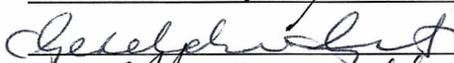
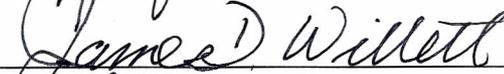
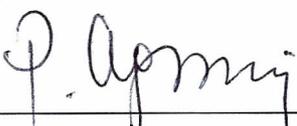


THE MECHANISM FOR BACILLUS CEREUS TOXICITY UNDER
MICROAEROBIC CONDITIONS

by

Kathleen Kilcullen
A Thesis
Submitted to the
Graduate Faculty
of
George Mason University
in Partial Fulfillment of
The Requirements for the Degree
of
Master of Science
Biology

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DEDICATION

This is dedicated to my supportive family, my loving boyfriend Mike, and all the teachers that inspired me to pursue a career in science.

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LIST OF ABBREVIATIONS

Alkylated Bovine Serum Albumin.....	Alky-BSA
Anthrolysin O.....	ALO
American Type Culture Collection.....	ATCC
Amplex Red.....	AR
Ascorbic Acid.....	AA
Bovine Serum Albumin.....	BSA
Complete Serum-Free Medium.....	CSFM
Cereolysin AB.....	CerAB
Cereolysin O.....	CLO
Cholesterol-Dependent Cytolysin.....	CDC
Cytotoxin K.....	CytK
Dulbecco's Modified Eagle Medium.....	DMEM
Dithiothreitol.....	DTT
Hemolysin BL.....	Hbl
Hemolysin II.....	Hly II
Hemolysin III.....	HlyIII
Horseradish Peroxidase.....	HRP
Human Small Airway Epithelial Cell.....	HSAEC
Kilodalton.....	kDa
Immune Inhibitor A Metalloprotease.....	InhA
Lactate Dehydrogenase.....	LDH
Liquid Chromatography Tandem-Mass Spectrometry.....	LC-MS/MS
Nitric Oxide.....	NO
Nitrosylated Bovine Serum Albumin.....	Nitro-BSA
Non-Hemolytic Enterotoxin.....	Nhe
Nominal Molecular Weight Limit.....	NMWL
Phosphate-Buffered Saline.....	PBS
Phosphatidylcholine-Specific Phospholipase C.....	PC-PLC
Phospholipase C.....	PLC
Pleiotropic Regulator.....	PlcR
Polyacrylamide Gel Electrophoresis.....	PAGE
Pore-Forming Toxin.....	PFT
Reactive Oxygen Species.....	ROS
Reactive Nitrogen Species.....	RNS
Reduced Bovine Serum Albumin.....	DTT-BSA
Reduced Nitrosylated Bovine Serum Albumin.....	DTT-Nitro-BSA

Room Temperature.....	RT
S-Nitrosothiol.....	RS-NO
Sodium Dodecyl Sulfate	SDS
Sphingomyelinase	SMase
Succinic Acid	SA
Supernatant	Sup
Dulbecco's Phosphate-buffered Saline with Tween-20.....	DBST

ABSTRACT

THE MECHANISM FOR BACILLUS CEREUS TOXICITY UNDER MICROAEROBIC CONDITIONS

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George Mason University, 2014

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Bacillus cereus, an opportunistic bacterium that causes food poisoning, secretes a multitude of virulence factors that are influenced by environmental stimuli such as oxygen, pH, glucose, and bacterial density. The cytotoxicity of this species has been mainly characterized under aerobic conditions and is primarily attributed to its enterotoxins. This thesis discovers *B. cereus* and the closely related *B. anthracis* share a common mechanism of toxicity that exclusively occurs under microaerobic conditions. Toxicity was caused by the permeabilization of the cell membrane by the pore-forming hemolysin cereolysin O (CLO) which facilitated the access of toxic bacterial metabolic product into the cell. This synergistic toxicity appeared to be potentiated by the presence of bovine serum albumin (BSA). Cultures grown in medium containing BSA had an acidification of bacterial supernatants to a pH of 5.2-5.5 indicating albumin increased the generation of anaerobic fermentation products. The activity of CLO and the metabolic

product succinic acid were found to be individually inhibited under aerobic conditions.

The effect of oxygen availability on *B. cereus* virulence was strain specific, possibly due

to differences in bacterial density influencing the nature and level of enterotoxin

production. This thesis highlights a shared mechanism of toxicity in these species through

the synergistic effect of their cholesterol-dependent cytolysins and fermentation products.

BACKGROUND

The virulence factors of *B. cereus*

B. cereus is a beta hemolytic bacterium that is most well-known for causing gastroenteritis (Stenfors Arnesen et al., 2008). It is a sporulating facultative anaerobe that is closely related to *B. anthracis* and *B. thuringiensis*. *B. cereus* produces a multitude of pathogenic factors that have been suggested to contribute toward toxicity. *B. cereus* is not considered a homozygous species and strains exhibit a large diversity in the presence and expression of these virulence factors (Ehling-Schulz et al., 2006). Pathogenicity of this species is attributed to the secretion of bacterial toxins and enzymes (Simonen and Palva, 1993). Its enterotoxins and emetic toxin are responsible for inducing two different forms of food poisoning, the diarrheal and emetic syndromes respectively. Additionally, *B. cereus* is an opportunistic pathogen that can infrequently cause bacteremia, meningitis, endophthalmitis, pneumonia, and there have been documented cases of fatal infections mimicking the clinical presentation of anthrax (Hoffmaster et al., 2004; Turnbull et al., 1979).

B. cereus secretes many virulence factors that contribute to pathogenicity including metalloproteases, collagenase, phospholipases, emetic toxin, enterotoxins, and hemolysins. Metalloproteases, in particular immune inhibitor A (InhA), are zinc-dependent enzymes that are believed to facilitate bacterial infiltration at the intestinal

barrier and evasion from macrophages (Guillemet et al., 2010). Phospholipase and sphingomyelinase (SMase) are enzymes that hydrolyze phospholipids and sphingomyelin on the plasma membrane, respectively, and can synergistically interact to form a cytolytic and hemolytic determinant called cereolysin AB (CerAB) (Doll et al., 2013; Gilmore et al., 1989; Titball, 1993). Cereulide, an emetic toxin, acts as a cationic ionopore and inhibits fatty acid oxidation, however, only a few strains encode this extrachromosomal toxin (Mikkola et al., 1999). The diarrheal syndrome is attributed to three enterotoxins, hemolysin BL (Hbl), non-hemolytic enterotoxin (Nhe), and cytotoxin K (CytK). These pore-forming toxins (PFTs) disrupt the membrane of epithelial cells lining the gastrointestinal tract (Senesi and Ghelardi, 2010). Additionally, Hbl is also believed to be the primary contributor to symptoms of endophthalmitis, an infection of the eye that can lead to blindness (Beecher et al., 2000). Hbl and Nhe are tripartite enterotoxins in which each component is secreted independently, binds to lipid bilayer, and then subsequently assembles to form a transmembrane pore causing osmotic lysis (Beecher and Macmillan, 1991; Sastalla et al., 2013; Senesi and Ghelardi, 2010). It is necessary for the components of Hbl and Nhe to sequentially assemble on the cellular membrane to elicit a toxic effect (Sastalla et al., 2013). The Hbl-B component must first attach to the cell membrane, followed by L1 and L2 binding for Hbl activity whereas Nhe toxicity requires binding of the NheC component, followed by NheB and then NheA. Although these toxins contain a 40% sequence homology, their subunits are not interchangeable. Cytotoxin K (CytK), is a single-component cytolytic, hemolytic, and necrotic toxin belonging to the β -barrel pore-forming toxin family that causes cytolysis towards

intestinal epithelia (Fagerlund et al., 2004; Hardy et al., 2001). *B. cereus* secretes an additional two beta-barrel pore-forming toxins, cereolysin O (CLO) and hemolysin II (HlyII), that are non-diarrheal hemolysins. CLO belongs to the family of cholesterol-dependent cytolysins that binds specifically to cholesterol on the plasma membrane for pore formation (Jacobs et al., 1999). Once a CLO monomer has bound to this receptor, the toxin undergoes oligomerization to form a pre-pore complex. When complete, this complex forms a large transmembrane β -barrel pore on the membrane surface consisting of 35-50 monomers (Tweten, 2005). Hemolysin II is a heat-labile toxin hypothesized to function in suppressing the host immune response by causing macrophage apoptosis. The last toxin, hemolysin III, has yet to be characterized because it has not been purified; however, it appears to function as a pore-forming toxin as well (Baida and Kuzmin, 1996). The toxicity of *B. cereus* is believed to be caused by a synergistic action of these virulence factors (Stenfors Arnesen et al., 2008). Examples of this effect include the concerted actions of Nhe and Hbl with SMase to contribute to toxicity (Beecher and Wong, 2000; Doll et al., 2013).

The environmental factors affecting bacterial toxicity

The expression of these pathogenic factors, with the exception of hemolysin II and hemolysin III, are primarily controlled by PlcR a pleiotropic regulator that functions as part of a quorum sensing system (Gohar et al., 2008). PlcR regulates in a cell-density manner, activating virulence gene expression at the beginning of stationary phase of growth (Slamti and Lereclus, 2002). Bacterial density has been shown to have a stimulatory effect on the production of *B. cereus* enterotoxins (Duport et al., 2004).

Additionally, the regulation of toxin expression has been shown to depend on the complex interaction of environmental factors including oxygen availability, redox potential, temperature, glucose availability, and pH (Garcia-Arribas and Kramer, 1990; Glatz and Goepfert, 1976). The regulatory mechanisms influencing virulence factor production are shown in **Figure 1**. There are conflicting studies on the influence of oxygen on enterotoxin production. Both anaerobiosis and aerobic conditions have shown to have a stimulatory effect (Christiansson et al., 1989; Van Der Voort and Abee, 2009; Duport et al., 2004, 2006). This contradiction could be due to aerobic cultures having a higher density of bacteria and it is the increase in density, instead of oxygen availability, responsible for enhanced enterotoxin production. Low oxidation-reduction potential and warmer cultivation temperatures (optimal temperature being 30°C) have been shown to stimulate enterotoxin production (Duport et al., 2006; Rowan and Anderson, 1998). Glucose is required for enterotoxin production; however, a high glucose concentration (greater than 50 g/L) results in its complete inhibition (Sutherland and Limond, 1993). Regulation is also affected by pH, but it is difficult to investigate the role of this factor without the additional variables of bacterial density and nutrient availability. Acidity appears to decrease enterotoxin production, but significant expression can still occur in a pH of 5-6 even though there is a reduction in bacterial growth (Sutherland and Limond, 1993; Thomassin et al., 2006).

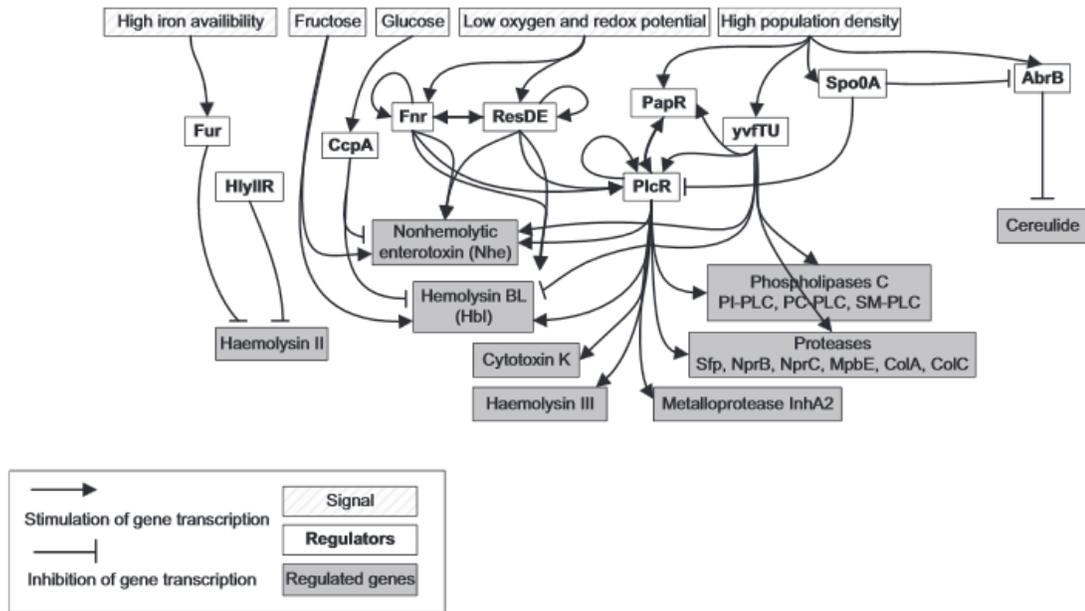


Figure 1 Regulation of toxin and virulence gene transcription in *B. cereus* (Ceuppens et al., 2011).

In addition to these factors, the presence of albumin has demonstrated a significant influence on the toxicity of the *Bacillus* species. Serum is believed to have bactericidal and bacteriolytic properties (Taylor, 1983), however, multiple studies have reported that bovine serum albumin (BSA) can significantly enhance the virulence of bacteria (Dubos, 1947; Elleboudy et al., 2011; Kruczek et al., 2014; Liu, 1973; St John et al., 2013). There are many proposed mechanisms for its influence including facilitating bacterial growth, increasing the production of virulence factors, or acting as a reservoir for toxic products (Dubos, 1947; Liu, 1973). Albumin was found to enhance the expression of quorum sensing-controlled virulence genes in *Pseudomonas aeruginosa* during late stationary phase (Kruczek et al., 2014). Research on *B. cereus* has found that medium supplemented with BSA increased the production of bacterial phospholipases

(Elleboudy et al., 2011). Additionally, albumin could function in chemical toxicity by concentrating nitric oxide (NO) and O₂ in its hydrophobic core followed by the micellar catalysis of NO into N₂O₃ or NO₂ which are more stable products (Nedospasov et al., 2000). This NO autooxidation can cause either direct nitrosylation or transnitrosylation of S-nitrosothiols (RS-NO) via albumin's cysteine and tryptophan residues. This reaction does not appear to be inhibited under oxygen-limited conditions (Bosworth et al., 2009; Ishima et al., 2007; Rafikova et al., 2002a). *B. anthracis* has been shown to depend on the NO-activation of catalase for survival in macrophages (Shatalin et al., 2008). Research further investigating this effect found nitric oxide may be responsible for chemical-mediated toxicity through its downstream formation of peroxynitrite and its protein modifications negatively interfering with the host cell (St John et al., 2013). Additionally, they found that the supplementation of culture medium with BSA increased *B. anthracis* toxicity via the ability of albumin to concentrate and stabilize volatile NO bacterial products.

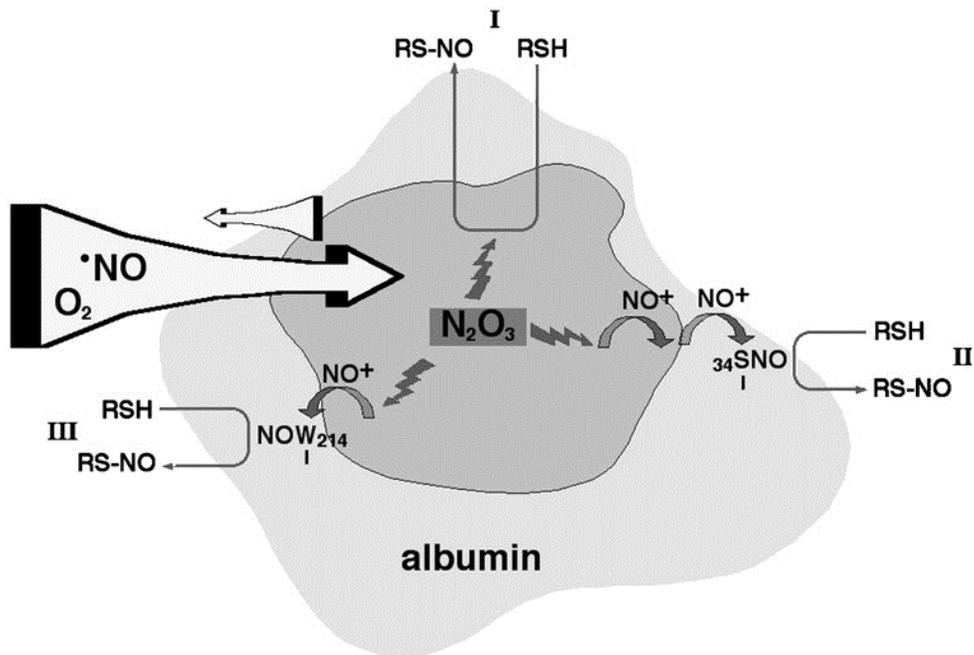


Figure 2 Micellar catalysis of NO oxidation and nitrosylation (Rafikova et al., 2002b).

Albumin can transfer NO⁺ to a thiol RSH (sulfhydryl group) by three different pathways: (I) directly, (II) via cysteine-34 residue, or (III) via tryptophan-214 residue.

Most of the toxin characterization for *B. cereus* has been conducted under strictly aerobic conditions. However, this characterization may be incomplete since *B. cereus* infections typically occur in an anaerobic environment, the gastrointestinal tract. It is possible that the virulence of this bacteria changes in response to oxygen deprivation. Studies exploring the effects of cultivating *B. cereus* in oxygen-limited conditions have focused exclusively on the transcription and expression of these pathogenic factors. Enterotoxin production appears to increase during fermentative growth compared to cultures grown under aerobic conditions (Duport et al., 2004; Finlay et al., 2002; Zigha et

al., 2006). Understanding the mechanisms for the production of these virulence factors plays a key role in unraveling the pathogenesis of *B. cereus*.

Comparing the pathogenicity of *B. cereus* and *B. anthracis*

B. anthracis is considered closely related to *B. cereus* with a 99% genetic similarity in their 16S rRNA sequences (Helgason et al., 2000). Species classification is primarily determined by the presence of extrachromosomal virulence genes located on plasmids, however, there is a debate on whether these bacteria should be considered separate species. *B. anthracis* contains two plasmids, pXO1 and pXO2, which encode for edema toxin and lethal toxin and a poly- γ -D-glutamic acid capsule, respectively.

Although pulmonary infections by *B. cereus* are rare, there have been a few documented cases of these bacteria causing a fatal respiratory illness that mimicked the symptoms of an anthrax infection. Sequencing this lethal isolate of *B. cereus* found the presence of a plasmid with 99.6% genetic similarity to pXO1 and an additional second plasmid that encodes a polysaccharide capsule (Hoffmaster et al., 2004). It has been demonstrated that a horizontal gene transfer of these plasmids is possible between these species (Battisti et al., 1985). Until recently, the virulence of *B. anthracis* has been solely attributed to these extrachromosomal virulence factors. However, *in vivo* toxicity studies using a derivative of the Ames strain [pXO1⁻/pXO2⁺] found that this strain of *B. anthracis* caused high levels of virulence that were independent of lethal and edema toxin (Welkos et al., 1993). This finding stresses the potential importance of chromosomally located virulence factors in contributing towards toxicity.

Research comparing the toxicity of *B. anthracis* and *B. cereus* filtrates found marked differences between these two species. Surprisingly, *B. cereus* filtrates rapidly destroyed the monolayers of three types of tissue-culture cells whereas *B. anthracis* demonstrated no cytopathic effect after 24 h (Bonventre, 1965). Additionally, *in vivo* studies using rat and mouse models found when *B. cereus* filtrates were administered intravenously it caused almost 100% lethality after injection (Bonventre and Eckert, 1963). Injections of *B. anthracis* filtrates were lethal only towards susceptible genetically inbred Fischer albino rats after a lag period of several hours. During late-stage inhalation infection, *B. anthracis* produces bacterial products that damage the lungs and cause pleural effusion, hypoxia, and metabolic acidosis. This suggests that there are anaerobic products being generated that cause cytotoxicity towards pulmonary epithelial cells. Research conducted on the avirulent Sterne strain [pXO1⁺/pXO2⁻] found that its secreted products were highly toxic towards cells when *B. anthracis* was grown under oxygen-limited conditions, and the cultivation under aerobic conditions resulted in a considerable loss of toxicity (Klichko et al., 2003; Popova et al., 2011). The proposed mechanism behind this enhanced virulence is hypothesized to be a synergistic effect of anthrolysin O (a homolog of *B. cereus* cereolysin O) and succinic acid, a byproduct of fermentation.

Current Study

Since the expression of virulence factors in *Bacillus* species appears to be affected by the presence of oxygen, this thesis tested if *B. cereus* demonstrates enhanced toxicity when cultivated in an oxygen-limited environment. Determining the role of oxygen,

especially for an enteric pathogen, is a critical component to understanding its pathogenicity. Additionally, *B. cereus* could demonstrate a similar mechanism of metabolic toxicity seen by *B. anthracis*. Although there are likely to be differences in the toxicity between these two species, they could still share the same mechanism for virulence. *B. cereus* secretes pore-forming toxins that could increase the permeability of cell membranes which would allow metabolic products to enter the cell and influence the production of reactive oxygen species. This would be a novel mechanism of toxicity for *B. cereus*. Understanding the commonalities and differences between the toxicity of *B. cereus* and *B. anthracis* is necessary for accurate bacterial identification in patients. *B. cereus* has historically been considered a minor disease-causing pathogen, and hospital specimens identified as containing *B. cereus* are frequently discarded as a contaminant. The increasing discovery of strains causing debilitating and occasionally lethal outcomes stresses the importance of fully characterizing this pathogen for diagnostic and treatment purposes.

RESEARCH AIMS

For this thesis, the cytotoxicity of *B. cereus* cultivated under microaerobic and aerobic conditions was determined towards human small airway epithelial cells (HSAECs). HSAECs were chosen as a model cell line because epithelial cells are well known targets of PFTs (Ratner et al., 2006). Additionally, lethal isolates of *B. cereus* have been documented to cause severe respiratory distress indicating a toxic effect directed at lung cells (Hoffmaster et al., 2006; Miller et al., 1997; Popova et al., 2011). Two strains were used for the characterization of *B. cereus*. Strain ATCC 14579 encodes three enterotoxins, Hbl, Nhe, and CytK, which are implicated in contributing to the diarrheal syndrome. Strain ATCC 11778, similar to *B. anthracis*, does not contain Hbl or CytK.

The mechanism behind cellular lysis was investigated to identify if a pore-forming toxin increases cell permeability allowing toxic bacterial products to enter the cell. In order to characterize these two strains, several variables including medium composition, length of cultivation, oxygen availability, pH, and toxin inhibitors were manipulated to analyze what factors contributed to bacterial virulence. Particularly BSA was investigated to determine its effect on pathogenicity.

After the general characterization of this bacterium using toxicity assays, culture supernatants were separated by nominal molecular weight limit (NMWL) and fractions of

each were used to determine the general characteristics of various sized virulence factors. These proteins were further separated by column chromatography and eluted fractions were analyzed for toxicity. Finally, mass spectrometry was conducted on the fractions to identify individual proteins and for semi-quantitative analysis. Comparisons were made between *B. cereus* and *B. anthracis* to analyze the similarities and differences in the cytotoxic potential of these two species.

Table 1 Estimated size of virulence factors in *B. cereus* ATCC 11778 and 14579.

+/- Denotes whether gene is present in strain (Brillard and Lereclus, 2004; Hansen and Hendriksen, 2001; Rajkovic et al., 2008)

Virulence factor	Size (kDa)	Strain	
		ATCC 11778	ATCC 14579
Immune inhibitor A (InhA)	87.9	+	+
Phospholipase C (PLC)	23.0	+	+
Sphingomyelinase (SMase)	34.0	+	+
Cereolysin AB (CerAB)	67.0	+	+
Collagenase	109.0	+	+
Cereulide	1.2	-	-
Hemolysin BL (Hbl)	45.0 (L2) 36.0 (L1) 35.0 (B)	-	+
Non-hemolysin E (Nhe)	41.0 (A) 39.8 (B) 36.5 (C)	+	+
Cytotoxin K (CytK, HlyIV)	34.0	-	+
Cereolysin O (CLO,	52.5	+	+

Virulence factor	Size (kDa)	Strain	
		ATCC 11778	ATCC 14579
HlyI)			
Hemolysin II (HlyII)	45.6	+	+
Hemolysin III (HlyIII)	24.4	+	+

RESEARCH DESIGN

Reagents

All reagents used were from Sigma-Aldrich unless specified otherwise.

Cholesterol was dissolved in ethanol and then further diluted into bacterial supernatants unless indicated. Ham's F-12 cell culture medium and Complete Serum-Free Medium (CSFM) were from Mediatech Inc., VA. Formulated Dulbecco's Modified Eagle Medium (DMEM) came from Sigma-Aldrich. BSA was of > 98% purity and essentially free from globulins and fatty acids. Amplex Red dye was from Invitrogen. The CytoTox-ONE Homogeneous Membrane Integrity Assay came from Promega, WI. Succinic acid concentration was measured using Succinic Acid assay kit from Megazyme. Protein concentration was estimated using the Bradford protein assay from Bio-Rad. Rabbit anti-streptolysin O antibody was from B-Bridge International, CA. Anti-rabbit IgG, HRP-linked antibody came from Cell Signaling Technology.

Bacterial strains, culture conditions, and preparation of culture supernatants (Sups)

The following procedure for bacterial propagation was used for every experiment unless stated otherwise. *B. cereus* ATCC 11778 and 14579 were grown on agar plates containing Luria broth. Agar plates were re-streaked every seven days and were kept at 8°C until inoculation. Single colonies were used to inoculate into Luria broth and

cultures were kept in an incubator shaker at 200 rpm, 37°C for 18 h. Overnight culture at 1:100 dilution was inoculated into either 10 mL of Complete Serum Free Medium (CSFM), a nitrate rich medium containing 1 g/L of bovine serum albumin (BSA), or DMEM supplemented with 1 g/L of BSA. Cultures were grown either under microaerobic conditions, in a stationary 6-well plate in an incubator at 37°C 5% CO₂, or under aerobic conditions, in loosely capped 50 mL tubes shaken at 200 rpm at 37°C for 20 h unless specified otherwise. Under stationary conditions, the bacteria consume available oxygen and gradually become hypoxic, thus representing a microaerobic environment. Cultures were collected and the optical density of 200 µL of bacterial suspension in a 96-well plate was measured in triplicates using a microplate reader at 600 nm.

Bacterial suspensions were centrifuged at 3000 x g for 15 min and supernatants were removed from the bacterial pellet. Penicillin (100 µg mL⁻¹) and streptomycin (100 U mL⁻¹) were added to supernatants to prevent any bacterial contamination. Fresh Sups were used immediately for challenge experiments. Sups were diluted prior to cell exposure to prevent an excessive amount of toxicity that could obfuscate the effect of an intervention.

Cell cultivation and toxicity studies

HSAECs (human small airway epithelial cells) were from Cambrex Inc., MD. Cells were cultured in Ham's F-12 medium containing 10% fetal bovine serum, non-essential amino acids, L-glutamine, and pyruvate and grown in 37°C in a 5% CO₂ atmosphere. Cells were seeded into a 96-well plate at a density of 2.5 x 10⁴ per well and

grown to confluence. For cytotoxicity assays, 200 μL of bacterial culture supernatants were added per well and incubated for 20 min at 37°C, 5% CO_2 without shaking. After cell exposure, the plate was spun at 2000 x g for 5 min, Sups were removed, and 200 μL of 5% resazurin, a redox dye, was added to each well. Resazurin measures cell survival because cellular metabolism breaks down the dye changing its color and fluorescence. Fluorescence was measured with an excitation at 530 nm and emission at 590 nm via spectrometer to determine the differences in cell viability after 2 h incubation with resazurin.

Assay kits

The listed kits were performed according to the manufacturers' protocols unless otherwise indicated. Cell permeability was measured using the CytoTox-ONE Homogeneous Membrane Integrity Assay which indicated the amount of lactate dehydrogenase (LDH) secreted from cells with a damaged membrane. For determining the presence of hydrogen peroxide and peroxyxynitrite the dye Amplex Red from Invitrogen was used with a modified protocol. Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine, AR) is a colorless derivative of dihydroresorufin that when oxidized produces the colorful fluorescent product resorufin which is detectable at 571 nm. AR and horseradish peroxidase (HRP) were added to bacterial medium prior to inoculation at a concentration of 0.1 mM and 0.2 U/mL, respectively. Cultures were collected every hour, centrifuged at 10,000 x g for 5 min, and the supernatants were removed. The absorbance of Sups was read at 571 nm via spectrometer. Penicillin (100 $\mu\text{g mL}^{-1}$) and streptomycin (100 U mL^{-1}) were added, and then Sups were stored at 8°C

overnight for AR challenge experiments. For toxicity experiments cells were exposed to 100 μ l of Sups for 20 min.

Column filtration of Sups

Supernatants were separated by filtration using Amicon Ultra-4 Centrifugal Filter Units with 3, 30, 50, and 100 kDa NMWL membranes (Millipore). 1.4 mL of Sups were added to each filter and samples were spun according to the manufacturer protocols unless indicated otherwise. Only filtrates from the 100 kDa and 30 kDa filters were assessed for toxicity. Retentates separated by the 50 kDa filter were diluted in medium titrated using HCl to be equal in volume and pH to the filtrate. The cytotoxicity assay on these filtered Sups was conducted as described above.

Supernatant fractionation

For size exclusion chromatography experiments, bacteria were cultured in DMEM with or without BSA (1 g/L) in 6-well plates under stationary conditions at 37°C, 5% CO₂ for 20 h. Sups were collected and concentrated 8-fold via SpeedVac at 32°C. 1 mL of supernatant concentrate was injected into a HiPrep 16/60 Sephacryl S-200 HR column (GE Healthcare Life Sciences) and run at a rate of 4 mL/min in a 50 mM Tris running buffer. Flow-through fractions were collected every 2 min between a run time of 13 and 135 min. The toxicity of fractions was assessed immediately and then fractions were stored at -20°C for mass spectrometry analysis. To test the toxicity of fractions, 100 μ L of fractions were added to a confluent 96-well plate of HSAECs containing 100 μ L of

DMEM. Cells were exposed for 1 h and resazurin was used as an indicator for cell survival.

SDS-PAGE, native PAGE, and Western blotting

For native PAGE and SDS-PAGE, protein fractions were mixed with a DNA loading buffer for native PAGE or with Laemmli sample buffer supplemented with 50 mM DTT for SDS-PAGE. Fractions for SDS-PAGE were boiled with the buffer for 5 min. Samples were separated using 4-20% Tris-Glycine gels and then transferred to nitrocellulose membranes using iBlot Dry Blotting System (Life Technologies). Membranes were blocked with 5% BSA in PBST for 1 h, and then incubated in a rabbit anti-streptolysin O antibody (1:1000) overnight. Membranes were washed in DBS + 0.05% Tween-20 and then incubated with an anti-rabbit IgG, HRP-linked antibody (1:5000) for 1 h at room temperature. Western blots were developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific) and then imaged using ChemiDoc XRS+ System (Bio-Rad).

Mass Spectrometry

To determine the protein composition of fractions, samples were prepared for liquid chromatography tandem-mass spectrometry (LC-MS/MS). Fractions were first concentrated 10-fold via SpeedVac at 32°C. Samples were resuspended and reduced in a 8 M urea/10 mM DTT mix for 30 min, alkylated by 50 mM iodoacetamide in the dark for 30 min, and then finally digested by trypsin at 37°C overnight. Peptides were purified via Zip-Tip (Millipore) and then samples were sent to the Center for Applied Proteomics

and Molecular Medicine (George Mason University, Manassas, Va) and analyzed by LC-MS/MS using a linear ion-trap mass spectrometer (LTQ, Orbitrap).

Modified BSA preparation

For the modification of BSA, 200 mg of BSA was added to 1 mL of 50 mM Tris-HCl pH 7.3. BSA was either alkylated by incubation with a final concentration of .0303 M of iodoacetamide at 37°C for 1 h in the dark or reduced using .0091 M of DTT at RT for 1 h. 1 mL of samples was extensively dialyzed using 1 L of PBS overnight at 8°C to remove DTT and iodoacetamide. Unmodified BSA was included as a control. BSA concentration was estimated using Bradford protein assay. For nitrosylation of BSA, 200 mg/mL of sodium nitrite was added to water and titrated to pH 5 using HCl. Alkylated, reduced, or unmodified BSA at 100 mg/mL concentration was mixed with equal amounts with the sodium nitrite solution and incubated in the dark for 1 h at RT. Ellman's Reagent (5,5'-dithio-bis-(2-nitrobenzoic acid)) also known as DTNB was used to estimate the amount of free sulfhydryl groups in BSA. DTNB reacts with free sulfhydryl groups producing a disulfide and 2-nitro-5-thiobenzoic acid, a yellow-colored product that can be measured at 412 nm by a spectrometer. BSA sulfhydryl groups were estimated by comparison to a cysteine standard curve.

Statistical analysis

Each measurement was done in triplicate and experiments were performed at least twice for consistency. In figures, error bars indicate 95% confidence intervals (two-tail *t*-test).

RESULTS

***B. cereus* generates cytotoxic proteins during exponential growth phase**

To determine the timeline of exotoxin production, the toxicity of *B. cereus* supernatants towards HSAECs was assessed every hour post-inoculation of overnight culture. Two strains, ATCC 14579 and 11778, were used for comparison. Microaerobic growth conditions were defined as static cultures incubated at 37°C and 5% CO₂ and aerobic conditions were incubated in a shaker at 37°C. Differences in the pH of microaerobic Sups indicated secretion of fermentation products started approximately 4 h after the inoculation of culture. The generation of fermentation products appeared to correlate with the toxicity of strain 11778 (**Figure 3D**). Additionally, strain 14579 generated more acidic sups (pH 5.21) than 11778 (pH 5.54) after a period of 10 h. Aerobic Sups were slightly acidic after 7 h with a pH of 6.8 for both strains. Results in **Figure 3C,D** show the Sups from cultures collected after 5 h demonstrated high levels of cytotoxicity. Additionally, both strains destroyed the cell monolayer after an exposure period of approximately 20 min. This suggests highly toxic virulence factors are expressed around mid-exponential phase. Previous studies on *B. cereus* have reported in anaerobic conditions Nhe is expressed early in exponential phase, whereas Hbl expression occurs at stationary phase (Zigha et al., 2006). Therefore, this early toxicity could be attributed to Nhe. In comparison, *B. anthracis* demonstrates marked differences

in its exotoxin production. *B. anthracis* displayed no toxicity until stationary phase and Sups required a 2 h cell exposure to elicit a toxic effect (Popova et al., 2011).

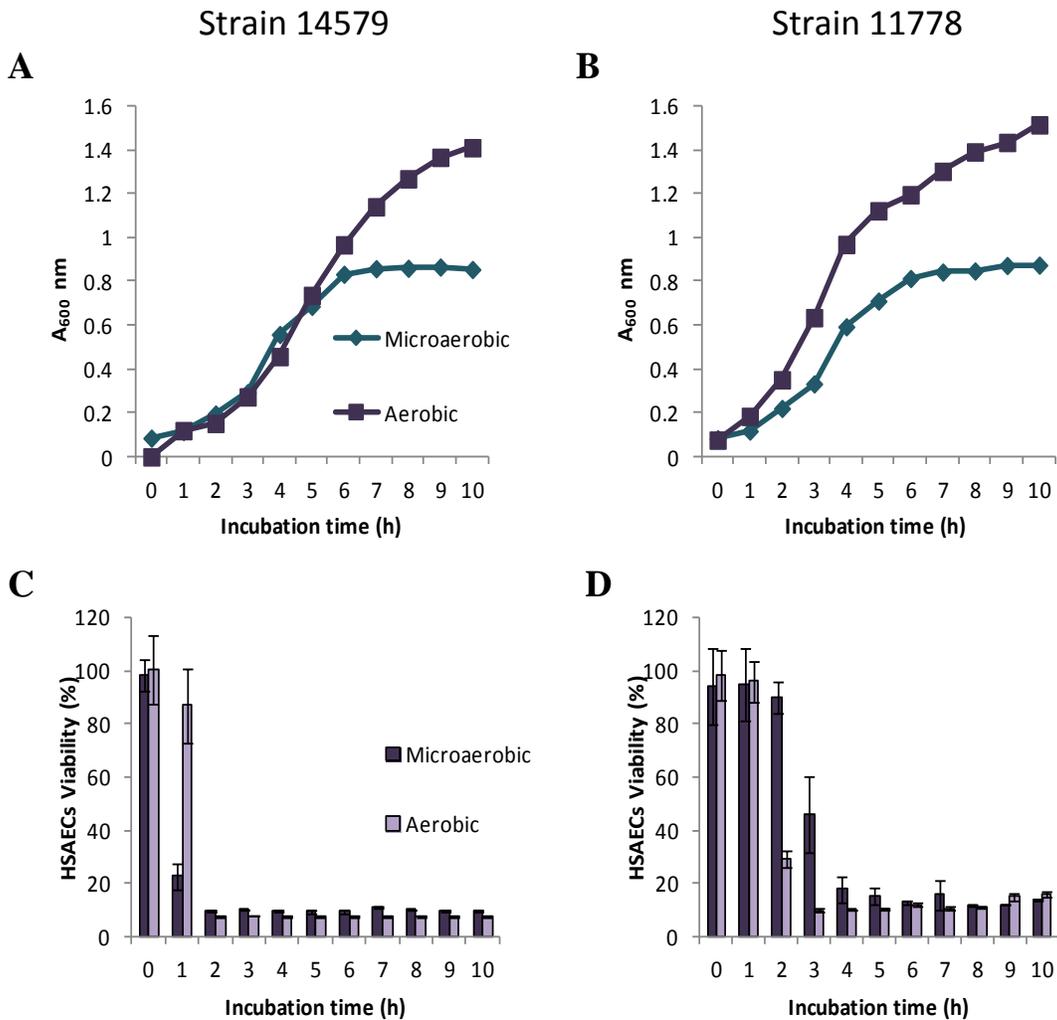


Figure 3 The growth and toxicity of *B. cereus* under microaerobic and aerobic conditions. Overnight culture was diluted 1:100 in 2 mL CSFM on a 24-well plate and the cultivated at 37°C and 5% CO₂ for microaerobic conditions. Microaerobic cultures were not agitated between collection times. Aerobic cultures were overnight culture diluted 1:100 in 20 mL of CSFM in a 50 mL tube and aerated by shaking 200 r.p.m. at 37°C. Strain 14579 (A,C) and 11778 (B,D) cultures were harvested every hour post-inoculation and their optical density was measured at 600 nm using a spectrometer. Sups were separated

from bacterial pellets and then exposed to HSAECs for 20 min. Cell viability was assessed using resazurin as described above.

An oxygen-limited environment affects the toxicity of *B. cereus* in a strain-dependent manner

The cytotoxicity of Sups from strains 14579 and 11778 were analyzed after propagation in CSFM for 20 h in microaerobic and aerobic conditions. **Figure 4** illustrates oxygen availability exhibits a strain specific influence on cytotoxicity. Strain 14579 Sups displayed reduced toxicity when cultivated under microaerobic conditions (**Figure 4A**). Sups from 11778 cultures demonstrated an opposite effect with enhanced toxicity occurring in microaerobic conditions (**Figure 4B**). This latter strain appears more similar to *B. anthracis* which also found greater toxicity in cultures propagated under microaerobic conditions (Popova et al., 2011). Differences in bacterial density between aerobic and microaerobic cultures were controlled as followed. The viability of cells exposed to Sups was divided by the viability of cells exposed to controls to normalize the data and allow for comparisons between different experiments. The normalized Sup viability (X) was subtracted from the normalized control viability (Y=1) to calculate normalized toxicity. This value was then divided by the optical density of Sup culture (Z). The formula $(1-X)/Z$ was used to calculate the individual bacterial contribution to toxicity. Results found aerobic cultures still had a greater contribution per bacteria to toxicity than microaerobic cultures for strain 14579 (data not shown).

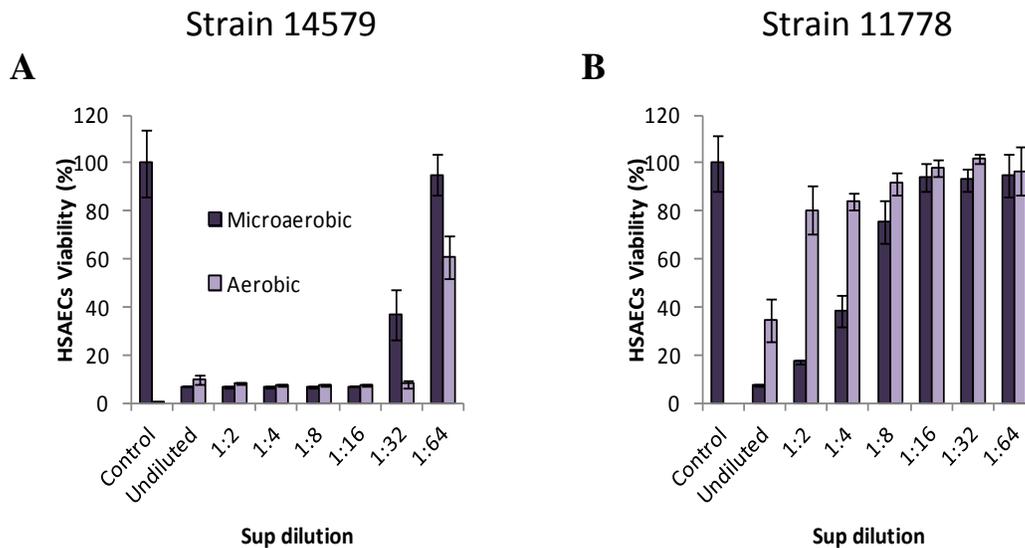


Figure 4 The effect of oxygen availability on *B. cereus* toxicity.

Sups from strains 14579 (A) or 11778 (B) were collected from cultures grown for 20 h in CSFM. Static cultures were grown at 37°C, 5% CO₂ and aerobic cultures at 37°C in shaker at 200 rpm. Supernatants were diluted in CSFM and then assessed for toxicity.

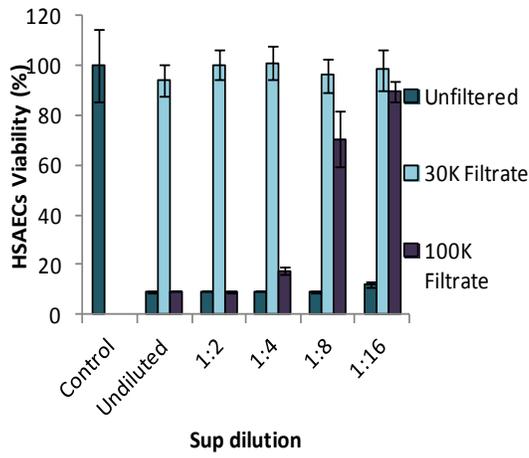
The filtration of sups into 30-, 50-, and 100-K NMWL fractions causes differential patterns of toxicity

To investigate the virulence factor(s) responsible for toxicity, Sups from strains 14579 and 11778 were separated using 30-K, 50-K, and 100-K NMWL Amicon filters. Experiments were performed with bacterial cultures grown in CSFM for 5 h or 20 h in the microaerobic or aerobic conditions outlined previously. **Figure 5A** illustrates for strain 14579 all the toxic factors produced by 5 h were greater than 30K and some of them are less than 100K. **Figure 5B** shows the toxic factors for strain 11778 at 5 h were greater than 30K and less than 100K. Microaerobic 20 h Sups from strain 14579 contained a virulence factor less than 30K that contributed to a minor amount of toxicity, and very toxic virulence factors that were less than 100K (**Figure 5C**). Additionally, this

strain had toxic factors less than and greater than 50K but the latter appears to contribute more to toxicity (**Figure 5E**). **Figure 5D** shows Sups from strain 11778 20 h microaerobic cultures had toxic factors less than 100K. For strain 11778 virulence factors less than and greater than 50K appeared equally toxic toward HSAECs (**Figure 5F**). Interestingly, the separation of a virulence factor greater than 100K caused a significant decrease in toxicity compared to unfiltered Sups of strain 14579, but not 11778 (**Figure 5C,D**). **Figure 5G,I** illustrates Sups from 14579 20 h aerobic cultures contained virulence factors greater than 30K and had greater toxicity from a protein larger than 50K. For 11778, all virulence factors in 20 h aerobic cultures were between 30K and 50K in size (**Figure 5H,J**). This suggests a virulence factor in 11778 Sups that is larger than 50K in size and contributes to toxicity exclusively in microaerobic conditions. These results do not exclude the contribution of lower molecular weight proteins toward virulence, but indicate they could require a larger toxin for increased activity.

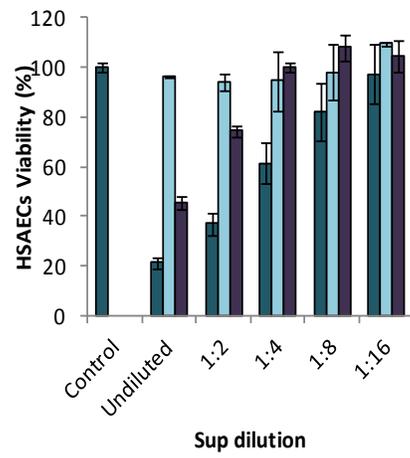
Strain 14579

A Microaerobic 5 h

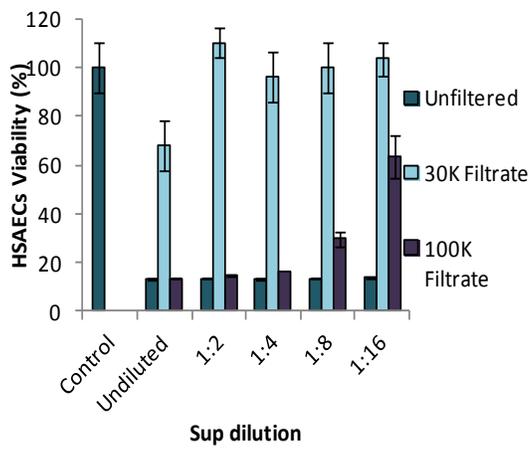


Strain 11778

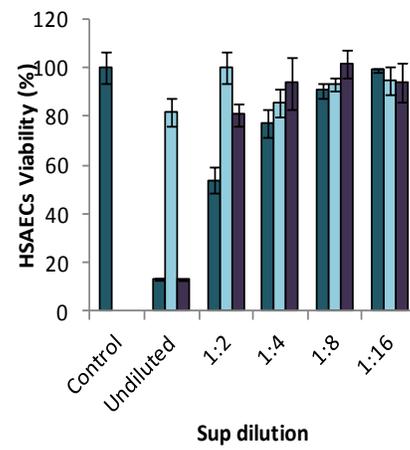
B



C Microaerobic 20 h



D



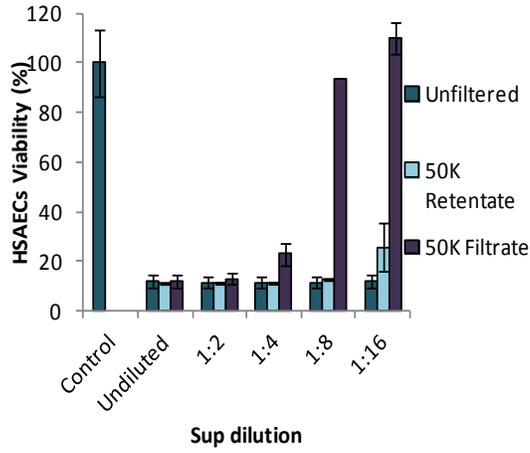
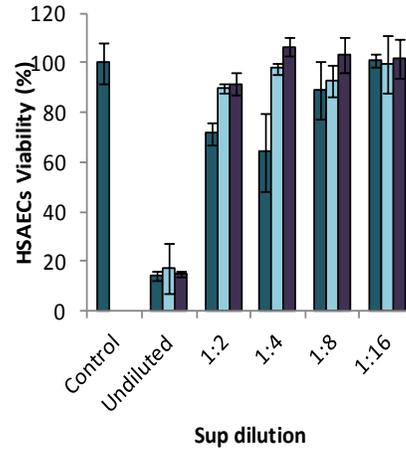
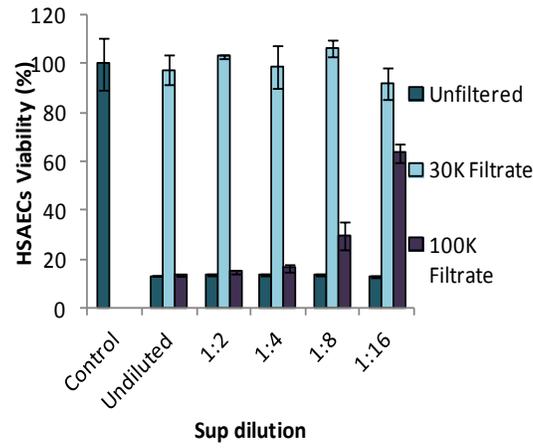
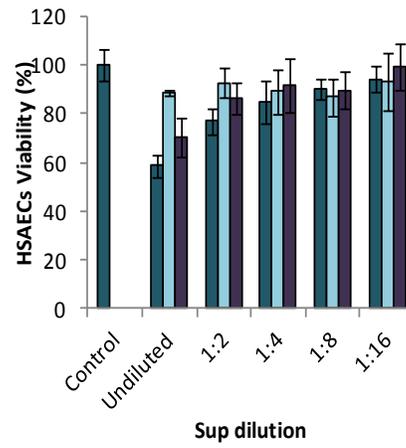
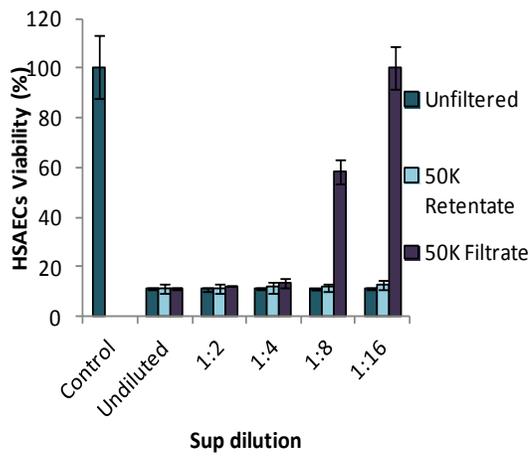
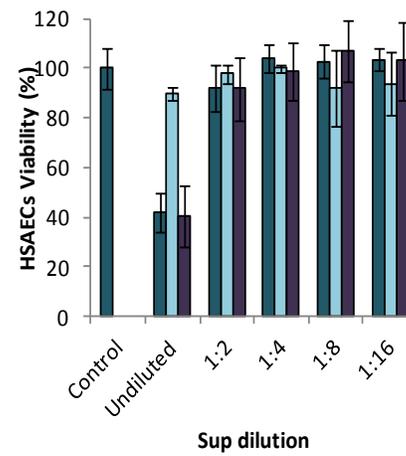
E Microaerobic 20 h**F****G Aerobic 20 h****H****I Aerobic 20 h****J**

Figure 5 The differential toxicity of filtered sups.

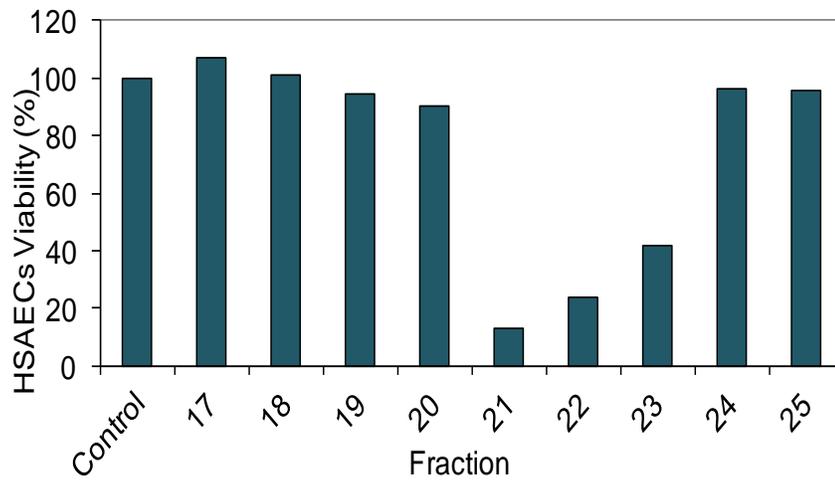
Sups from strains 14579 (A,C,E,G,I) and 11778 (B, D,F,H,J) were grown in CSFM under the microaerobic and aerobic conditions and collected after 5 (A,B) or 20 (C-J) h. (A-F) Sups were separated using Amicon 30-K and 100-K NMWL filters. Only the filtrates were analyzed for toxicity. (G-J) Sups were separated using Amicon 50-K NMWL filter. Retentate separated using the 50-K NMWL filter was diluted in medium titrated with HCl to the pH and volume of the filtrate.

The column filtration of Sups from strains 14579 and 11778 show similar toxicity profiles

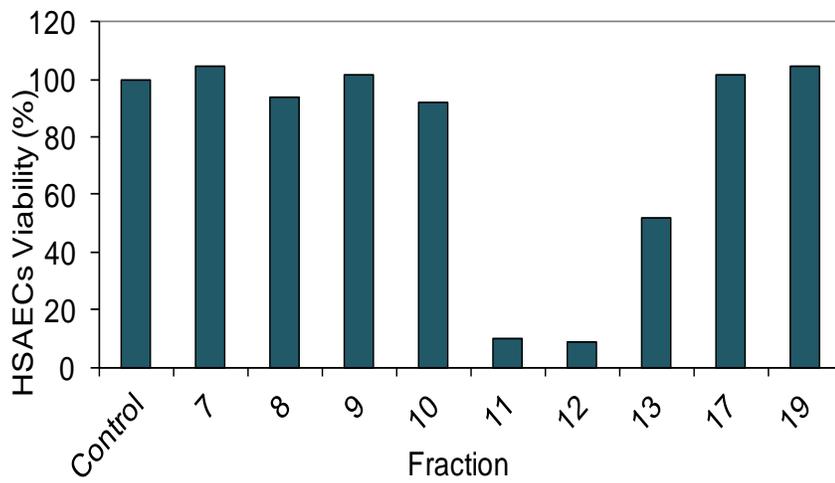
For a better separation of individual toxic proteins, Sups were filtered by size exclusion chromatography. Sups from strain 14579 were grown in DMEM with or without 1 g/L BSA. Sups from 11778 were grown in DMEM with 1 g/L of BSA and cultures grown without BSA were not evaluated for this strain due to the expected lack of toxicity in fractions post-filtration. Sups were then concentrated via SpeedVac 8-fold for a final volume of 1 mL. Concentrate was injected into a HiPrep 16/60 Sephacryl S-200 HR column and run for 90 min. Eluted fractions were diluted 2-fold in medium, exposed to cells for 1 h, and then viability was assessed. The start time of collected fractions differed between experiments; however, significant toxicity was only found in fractions collected about 40 min into the run. **Figure 6** illustrates the toxicity of all the fractions eluted between 30-45 min. Both strains exhibited a similar pattern on the detected OD profile with highly toxic fractions overlapping with the tail of the BSA peak (data not shown). This suggests a toxic protein is similar in size to BSA. Strain 14579 fractions demonstrated increased toxicity compared to those from 11778 (**Figure 6B,C**).

Strain 14579

A DMEM



B DMEM with BSA



Strain 11778

C DMEM with BSA

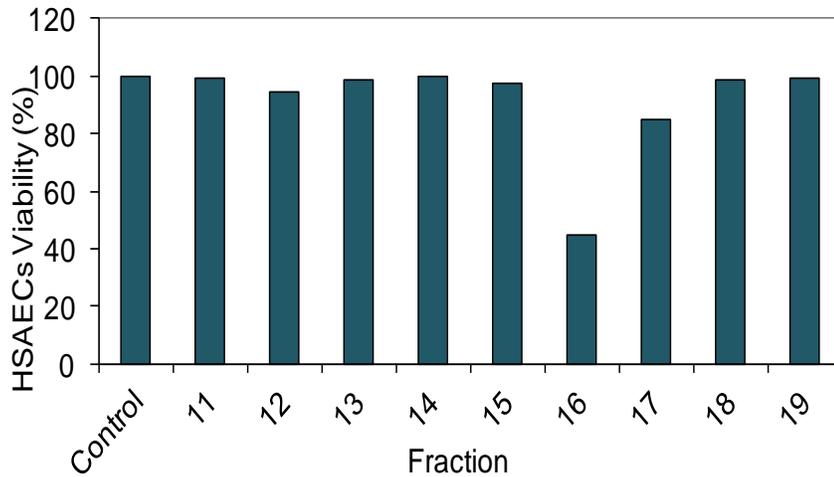


Figure 6 The cytotoxicity of fractioned Sups separated by size exclusion chromatography.

Sups from strain 14579 were grown in DMEM without (A) or with (B) 1 g/L BSA under the microaerobic conditions for 20 h. (C) Sups from strain 11778 were grown in DMEM with 1 g/L of BSA in the same conditions. Sups were concentrated 8-fold by SpeedVac and 1 mL of concentrate was injected into the column. Sups were separated using HiPrep 16/60 Sephacryl S-200 HR column. Figures include the toxicity of fractions eluted between 30 and 45 min.

Toxic fractions 16 (11779), 12 (14579), and 21 (14579) and nontoxic fraction 20 (14579) were analyzed by LC-MS/MS. Spectra counts in each sample quantify the detected peptides from each virulence factor. Results from **Table 2** shows fractions from cultures grown in medium supplemented with BSA contained large amounts of collagenase, Nhe, and CLO, indicating these proteins are potential candidates responsible for toxicity. Additionally, strain 14579 secreted significant amounts of Hbl. The nontoxic fraction 20 (14579 grown without BSA) contained all the components needed for Hbl activity. The toxic fraction 21 (14579 without BSA) did not contain all the subunits needed for Hbl or Nhe activity indicating CLO or InhA are the only possible

toxic proteins in this fraction. Interestingly, the Nhe binding subunit, NheC, was not identified in any fraction.

Table 2 Virulence factors identified via mass spectrometry of *B. cereus* strain 11778 and 14579 column fractions (spectra count). Toxic fractions are indicated with * and strains grown in the presence of 1 g/L BSA are identified as +.

Known virulence factor	Fraction 16* 11778 ⁺	Fraction 12* 14579 ⁺	Fraction 20 14579	Fraction 21* 14579
Bacillolysin	1	0	0	0
Collagenase	22	25	0	0
Hemolysin BL binding component precursor	0	10	3	4
Hemolysin BL lytic component L1	0	12	1	0
Hemolysin BL lytic component L2	0	15	22	14
Immune Inhibitor A precursor	0	0	1	3
Non-hemolytic enterotoxin lytic component A	19	10	4	3
Non-hemolytic enterotoxin lytic component B	12	5	0	0
Cereolysin O precursor	11	5	0	1
Sphingomyelin phosphodiesterase	2	0	0	0

Cholesterol inhibits the toxicity of microaerobic Sups

Cereolysin O is a 52.5 kDa cholesterol-dependent PFT that is known to be inhibited by the addition of cholesterol. To identify the contribution of CLO to toxicity Sups from microaerobic and aerobic cultures were incubated with 10 µg/mL of cholesterol for 1 h prior to cell exposure at RT. Results from **Figure 7A,B** demonstrate cholesterol caused an almost 2-fold reduction in the toxicity of Sups from both strains.

Additionally, cholesterol had no effect in inhibiting the toxicity of aerobic Sups (**Figure 7C,D**). **Figure 8** illustrates the supplementation of cholesterol into medium prior to bacterial inoculation slightly increased its effectiveness as an inhibitor. Since cholesterol did not completely abrogate Sup toxicity, it suggests there are additional factors independent of CLO contributing to virulence. Similar experiments with *B. anthracis* found the addition of cholesterol caused an approximate 2-fold reduction in toxicity in microaerobic, and not aerobic, supernatants (Popova et al., 2011).

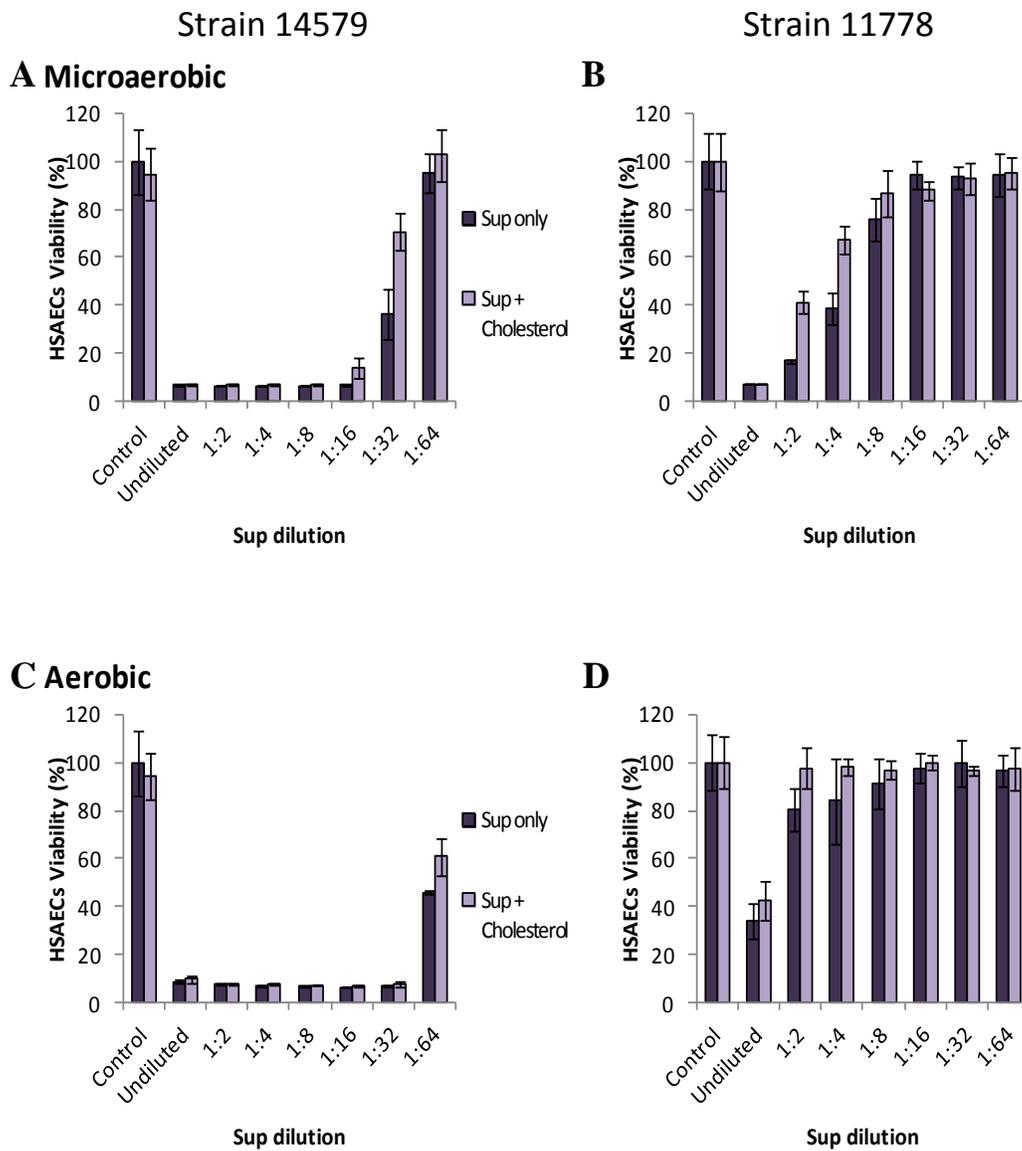


Figure 7 Cholesterol exhibits a protective effect against Sups from microaerobic cultures. Sups from strains 14579 (A,C) and 11778 (B,D) aerobic and microaerobic cultures were cultivated in CSFM. Cholesterol was added to Sups at a concentration of 10 $\mu\text{g/ml}$ and incubated at room temperature for 1 h prior to cell exposure. A control of medium with cholesterol was included.

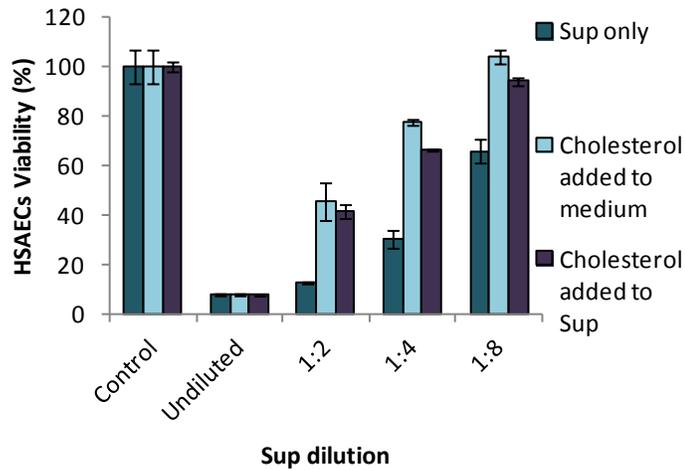


Figure 8 The direct addition of cholesterol into Sups is slightly less inhibitive than its supplementation into inoculation medium.

Sups were from strain 11778 microaerobic cultures grown in DMEM supplemented with 1 g/L of BSA. Cholesterol was added to Sups or inoculation medium at a final concentration of 10 µg/ml. A control of medium was included under the same conditions.

Cereolysin O is a pore-forming toxin that contributes to cytotoxicity via membrane permeabilization. Therefore, the amount of permeability was measured and compared to cell viability. Sups from 11778 and 14579 microaerobic cultures were incubated with cholesterol at a 10 µg/ml concentration. Permeability and viability of HSAECs were assessed using Cytotox Homogenous Membrane Integrity Assay kit and resazurin, respectively. Results from **Figure 9** indicate cell permeability strongly correlated with toxicity for both strains of *B. cereus*. These data suggest membrane permeability is the major, but not exclusive, mechanism of cytotoxicity. There is still residual toxicity, especially in strain 11778, found in the absence of permeability indicating another mechanism of toxicity (**Figure 9B**). Permeability in strain 11778 was almost completely inhibited by cholesterol demonstrating CLO is the major PFT in this

strain. Since the other enterotoxins are absent in this strain, the remaining permeability could be attributed to Nhe. The permeability of 14579 was only slightly inhibited by cholesterol suggesting CLO plays a minimal role and other PFTs were responsible for the majority of induced permeability (**Figure 9A**). Interestingly, CLO appeared to be more potent in strain 14579 since the toxin remained active even when diluted 64-fold.

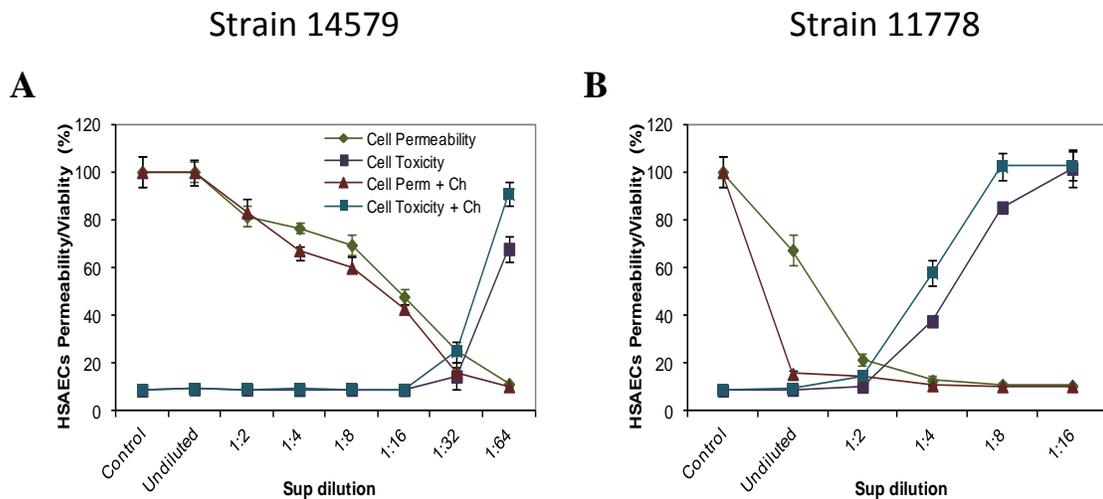


Figure 9 Cell permeability is major mechanism for cytotoxicity, however, CLO contribution towards permeability is strain dependent.

Sups from microaerobic 14579 (A) and 11778 (B) culture were incubated with 10 µg/ml cholesterol for 1 h at room temperature. 100 µl of Sups were added to cells and incubated for 20 min. To assess permeability, the CytoTox-ONE Homogeneous Membrane Integrity kit was used per manufacturer's instructions. Medium and the provided lysis solution were used as controls for permeability and viability assays. Toxicity towards HSAECs was analyzed using resazurin.

Bacterial propagation in medium supplemented with BSA potentiates the cytotoxicity of *B. cereus* supernatants

Previous research on bovine serum albumin (BSA) reported this molecule can increase the toxicity of *Bacillus* species (Elleboudy et al., 2011; Popova et al., 2011; St John et al., 2013). To investigate this effect, cultures of ATCC 14579 and 11778 were

grown in microaerobic conditions in DMEM supplemented with or without 1 g/L of BSA. **Figure 10** demonstrates BSA significantly increased the toxicity of bacterial supernatants from both strains. The optical density of the cultures indicated only minor differences in growth (0.72 OD₆₀₀ in DMEM versus 0.80 OD₆₀₀ in DMEM with BSA). However, variations in Sup pH were noted with a pH of 5.4 and 6.9 in cultures grown in the presence and absence of BSA.

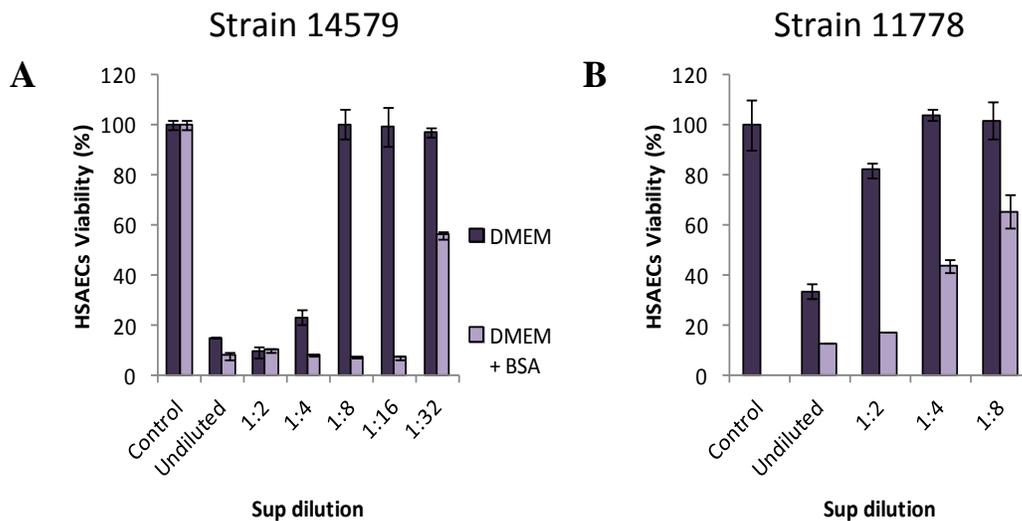


Figure 10 The cultivation of *B. cereus* in DMEM supplemented with BSA increases the cytotoxicity of secreted products.

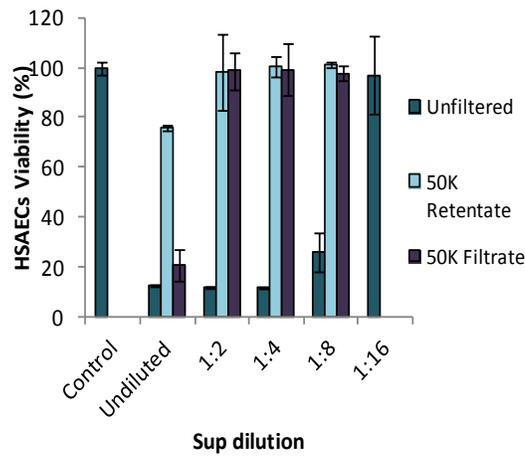
Inoculation medium was supplemented with or without 1 g/L of BSA. Sups were generated from 14579 (A) and 11778 (B) microaerobic cultures. Dilutions were prepared with the same medium used for growing cultures.

To determine if BSA influences virulence factor production Sups from strain ATCC 14579 were grown in medium supplemented with or without BSA at a concentration of 1 g/L. Strain 11778 was not evaluated in these experiments due to its

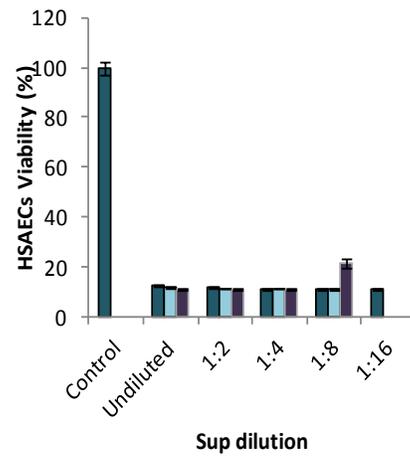
minimal amount of toxicity found in the absence of BSA. Supers were then separated using 50-K or 100-K NMWL filters and retentate fractions were diluted in the medium titrated with HCl to match the volume and pH of the filtrate. **Figure 11A-D** illustrates Supers cultivated without BSA have a considerable loss of biological activity upon filtration compared to those grown with BSA. Mass spectrometry of these fractions without BSA identified almost all virulence factors were in the retentate with minimal proteins filtering through (data not shown). This suggests proteins could be aggregating on top of the filter resulting in their inactivation. **Figure 11E,F** demonstrates when 1 g/L of BSA was supplemented into supernatants prior to filtration, the toxicity of individual fractions was enhanced, especially in the retentate. The supplementation of BSA prior to filtration does not restore toxicity to the amount found in Supers from cultures grown in the presence of BSA (**Figure 12B,F**). To determine the loss of biological activity in Supers filtered without BSA, post-filtration the fractions were combined and assessed for toxicity. **Figure 12A** shows that Supers cultivated in the absence of BSA had a substantial loss of activity upon filtration, whereas when cultivated in medium supplemented with BSA Supers retained most of its toxicity post-separation. To further elucidate if the addition of BSA could be influencing the toxicity of cultures during growth rather than during filtration, BSA at 1 g/L was added to Supers cultivated in DMEM either immediately before or after filtration. As a control, BSA was also supplemented into unfiltered Supers. Results from **Figure 12B** indicate the addition of BSA prior to filtration substantially increased the toxicity of Supers. The toxicity of the filtered BSA-supplemented Supers appeared to have only a slight loss of activity compared to Supers

filtered without BSA. Surprisingly, a simple addition of BSA to unfiltered sups prior to cell exposure enhanced toxicity as well. This suggests the process of filtration caused an inactivation of virulence factors and the presence of BSA mitigated this effect.

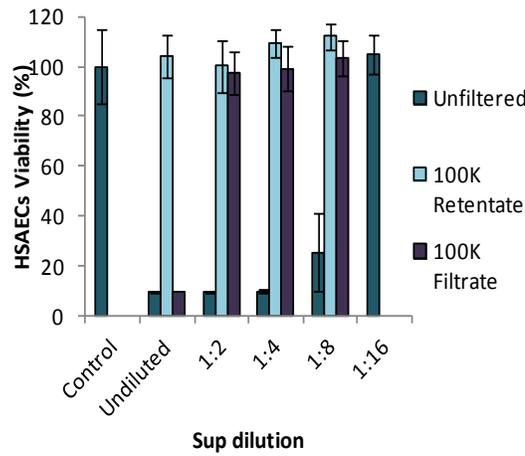
A DMEM



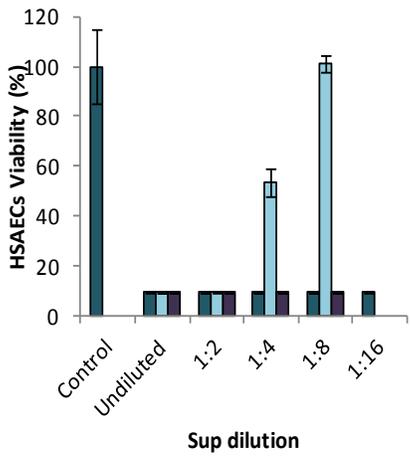
B DMEM with BSA



C



D



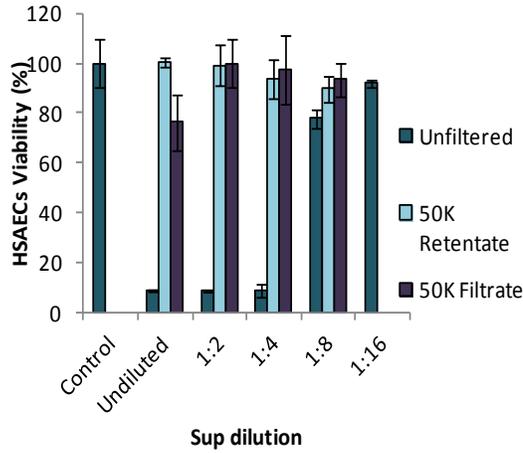
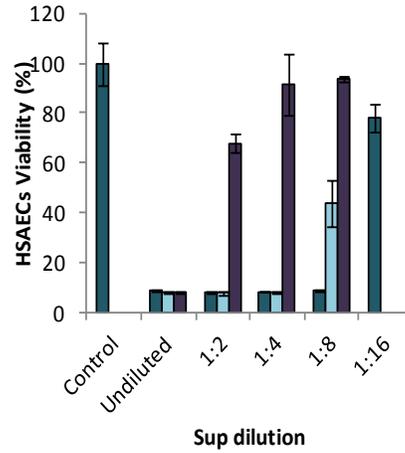
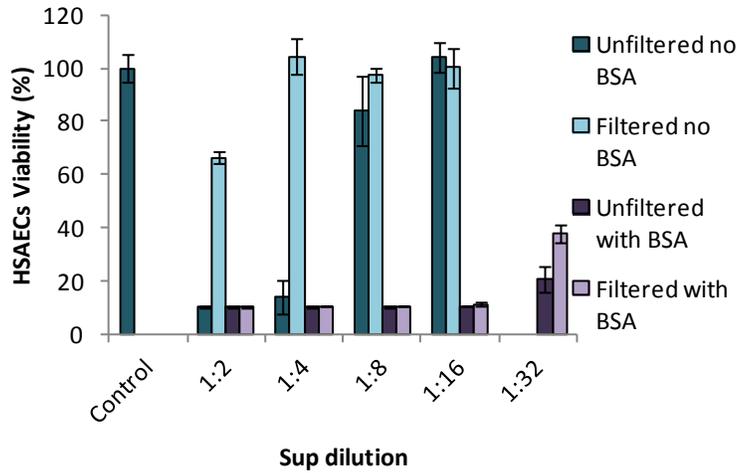
E**F**

Figure 11 BSA prevents the loss of toxicity found in individual filtered fractions.

Sups from ATCC 14579 were grown in DMEM under the microaerobic conditions described previously. DMEM was supplemented with BSA at a concentration of 1 g/L to either (A-D) medium prior to inoculation or (E,F) sups prior to filtration. Sups were then separated using Amicon 50 K and 100 K NMWL filters. Retentates were diluted in DMEM titrated with HCl to match the pH and volume of the filtrate.

A

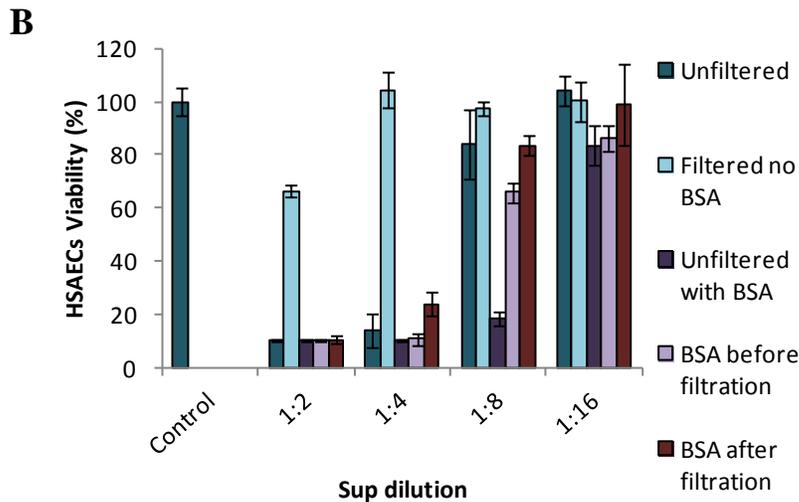
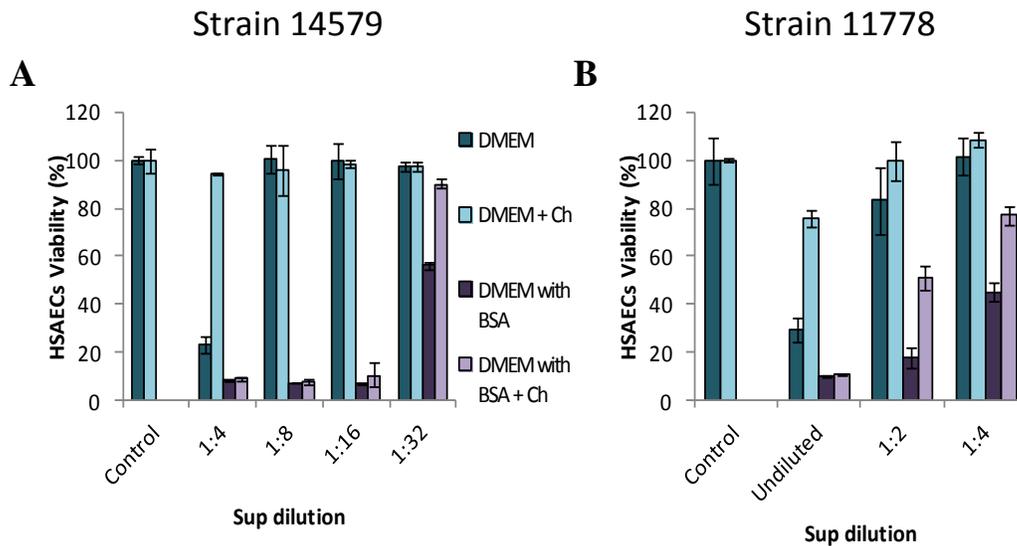


Figure 12 The effect of BSA in retaining the toxicity of combined fractions post-filtration. Sups from ATCC 14579 were grown in DMEM under the microaerobic conditions. DMEM was supplemented with BSA at a concentration of 1 g/L to either (A) medium prior to inoculation or (B) Sups pre- or post-filtration. Sups were then separated using Amicon 50-K NMWL filters. Retentates were combined with the filtrate after separation and the combined fractions were tested for toxicity using resazurin.

BSA prevents the inactivation of the reduced isoform of cereolysin O

Cereolysin O was investigated as a potential candidate responsible for this loss of activity. This cholesterol-dependent cytotoxin has previously been found to be inactivated by two possible mechanisms. Under specific conditions, CLO monomers spontaneously undergo pre-oligomerization which renders the toxin unable to bind to cholesterol located on the cell membrane (Cowell et al., 1978; Gilbert, 2005). Additionally, CLO is reported to have two conformations depending on the state of its cysteine residue. The active form has a free sulfhydryl group and, if oxidized, the protein then becomes inactivated. To investigate the influence of BSA on CLO activity, cholesterol was incubated with Sups from 14579 and 11778 microaerobic cultures grown in the presence or absence of BSA. **Figure 13A,B** demonstrates CLO is active in both

strains regardless of BSA supplementation, however, this activity appears to be higher in the absence of BSA. The activity of CLO post-separation by a filter or size-exclusion column was tested as well. Sups from 11778 cultures grown with or without 1 g/L of BSA were separated in a 3-K NMWL filter to concentrate the sample and to remove the salt present in the medium. The column fraction for 11778 had been stored at 8°C overnight before tested for toxicity. Post-separation, CLO only remained functional in Sups from cultures grown in the presence of BSA (**Figure 13C**).



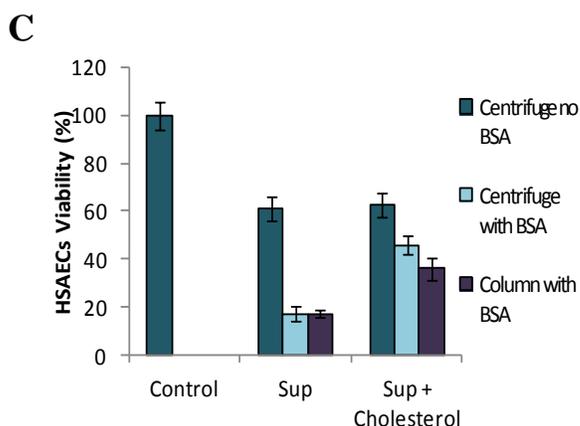


Figure 13 CLO remains active in Sups cultured in the presence or absence of BSA but becomes inactive after separation without BSA.

Cholesterol at 10 $\mu\text{g/ml}$ was incubated for 1 h at RT with Sups from 14579 (A) and 11778 (B) microaerobic cultures grown with or without 1 g/L of BSA in DMEM. (C) Sups from 11778 microaerobic cultures grown in DMEM with or without 1 g/L of BSA. 4 mL of Sups were then separated using Amicon 3-K NMWL centrifugal filters and the individual fractions were combined. Column fractions from earlier experiments stored at 8°C overnight were also assessed for CLO activity. Samples were diluted in medium and then incubated with cholesterol at a concentration of 10 $\mu\text{g/ml}$ for 1 h at room temperature.

To determine the mechanism behind the inactivation of CLO, 11778 and 14579 cultures were grown in the presence or absence of 1 g/L BSA and Sups were concentrated using a 3-K NMWL filter. Retentate fractions were run on SDS-PAGE gels with or without buffer containing DTT and SDS. A rabbit antibody against streptolysin O, which recognizes homologous ALO, was incubated with the membrane (Heffernan et al., 2007). An anti-rabbit goat secondary antibody was then used followed by chemiluminescent detection. **Figure 14A** illustrates samples run on SDS-PAGE contained a CLO dimer post-filtration with a single band detected approximately 120 kDa in size. Using BSA in the blocking buffer could have prevented the detection of bands in some lanes and could be responsible for the absence of a CLO band in strain 14579

grown in BSA. Interestingly, CLO is believed to be primarily monomeric in solution (Cowell et al., 1978; Gilbert, 2005). The presence or absence of BSA had no effect on dimer formation in Sups. Only when Sups were filtered in the presence of 20% SDS did they retain the monomeric form (**Figure 14C**). **Figure 14B** demonstrates native PAGE conditions which contained samples run without a reducing buffer. Sups from cultures grown in 1 g/L of BSA had two bands of CLO, indicating there were different charged forms of the toxin. Previous research has identified the oxidized form of CLO migrates faster than its reduced form (Cowell et al., 1976). These results suggest BSA does not prevent the aggregation of CLO but instead protected an active conformation from being oxidized. Additionally, **Figure 14** illustrates the production of CLO was greater in strain 11778 than 14579.

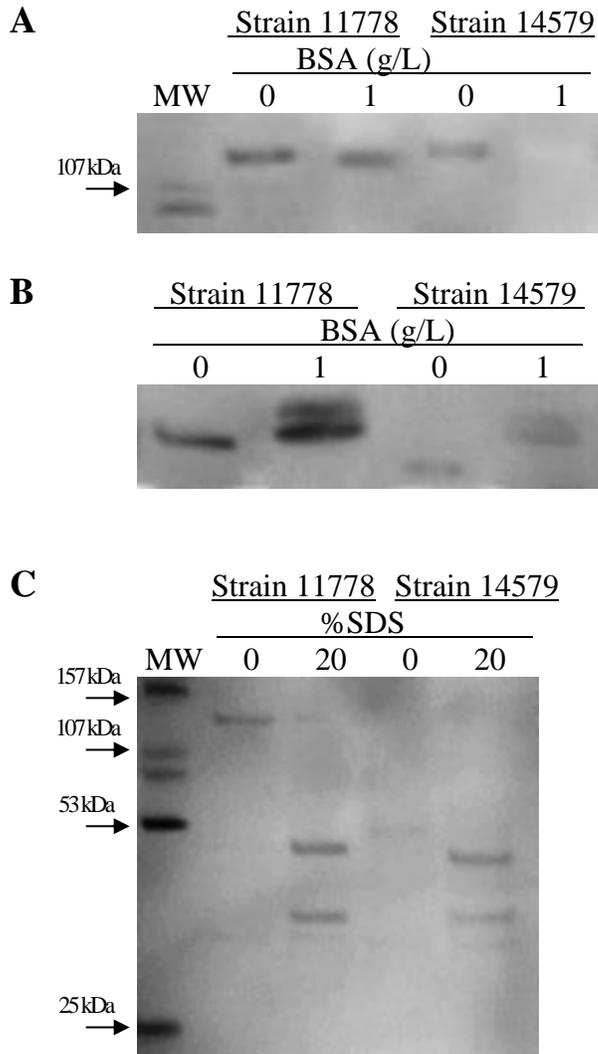


Figure 14 BSA influences the conformation of concentrated cereolysin O in strains 11778 and 14579. Sups from 14579 and 11778 cultures were grown in medium with or without 1 g/L of BSA. Supernatants were concentrated 6-fold using Amicon 3-K filters. Retentates from filtration were run on SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked with 5% BSA in PBST and reacted with rabbit polyclonal anti-streptolysin O antibody followed by an anti-rabbit IgG, HRP-linked antibody. Blot was developed using SuperSignal West Dura Extended Duration Substrate (Thermo Scientific). **(A)** Samples were incubated and heated with a buffer containing SDS and DTT and then run in SDS-PAGE. **(B)** Samples were run in a DNA loading buffer without SDS. **(C)** Sups were filtered in the presence of 20% SDS to prevent dimerization.

Metabolic product succinic acid acts as a pathogenic factor and is enhanced by BSA

The *Bacillus* species can undergo anaerobic fermentation that results in the generation and secretion of acidic metabolic products. The pH value of 5.4 in Sups indicated succinic acid (SA) in its partially protonated state with a pK_a of 4.19 and 5.57 could be a potential contributor. Succinic acid is a metabolic by-product of *Bacteroides* species generated under anaerobic conditions that contributes to virulence at a pH of 5.5 but not at a pH of 7.0 (Rotstein et al., 1987). The mechanism behind SA-induced toxicity involves the fatty acid causing a reduction of intracellular pH in the cell that facilitates irreversible respiratory burst. The concentration of SA in Sups was found to be 1.43 ± 0.1 mM (mean \pm SD) regardless of BSA supplementation. A stock solution of 100 mM succinic acid in water was prepared. To determine if succinate could influence *B. cereus* virulence, SA was directly added to bacterial supernatants at a concentration of 0, 2, and 5 mM for 1 h at RT and then assessed for toxicity. **Figure 15A,B** illustrates Sups supplemented with succinic acid displayed no increase in cytotoxicity. Previous experiments with *B. anthracis* found SA enhanced Sup toxicity; however, Sups had a 10x longer exposure time compared to this experiment with *B. cereus*. To control for this variable, cells were exposed for 20 min, Sups were then removed, and medium titrated to the pH of Sups was supplemented with 0, 2, or 5 mM of SA was incubated with cells for 2 h at RT. Results from **Figure 15D** demonstrate succinic acid significantly enhanced the cytotoxicity of Sups from cultures grown in a medium containing BSA. The addition of SA in the absence of BSA appeared to have no effect (**Figure 15C**). One reason for

this loss of activity is that the pH of these Sups (6.9 in DMEM without BSA versus 5.4 in DMEM with BSA) was too basic for SA.

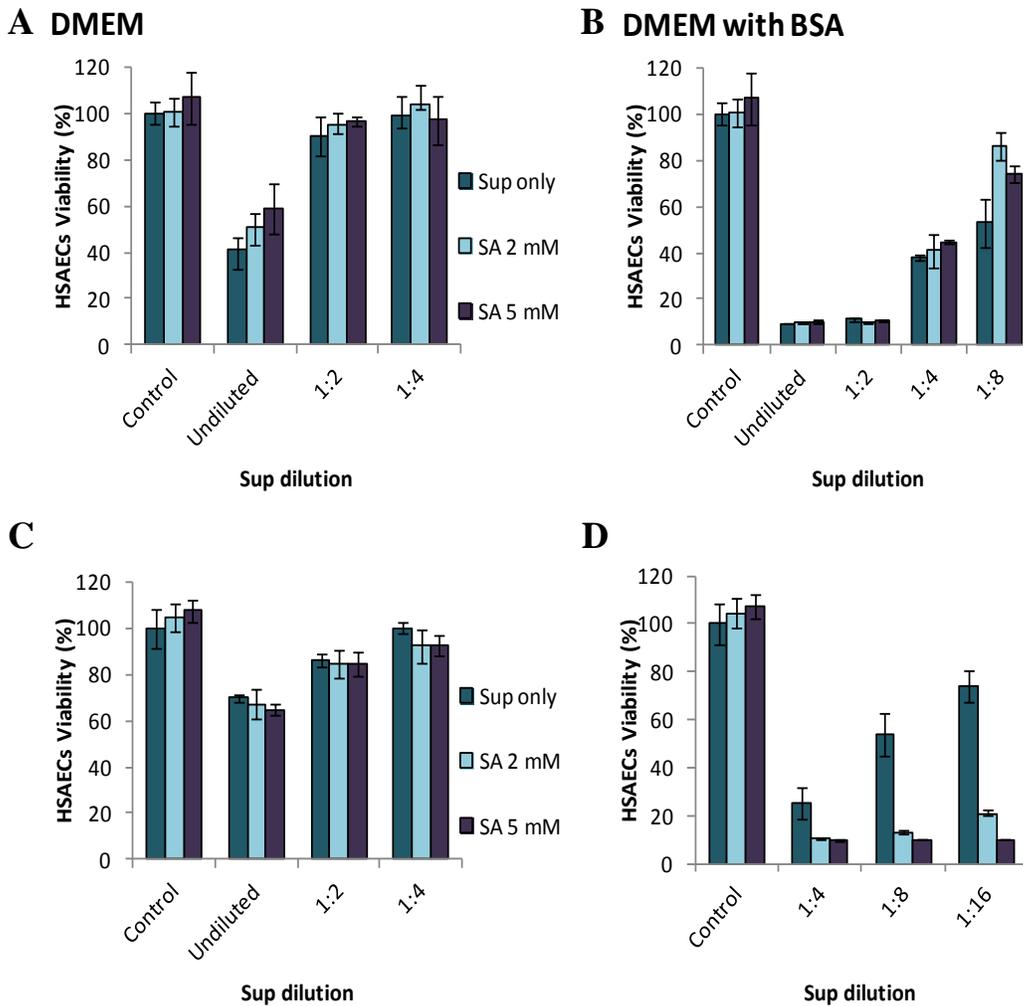


Figure 15 Fermentation product succinic acid increases the toxicity of microaerobic Sups grown in medium containing albumin.

Strain 11778 was inoculated into DMEM medium with and without 1 g/L of BSA and grown under microaerobic conditions. Sups were diluted in DMEM titrated to the pH of Sups using HCl. (A,B) Succinate at a concentration of 0, 2, and 5 mM in water was added directly to Sups and incubated for 1 h at room temperature. Cells were exposed to Sups for 20 min and then viability was determined using resazurin. (C,D) After exposure, Sups were removed and DMEM was supplemented with 0, 2, or 5 mM succinic acid incubated for 2 h at 37°C 5% CO₂. After incubation, medium was removed and viability was assessed using resazurin. Controls included the same concentration of succinic acid in titrated medium.

To determine the contribution of SA to *B. cereus* toxicity, Sups from 11778 cultivated in 1 g/L BSA were diluted in medium titrated to a pH of 5.4 or 7 using HCl. Results from **Figure 16** show Sups demonstrated enhanced toxicity at pH 5.4 after a 120 min cell exposure compared to the 20 min exposure. Additionally, cholesterol had a greater effect in inhibiting the toxicity of acidic Sups with a longer cell exposure (**Figure 16B**). These results suggest SA is a pathogenic factor for *B. cereus* and its contribution to toxicity depends on the pore-forming activity of cereolysin O. This identifies a shared mechanism of toxicity between *B. cereus* and *B. anthracis* through the synergistic effect of a cholesterol-dependent cytolysin and metabolic product succinate under microaerobic conditions (Popova et al., 2011).

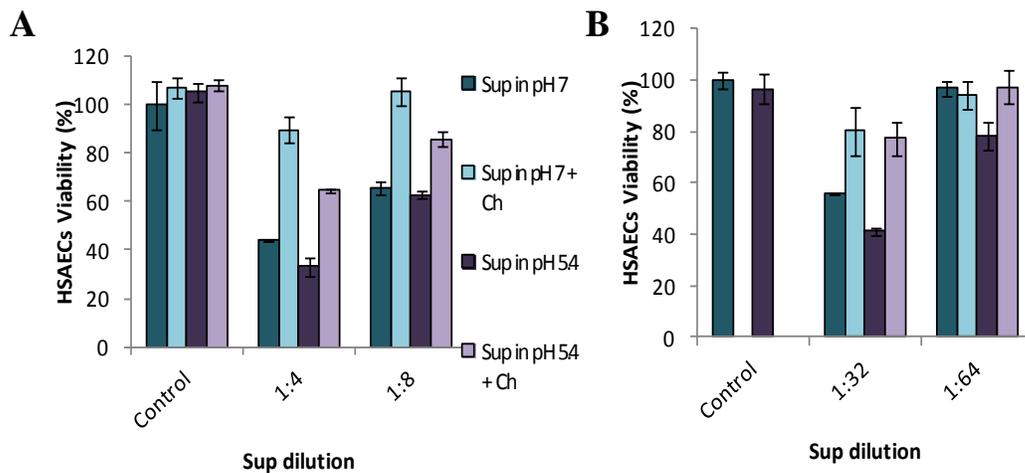


Figure 16 *B. cereus* ATCC 11778 demonstrates enhanced toxicity in a pH of 5.4 that is dependent of CLO.

Sups were collected from cultures grown in microaerobic conditions for 20 h in DMEM with 1 g/L BSA. Dilutions were made in medium titrated to a pH of 5.4 or 7 using HCl. Cells were exposed to Sups for either (A) 20 min or (B) 120 min. Controls included medium titrated to a pH of 5.4 and 7.

BSA could potentiate the chemically-mediated toxicity of *B. cereus*

The experiments above indicate BSA influences toxin aggregation and activity; however, its potential on enhancing chemical toxicity remained to be tested. Previous research suggested albumin can enhance bacterial virulence by capturing and stabilizing *B. anthracis* NO products (St. John et al., 2013). To determine the level of ROS/RNS production in *B. cereus*, the dye Amplex Red (AR) was used. Amplex Red is a colorless and non-fluorescent derivative of resorufin that when coupled with HRP, will fluoresce when oxidized by hydrogen peroxide and/or peroxynitrite. Bacterial culture was inoculated into DMEM containing 1 g/L of BSA, 0.1 mM of AR and 0.2 U/mL of HRP. Aliquots were collected every hour for 10 h and then Supers were removed and analyzed by spectrometer. Controls included medium containing both AR and HRP reagents. **Figure 17A** illustrates 11778 and 14579 microaerobic cultures generated significant amounts of ROS/RNS. The declination curve present in 14579 data could indicate further oxidation of resorufin into a colorless product by peroxidase or that the onset of microaerobic conditions had insufficient oxygenation needed for the oxidation of Amplex Red. The toxicity and cholesterol inhibition of the Supers from ATCC 11778 was assessed. The generation of oxidizing species correlated with an increase in Super toxicity that could not be explained by increasing amounts of CLO contribution (**Figure 17B**).

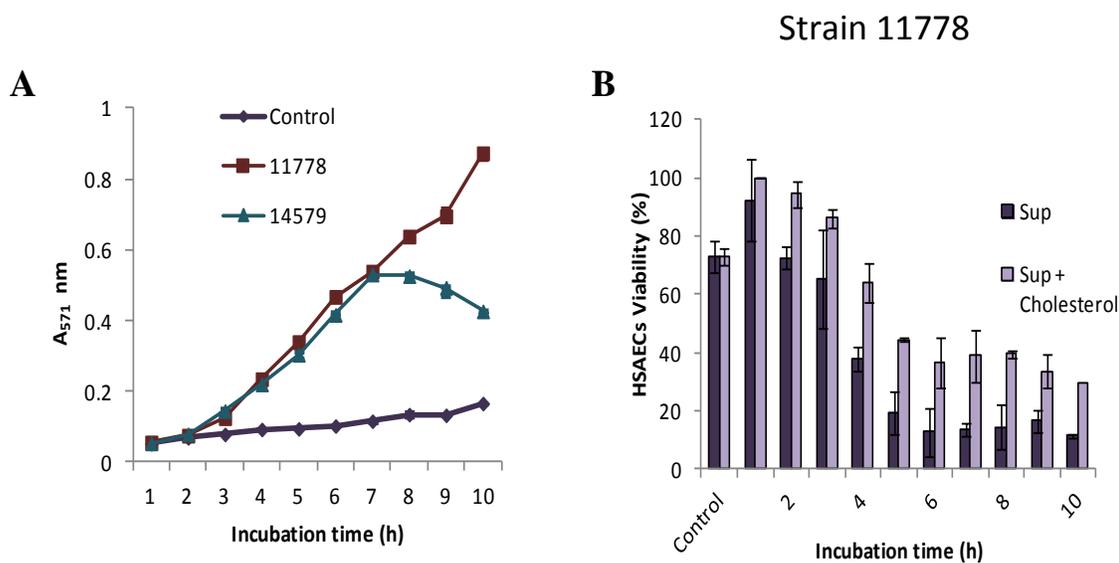


Figure 17 Amplex Red indicate ROS/RNS production from bacterial cultures correlated with increased toxicity.

ATCC 11778 and 14579 microaerobic cultures were grown in DMEM with BSA 1 g/L supplemented with 0.1 mM of Amplex Red reagent and 0.2 U/mL of HRP. (A) Sups were collected every hour and their absorbance read via spectrometer at 571 nm. (B) 11778 Sups were stored overnight at 8°C prior to the toxicity assay. Cholesterol was incubated with Sups at a concentration of 10 µg/mL for 1 hr at RT before cell exposure.

Uric acid is a natural scavenger that selectively and irreversibly binds to peroxynitrite causing its inactivation. To determine the presence and contribution of peroxynitrite in Sups, uric acid was supplemented into bacterial medium prior to inoculation. To prevent precipitation, 50 mg of uric acid was added to 10 mL of .1 M NaOH for a stock concentration of 30 mM. Uric acid was added to culture medium at a final concentration of 10 µM, 100 µM, and 1 mM concentrations and was titrated to the original pH of medium using NaOH. Sups were slightly more acidic when supplemented with uric acid (pH 5.35 no uric acid and pH 5.26 with 1 mM uric acid). **Figure 18** shows that the addition of uric acid in 10 µM, 100 µM, and 1 mM concentrations had no effect on HSAEC viability. This suggests peroxynitrite is not an important virulence factor in

B. cereus. Upon further investigation the activity of uric acid was relatively low towards radicals derived from peroxyntrous acid when compared with other antioxidants, such as ascorbate (Balavoine and Geletii, 1999).

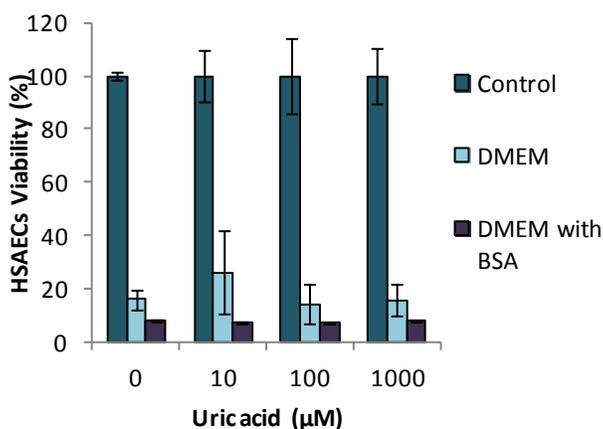


Figure 18 The supplementation of uric acid into medium does not reduce the cytotoxicity of 14579 supernatants.

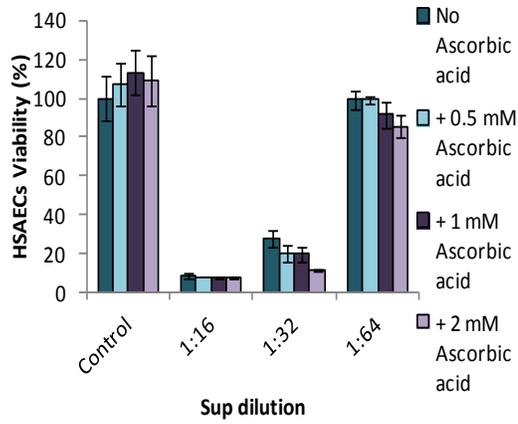
A concentration of 10 µM, 100 µM, and 1 mM of uric acid was added to DMEM medium with and without 1 g/L of BSA. Medium was inoculated with strain 14579 and cultures were grown under microaerobic conditions. Sups were diluted using the same medium used for inoculation. Controls containing the same concentration of uric acid were included.

Ascorbic acid (AA), also known as vitamin C, was found to have the highest level of antioxidant activity causing complete protection against NO-derived radicals (Balavoine and Geletii, 1999). Interestingly, ascorbate can also act as a reducing agent reacting with S-nitroso albumin to produce thiol, dehydroascorbic acid, and NO (Holmes and Williams, 2000). S-nitrosothiols will typically undergo a small amount of decomposition (<10%) over the course of 3 h. However, in the presence of ascorbate,

this reaction is accelerated and is completed with 50% of thiols decomposed after 20 min (Smith and Dasgupta, 2000). Ascorbic acid has been shown to have pro-oxidant properties under specific conditions (Guidarelli et al., 2001; Inai et al., 2005). A stock solution of 100 mM ascorbate in water was prepared. To determine the effect of ascorbic acid on Sup toxicity, AA was incubated with microaerobic Sups for 2 h at 0, 0.5, 1, and 2 mM concentrations. Sups supplemented with ascorbate were more acidic with a pH from 5.42 to 5.34, 5.28, and 5.08 in 11778 and a pH from 5.35 to 5.29, 5.11, and 4.96 in 14579 containing .5, 1, and 2 mM of ascorbate, respectively. **Figure 19A** shows ascorbate, like uric acid, had a minimal effect on 14579 Sups. Interestingly, it potentiated the toxicity of 11778 (**Figure 19B**). **Figure 19C,D** demonstrates the contribution of ascorbate in 11778 was dependent on the presence of BSA. Additionally, the effect of ascorbate was only partially reduced upon CLO inhibition (**Figure 19E**). These results suggest nitrosylated BSA could be present in Sups and if a contributor to toxicity, then this effect could be exacerbated by ascorbic acid.

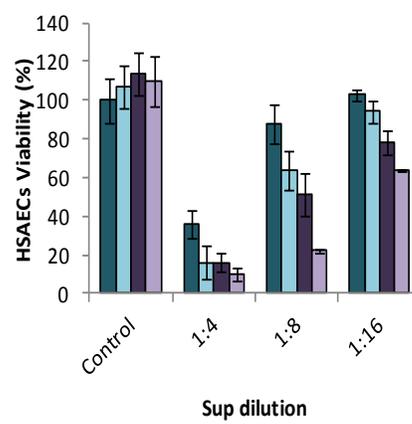
Strain 14579

A

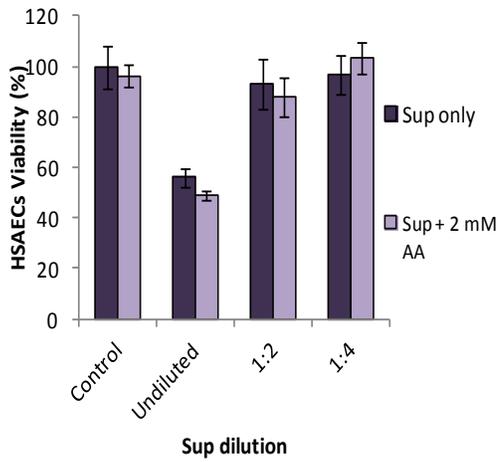


Strain 11778

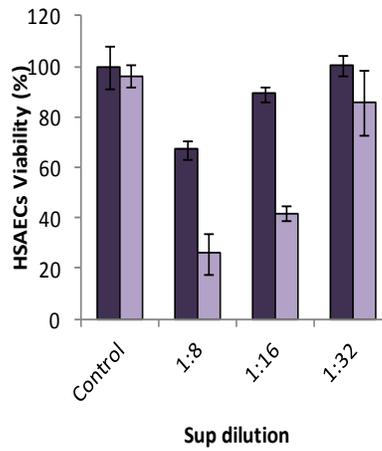
B



C DMEM



D DMEM with BSA



E

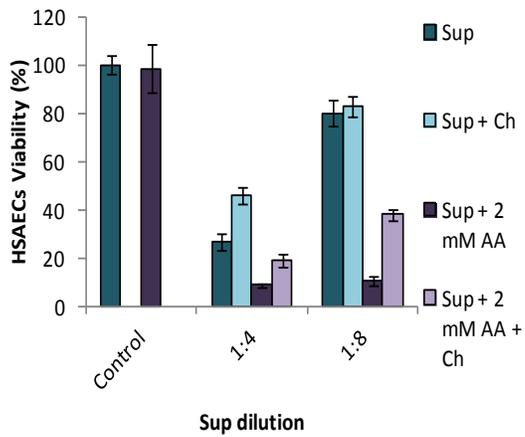
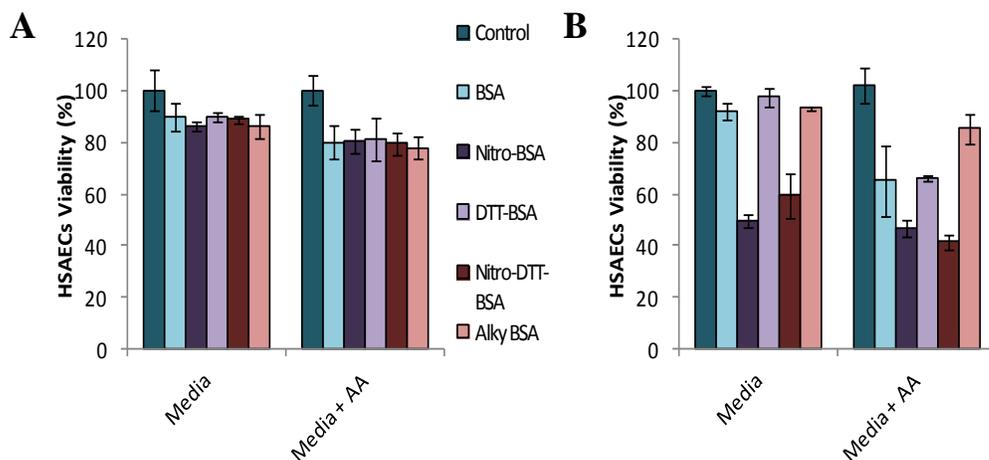


Figure 19 The addition of ascorbic acid exacerbates Sup toxicity and is dependent on the presence of BSA.

(A,B) Strains 14579 and 11778 were inoculated into DMEM medium with 1 g/L of BSA and grown under microaerobic conditions. Dilutions were made with medium titrated using HCl to the pH of grown cultures (5.2 for 14579 and 5.4 for 11778). 100 mM stock of Ascorbic acid in water was prepared and then added to Sups at a concentration of 0, 0.5, 1, and 2 mM and incubated for 2 h at room temperature. Cells were exposed for 20 min and viability was determined using resazurin. (C,D) Strain 11778 cultures were grown in DMEM with or without 1 g/L of BSA and the toxicity of 2 mM ascorbate in Sups was tested. (E) Cholesterol at a concentration of 10 µg/ml and ascorbate at a 2 mM concentrations were incubated with Sups from 11778 and cytotoxicity was assessed. Controls included the same concentrations of ascorbic acid.

BSA can interact with reactive species through multiple pathways, one of which being the nitrosylation of albumin's thiol groups. BSA has 35 cysteine residues, 34 of which are in disulfide bridges (Peters Jr., 1985). Cys-34 is the one active thiol group that can interact with NO. Such a reaction could concentrate and stabilize nitric oxide (NO) facilitating the downstream formation of toxic NO derivatives, therefore, nitrosylated BSA was investigated as a potential toxic factor. Prior to nitrosylation, BSA was modified in two ways. The sulfhydryl residues in 200 mg/mL of BSA in a 50 mM Tris-HCl pH 7.3 solution were reduced or alkylated using .009 M of DTT or .03 M of iodoacetamide, respectively. Reduced BSA affected its structure causing the sample to become viscous. Both modified and unmodified BSA were extensively dialyzed overnight using PBS. For nitrosylation of BSA, 200 mg/mL of sodium nitrite was added to water and then titrated to pH 5 using HCl. Equal parts of titrated sodium nitrite and 100 mg/mL modified or unmodified BSA were mixed and incubated for 1 h. The concentration of free thiol groups in BSA was determined using Ellman's reagent. The percentage of free cysteine residues was 30% in unmodified BSA meaning a 30% average of BSA molecules have a free thiol group. 15% of cysteine residues were free in

nitrosylated BSA (Nitro-BSA) indicating a partial nitrosylation of BSA molecules. BSA reduced with DTT (DTT-BSA) and nitrosylated reduced BSA (Nitro-DTT-BSA) had free thiol content of 380% and 400%, respectively. This suggests DTT freed many cysteine groups that were previously in disulfide bridges. It is unclear why the nitrosylation of reduced BSA caused an increase in the percentage of free thiol groups. Alkylated BSA (Alky-BSA) contained only 2% free cysteine residues. **Figure 20A,B** demonstrates nitrosylated BSA in titrated medium was toxic after a cell exposure of 120 min and not 20 min. Supplementation of 2 mM ascorbate appeared to potentiate BSA toxicity regardless of nitrosylation. **Figure 20C** shows enhancement of ascorbic acid is dependent on the presence of BSA in Sups, however the modification of BSA has no effect.



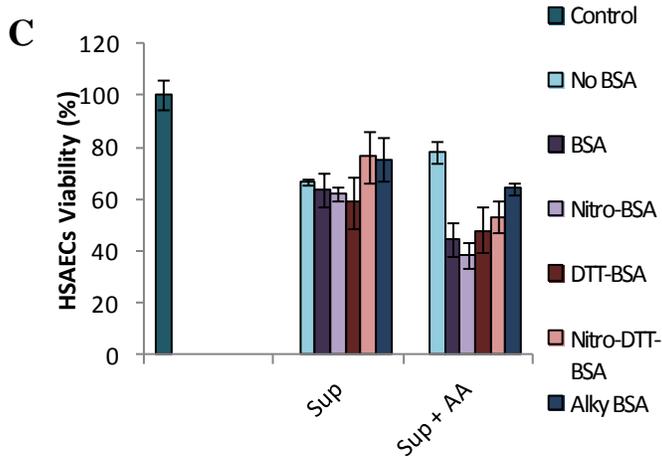


Figure 20 Nitrosylated BSA independently contributes to cytotoxicity, but does not appear to be a factor for the enhanced toxicity in Sups when supplemented with ascorbate.

(A,B) Medium titrated to pH 5.4 using HCl was supplemented with 1 g/L of modified or unmodified BSA. A stock solution of 100 mM Ascorbate was prepared in water. Ascorbic acid was added to samples at 2 mM concentration and incubated for 2 h at RT. Samples were exposed to cells for (A) 20 min or (B) 120 min. (C) 11778 microaerobic cultures were grown without BSA and Sups were supplemented with unmodified or modified BSA at a final concentration of 1 g/L. Sups were diluted two-fold in medium titrated to the pH of grown cultures and then incubated with 2 mM ascorbate for 2 h at RT. Cells were exposed to Sups for 20 min and then assessed for viability. Controls included the same concentration of ascorbic acid.

DISCUSSION

In this thesis, *B. cereus* was discovered to secrete very toxic products in the beginning of the exponential growth phase that can destroy the cell monolayer after an exposure period of 20 minutes. ATCC 14579, compared to 11778, appeared to be the more virulent strain of *B. cereus*, demonstrating high levels of toxicity even after supernatants were diluted 32-fold. The mechanism of cytotoxicity was investigated and found to be primarily caused via permeabilization of the cell membrane by a pore-forming toxin (**Figure 9**). Filtration experiments identified a toxin between 30 and 100 kDa in size was responsible for the majority of the toxicity in both strains and its production was greater during stationary compared to mid-exponential growth phase. The effect of oxygen on the toxicity of *B. cereus* bacterial supernatants was found to be strain specific. A virulence factor larger than 50 kDa from ATCC 11778 demonstrated a dependence on microaerobic conditions for activity. The major PFT of ATCC 11778 was identified as cereolysin O with an almost complete loss of permeability upon cholesterol inhibition (**Figure 9**). CLO activity or expression was undetectable in SupS from aerobic cultures indicating this oxygen-labile toxin was the virulence factor from the 50 kDa retentate.

The toxicity of ATCC 14579 appeared to not be affected by the presence of oxygen, possibly due to Hbl, a toxin absent from 11778. Hbl production has been shown

to be greatly influenced by bacterial density with higher levels secreted from cultures with greater bacterial numbers (Duport et al., 2004). Since aerobic versus microaerobic cultures generally had twice the bacterial count (averages were 1.6 OD₆₀₀ for aerobic compared to 0.8 OD₆₀₀ for microaerobic), the production of Hbl could explain this difference. Filtration experiments on this strain found an increase in aerobic toxicity from a virulence factor between 30 and 50 kDa in size supported this. CLO appeared to play a minor role in the permeability caused by ATCC 14579, yet still contributed to toxicity even when diluted 64-fold. This suggests CLO is potent toxin which is supported by previous research (Bernheimer and Grushoff, 1967). Interestingly, although CLO contributed the toxicity of 14579, this strain displayed no loss of toxicity in its 50 kDa retentate under aerobic conditions. This suggests there is an oxygen-independent virulence factor larger than 50 kDa in this strain that does not contribute in 11778.

Microaerobic Sups fractioned via a size exclusion column had the highest toxicity occurring in fractions eluted at the same time. Mass spectrometry of these fractions from cultures grown in the presence of BSA identified both strains produced significant amounts of collagenase, Nhe, and CLO. Production of Hbl was discovered in fractions from 14579. Additionally, a nontoxic fraction without BSA had the all three subunits for Hbl activity and a toxic fraction contained only CLO and InhA. Surprisingly, the binding component of the Nhe enterotoxin was not identified in fractions using mass spectrometry analysis. Since all three components are necessary for toxin virulence, the toxicity in these fractions cannot be attributed to Nhe (Sastalla et al., 2013). However,

the individual contribution of Nhe in Sups remains unknown since it could not be differentiated in filtration experiments from other virulence factors.

One distinguishing characteristic between *B. cereus* and *B. anthracis* that is used in the clinical setting to identify the pathogen is its ability to induce hemolysis in erythrocytes (Ramarao and Sanchis, 2013). It was indicated that the mechanism behind this difference is the presence of a nonsense mutation silences the PlcR regulator in *B. anthracis*. Interestingly, *B. anthracis* hemolytic activity can be induced under anaerobic conditions and is predominantly attributed to its cholesterol-dependent cytolysin and phospholipases (Klichko et al., 2003; Shannon et al., 2003). This study also found CLO activity was dependent on oxygen-limited conditions. The persistence of *B. cereus* strain 14579 hemolysis in aerobic conditions is likely attributed to another hemolysin such as Hbl or CytK, which is not present in *B. anthracis*.

One difference noted between these two bacteria is their dependence on pH for virulence. *B. anthracis* Sups required a pH < 6 for pathogenicity and anything less acidic abrogated toxicity completely (Popova et al., 2011). From these experiments, recombinant ALO displayed almost no toxicity in an acidic pH until it was combined with the fermentation product succinic acid. Therefore, the pH dependence of this bacterium was attributed to the synergistic effect of ALO and SA. Investigation in *B. cereus* found that SA did enhance Sup toxicity and its activity was dependent on CLO pore-formation. However, CLO appeared to contribute toward toxicity even at a pH of 7. This suggests there could be differences in regulatory mechanisms of CDC production between *Bacillus* species or the presence of other virulence factors, possibly

phospholipases, in *B. cereus* that synergistically interact with CLO and remain functional under basic conditions.

BSA appeared to influence multiple factors in *B. cereus* to enhance bacterial toxicity. Secreted products from cultures grown in the presence of BSA were significantly more acidic, indicating albumin could function as a metabolic regulator. SA production was unaffected by BSA; however, other fermentation products were not investigated and could be responsible for the change in pH. BSA still indirectly influenced SA since the acidification of Sups is necessary for SA-mediated activity.

BSA also appears to play an important role in preventing the inactivation of toxins upon experimental manipulation. The filtration of bacterial sups without BSA resulted in toxin aggregation on the top of the filter. The activity of CLO in these fractions post-separation depended on the presence of BSA. Cereolysin O typically exists as a monomer in solution, however it can occasionally self-associate spontaneously under low concentrations (Cowell et al., 1978). Concentrating this toxin more than 6 $\mu\text{g/mL}$ has been shown to exacerbate this effect. Results from this thesis found concentrated Sups isolated from cultures grown in the presence of 1 g/L BSA contained two different isoforms of CLO that were identical in size. The presence of the CLO dimer occurred in Sups regardless of BSA supplementation, however, this dimer continued to display toxic activity only in Sups with BSA. Previous research described these two forms as an inactive oxidized form containing disulfide bonds in its cysteine residues and an active reduced form with free sulfhydryl (SH) groups (Cowell et al., 1976). This SH group is in the C-terminal region of the CDC located on domain 4, the

domain responsible for the initial monomer attachment to the cell membrane (Jacobs et al., 1999). Blocking this thiol causes a steric hindrance resulting in the toxin being unable to bind to cholesterol in the cell membrane but does not interfere with the interaction of the toxin and free cholesterol (Alouf, J et al., 2006). BSA appeared to prevent total oxidation of the toxin allowing CLO to remain partially functional. Additionally, BSA is likely to promote the activation of other virulence factors since the loss of CLO activity does not completely explain the post-filtration loss of activity in Sups without BSA.

Interestingly, CLO appeared to have a greater contribution in Sups without BSA when cell exposure was 20 min. Hemolysis by ALO is enhanced by the contribution of phospholipases exclusively under anaerobic conditions (Klichko et al., 2003). BSA has been shown to influence the inactivation of alkaline phosphatase activity, an enzyme which causes the downstream effect of PLC inhibition. The mechanism suggests the supplementation of medium with BSA would stimulate phospholipase activity. However, phospholipase production in *B. cereus* occurs regardless of oxygen availability, but is inhibited in an acidic pH (Elleboudy et al., 2011; Pomerantsev et al., 2003). BSA could be indirectly inhibiting phospholipase activity through the acidification of Sups which would lower the contribution of CLO to toxicity. Therefore, BSA could play a complex role in inhibiting the synergistic toxicity of CLO and phospholipases and facilitating CLO and SA-mediated toxicity.

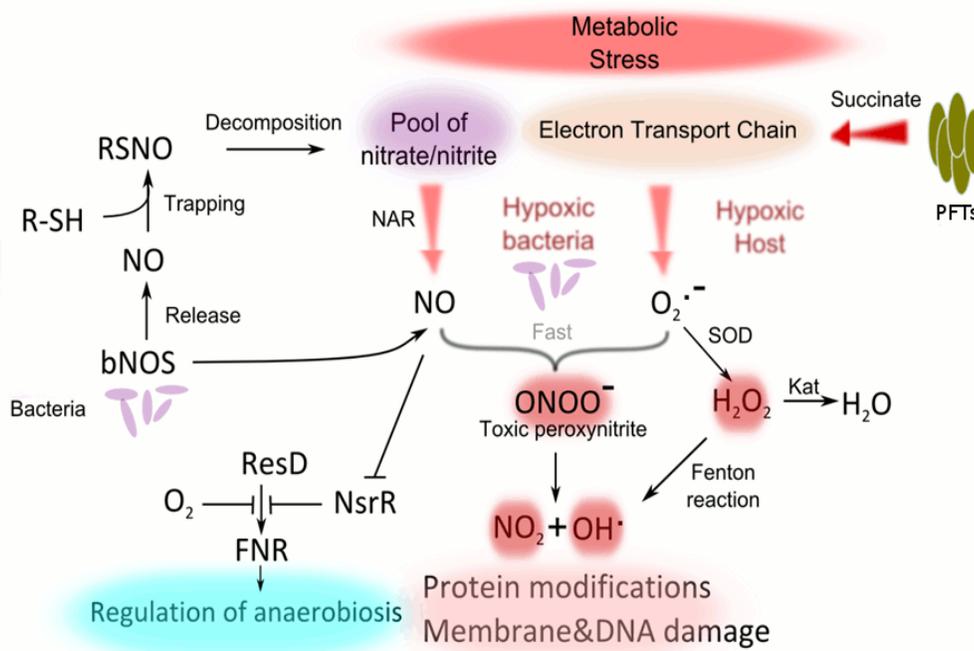


Figure 21 The proposed mechanism of metabolic toxicity by *B. cereus* in hypoxic conditions.

BSA was investigated as a potential contributor towards RNS/ROS toxicity in *B. cereus*. Research on *B. anthracis* found bacteria generate NO that contributed to cytotoxicity through their downstream transformation into peroxyntirite when stabilized by BSA (St. John et al., 2013). Similar to *B. anthracis*, both strains of *B. cereus* generated significant amounts of reactive species that correlated with enhanced bacterial toxicity. **Figure 21** illustrates the possible mechanism of metabolic toxicity for this species. Bacteria generate NO that could be stabilized by an albumin thiol (R-SH) essentially acting as a reservoir for these products. The release of NO products would cause an accumulation of nitrate and nitrite which serves as a regulator for anaerobic fermentation. The onset of anaerobic growth would trigger the production of CLO and succinate. CLO causes cell membrane permeabilization which would allow succinate to

acidify the cytoplasm resulting in the burst of ROS from the cell. Additionally, released NO from albumin could react with this cellular ROS and form into toxic peroxynitrite.

Ascorbic acid was investigated as a potential peroxynitrite inhibitor; however, it potentiated the toxicity of Sups but was dependent on the presence of BSA for this activity. Interestingly, ascorbate can also act as a reducing agent towards S-nitroso-albumin facilitating the release of NO resulting in free sulfhydryl residues (Scorza et al., 1997). By itself, nitrosylated BSA was found to be toxic towards HSAECs but required a cell exposure time of 120 min. The toxicity of ascorbate depended on the presence of modified or unmodified BSA but was not affected by cysteine nitrosylation. However, peroxynitrite can also interact with albumin via nitrosation of its tryptophan residues (Nedospasov et al., 2000; Suzuki et al., 2004). Additionally, ascorbate can interact with tryptophan in BSA facilitating the release NO from n-nitrosotryptophan (Kytzia et al., 2006; Steinhart et al., 1993; Uchidi and Kawakishi, 1988). Tryptophan is currently being investigated as the target involved in ascorbate-mediated toxicity. Additionally, ascorbate has been shown to reduce disulfide bridges for numerous compounds containing cysteine residues (Giustarini et al., 2008). Therefore, it is possible that ascorbate is reducing the disulfide residues in CLO, increasing its activity. **Figure 19E** shows cholesterol inhibition appeared greater in Sups with ascorbate but it is unclear why ascorbate had no effect in Sups without BSA when CLO remains active (**Figure 19C**).

Historically, *B. cereus* has been considered a minor food pathogen and research has focused on its enterotoxins. However, highly pathogenic and lethal strains of this species have been identified and causing disease that mimicked symptoms of anthrax

(Hoffmaster et al., 2004; Klee et al., 2010; Oh et al., 2011). Interestingly, *B. anthracis* and *B. cereus* appear to contain many of the same chromosomally-located virulence factors and the contribution of these factors towards *B. anthracis* toxicity has been emphasized (Ivanova et al., 2003; Welkos et al., 1993). This thesis discovered *B. cereus* and *B. anthracis* share a common virulence mechanism involving pore-formation by their oxygen-labile CDC and its synergistic toxicity with metabolic products. This effect appeared to be directly or indirectly potentiated by BSA.

CONCLUSION

The major findings of this thesis include the following:

1. The cultivation of *B. cereus* in microaerobic conditions caused an increased toxicity of Sups from strain 11778 but not 14579. The activity of CLO was discovered to be dependent on the absence of oxygen.
2. The cytotoxicity of microaerobic Sups was determined to be dependent on the pore-forming activity of bacterial toxins.
3. The nature of toxic products in the strain 11778 was indicated as CLO, and in the strain 14579 as CLO and possibly Hbl and collagenase. A novel mechanism of metabolic toxicity was found for *B. cereus* that involved the synergistic activity of CLO and succinic acid as a bacterial fermentation product.
4. BSA significantly increased the toxicity of Sups by its stimulation of anaerobic fermentation, prevention of virulence factor aggregation, and possibly by concentration and stabilization of bacterial ROS/RNS within its globule.
5. For the first time, ascorbate was discovered to enhance the toxicity of *B. cereus* Sups in the presence of BSA. The mechanism behind this effect does not involve interaction of ascorbic acid with S-nitrosylated albumin,

indicating the potential involvement of other BSA residues in generation of toxic species.

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