained for WST-1 assays were converted to cell inhibition via the formula: % inhibition = 100 - % viability. Hence, a higher % inhibition would indicate a higher cytotoxicity of the isolate towards cells via the WST-1 assay. A higher ratio obtained in the LDH assay results would also indicate a higher cytotoxicity of the isolate towards cells as assessed by the LDH assay. During analysis in R version 3.5.2 (R Core Team, 2018), the presence or absence of genes were indicated by numeric vectors (Appendix B), with '1' corresponding to the gene absent, and '2' corresponding to the gene present. A similar system was adopted for the presence ('2') or absence ('1') of Hbl-L_2 and NheB components in the supernatants of the isolates, detected using the Duopath immunoassay kits.

The time of culture harvest (i.e., time taken for isolates to reach stationary phase) as well as the presence of the virulence gene *cytK-2* did not correlate with any of the tested factors (Fig. 4-37) at significance level of 0.05.

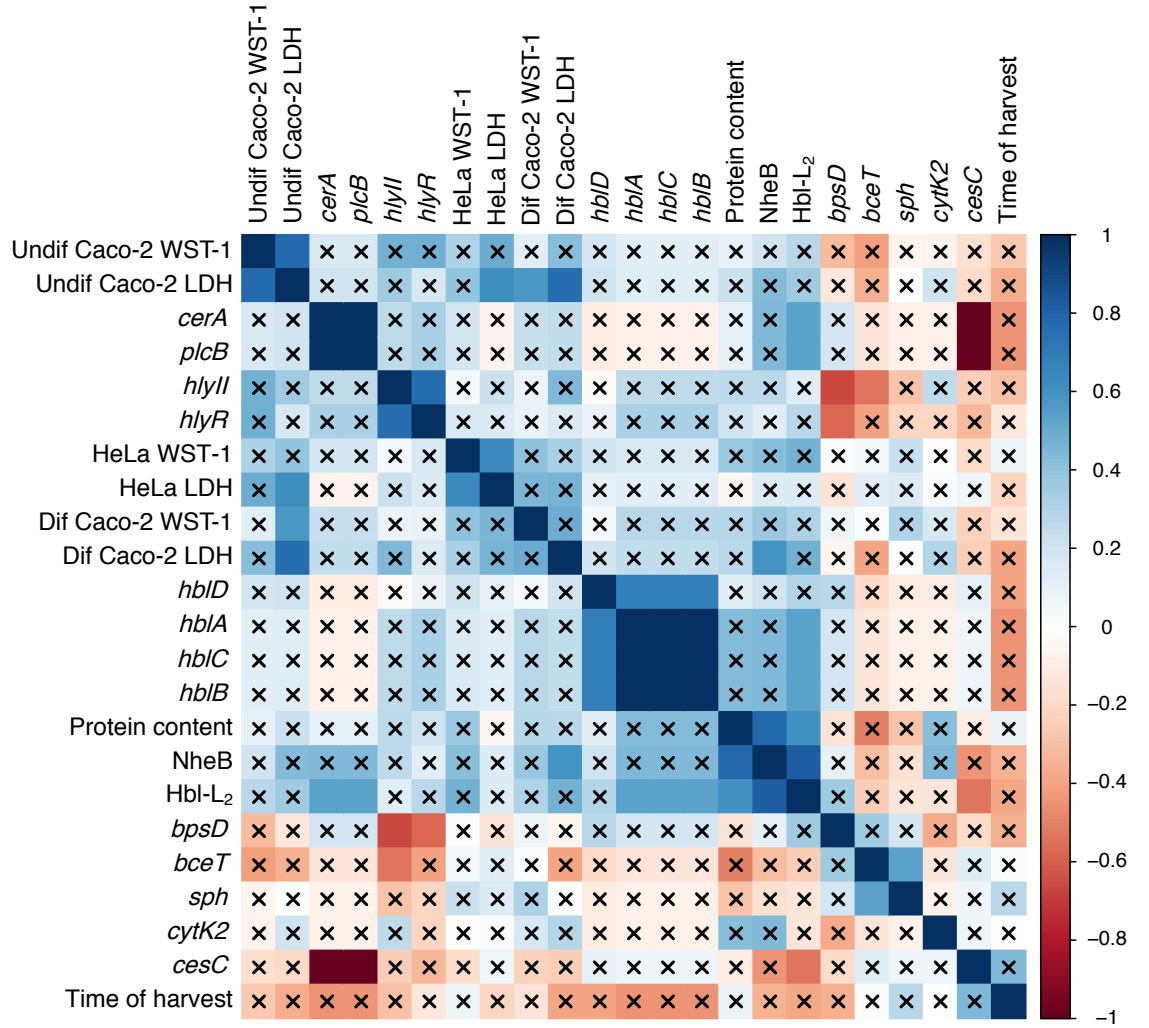

Figure **4-37**: Correlation plot of the WST-1 and LDH assay results for different cell lines with the virulence genes, protein content in supernatant, detection of NheB and Hbl-L_2 in supernatant, and time of harvest. The WST-1 assay mean viabilities were converted to mean inhibition percentages (% inhibition = 100 - % viability) to make the correlations with LDH assay results easier to read. Blue boxes show positive correlations, while red boxes show negative correlations. The intensity of the color represents the magnitude of the respective correlation coefficient – higher the intensity of color, higher is the magnitude of correlation coefficient. This is shown by the scale on the right of the plot. The color on the scale indicates positive (blue) or negative (red) correlations. Boxes which are not crossed out have statistically significant ($P < 0.05$) correlations, as determined by the Pearson's correlation test.

A number of statistically significant associations were found (correlations that are not crossed out in Fig. 4-37). These are described below.

The results of the LDH assay with undifferentiated Caco-2 cells were found to be concordant (P = 0.0008) with the results from the WST-1 assay with undifferentiated Caco-2 cells (correlation coefficient, r = + 0.7712), which could indicate that in undifferentiated Caco-2 cells, the pore-forming nature of the toxins present in the supernatants could contribute to a large extent to the reduced viability of the cells. The same trend was observed with HeLa cells (P = 0.0093; r = + 0.6460). In addition, the results from the LDH assay with undifferentiated Caco-2 cells were also concordant with the LDH assay results from the HeLa cells (P = 0.0149; r = + 0.6140) as well as those from the differentiated Caco-2 cells (P = 0.0008; r = + 0.7680). This could hint at the nature of the pore-forming toxins, which seem to affect all the cell lines studied in a similar manner. Interestingly, the WST-1 assay results from the differentiated Caco-2 cells were also concordant with the LDH assay results with undifferentiated Caco-2 cells (P = 0.0255; r = + 0.5731), which could just be an association observed. We also ran the Pearson's correlation test for checking concordance of the WST-1 and LDH assay results we obtained in HeLa cells with those of the PI assay results in HeLa cells as published previously (Miller et al., 2018). The HeLa cell results from PI assay were not concordant with those of LDH and WST-1 assays at a significance level of 0.05. The non-agreement in results could be driven in part by the differences in the growth medium used in the two cases (Miller et al. (2018) cultured the isolates in BHI medium, while we cultured them in MOD medium).

The cytotoxicity assay results obtained in HeLa, undifferentiated, and differentiated Caco-2 results were not found to be neither associated with presence of virulence genes nor with the detection of Hbl-L_2. Further, the LDH assay results obtained from the differentiated Caco-2 cells were found to associate with the qualitative detection of NheB (P = 0.0182; r = + 0.5995) in the supernatant using Duopath immunoassay kits, which could hint at the role of NheB in the pore-formation of differentiated Caco-2 cells. In all other assays and cell lines studied, there was no significant association between cytotoxicity results and NheB detection.

In some cases, virulence gene and enterotoxin (Hbl-L_2 and NheB) component detection were significantly associated with each other. With reference to the *hbl* operon, the presence of all constituent genes of the operon (*hblABCD*) were found to be positively correlated with each other ($P < 0.05$; Table 4-8). The immunoassay-based detection of the Hbl-L_2 component (encoded by *hblC*) in the supernatant of the isolates was also positively correlated with all the genes of the *hbl* operon, although with *hblD* (which encodes Hbl-L_1), the effect was not found to be significant. The detection of Hbl-L_2 in the supernatant was also positively correlated ($P = 0.0401$) with the *cerA* and *plcB* genes ($r = + 0.5345$ for both associations), and negatively correlated with the *cesC* gene ($r = - 0.5345$). The *cesC* gene is actually a part of the plasmid pCER270 and forms a part of the *ces* operon that is responsible for cereulide biosynthesis (Ehling-Schulz et al., 2006, 2015). This gene was detected in only one of the isolates (FSL K6-1030; Fig. 4-36); this isolate needs to be further studied to determine if it is indeed an emetic isolate. The presence of *cesC* gene was also found to negatively correlate with the presence of *cerA* and *plcB* genes ($P < 2.2 \times 10^{-16}$; $r = - 1$), with the presence of *cerA* and *plcB* genes being positively correlated to one another ($P < 2.2 \times 10^{-16}$; $r = + 1$). The latter can be explained because with *B. cereus*, the cytolytic determinant cereolysin AB comprises of nothing but phospholipase C (encoded by *plcB*) and sphingomyelinase (*sph*), and hence the genes names *cerA* and *cerB* can be used interchangeably with *plcB* and *sph*, respectively (Gilmore et al., 1989). The hemolysin II regulator protein gene *hlyR* was positively correlated with the hemolysin II gene *hlyII* as expected ($P = 0.0009$; $r = + 0.7638$). The presence of *hlyII* and *hlyR* genes was negatively associated with that of the *bpsD* gene ($P < 0.05$; Table 4-8). Additionally, the *bceT* gene presence was negatively associated with the *hlyII* gene presence, but positively associated with the presence of the *sph* gene ($P < 0.05$; Table 4-8). These could be chance associations.

Lastly, we observed that the detection of Hbl-L_2 component in the supernatant, the detection of NheB component in the supernatant, and the protein content of the supernatant, were

all positively correlated with one another ($P < 0.05$; Table 4-8). A summary of the all the statistically-significant associations is presented in Table 4-8.

Table **4-8**: Pearson correlation coefficients for significant ($P < 0.05$) associations between WST-1 and LDH assay results for different cell lines, the presence of various virulence genes, protein content in supernatant, and detection of detection of NheB and Hbl-L_2 in supernatants of *B. cereus* clade IV isolates

Factor-1	Factor-2	Correlation coefficient, r	P value
% Inhibition of undifferentiated Caco-2 cells via WST-1 assay	Ratio obtained for undifferentiated Caco-2 cells in LDH assay	+ 0.7712	0.0008
Ratio obtained for undifferentiated Caco-2 cells in LDH assay	Ratio obtained for HeLa cells in LDH assay	+ 0.6140	0.0149
Ratio obtained for undifferentiated Caco-2 cells in LDH assay	% Inhibition of differentiated Caco-2 cells via WST-1 assay	+ 0.5731	0.0255
Ratio obtained for undifferentiated Caco-2 cells in LDH assay	Ratio obtained for differentiated Caco-2 cells in LDH assay	+ 0.7680	0.0008
Presence of *cerA* gene	Presence of *plcB* gene	+ 1.0000	$< 2.2 \times 10^{-16}$
Presence of *cerA* gene	Presence of Hbl-L_2 in supernatant	+ 0.5345	0.0401
Presence of *cerA* gene	Presence of *cesC* gene	- 1.0000	$< 2.2 \times 10^{-16}$

Presence of *plcB* gene	Presence of Hbl-L_2 in supernatant	+ 0.5345	0.0401
Presence of *plcB* gene	Presence of *cesC* gene	- 1.0000	$< 2.2 \times 10^{-16}$
Presence of *hlyII* gene	Presence of *hlyR* gene	+ 0.7638	0.0009
Presence of *hlyII* gene	Presence of *bpsD* gene	- 0.6614	0.0072
Presence of *hlyII* gene	Presence of *bceT* gene	- 0.5345	0.0401
Presence of *hlyR* gene	Presence of *bpsD* gene	- 0.5774	0.0242
% Inhibition of HeLa cells via WST-1 assay	Ratio obtained for HeLa cells in LDH assay	+ 0.6460	0.0093
Ratio obtained for differentiated Caco-2 cells in LDH assay	Presence of NheB in supernatant	+ 0.5995	0.0182
Presence of *hblD* gene	Presence of *hblA* gene	+ 0.6814	0.0052
Presence of *hblD* gene	Presence of *hblC* gene	+ 0.6814	0.0052
Presence of *hblD* gene	Presence of *hblB* gene	+ 0.6814	0.0052
Presence of *hblA* gene	Presence of *hblC* gene	+ 1.0000	$< 2.2 \times 10^{-16}$
Presence of *hblA* gene	Presence of *hblB* gene	+ 1.0000	$< 2.2 \times 10^{-16}$
Presence of *hblA* gene	Presence of Hbl-L_2 in supernatant	+ 0.5345	0.0401
Presence of *hblC* gene	Presence of *hblB* gene	+ 1.0000	$< 2.2 \times 10^{-16}$
Presence of *hblC* gene	Presence of Hbl-L_2 in supernatant	+ 0.5345	0.0401
Presence of *hblB* gene	Presence of Hbl-L_2 in supernatant	+ 0.5345	0.0401

Protein content in supernatant	Presence of NheB in supernatant	+ 0.7834	0.0005
Protein content in supernatant	Presence of Hbl-L_2 in supernatant	+ 0.6016	0.0177
Presence of NheB in supernatant	Presence of Hbl-L_2 in supernatant	+ 0.8292	0.0001
Presence of Hbl-L_2 in supernatant	Presence of *cesC* gene	- 0.5345	0.0401
Presence of *bceT* gene	Presence of *sph* gene	+ 0.5345	0.0401

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Chapter 5.

Conclusions and Future Directions

The *Bacillus cereus* group of pathogens is widespread and comprises of several different species. While the mechanism of pathogenicity is well understood for the emetic strains of *B. cereus*, those causing diarrhea are poorly characterized. It is seen from literature that the toxicity depends on growth conditions of the bacteria, and the cell lines used. Hence, we aimed to optimize cytotoxicity assays for *B. cereus* group isolates. The 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assay was found to be a more appropriate assay for assessing cytotoxicity of *B. cereus* supernatants than the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay when HeLa cervical cancer cells were used, as the detachment of the cells did not affect the results as it did in the MTT assay. We optimized the WST-1 assay specific for rapid screening of *B. cereus* isolate supernatants on HeLa cells and used it to assess cytotoxicity of the 2016 foodborne outbreak-associated isolates, and *B. cereus* group type strains, including the type strains of the 9 novel *B. cereus* group species. Using an immunoassay-based method, we identified *B. albus* N35-10-2 as the only novel species that produced detectable amounts of Hbl.

We also optimized the WST-1 and lactate dehydrogenase (LDH) assays to characterize cytotoxicity of *B. cereus* clade IV dairy isolates, using both differentiated and undifferentiated Caco-2 human epithelial colorectal adenocarcinoma cells, as well as HeLa cells. Across all the assays, *B. cereus sensu stricto* ATCC 14579 was found to be one of the most toxic.

Future studies could utilize the WST-1 and LDH assays, optimized here, for screening more *B. cereus* isolates, both from clade IV, and the *B. cereus* group in general, for a comparative analysis of their cytotoxic potential. Having a larger dataset to compare within, would give more statistical power for associations, if any, which can be further investigated in terms of the underlying mechanisms of cytotoxicity. Since we investigated the dairy (environmental) isolates, it may be worthwhile to investigate the cytotoxicity of isolates that have been implicated in outbreaks in the past, in order to find whether the optimized assays, either individually, or taken together, can lead to classifying these as cytotoxic. We saw from our studies and literature that changing the bacterial culturing medium could have an effect on the observed cytotoxicities. It would hence be good to investigate the cytotoxicities of the same set of isolates, say, the clade IV isolates, when grown in different food matrices, e.g., milk, rice, meat. The clade-specific effect of temperature on the expression of diarrheal toxins and cytotoxicities is largely missing from literature. This could be another area of potential research. Being implicated in foodborne illnesses and a spore-former, it is critical to evaluate the pathogenic potential of isolates after heat treatment, or at different holding temperatures of food. Finally, we have optimized the assays to assess cytotoxicity of supernatants of isolates grown in MOD medium. Currently in literature, we do not see conclusive associations between the presence/absence of virulence genes, gene expression, and cytotoxicity. Having the cytotoxicity assays optimized for isolates grown in MOD medium paves way for future studies investigating the proteomic and metabolomic profiles of the supernatants of *B. cereus* isolates simultaneously. When closely related *B. cereus* isolates are profiled, we could hypothesize some proteins/small molecules to be differentially produced by isolates that show high versus those that show low toxicity. This could lead to better understanding of the underlying mechanisms driving *B. cereus* pathogenicity.

Appendix A

Concentrating proteins in supernatants for proteomics studies

A.1. Background

We aimed to characterize the proteome profile of representative isolates from *B. cereus* clade IV in order to test for associations between the proteome profiles and cytotoxic effects on the differentiated Caco-2 human epithelial colorectal adenocarcinoma cell line. We hypothesized that proteins expressed by all cytotoxic isolates but not by any of the non-cytotoxic isolates are likely potential virulence factors involved in the diarrheal disease. We also aimed to compare the proteins expressed across samples quantitatively (using the isobaric tags for relative and absolute quantitation (iTRAQ) method), in order to determine whether the proteins that were being expressed across all samples were being expressed in different quantities, which could have had a bearing on the cytotoxicity of differentiated Caco-2 cells. The iTRAQ is an isobaric labeling method used in proteomics (using mass spectrometry) in which multiple protein samples can be quantified simultaneously in a multiplexed reaction (Ross et al., 2004). The tags work by binding the N-terminus and side chain amines of peptides generated during the trypsin digestion of protein samples (Ross et al., 2004). The maximum number of samples that can be characterized by quantitative proteomics using iTRAQ labelling is 8.

We first selected 8 isolates for the characterization of their proteome profile in a multiplexed reaction. We first screened isolates for the detection of non-hemolytic enterotoxin (Nhe) and hemolysin BL (Hbl) toxin components in their supernatants. From the group of isolates where both Nhe and Hbl were detected in their supernatants, we selected 8 isolates based on their

cytotoxicity in differentiated Caco-2 cells. From the lactate dehydrogenase (LDH) assays, we saw the lowest absorbance ratios (i.e., lowest degree of pore formation in cell membranes) with isolates FSL W7-1101 and FSL W8-0640, and the highest absorbance ratios (i.e., highest degree of pore formation in cell membranes) with isolates *B. cereus sensu stricto* (*s.s.*) ATCC 14579 and FSL R5-0184. From the 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1) assays, we saw the highest mean viabilities with isolates FSL K6-0040 and *B. thuringiensis* ATCC 10792, and the lowest mean viabilities with the isolates *B. cereus s.s.* ATCC 14579 and FSL R5-0184. Hence, we chose the isolates FSL W7-1101, FSL W8-0640, *B. cereus s.s.* ATCC 14579, FSL R5-0184, FSL K6-0040, and *B. thuringiensis* ATCC 10792 as part of the 8 isolates. We chose FSL K6-0073 and FSL K6-0043 as the remaining two isolates, as these had absorbance ratios (LDH assay) and viabilities (WST-1 assay) intermediate to those of the 6 chosen isolates.

Proteomics studies at the Mass Spectrometry & Proteomics core facility at Penn State College of Medicine (Hershey, Pennsylvania) required samples with a protein concentration of 1-2 µg/µl, with at least 10 µl of the samples. Using the Bradford assay, we determined that the protein concentration in the harvested supernatants of chosen isolates ranged from approximately 13 to 18 µg/ml (Table 4-7). Hence, the proteins had to be concentrated before submitting the samples for secretome characterization. In order to achieve the target protein concentrations, we used two different methods: acetone precipitation, and protein concentrators. Both of these methods are discussed in the following sections.

A.2. Acetone precipitation and re-solubilization

We thawed the supernatant of *B. cereus s.s.* ATCC 14579 and added the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) at 1 mM final concentration (Bhadauria et al., 2007; Florens

et al., 2006; Lai et al., 2006; Maguire et al., 2002; Zischka et al., 2003). To 8 ml of thawed supernatant, we added 32 ml of chilled (temperature of – 20 °C) acetone and kept the suspension overnight in – 20 °C as per standard protocol (Barritault et al., 1976; Bonn et al., 2018; Trzeciecka and Bhattacharya, 2019). We centrifuged (Beckman Coulter Avanti® J-26 XPI) the samples at 11,000 g for 10 min at 4 °C to obtain a firm pellet. After decanting the acetone, we air dried the pellet in a chemical fumehood.

We re-suspended the pellet in 0.1% formic acid solution as per recommendations from the Mass Spectrometry & Proteomics core facility at Penn State College of Medicine (Hershey, Pennsylvania). Even on adding 5 ml of 0.1% formic acid solution, the pellet did not dissolve, and the suspension was hazy. We spun down the suspension at 11,000 g for 30 min, but the precipitate was cloudy, and we had problems recovering the solution without pipetting some of the precipitate up.

We hence proceeded by following an alternate protocol (Doucette et al., 2014) for re-solubilization of the protein pellet obtained after acetone precipitation. Briefly, acetone was decanted and the pellet was air dried in fumehood. The pellet was incubated in – 20 °C for 2 min. 50 µl of 80% formic acid was added, and the suspension was incubated in – 20 °C for 2 min. The suspension was pipetted up and down five times, and incubated for 8 min at – 20 °C. The samples were diluted 10-fold in high performance liquid chromatography (HPLC)-grade water (Millipak Express 20). This was to be followed by a centrifugation step to recover the proteins. However, the pellet still did not dissolve.

We attempted to use 1 ml of 80% formic acid to dissolve the pellet instead of 50 µl and repeated the procedure. The haziness at the end of the 10-fold dilution in water was to a lesser extent. We centrifuged the suspension (10 ml) at 11,000 g for 30 min at 14 °C (Doucette et al., 2014), and retrieved the upper 7 ml of the fraction (the upper 70% volume of the solution, containing the soluble proteins, as per Doucette et al., 2014). We found that the protein yield was

27% of the theoretical yield on a weight/weight basis, i.e. from 178.16 µg of protein contained in 8 ml of supernatant, we obtained 48.10 µg of protein contained in 7 ml of formic acid-water mixture. Hence, this approach did not work in terms of obtaining the target concentration and amount of protein.

We repeated the protocol by Doucette et al., 2014 again, this time dissolving the protein pellet obtained from 8 ml of spent bacterial medium (178.16 µg total protein) in 8.9 µl of 80% formic acid, such that after 10x dilution in water, 89 µl of suspension was obtained. This suspension would have theoretically had a protein concentration of 2 µg/µl. However, the pellet was very difficult to dissolve, and resulted in 7.2% of the expected theoretical yield in the solution recovered after spinning down the suspension.

A.3. Use of protein concentrators

Since the acetone precipitation method did not yield proteins that could be re-solubilized, we concentrated the proteins using physical means. For this purpose, we used protein concentrators (Elhosseiny et al., 2016, 2019; Silva et al., 2012) of the lowest molecular size cutoff available (Pierce, 3 kDa). Briefly, we loaded 6 ml of supernatants onto the filter membranes of the concentrators and spun the concentrators at 7,500 g until the volume of retentate reduced to about 100 µl (12 to 16 h). We collected the flow-through (filtrate) and stored in – 80 °C. To the top of the filter containing the retentate, we added 100 µl of HPLC grade water and spun the filters again. We collected the retentates from the tube (volumes ranging from 48 µl to 108 µl), and measured the protein concentration. The concentrations ranged from 0.54 µg/µl to 1.99 µg/µl. We applied the filtrates and retentates of two highly toxic isolates (*B. cereus s.s.* ATCC 14579 and FSL R5-0184) on a single well of differentiated Caco-2 cells at 1% v/v level, to measure the cytotoxicity of both fractions using the LDH assay where higher degree of pore formation would indicate higher

toxicity. In case of both isolates, we saw that the retentate fraction that contained proteins was the toxic fraction.

Half of the volume of retentate obtained for each isolate was stored separately in – 80 °C. We worked with the rest half of the volume. For isolates that yielded below 1 µg/µl protein concentration, we attempted to evaporate the water under vacuum using a Speed Vac (Eppendorf Vacufuge), to attain a target protein concentration between 1-2 µg/µl. We sent the samples for proteomics to the Mass Spectrometry & Proteomics core facility at Penn State College of Medicine (Hershey, Pennsylvania).

While running the trypsin digested and iTRAQ labelled samples through the mass spectrometer, the technician noticed that a large proportion of the iTRAQ tags were being consumed by small molecular weight (166 and 132 Da) products. Probable causes included the protease inhibitor used (which was ruled out because PMSF did not contain nitrogen), and the presence of large amounts of amino acids (which was hypothesized to be the case here).

We thawed the spare concentrated samples stored in – 80 °C, and loaded them onto the protein concentrator filters. We washed the sample 4 times with HPLC grade water, each time making the volume up to 3 ml, the volume of the supernatant from which the samples were derived. This step was carried out to remove salts, amino acids and other undesired components that were hypothesized to interfere with proteomics. We did not proceed with further washing, as four washes resulted in a 40% loss in protein content of one of the samples (*B. thuringiensis* ATCC 10792), found by calculating the weight by weight percentage of protein in sample before the first wash and after the fourth wash. We collected the retentates ranging from 73 µl to 109 µl, and measured the protein concentration. The concentrations ranged from 0.19 µg/µl to 0.64 µg/µl. As all isolates yielded below 1 µg/µl protein concentration, we attempted to evaporate the water under vacuum using a Speed Vac, to attain a target protein concentration between 1-2 µg/µl. To confirm that the Nhe and Hbl toxins were not lost during the washing process, we applied 150 µl of diluted sample

(1 µl of concentrated sample diluted to 150 µl) of two isolates (*B. cereus s.s*. ATCC 14579 and FSL R5-0184) to the immunoassay port of the Duopath® Cereus Enterotoxins kit. Both isolates tested positive for Hbl and Nhe toxins, confirming that they were not lost during the washing step. We sent the samples again for proteomics to the Mass Spectrometry & Proteomics core facility at Penn State College of Medicine (Hershey, Pennsylvania). This time, we requested them to not use iTRAQ labels, as the amino acids could still interfere with the tags (Kaspar et al., 2009), depending on the sensitivity of the tags and the amount of residual amino acids still present in the samples.

An unknown component of the trypsin digested samples was found to be clogging the Polywax HPLC (weak anion exchange) columns when separating the samples before loading into the mass spectrometer. They attempted to use a different column, the SCX HPLC (strong cation exchange) column instead. They used the concentrated protein sample of *B. cereus s.s.* ATCC 14579 supernatant, and saw an increase in the number of confident identities of proteins (17 in number, as opposed to 2 that they got when using Polywax columns), but it was still less than the expected number of 100 for *B. cereus s.s.* ATCC 14579 grown aerobically in MOD medium as reported by Clair et al., 2010. Further optimization of the protein extraction and purification is needed prior to secretome characterization.

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Appendix B

R programming language codes

B.1. Paired Student's t-tests

```
library(stats)
data1 <- read.table('name_of_spreadsheet.csv', header = TRUE, sep = ",")
res <- t.test(Viability ~ Isolate, data = data1, paired = TRUE)
res
```

B.2. Bartlett's test for homogeneity of variances

```
library(stats)
data1 <- read.table('name_of_spreadsheet.csv', header = TRUE, sep = ",")
res2 <- bartlett.test(Viability ~ Isolate, data = data1)
res2
```

B.3. Welch test and Games-Howell posthoc test

```
library(onewaytests)
library(userfriendlyscience)
data1 <- read.table('name_of_spreadsheet.csv', header = TRUE, sep = ",")
welch.test(Viability~Isolate, data1, rate = 0, alpha = 0.05, na.rm = TRUE, verbose =
TRUE)
```

```
posthocTGH(data1$Viability, data1$Isolate, method=c("games-howell"), conf.level =
0.95, digits=3, p.adjust="bonferroni", formatPvalue = TRUE)
```

B.4. One-way ANOVA and Tukey post-hoc test

```
library(stats)
library(agricolae)
data1 <- read.table('name_of_spreadsheet.csv', header = TRUE, sep = ",")
data.lm <- lm(Viability ~ Isolate, data = data1)
summary(aov(data.lm))
tukey.test <- HSD.test(aov(data.lm), trt='Isolate')
tukey.test
```

B.5. Pearson's correlation tests and correlation plot

```
library(stats)
library(corrplot)
data1 <- read.csv('name_of_spreadsheet.csv', header = TRUE, sep = ",", row.names =
c(1))
data1[7:21] <- lapply(data1[7:21], as.numeric)
res <- cor.test(data1$bceT, data1$sph, method = "pearson")
res
signitest2019<-cor.mtest(data1, conf.level = .95)
corrplot(cor2019, p.mat = signitest2019$p, method = "color", sig.level = .05, pch.cex = 1,
pch.col = "black", insig = "pch", order = "hclust", tl.col="black")
```