

Original Article

The dangers of sublethal carvacrol exposure: increases in virulence of *Bacillus cereus* during endophthalmitis

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Abstract: *Bacillus cereus* can cause endophthalmitis through secretion of virulence factors, including hemolysin BL (Hbl) and nonhemolytic enterotoxin (Nhe). Carvacrol is an extract from oregano oil, with potential for curtailing *B. cereus* endophthalmitis, due to antimicrobial and anti-inflammatory qualities. However, sublethal levels of carvacrol increases *B. cereus* virulence. The goal of this study was to investigate the increase in *B. cereus* virulence potential in response stress induced by a subinhibitory concentration (SIC) of carvacrol. Enterotoxin production and tissue damage were examined during ocular infections *in vitro* and *in vivo*. We hypothesized that the SIC of carvacrol would significantly increase toxin production in *B. cereus* without progressing systemically. RT-PCR determined SIC carvacrol-treated *B. cereus* had significantly higher *hblC* and *nheA* mRNA expression levels than controls *in vitro*. ELISA and RPLA analysis revealed a 46.8% and 50% increase in NheA and HblC toxin levels, respectively, in SIC-treated cultures. *Caenorhabditis elegans*-fed SIC carvacrol-treated *B. cereus* had a significantly higher mean mortality rate than nematodes fed untreated *B. cereus*. Significantly higher TNF- α levels were observed in SIC carvacrol-treated *B. cereus* mice compared to other treatment groups except for mice infected with *B. cereus* alone. Significantly higher IL-6 levels were also found in SIC-*B. cereus* mice. Histological analysis using Rose-Bengal and DAPI determined that the eyes of mice infected with SIC carvacrol-treated *B. cereus* had significantly more damage than eyes treated with *B. cereus* alone. The SIC of carvacrol increased *B. cereus* virulence *in vitro* and *in vivo*, with a mild systemic infection noted.

Keywords: *Bacillus cereus*, enterotoxin, endophthalmitis, carvacrol

Introduction

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming bacterium that, in addition to being well-known in foodborne illness [1], is capable of causing endophthalmitis [2, 3]. *B. cereus* produces a variety of damaging toxins that play an important role in virulence during infection with endophthalmitis. Most of these toxins are under control of the global transcriptional regulator PlcR, including hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) [4]. *B. cereus* is the second most common source of posttraumatic endophthalmitis, next to *Staphylococcus aureus* [3]. Of the exotoxins and enzymes known to be produced by *B. cereus*, only Hbl has been studied in the context of endophthalmitis [5, 6], where it has

been shown to cause irreversible tissue damage to retinal photoreceptors in less than 24 hours, resulting in blindness [7, 8]. However, additional virulence determinants are possibly involved in *B. cereus* endophthalmitis, some of which may facilitate localized inflammation and/or dissemination of bacteria into the peripheral blood.

Endophthalmitis is an inflammatory infection that occurs after introduction of bacteria into the posterior segment of the eye [3]. Because of its motile nature, *B. cereus* has been known to rapidly migrate from the vitreous of the eye to other intraocular structures as early as nine hours after initial infection. Although damage to the blood-retinal barrier is often a consequence of *B. cereus* endophthalmitis, a systemic infection is not typically observed.

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The observed eye infection is difficult for health professionals to immediately diagnose precisely. Thus, a broad-spectrum of antibiotics is commonly administered [3]. However, the eye is an immunoprivileged site and oral or intravenous antibiotics are typically not effective. The most common treatment is the injection of vancomycin and an aminoglycoside or cephalosporin directly into the vitreous of the eye. Because amikacin (an aminoglycoside) is toxic to sensitive retinal cells, ceftazidime (a cephalosporin) is often preferred. Unfortunately, *B. cereus* is not always responsive to this drug and in worse case scenarios a vitrectomy may be done as a fast and effective way to drain the eye of fluid containing the causative bacteria, toxins, and host inflammatory cells [9].

While intravitreal antibiotics are capable of effectively controlling *B. cereus* endophthalmitis, potential problems caused by the host inflammatory response remain. In response to ocular infection with *B. cereus*, a variety of pro-inflammatory cytokines are produced by Müller cells, astrocytes, and pericytes [1, 6]. TNF- α and IL-6 are two pro-inflammatory cytokines produced in the eye during endophthalmitis [3]. TNF- α signals polymorphonuclear leukocytes to rapidly migrate to the site of infection within the eye, causing extensive inflammatory damage along the way [6]. IL-6 is stimulated by TNF- α , and plays a role in the differentiation of B- and T-lymphocytes [6]. Antibiotic treatment frequently causes structural damage to bacteria, and release of various components, especially peptidoglycan antigens, augmenting the host inflammatory response. For this reason, many doctors choose to supplement their antibiotic regime with a corticosteroid such as dexamethasone in an attempt to suppress additional ocular damage caused by inflammation. However, studies have shown that the addition of dexamethasone is futile, if not detrimental, to intraocular antibiotic treatment [10]. Because host inflammatory cells cause so much collateral damage during infection with endophthalmitis, there is a great need for a drug capable of inhibiting the inflammatory response within the eye.

Many phenolic compounds found in plants have antimicrobial, antioxidant, and anti-inflammatory capabilities [11]. Carvacrol is a component of oregano oil that displays all of these

qualities. Carvacrol works by disrupting the cell membrane of *B. cereus* and causing it to swell while releasing intracellular ATP [12, 13]. Carvacrol also disrupts the K⁺ ion gradient within the cell membrane and is lethal to *B. cereus* at concentrations exceeding 1 mM [14]. However, it is the anti-inflammatory qualities of carvacrol that render it most useful as a possible treatment for endophthalmitis. In particular, carvacrol is known to inhibit TNF- α and IL-6 expression at both transcriptional and translational levels, along with other pro-inflammatory cytokines *in vitro* [15]. Carvacrol may be an alternative treatment to antibiotics because of its ability to kill bacterial cells while also suppressing the host inflammatory response.

Although carvacrol can effectively control *B. cereus* growth, it is very important to administer this chemical compound at the correct concentration. *B. cereus* has been documented to adjust to carvacrol at concentrations of 0.4 mM or below [16]; changing its fatty acid content after exposure to these subinhibitory concentrations (SIC). This mechanism most likely is an attempt to protect its intracellular ATP and K⁺ ions. Studies performed in this laboratory have determined that the SIC of carvacrol against *B. cereus* ATCC14579 is 1 mM, and early experiments indicated that *in vitro* toxin production increased compared to that in non-stressed cultures.

In this study, we hypothesized that the addition of carvacrol at the SIC would increase the virulence of *B. cereus* and result in more extensive eye tissue damage than *B. cereus* alone. We also hypothesized that the endophthalmitis infection would not progress into a systemic infection. This study is important because it investigates the behavior of *B. cereus* in response to a potential alternative treatment for endophthalmitis. This study is also unique because the antimicrobial and anti-inflammatory qualities of carvacrol have yet to be tested in an ocular infection model.

Materials and methods

Determination of carvacrol subinhibitory concentration (SIC)

Bacillus cereus ATCC 14579 was cultured in Mueller-Hinton broth (Sigma-Aldrich, St. Louis, MO) at 32°C while shaking at 200 rpm. This

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Table 1. Forward and reverse RT-PCR primers used in this study

Designation	5'→3' Primer Sequence	GenBank Accession or reference
<i>hblC</i> forward	AATGGTCATCGGAACTCTAT	17
<i>hblC</i> reverse	CTCGCTGTTCTGCTGTTAAT	76
<i>nheA</i> forward	TACGCTAAGGAGGGGCA	76
<i>nheA</i> reverse	GTTTTATTGCTTCATCGGCT	76
<i>plcR</i> forward	ACTAGGATCCATGCAAGCAGAGAAATTAG	91897266
<i>plcR</i> reverse	ACTAAGGTCCTTATCTGCTGATTTTATTAC	91897267
<i>gyrB</i> forward	GCGTTAGAAGTTTCAAGTTTAC	AF090330
<i>gyrB</i> reverse	CCGATTGCCGTAATAATTGTAC	AF090330

strain has previously been shown to harbor and express the Hbl and Nhe enterotoxin operons [17]. Mueller-Hinton broth tubes (fifteen) were seeded with *B. cereus* at a concentration of 10^2 CFU/ml (colony-forming units ml⁻¹, mid-log phase 32°C saline-washed cells). Carvacrol (SAFC Supply Solutions, St. Louis, MO) was added to each tube in a 1:2 dilution series beginning at 256 mM. All tubes were assessed by O.D. (600 nm) measurements taken after dilutions had been performed as well as by standard plate count (SPC) onto Mueller-Hinton agar (Sigma). Tubes were incubated at 32°C for 24 h (shaking at 200 rpm) after which time growth was assessed by again measuring O.D. (600 nm) and SPC. The highest carvacrol concentration allowing for bacterial growth was determined to be the SIC (whereas the lowest carvacrol concentration preventing measurable growth was the minimum inhibitory concentration; MIC). This and all experiments were performed in quadruplicate unless otherwise indicated.

nheA and *hblC* expression in vitro

Total RNA isolation from untreated and SIC carvacrol-grown *B. cereus* was performed as in Gracias and McKillip [18] using Trizol reagent (Life Technologies, Grand Island, NY) and 1.5 mL of culture incubated as previously described. Final RNA pellets were resuspended in sterile nuclease-free water DNase-treated, quantified spectrophotometrically, and purity determined using A_{260}/A_{280} ratio values prior to reverse transcriptase polymerase chain reaction (RT-PCR) setup.

All RNA samples from SIC carvacrol-treated cultures were standardized to a concentration of 0.5 µg/µl for RT-PCR using a MasterAmp™ High

Fidelity RT-PCR Kit (Epicentre Biotechnologies, Madison, WI) and included 12.5 µl MasterAmp 2X RT-PCR pre-mix, 0.5 µl MMLV RT Plus enzyme, 0.5 µl MasterAmp TAQurate DNA Polymerase Mix, 100 pmol each primer (Table 1), RNA template, and sterile nuclease-free water to a total volume of 25 µl. Cycling parameters were 42 cycles of a 94°C 1 min. denaturation, 53°C 1 min. primer

annealing, and 72°C extension phase. A confirmatory melting curve analysis was included at the end of each run. All PCR was performed on a Cepheid SmartCycler (Cepheid, Sunnyvale, CA). All RT-PCR reactions were completed in quadruplicate and C_T value means were analyzed using a Student's t-test ($P < 0.05$).

Standard curves were completed for relative quantification of *nheA* and *hblC* expression. Mid-log phase *B. cereus* total RNA was extracted and serially diluted 1:2 in sterile nuclease-free water, starting at 10 µg/µl of RNA to 0.31 µg/µl, with 1 µl of RNA being used for each of triplicate reactions at each dilution using the conditions described below. Resulting mean cycle threshold (C_T) values were plotted against RNA concentrations to provide a standard curve against which relative expression levels were compared versus a housekeeping gene control, *gyrB*.

HblC and *NheA* toxin Immunoassays

B. cereus control samples were inoculated in Mueller Hinton broth 6 h prior to assay. For stress treatments, *B. cereus* stress samples were inoculated with carvacrol at the desired concentration as described previously (1 mM, 2 mM, 8 mM) 6 h prior to the assay. *B. cereus* control samples (1 ml) from each tube sample per replicate lane was centrifuged at 1,000xg for 20 min at 4°C.

In order to ascertain presence of the *B. cereus* L₂ component of Hbl toxin in treatments, the BCET-RPLA kit (Oxoid Limited #TDO950, Basingstoke Hampshire, UK) was performed according to manufacturer's instructions, using serially diluted culture supernatant for relative quantification of toxin. Additionally, the NheA

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component of the *B. cereus* Nhe toxin complex was quantified by enzyme-linked immunosorbent assay (ELISA) using the TECRA-BDE kit (Tecra #BDEVIA48, Frenchs Forest, Australia) according to instructions provided. All immunoassays were performed in quadruplicate and results used for statistical analyses using a one-way t-test ($P < 0.05$).

C. elegans in vivo bioassay model

Overnight *B. cereus* ATCC14579 control samples were inoculated in Tryptic Soy Broth (TSB, Weber Scientific, Hamilton, NJ) 6 h prior to the assay and incubated at 32°C while shaking at 200 rpm. For stress treatments, *B. cereus* stress samples were inoculated into TSB with SIC carvacrol as described previously. *Caenorhabditis elegans* (Carolina Biological, Durham, NC USA) were propagated on Nematode Growth Agar (NGA) plates (Carolina) containing a lawn of *E. coli* strain OP50 for approximately 48 h prior to separation into different treatment groups. *B. cereus* untreated, *B. cereus* with SIC carvacrol, *B. cereus* with MIC carvacrol, and *E. coli* alone were plated onto separate NGA plates (Carolina) after reaching an OD_{600} of 0.25. Starting at 48 h after the separation into treatment groups, *C. elegans* were monitored for mortality every 24 h for 120 h total. Data were collected at 48 h, 72 h, 96 h, and 120 h by averaging ten replicates per treatment/time-point and statistically analysed using one-way ANOVA ($P < 0.05$). Mean internal volume calculations were also performed for each nematode treatment group (using mean πr^2 h values) but were not used for data analysis comparisons.

Mouse in vivo bioassay model-bacteria preparation

Bacillus cereus ATCC14579 was cultured in Tryptic Soy Broth (TSB) (Weber Scientific) and incubated at 30°C under aeration at 150 rpm. *B. cereus* was also cultured in the same way with the 1 mM SIC of carvacrol. The bacteria were sub-cultured twice before being administered to mice at 5×10^7 CFU in sterile saline.

In vivo infection with *Bacillus cereus* + SIC of carvacrol

All experiments were performed following approval from the Ball State University Institutional Biosafety Committee (IBC), as well as the

Institutional Animal Care and Use Committee (IACUC). CITI certification was also obtained. Fifteen BALB/c mice approximately 10 weeks of age were acquired from Jackson Laboratory (Bar Harbor, ME) and placed in separate cages according to gender. Three BALB/c mice were randomly assigned to each of five different treatment groups. Mice were anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) (Henry Schein, Melville, NY) prior to infection in order to facilitate bacterial inoculation by eye drops in each eye. The five treatment groups consisted of 1) a negative control group receiving only saline, 2) an experimental control group receiving only the SIC of carvacrol without bacteria, 3) a positive control group receiving 5×10^7 CFU of *B. cereus* in saline, 4) an experimental group receiving 5×10^7 CFU of *B. cereus* cultured with the SIC of carvacrol, and 5) a second positive control group receiving an intraperitoneal injection of 5×10^7 CFU of *B. cereus* alone (the latter serving as a cytokine-induced positive control group). Mice were infected with the same volume (15 μ l) in each eye by micropipet. The mice were closely monitored throughout the infection period of 14 days, after which they were sacrificed.

In vivo infection-tissue preparation

After a 14 day immune challenge, spleens were immediately harvested from each mouse and stored in ice-cold lysis buffer (150 mM NaCl; 50 mM Tris pH 7.4; 1 mM EDTA) (Amresco, Solon, OH). The spleen tissue was homogenized and centrifuged at $2K \times G$ for three minutes at 4°C, after which the supernatant was collected and stored at -20°C for future use in ELISA experiments. The eyes were also harvested immediately and stored in an eye tissue fixative solution (4% paraformaldehyde (Thermo Scientific, Rockford, IL) containing 20 μ l 10 N NaOH added dropwise (Sigma-Aldrich). After removing the lens, the eyes were stored at -20°C for future histological analysis.

In vivo infection-ELISA-based TNF- α , IL-6, and IgG measurement

Spleen lysates for each mouse were used to detect the presence of pro-inflammatory cytokines TNF- α and IL-6, as well as to ascertain IgG induction against *B. cereus* in a systemic immune response. Direct ELISAs were per-

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formed using the Mouse TNF- α ELISA Ready-SET-Go![®] (eBioscience, San Diego, CA) and Mouse IL-6 ELISA Ready-SET-Go![®] (eBioscience) kits following the manufacturer's instructions. A TNF- α protein standard (eBioscience) and IL-6 protein standard (eBioscience) were diluted according to the manufacturer's instructions and used for the construction of standard curves in order to determine protein concentrations. An indirect ELISA was also performed by coating the 96-well plate (Falcon 3911 MicroTest III Flexible Assay Plate, Becton Dickinson Labware, Oxnard, CA) with a combination of 86% phenol (Sigma-Aldrich), 0.25% glutaraldehyde (Eastman Kodak Company, Rochester, NY) and 5×10^7 CFU *B. cereus* suspended in sterile saline. After coating the plate, the ELISA was performed following the manufacturer's instructions. Absorbance values were measured by the Bio-Rad Model 680 Microplate Reader at 450 nm. All readings were taken in triplicate for statistical analyses.

In vivo infection - histological analysis of eye tissue

Fixed mouse eyes were sectioned at 12 μ m thickness using a MICROM HM 505 N cryostat. Each eye was stored in 30% sucrose (Life Technologies, Carlsbad, CA) cryoprotectant at least 24 hours prior to sectioning and transferred into OTC prior to slicing. For each eye, five slides were prepared using TRUBOND 380 Microscope Slides (Tru Scientific, Bellingham, WA). Each slide contained three slices of eye tissue.

Eye tissue slides were then stained with 1% DAPI (Life Technologies) for four minutes and 0.001% Rose Bengal (Sigma-Aldrich) for 12 minutes while gently rocking. Undamaged eye tissue is stained blue by DAPI, while Rose Bengal stains damaged tissue red. After washing with $1 \times$ PBS (Fisher Scientific), the slides were imaged using a Cannon powershot A650IS digital camera attached to a Zeiss 25 ICS TLM (with fluorescence filters). The images acquired at $100 \times$ magnification were analyzed using Image-Pro Express 6.0 software. Data for each slide were averaged, resulting in a single mean intensity value for red spectrum fluorescence and blue spectrum fluorescence in each eye.

In vivo infection-statistical analysis

A one-way ANOVA using the Tukey's Pairwise Comparisons Test was performed on the log-

transformed mean protein concentrations obtained from TNF- α and IL-6 direct ELISAs in order to meet the required assumptions for this statistical test. A one-way ANOVA using the Tukey's Pairwise Comparisons Test was also performed on the mean O.D. values of the indirect ELISA without a need for data transformation. Mean red intensity values for fluorescently stained ocular tissue were transformed to the fourth power in order to meet the assumptions required for statistical analysis using a one-way ANOVA with Tukey's Pairwise Comparisons Test.

Results

SIC determination

After performing a carvacrol dilution series, testing indicated that 1 mM carvacrol was the SIC and 2 mM carvacrol was the minimum inhibitory concentration (MIC) based on OD measurements and recovered viable cell counts from broth dilutions. This finding is consistent with the literature. We therefore confirmed the carvacrol dose necessary to sublethally injure *B. cereus* so that we could assess if there is an increase or decrease in *hbIC* and *nheA* gene expression.

RNA standard curves

RNA primer standard curves were generated using total RNA extracted from TSB-grown *B. cereus* ATCC14579 and primers specific for target gene mRNA *gyrB* (positive control), *hbIC*, and *nheA*. RNA standard curves were performed in order to standardize the RNA concentrations among control vs carvacrol-treated cells in this study, allowing for relative quantification of expression compared to a housekeeping control. When assessing cycle threshold (C_T) values, results indicate that SIC levels of carvacrol down-regulate mRNA expression of *hbIC* on average 12.9% compared to the control cultures in *B. cereus* (**Table 2**), a statistically significant difference. The housekeeping gene *gyrB* was also significantly downregulated by 30.7% (**Table 2**) in response to SIC carvacrol, while the enterotoxin gene *nheA* was upregulated 4.9% (but was not considered statistically significant compared to untreated *B. cereus*).

Toxin protein production

Commercial immunoassay toxin kits were performed to determine if the carvacrol-mediated

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Table 2. 1 mM carvacrol (SIC) exposure causes mRNA down-regulation in *gyrB*, *hbIC*, and up-regulation of *nheA*

Gene target	<i>B. cereus</i> control mean C _T value	Carvacrol-treated <i>B. cereus</i> mean C _T value	Carvacrol treated <i>B. cereus</i> mean C _T value
<i>gyrB</i>	15.46 ± 0.94 (n = 14)	20.21 ± 1.9 (n = 14)	30.7% decrease*
<i>hbIC</i>	14.96 ± 0.46 (n = 36)	16.89 ± 0.81 (n = 36)	12.9% decrease**
<i>nheA</i>	14.56 ± 0.62 (n = 17)	13.85 ± 0.50 (n = 17)	4.9% increase

B. cereus gyrase subunit B gene was used as a housekeeping control. Relative gene expression changes were measured by mean cycle threshold (C_T) values. All RT-PCR reactions were completed in quadruplicate and C_T value means were analyzed using a Student's t-test. All data were expressed as + S.E.M. and the criterion used to assess significance was set at P<0.05. n = number of samples tested. *Statistically significant versus nonstressed control (P = 0.035). **Statistically significant versus nonstressed control (P = 0.04).

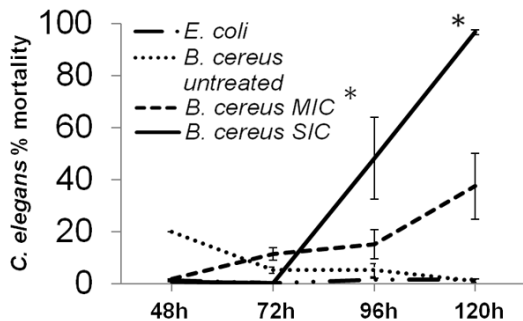


Figure 1. Mean mortality percentages were calculated for *C. elegans* fed with different treatments of bacteria at 48 h, 72 h, 96 h, and 120 h. Statistical analysis was performed on data obtained after 48 h. Asterisks denote a significant difference between *B. cereus* stressed with the SIC of carvacrol and all other treatment groups at that time point (P<0.05).

decrease in mRNA expression of *hbIC* would correspond to a decrease in HbIC protein expression as well. ELISA analysis revealed a 46.8% increase in NheA protein toxin in response to 1 mM carvacrol (SIC) exposure when compared to non-stressed bacteria. RPLA analysis showed a 50% increase in HbIC protein toxin expression in response to SIC levels of carvacrol (data not included). However, a 2 mM (MIC) or higher (8 mM concentration) of carvacrol inhibits any detectable level of either HbIC or NheA proteins.

Carvacrol effects on *B. cereus* virulence in *Caenorhabditis elegans*

C. elegans mortality was selected as the measure of choice for quantifying the effect of carvacrol-treated *B. cereus* *in vivo*. The SIC carvacrol/*B. cereus*-fed group showed a statisti-

cally significant increase in mortality after 96 h and 120 h compared to nematodes reared on only *E. coli* or only untreated *B. cereus* (**Figure 1**).

TNF- α pro-inflammatory cytokine expression during *B. cereus* ocular infection

Mice infected with SIC carvacrol-treated *B. cereus* had significantly higher detectable TNF- α levels in the spleen than all other treatment groups except for mice treated with *B. cereus*

alone (**Figure 2A**). Mice injected intraperitoneally with *B. cereus* displayed lower TNF- α levels, but not significantly different levels from mice treated with *B. cereus* alone. Mice treated with SIC carvacrol alone had significantly lower detectable TNF- α levels than any other group.

IL-6 pro-inflammatory cytokine expression during *B. cereus* ocular infection

Mice infected with SIC carvacrol-treated *B. cereus* had significantly higher detectable IL-6 levels in the spleen than those of all other treatment groups except for mice injected intraperitoneally with *B. cereus* (**Figure 2B**). No significant difference was observed in detectable IL-6 levels among the saline control mice, the mice infected with *B. cereus* alone, and the mice injected with *B. cereus*. Mice infected with SIC carvacrol alone had significantly lower detectable IL-6 levels than all other treatment groups.

Anti-*B. cereus* IgG levels during ocular infection

Mice infected with SIC carvacrol-treated *B. cereus* had significantly higher detectable IgG levels than mice infected with *B. cereus* alone (**Figure 2C**). No significant difference was observed between mice infected with SIC carvacrol-treated *B. cereus* via the eye and mice injected intraperitoneally with *B. cereus* however.

Histological analysis of tissue damage during *B. cereus* ocular infection

Verification of *B. cereus* infection was noted as a decrease in eye tissue integrity and by the

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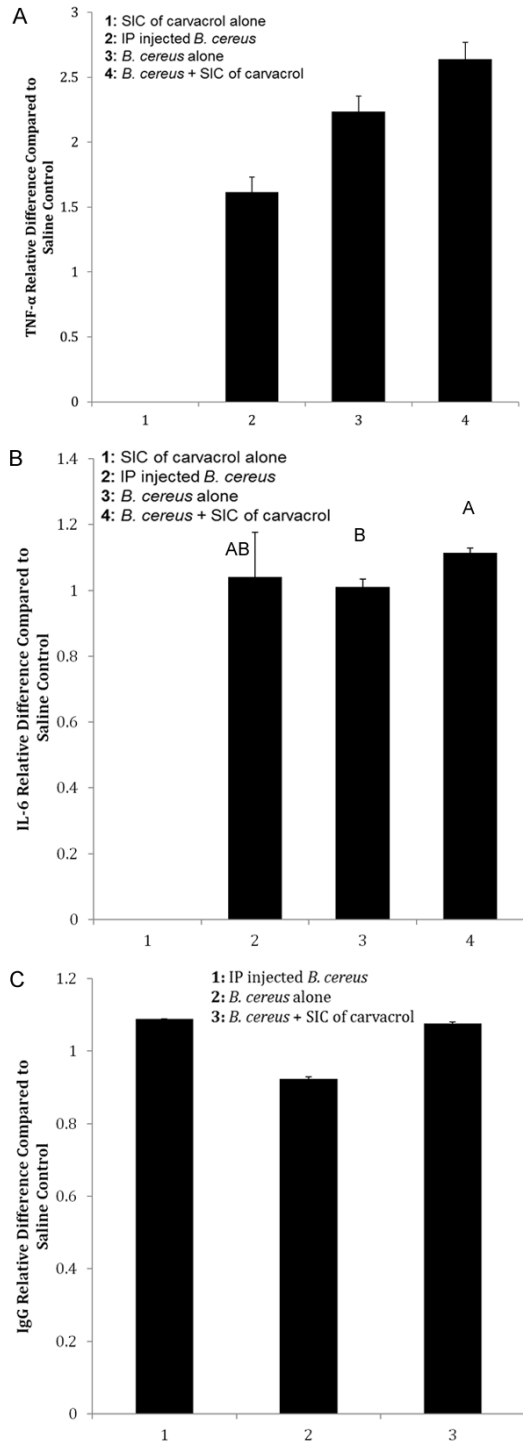


Figure 2. A. Relative difference of mean TNF- α levels in mice following ocular infection with *Bacillus cereus* ATCC14579 as indicated by treatment group on the x-axis: 1. SIC of carvacrol alone; 2. IP injected *B. cereus*; 3. *B. cereus* alone; 4. *B. cereus* + SIC carvacrol. Mean log TNF- α protein concentrations were obtained from ELISA analysis of spleen lysates for three mice within each treatment group and compared using a one-way ANOVA with Tukey's

Pairwise Comparisons Test (Mean log protein concentration \pm SEM). Treatment groups with different letters are significantly different ($P < 0.05$). B. Relative difference of mean IL-6 levels in mice following ocular infection with *Bacillus cereus* ATCC14579 as indicated by treatment group on the x-axis: 1 = IP injected *B. cereus*; 2 = *B. cereus* alone; 3 = *B. cereus* + SIC of carvacrol. Mean log IL-6 protein concentrations were obtained from ELISA analysis of spleen lysates for three mice within each treatment group and compared using a one-way ANOVA with Tukey's Pairwise Comparisons Test (Mean log protein concentration \pm SEM). Treatment groups with different letters are significantly different ($P < 0.05$). C. Mean A_{450} values were obtained using indirect ELISA of spleen lysates for three mice within each treatment group to assess relative levels of anti-*B. cereus* IgG. X-axis labels 1 = IP injected *B. cereus*; 2 = *B. cereus* alone; 3 = *B. cereus* + SIC of carvacrol. Mean A_{450} values were compared using one-way ANOVA with a Tukey's Pairwise Comparisons Test (Mean A_{450} value \pm SEM). Treatment groups with different letters are significantly different ($P < 0.05$).

presence of the bacteria in Gram-stained sections. Overall, mouse eyes treated with SIC of carvacrol alone had better tissue integrity, with more eye layers present than any other group (Figure 3, picture 1 insert). Mouse eyes infected with SIC carvacrol-treated *B. cereus* displayed a lower level of tissue integrity upon examination. Fluorescently staining the tissue with DAPI and Rose Bengal red resulted in significantly higher red saturation intensity values, and therefore, verified that the tissue displayed more damage when the eyes were infected with *B. cereus* alone than eyes treated with saline (Figure 3). However, eyes infected with SIC carvacrol-treated *B. cereus* and eyes treated with SIC carvacrol alone were not significantly different from each other. This may be due to the actual presence of more layers in the SIC carvacrol alone group which often fluoresced red.

Discussion

This study examined the effect of an essential oil (carvacrol) as a possible treatment option other than antibiotics for *B. cereus* induced endophthalmitis and the possible consequences of SIC use. Because retinal photoreceptor cells are sensitive to exogenous antigens, the inflammatory response, and high doses of antimicrobial agents [19-22], exploration of novel infection control strategies is vital. Since carvacrol has been previously shown to kill vegetative *B. cereus* [12, 14], along with exhibiting

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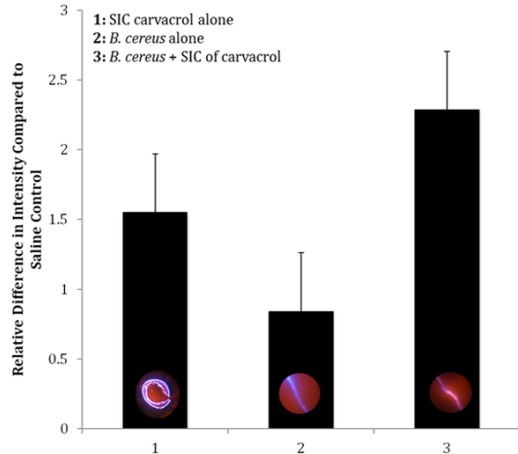


Figure 3. Relative difference of mean fluorescent red intensity values in mouse eyes ($n = 6$), representing ocular tissue damage after infection with *B. cereus*. X-axis labels 1. SIC carvacrol alone; 2. *B. cereus* alone; 3. *B. cereus* + SIC of carvacrol. Mean intensity values were obtained from mouse eye cryosections stained with DAPI and Rose Bengal using Image Pro Express 6.0 software. Picture inserts demonstrate the integrity of the tissue samples. 1. SIC carvacrol alone presents the best tissue integrity, Mean intensity values were transformed to the fourth power and compared using a one-way ANOVA with Tukey's Pairwise Comparisons Test (Mean intensity value \pm SEM). Treatment groups with different letters are significantly different ($P < 0.05$).

anti-inflammatory properties (COX-2 inhibitor) [12, 14], it may show further promise as a treatment option for endophthalmitis.

Our results show that SIC levels of carvacrol downregulate mRNA expression of *hblC* on average 12.9% compared to the control cultures in *B. cereus*. Interestingly, our housekeeping control gene (*gyrB*) was downregulated 30.7% in response to SIC carvacrol treatment as well, at the same cell density and total RNA yield as in other treatments, suggesting a global stress-induced down-regulation of gene expression when grown under this chemical stressor. Thus, *hblC* was downregulated to a significantly less degree compared to the housekeeping gene, underscoring the differential regulation of the Hbl operon. Moreover, even under these SIC carvacrol stress circumstances, *nheA* was upregulated by 4.9%, indicating some degree of expression of virulence genes are needed by the pathogen when presented with a sublethal chemical injury such as carvacrol, a phenomenon that has been suspected

in many bacterial pathogens but never conclusively measured [24, 25].

SIC-mediated effects on virulence gene mRNA levels in *B. cereus* were augmented by immunoassay of protein toxin production in broth under the same level of stress. ELISA analysis revealed a 46.8% increase in NheA protein toxin in response to 1 mM carvacrol (SIC) exposure compared to untreated controls while RPLA analysis revealed a mean 50% increase in HblC protein toxin expression in response to SIC levels of carvacrol. However, a 2 mM (MIC) or 8 mM concentration of carvacrol inhibited any detectable level of either the HblC or NheA proteins, as would be expected. This finding could be attributed to translation of existing mRNA toxin transcripts synthesized prior to and during carvacrol exposure. The possibility exists that SIC carvacrol stress decreases mRNA turnover in treated *B. cereus* compared to control cultures, an avenue we are currently exploring. It has been shown in a previous study that *B. cereus* enterotoxin gene transcription and Nhe/Hbl protein titers vary greatly in a strain-specific manner, even to the extent of great incongruence between mRNA levels and corresponding toxin levels within the same strain [26]. The complex regulatory network at play in this group of bacteria is only partially elucidated, involving the redox-sensitive ResDE network, PlcR, CodY, and likely many other yet-to-be identified transcriptional effectors, delaying our full understanding of quorum sensing and stress response mechanisms in *Bacillus* spp. [27, 28]. In light of the state of the field currently, our results demonstrate that a sublethal dose of carvacrol increases HblC and NheA enterotoxin production substantially over non-stressed bacteria while only modestly increasing (or opting to not downregulate) transcription of *hblC* and *nheA*. This is clinically relevant, as a sublethal dose (1 mM) of carvacrol administered clinically could easily exacerbate endophthalmitis tissue damage and perhaps result in a systemic infection.

In vivo work substantiated these findings. Since the SIC carvacrol-treated *C. elegans* group showed a statistically significant increase over any other treatment group in mortality rate after 96 h and 120 h, the hypothesis that SIC carvacrol increases the virulence of *B. cereus* is supported. The specific nature of these viru-

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lence factors was not elucidated in this study, which are likely a coordinately regulated set of products from multiple operons. If the *C. elegans* mortality rates for each treatment group are any indication of their health in the MIC and SIC concentrations of carvacrol, this experiment supports the hypothesis that SIC carvacrol increases the virulence of *B. cereus*, and thus increases the mortality rate of *C. elegans*. This finding is significant because it emphasizes the importance of treating *B. cereus* ocular infections with the appropriate concentration of carvacrol.

Our *in vivo* results from the mouse model, specifically on the ELISA-based detection of TNF- α and IL-6 from spleen lysates, support the notion that at least a modest systemic bacterial infection (and enhanced virulence) does occur from ocular introduction of SIC carvacrol-treated *B. cereus*. To the authors' knowledge, this is the first published report of a *B. cereus*-mediated ocular infection progressing systemically. The goal of this aspect of the study was to determine whether or not the SIC of carvacrol increases virulence of *B. cereus* during infection with endophthalmitis in mice.

We have determined that mice infected ocularly with *B. cereus* and the SIC of carvacrol have an increased systemic pro-inflammatory cytokine response compared to mice infected with *B. cereus* alone. TNF- α levels increased slightly, although the difference was not significant. However, mice infected with SIC carvacrol-treated *B. cereus* had significantly higher detectable IL-6 levels in the spleen than those of all other treatment groups. In fact, the SIC carvacrol-treated mice were not significantly different than the mice injected intraperitoneally with *B. cereus* in terms of IL-6 production; the latter of which served as a positive control for this cytokine ELISA. This observation indicates *B. cereus* escaped the confines of the eye and progressed systemically. Histological analysis confirmed that *B. cereus* stressed with the SIC of carvacrol resulted in more tissue damage to the eye than untreated *B. cereus*. However, mice infected with *B. cereus* and the SIC of carvacrol did not exhibit significantly more eye damage than mice treated with the SIC of carvacrol alone, suggesting that carvacrol alone was a modest irritant. However, mice treated with carvacrol alone did not display any external signs of irritation during the 14 d immune

challenge nor did this group display any detectable cytokines from ELISA.

The increased levels of pro-inflammatory cytokines and anti-*B. cereus* IgG levels show that the SIC of carvacrol did increase the virulence of *B. cereus* during endophthalmitis, consistent with our *C. elegans* data, and the *in vitro* experiments. However, another possibility in the mouse model is that carvacrol elicited damage to the blood-retinal barrier and allowed *B. cereus* to escape the eye more readily, rather than the escape being only mediated by bacterial products. The third and most likely possibility, all factors considered, is that the effects seen here are a combination of increased bacterial virulence from sublethal stress, and carvacrol effects on retinal tissue.

Rose Bengal was chosen for this experiment because it is commonly used to stain ocular tissue damage specifically, especially in the form of eye drops *in vivo* [29]. Prior to this experiment, it was not known how Rose Bengal would stain specific intraocular structures. Thus, overall damage to the eye was analyzed instead of specific region comparison. Images were normalized to the eyes treated with saline, to maintain consistency with the treatments used to obtain ELISA results.

It is important to understand the response of *B. cereus* to the SIC of carvacrol in the ocular environment because carvacrol may be a potential alternative treatment for endophthalmitis caused by *B. cereus*, due to its antimicrobial and anti-inflammatory qualities [30]. However, practitioners should be clear on the benefits and the consequences of using carvacrol as a possible treatment option. Although the minimum inhibitory concentration (MIC) would be the dose administered to patients, it is possible that carvacrol could have trouble penetrating the vitreous of the eye when given in the form of eye drops. It is therefore possible that patients treated with the MIC of carvacrol could actually be receiving lower levels intraocularly. For this reason, it is important to understand the effects of sublethal levels of carvacrol on the eye. This study, in combination with previous research done in this laboratory, has shown that the SIC of carvacrol may increase the virulence of *B. cereus*. Therefore, it is possible that patients receiving the SIC of carvacrol

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could have a more virulent *B. cereus* eye infection, resulting in more eye tissue damage than the bacteria would have caused by itself.

In order to answer some of the questions generated from this study, we propose the use of retinal pigment epithelial (RPE) cells *in vitro* to assess damage done to retinal cells by the SIC of carvacrol alone. RPE cells make up the outer layer of the blood-retinal barrier. We will also quantify tight junction protein expression in RPE cells infected with *B. cereus* grown in the SIC of carvacrol compared to cells infected with the bacteria alone. These studies will determine if the SIC of carvacrol causes damage by testing for cytotoxicity to RPE cells or disruption of tight junctions within the blood-retinal barrier. After assessing the effects of subinhibitory carvacrol concentrations on RPE cells *in vitro*, another *in vivo* mouse model experiment may be performed to determine if *B. cereus* stressed with the SIC of carvacrol causes the bacteria to increase its toxin production within the eye. This study would answer the question of whether or not *B. cereus* actively produces toxins in the ocular environment, and if so, at what time-point is toxin production at its highest. It is important to provide answers to these questions, because gaining a better understanding the way *B. cereus* behaves in the ocular environment could affect endophthalmitis treatment regimes.

Disclosure of conflict of interest

None.

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References

- [1] Dubouix A, Bonnet E, Alvarez M, Bensafi H, Archambaud M, Chaminade B, Chabanon G and Marty N. *Bacillus cereus* infections in traumatology orthopedics department: retrospective investigation and improvement of health practices. *J Infect* 2005; 50: 22-30.
- [2] Aaberg TM, Flynn HW, Schiffman J and Newton J. Nosocomial acute-onset postoperative endophthalmitis survey: a 10 year review of incidence and outcomes. *Ophthalmol* 1998; 105: 1004-1010.
- [3] Agata N, Mori M, Ohta M, Suwan S, Ohtani I and Isoobe M. A novel dodecadepsipeptide, cereulide, isolated from *Bacillus cereus* causes vacuole formation in Hep-2 cells. *FEMS Microbiol Lett* 1994; 121: 31-34.
- [4] Agaisse H, Gominet M, Okstead OA, Kolsto AB and Lereclus D. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. *Mol Microbiol* 1999; 32: 1043-1053.
- [5] Callegan MC, Jett BD, Hancock LE and Gilmore MS. Role of hemolysin BL in the pathogenesis of extraintestinal *Bacillus cereus* infection assessed in an endophthalmitis model. *Infect Immun* 1999; 67: 3357-3366.
- [6] Callegan MC, Booth MC, Jett BD and Gilmore MS. Pathogenesis of Gram-positive bacterial endophthalmitis. *Infect Immun* 1999; 67: 3348-3356.
- [7] Beecher DJ, Pulido JS, Barney NP and Wong AC. Extracellular virulence factors in *Bacillus cereus* endophthalmitis: methods and implication of involvement of hemolysin BL. *Infect Immun* 1995; 63: 632-639.
- [8] Beecher DJ, Olsen TW, Somers FB and Wong AC. Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of *Bacillus cereus* endophthalmitis. *Infect Immun* 2000; 68: 5269-5276.
- [9] Bamforth SD, Lightman SL and Greenwood J. Ultrastructural analysis of interleukin-1 beta-induced leukocyte recruitment to the rat retina. *Invest Ophthalmol Vis Sci* 1997; 38: 25-35.
- [10] Benson MT, Sheperd L, Rees RC and Rennie IG. Production of interleukin-6 by human retina pigment epithelium *in vitro* and its regulation by other cytokines. *Curr Eye Res* 1991; 11: 173-179.
- [11] Miguel MG. Antioxidant and anti-inflammatory activities of essential oils: a short review. *Molecules* 2010; 15: 9252-9287.
- [12] Ultee A, Bennik MH and Moezelaar R. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* 2002; 68: 1561-1568.
- [13] Ultee A and Smid EJ. Influence of carvacrol on growth and toxin production by *Bacillus cereus*. *Int J Food Microbiol* 2001; 64: 373-378.
- [14] Ultee A, Kets EP and Smid EJ. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus*. *Appl Environ Microbiol* 1999; 65: 4606-4610.
- [15] Aristatile B, Al-Assaf AH and Pugalendi KV. Carvacrol suppresses the expression of inflammatory marker genes in D-galactosamine-hepatotoxic rats. *Asian Pac J Trop Med* 2013; 6: 205-211.

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- [16] Ultee A, Kets EP, Alberda M, Hoekstra FA and Smid EJ. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch Microbiol 2000; 174: 233-238.
- [17] Phelps RJ and McKillip JL. Enterotoxin production in natural isolates of *Bacillaceae* outside the *Bacillus cereus* group. Appl Environ Microbiol 2002; 68: 3147-3151.
- [18] Gracias KS and McKillip JL. Nucleic acid sequence-based amplification (NASBA) in molecular bacteriology: a procedural guide. J Rapid Meth Automation Microbiol 2007; 15: 295-309.
- [19] Campochiaro PA and Lim JI. Aminoglycoside toxicity in the treatment of endophthalmitis. Arch Ophthalmol 1994; 112: 48-53.
- [20] D'Amico DJ, Caspers-Velers L, Libert J, Shanks E, Schrooyen M, Hanninen LA and Kenyon KR. Comparative toxicity of intravitreal aminoglycoside antibiotics. Am J Ophthalmol 1985; 100: 264-275.
- [21] Anonymous A randomized trial of immediate vitrectomy and of intravenous antibiotics for the treatment of postoperative bacterial endophthalmitis. Arch Ophthalmol 1995; 113: 1479-1496.
- [22] Wiechens B, Neumann D, Grammer JB, Pleyer U, Hedderich J and Duncker GI. Retinal toxicity of liposome-incorporated and free ofloxacin after intravitreal injection in rabbit eyes. Int Ophthalmol 1998; 22: 133-143.
- [23] Landa P, Kokoska L, Pribylova M, Vanek T and Marsik P. *In vitro* anti-inflammatory activity of carvacrol: COX-2 catalyzed prostaglandin E₂ biosynthesis. Arch Pharm Res 2009; 32: 75-78.
- [24] In: Fratamico P, Bhunia AK and Smith JL, editors. Foodborne pathogens: microbiology and molecular biology. Poole, UK: Horizon Scientific Press; 2008.
- [25] Smith AR, Ellison AL, Robinson AL, Drake MA, McDowell SA, Mitchell JK, Gerard PD, Heckler RA and McKillip JL. Enumeration of sublethally injured *Escherichia coli* O157:H7 and *E. coli* B-41560 using selective agar overlays versus commercial methods. J Food Prot 2013; 76: 674-679.
- [26] Jeßberger N, Krey VM, Rademacher C, Böhm ME, Mohr AK, Ehling-Schulz M, Scherer M and Märtilbauer E. From genome to toxicity: a combinatory approach to highlights the complexity of enterotoxin production in *Bacillus cereus*. Frontiers Microbiol 2015; 6: 560- 575.
- [27] Duport C, Zigha A, Rosenfeld E and Schmitt P. Control of enterotoxin gene expression in *Bacillus cereus* F4430/73 involves the redox-sensitive ResDE signal transduction system. J Bacteriol 2006; 188: 6640-6651.
- [28] Nimmer PS, Beer MR and McKillip JL. *Bacillus cereus*: a bacterial species of environmental and clinical significance. J Liberal Arts Sci 2013; 18: 21-32.
- [29] Khan-Lim D and Berry M. Still confused about rose bengal? Curr Eye Res 2004; 29: 311-317.
- [30] Russo M, Galletti GC, Bocchini P and Carnacini A. Essential oil chemical composition of wild populations of italian oregano spice (*Origanum vulgare* ssp. *hirtum* (Link) letsvaart): a preliminary evaluation of their use in chemotaxonomy by cluster analysis. 1. inflorescences. J Agric Food Chem 1998; 46: 3741-3746.