EFFECTS OF SUBINHIBITORY CARVACROL LEVELS ON *BACILLUS CEREUS* VIRULENCE DURING ENDOPHTHALMITIS *IN VIVO*

A THESIS SUBMITTED TO THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE

MASTER OF SCIENCE

BY

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MUNCIE, INDIANA

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TABLE OF CONTENTS

ABSTRACT

THESIS: Effects of subinhibitory carvacrol levels on *Bacillus cereus* virulence during endophthalmitis *in vivo*

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Bacillus cereus is a Gram-positive, pathogenic bacterium capable of causing an ocular infection known as endophthalmitis. The virulence of *B. cereus* during endophthalmitis is largely attributed to the presence of toxins such as Hbl and Nhe. Although broad-spectrum antibiotics such as vancomycin are able to control *B. cereus* in the eye during infection, these antibiotics can be toxic to sensitive retinal cells, and they do not control the damaging inflammatory response mounted by the host. Carvacrol is an extract from oregano oil with both antimicrobial and anti-inflammatory qualities that may serve as a possible alternative treatment for *B. cereus* endophthalmitis. However, at subinhibitory levels, carvacrol increases the virulence of *B. cereus*. We hypothesize that *B. cereus* exposed to subinhibitory carvacrol concentrations will cause more damage to the eye than the bacteria alone without progressing into a systemic infection in an *in vivo* mouse model. Systemic pro-inflammatory cytokines (TNF-α and IL-6) and anti-*B. cereus* IgG levels were measured by enzyme-linked immunosorbent assays. We found that mice infected with *B. cereus* and the subinhibitory concentration (SIC) of carvacrol had higher systemic levels of TNF-α, IL-6, and anti-*B. cereus* IgG. Ocular damage caused by infection

with *B. cereus* was quantified by histological analysis. We found that eyes infected with *B. cereus* stressed with the SIC of carvacrol had more damage than eyes infected with the bacteria alone. However, ocular damage was not significantly different in mice treated with *B. cereus* stressed with the SIC of carvacrol and mice treated with the SIC of carvacrol alone. We determined that endophthalmitis caused by *B. cereus* stressed with the SIC of carvacrol results in an increased systemic immune response and increased ocular damage, but we are unable to confirm if these increases are due to bacterial virulence or irritation caused by carvacrol. Future studies will investigate the effects of carvacrol on retinal pigment epithelial (RPE) cells found in the blood-retinal barrier.

I. Introduction

Bacillus cereus

Bacillus cereus is a Gram positive, spore-forming, rod-shaped bacterium found naturally in a wide variety of environments (1). Although vegetative *B. cereus* grows optimally within a temperature range of 25 to 37°C (2), their endospores are capable of surviving in extreme temperatures (1). Because *B. cereus* endospores are also able to survive hostile conditions such as pasteurization and gamma radiation, these bacteria are often problematic in the food industry and result in high levels of foodborne illness. *B. cereus* endospores are capable of adhering to many surfaces, allowing them to spread to other food sources with ease (3). Once *B. cereus* endospores are ingested, they germinate within the small intestine and produce enterotoxins that cause the diarrheal symptoms often referred to as food poisoning. The symptoms involved in *B. cereus* foodborne illness are similar to those of *Clostridium perfringens*, but the illness is caused by entirely different enterotoxins produced by the two species.

In addition to causing foodborne illness, *B. cereus* has been implicated in other non-gastrointestinal infections, such as endophthalmitis (4), endocarditis (5), meningitis (6), and bacteremia. *B. cereus* is difficult to eradicate due to the resilient nature of their endospores, and hospital contamination is a problem that can lead to infections in immunocompromised individuals and patients with catheters (7). Sasahara *et al.* reported that one hospital's linens and washing machine were contaminated with *B. cereus*, which led to an outbreak of bacteremia among patients (8).

Virulence Factors of *B. cereus*

B. cereus produces a wide array of virulence factors that render it pathogenic. *B. cereus* is known to produce up to three antigenically distinct enterotoxins, including hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) (1, 3). Hbl and Nhe are comprised of three toxin components while CytK consists of a single component. The Hbl enterotoxin consists of L_1, L_2, and B proteins, which originate from the genes *hblD*, *hblC*, and *hblA* within the same operon (Fig. 1). The Nhe enterotoxin consists of NheA, NheB, and NheC proteins, each encoded by genes in the *nheABC* operon (Fig. 1).

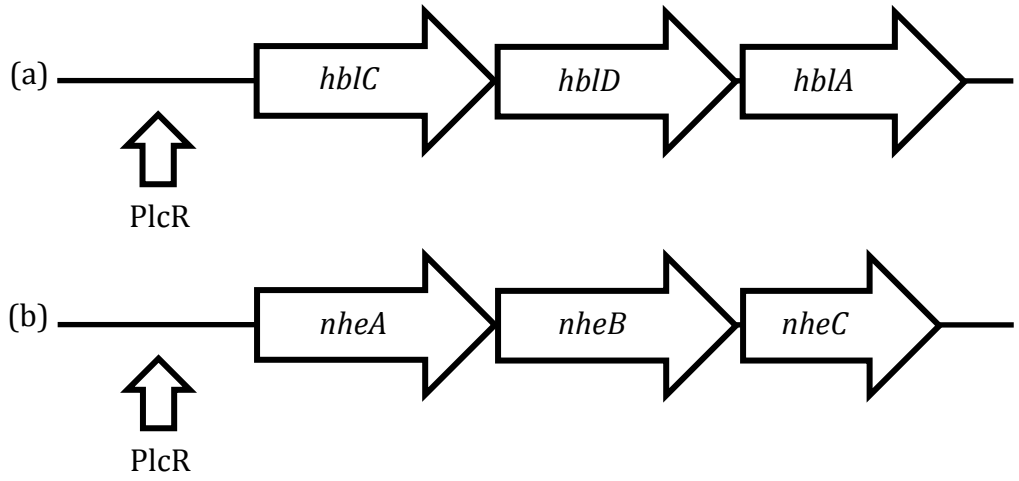

Figure 1. A schematic representing the *hbl* operon (a) and *nhe* operon (b), both of which are positively regulated by the global effector PlcR.

The Hbl, Nhe, and CytK enterotoxins are activated by the transcriptional regulator PlcR (Fig. 1) (9) and cause damage to host cells by forming membrane-spanning pores (3). The formation of pores by these enterotoxins disrupts membrane integrity of host cells and local membrane physiology, including ion gradient regulation. *B. cereus* secretes additional toxins under the regulation of PlcR such as hemolysins and phospholipases which may contribute to pathogenicity by working in conjunction with Hbl, Nhe, and CytK.

Endophthalmitis

Endophthalmitis is an inflammatory ocular infection that occurs when bacteria enter into the posterior segment of the eye (Fig. 2) (4). Endophthalmitis often causes permanent damage to photoreceptor cells, resulting in partial or complete loss of vision. One reason why this eye infection causes so much damage is because while the aqueous humor of the eye is replaced approximately every 100 minutes, the vitreous does not undergo regeneration (10). Therefore, the vitreous is more susceptible to infection if bacteria are introduced. This devastating eye infection is categorized into three groups based on the bacterial method of entry: postoperative, endogenous, and posttraumatic.

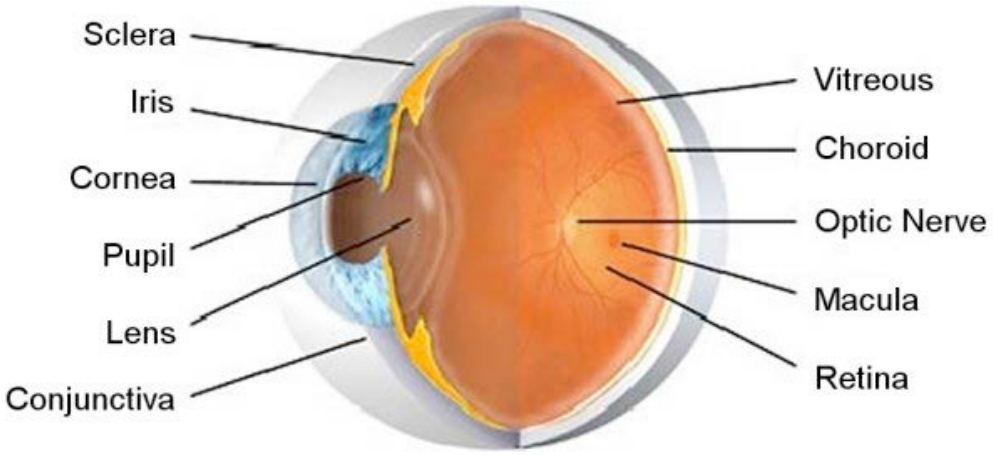

Figure 2. An illustration of the anatomy of the eye. (http://www.harvard-wm.org/wp-content/uploads/2014/11/Human-eyesight-facts.jpg)

<u>Postoperative Endophthalmitis</u>

Postoperative endophthalmitis occurs after approximately 0.1% of cataract surgeries in the United States (10). Gram-positive bacteria are the cause of over 95% of postoperative endophthalmitis cases, including 70% of cases caused by *Staphylococcus aureus*. This is likely due to contamination from the skin of the doctors performing the

surgery, because *S. aureus* commonly resides on the skin. Bacteria can also be introduced by the eyelid and preocular tear film, which protects the cornea (4). These bacteria that colonize the surface of the eye make their way into the aqueous humor in 7-34% of cataract surgeries (10). However, endophthalmitis does not result every time the aqueous humor is contaminated, but only when the bacteria spread from the aqueous humor into the vitreous of the eye.

Although inflammation within the eye is a common side effect on the first day of recovery from cataract surgery, inflammation observed two days after surgery is typically a symptom of postoperative endophthalmitis (10). This eye infection occurs within the first week after having cataract surgery in 75% of patients with postoperative endophthalmitis. Symptoms include loss of vision, eye redness, swelling, and pain. Upon initial examination, the retina is often not visible due to the aggregation of white blood cells during infection. Approximately 10% of postoperative endophthalmitis patients are left with no functional vision.

Postoperative endophthalmitis can be diagnosed by culturing bacteria from intraocular fluid, but 30% of cases cannot successfully culture bacteria due to low sensitivity of recovery methods used (10). One alternative diagnostic tool is the use of polymerase chain reaction (PCR) to detect lower levels of bacteria that may be too low to successfully culture. In a study of 29 patients with endophthalmitis, PCR resulted in diagnoses with 20% more accuracy than culture methods (11).

Endogenous Endophthalmitis

Endogenous endophthalmitis results from bacteria in the bloodstream that have spread to the eye (4). This route of infection accounts for 2-8% of endophthalmitis cases

and is more common in immunocompromised individuals, patients undergoing immunosuppressive drug treatment, patients with indwelling catheters, and intravenous drug users. The latter group is infected with a wider variety of bacteria, including *Bacillus* species, due to contamination of needles or the drugs themselves. The most common microbe responsible for endogenous endophthalmitis is the opportunistic fungus *Candida albicans*. Because endogenous endophthalmitis originates from the bloodstream, approximately 75% of blood cultures test positive for the causative bacterium along with a positive culture from the vitreous of the eye (10).

Posttraumatic Endophthalmitis

In comparison to postoperative and endogenous forms, posttraumatic endophthalmitis is caused by a wider variety of microbes (4). This diversity occurs because an object from the environment penetrates the eye and causes injury as opposed to infection due to contamination from bacteria that typically colonize the skin. For this reason, the ubiquitous *B. cereus* is one of the most common species responsible for infection in posttraumatic endophthalmitis. In a study by Callegan *et al.*, *B. cereus* accounted for 52.6% of 39 ocular isolates known to be infected with a *Bacillus* species (12). *Bacillus thuringiensis* accounted for 26.3% of isolates. In a study by Miller *et al.*, 83% of patients with endophthalmitis were infected with *B. cereus* (13).

Because *B. cereus* is ubiquitous and found naturally in the soil (3), objects in the work environment, such as metal, may easily be contaminated and cause infection when introduced into the eye (14). Enduring a traumatic injury involving the introduction of a foreign body, especially metal, into the eye greatly increases the chances of infection with posttraumatic endophthalmitis (10). In a retrospective study of 13 individuals with

posttraumatic endophthalmitis caused by *B. cereus*, 77% of patients had a traumatic injury to the eye in which a foreign body was introduced (14). Of these patients, 90% of the intraocular foreign bodies were metallic. This type of injury often occurs after two metal objects come into contact under force, resulting in airborne metal fragments penetrating the eye. Injuries resulting in posttraumatic endophthalmitis often occur in work environments with access to soil, dust, or hay, such as a farm. Another likely explanation for the correlation between an eye injury with a foreign body and endophthalmitis caused by *B. cereus* is that the foreign body penetrates the eye and allows bacteria that colonize the outer eye inside to facilitate an opportunistic infection. A study by Vanbijsterveld *et al*. swabbed the eyes of 20 healthy people who worked in rural environments with access to hay and soil (15). Seven different *Bacillus* species were isolated from the conjunctiva of 40 eyes, and four were from the *B. cereus* family. This study suggests that the foreign body does not necessarily have to be contaminated with the bacteria in order to introduce an endophthalmitis infection.

While other Gram-positive bacteria remain localized in the vitreous of the eye during endophthalmitis, *B. cereus* is a motile organism, which allows it to spread to retinal tissue and the aqueous humor as early as nine hours after initial infection (4). The extreme and sudden decline of the eye during *B. cereus* endophthalmitis is due to the damaging toxins produced by this bacterium. A study by Moyer *et al*. found that after eight hours of infection with *B. cereus*, 86-89% of retinal epithelial (RPE) cells were necrotic or undergoing necrosis (16). However, only 4% of RPE cells were undergoing apoptosis, which suggests that *B. cereus* toxins play a bigger role in tissue damage than the host inflammatory response does. Studies from Beecher *et al*. have determined that no single *B.*

cereus toxin is responsible for the rapid deterioration of the eye, but rather a mixture of toxins working together (17, 18). Beecher observed that eyes infected with a crude supernatant from *B. cereus* caused more tissue damage than eyes infected with pure HBL toxin, and when this toxin was neutralized in the crude *B. cereus* supernatant, lactate dehydrogenase (LDH) release only decreased by 55% (18). LDH normally resides inside the cell but is released when cells are damaged by bacterial toxins (19). Although this information reveals that HBL does in fact play an important role in the pathogenesis of *B. cereus* endophthalmitis, it also shows that approximately 45% of eye tissue damage is due to the work of other toxins and enzymes.

Treatment

Unfortunately, proper identification of the bacterial species causing endophthalmitis upon initial diagnosis can be challenging, so it becomes difficult to know which antibiotic will work best. Broad-spectrum antibiotics are typical chosen for this reason and directly injected into the eye (4). The conventional treatment for *B. cereus* endophthalmitis is the direct intraocular injection of antibiotics, most commonly vancomycin, in addition to an aminoglycoside or cephalosporin (10). Systemic antibiotics are also commonly administered, although they do not always make it into the vitreous of the eye due to the protection of the blood-ocular barrier (4). In a study performed by Ferencz *et al.*, vitreous samples taken from patients given intravenous vancomycin had either no vancomycin present in the vitreous, or levels that fell beneath the minimum inhibitory concentration (MIC) required for most Gram-positive bacteria (20). However, advanced endopthalmitis with high levels of intraocular inflammation results in the degradation of the blood-ocular barrier, potentially allowing systemic treatments more access to the vitreous of the eye (4).

In a study by Miller *et al*., 42% of patients given systemic vancomycin or cephalosporins such as cefazolin or ceftazidime lost their ability to see even light (13). On the other hand, only 17% of patients lost their light perception sight after undergoing treatment with systemic fluoroquinolones (ciprofloxacin, ofloxacin, levofloxacin, gatifloxacin, moxifloxacin). This study supports the conclusion drawn by Callegan *et al*. that although vancomycin is an effective intraocular treatment, it is not beneficial when given intravenously (4).

Miller *et al*. found that *B. cereus* is susceptible to vancomycin, gentamicin, and fluoroquinolones when in the vitreous of the eye (13). However, only 14% of *B. cereus* vitreous isolates were susceptible to penicillin or cephalosporins (cefazolin and ceftazidime). The results of this study were supported by Callegan *et al*., who found that *B. cereus* was most susceptible to ciprofloxacin and vancomycin, but not ceftazidime (12). Callegan also found that *B. cereus* was moderately susceptible to amikacin, an aminoglycoside that is commonly used in combination with vancomycin. Although ceftazidime is not as sensitive, many doctors prefer it over amikacin because the latter can be cytotoxic to retinal tissue (4, 10). However, a study by Wiskur *et al*. suggested that gatifloxacin is more effective than vancomycin because of the former's ability to clear *B. cereus* after six hours of infection in the vitreous of the eye while vancomycin was only bactericidal when administered after four hours of infection (21). Although gatifloxacin was more effective after six hours due to its ability to migrate into the aqueous humor, this antibiotic resulted in a lower level of retinal retention than that of vancomycin. Overall, vancomycin is a better treatment option when endophthalmitis is caught early and is still

localized in the vitreous of the eye. It is important to treat *B. cereus* as early as possible in order to eliminate bacteria before they can cause overwhelming damage to the eye.

The use of antibiotics can also cause damage by inducing intraocular inflammation while trying to eliminate the bacteria (4). Photoreceptor cells are extremely sensitive to both the invading microbe and the host response mounted against it. These retinal cells can also be sensitive to some antibiotic treatments. For this reason, the corticosteroid dexamethasone is often used in conjunction with antibiotics in an attempt to relieve some of the antibiotic-induced inflammation and prevent unnecessary tissue damage during treatment. The use of corticosteroids remains controversial however, as a study by Wiskur *et al*. concluded that dexamethasone did not significantly benefit the antibiotic treatment, but instead caused vancomycin to be less effective after six hours of infection with *B. cereus* (21).

Another treatment option for endophthalmitis is a vitrectomy, or the surgical removal of vitreous fluid and replacement with sterile saline (4). A vitrectomy is often used in combination with antibiotics, and it is the most aggressive way to treat severe endophthalmitis (10). By draining infected vitreous fluid, the bacteria are removed or diluted, along with detrimental host inflammatory cells and bacterial toxins that cannot be eliminated by antibiotics. A similar method is the vitreous needle tap, which is a less invasive way to sample the vitreous of the eye with a needle. Although this method is simpler and does not require an operating room, the vitreous needle tap is not as beneficial as the vitrectomy. Patients treated with a vitreous needle tap had 27% less functional vision when compared to patients treated with a vitrectomy according to the Endophthalmitis Vitrectomy Study (EVS). In a study by Miller *et al*., 71% of eyes treated

with a vitreous needle tap and intravitreal antibiotics lost all functional eyesight (13). On the other hand, only 29% of eyes treated with a vitrectomy and intravitreal antibiotics lost all functional eyesight. Although some doctors are against performing a vitrectomy at the risk of further traumatizing the eye, the general consensus is to perform a vitrectomy and administer intraocular antibiotics as soon as possible (4).

Prognosis

Overall, the visual outcome of patients with endophthalmitis varies according to the bacterium responsible and the length of time between infection and treatment (10). *B. cereus* endophthalmitis has particularly low rates of visual recovery due to the aggressive, irreversible damage done by toxins in a short amount of time (4). Callegan *et al.* concluded from various studies that overall, only 9% of patients with posttraumatic endophthalmitis recovered with functional eyesight of 20/70 or better. Of the patients with endophthalmitis, 70% lost all functional eyesight, and half of these patients had to have their eyes removed entirely due to severe necrosis.

Blood-Retinal Barrier

The blood-retinal barrier is a stringent regulatory system in the posterior of the eye that defines an immunoprivileged region protected from various macromolecules and leukocytes found in the bloodstream (22, 23). The blood-retinal barrier consists of an inner and outer layer. The inner blood-retinal barrier is composed of retinal endothelial cells connected by tight junctions (22). This layer of endothelial cells lies on top of a basal lamina consisting of astrocytes, Müller cells, and pericytes. Astrocytes are cells responsible for preserving the structure of retinal vessels, and they also induce expression of proteins involved in tight junction formation. Müller cells aid retinal endothelial cells in the

regulation of nutrient intake and waste elimination across the blood-retinal barrier. Pericytes strengthen the inner blood-retinal barrier by inducing the expression of occludin, a tight junction protein. These three cell types help preserve the blood-retinal barrier by signaling to retinal endothelial cells when changes occur within the isolated intraocular environment. The inner blood-retinal barrier separates the retinal neurons from the capillary blood supply. The outer blood-retinal barrier is composed of retinal pigment epithelial (RPE) cells joined together by tight junctions (22). The outer blood-retinal barrier separates the highly sensitive photoreceptor cells from the blood supply found in the choroid. RPE cells take up nutrients from the blood supply found in the choroid and deliver them to photoreceptor cells. These retinal cells are also capable of removing waste by undergoing phagocytosis when photoreceptor cells have been worn out or damaged. Another important function of RPE cells is their ability to supply photoreceptor cells with retinol, a chemical necessary for functional eyesight. RPE cells are also responsible for absorbing light and protecting photoreceptor cells from damage due to overexposure. RPE cells are known to produce growth factors and cytokines such as pigment epithelium-derived factor (PEDF), VEGF, transforming growth factor-β (TGF-β), various interleukins and TNF-α (24).

Tight junctions, also known as zonula occludens, consist of over 40 proteins responsible for tightly joining retinal cells together and preventing gaps between these cells that may allow for harmful molecules to flow through (23). Some of the main proteins involved are occludin and 24 different types of claudins. When occludin binds to one of the three zonula occludens (ZO) proteins, a tight junction is formed. Junctional adhesion molecules, symplekin, tricellulin, cingulin, and 7H6 also aid the formation of tight junctions.

When tight junctions become damaged, the connection between retinal cells is lost, and therefore permeability of the blood-retinal barrier increases (23). In a study by Erickson *et al*., RPE cells treated with occludin siRNA had 65% less occludin present and a 15% increase in permeability within tight junctions (25). Blood-retinal permeability is also increased when occludin is phosphorylated (23). Cell-signaling proteins, such as adenosine, PGE1, IL-1β, VEGF, and TNF-α, are capable of dissociating tight junctions and therefore increasing blood-retinal permeability. Of these pro-inflammatory proteins, TNF-α and IL-1β cause the most damage to the blood-retinal barrier. When leukocytes are recruited during inflammation, they disrupt tight junction protein expression and cause the blood-retinal barrier to deteriorate. Tight junction formation is also deterred when Na^+/K^+ ATPase activity is disrupted due to changes in cell polarity (26).

Host Inflammatory Response

They eye is often referred to as an immunoprivileged region because of the blood-ocular barrier, but another reason for its unique environment is its function in immunosuppression (27). Although immunosuppression mechanisms are often employed by the body to prevent autoimmunity, the eye is unique because these mechanisms are always active. Evidence for this immunosuppressive response is observed in the high acceptance rate of corneal grafts in the eye (28). In the aqueous humor, antigen-presenting cells are able to take up the antigen and activate regulatory CD4+ T cells. Antigen presenting cells then migrate out of the trabecular meshwork of the eye into the venous bloodstream towards the spleen (4). This immune response is known as the anterior chamber-associated immune deviation (ACAID). The immunoprivileged microenvironment

of the eye allows for the removal of antigens without initiating a destructive inflammatory response that could cause irreversible damage to sensitive intraocular tissue.

Although the immunosuppressive response of the eye is often beneficial, it can allow bacteria to grow unimpeded and gain a foothold without a threat from ocular immune cells (4). Eventually the ACAID immune response becomes overwhelmed, and a devastating inflammatory response is initiated. The inflammatory response is especially robust in endophthalmitis caused by *B. cereus*. Peptidoglycan, a component found in cell wall of *B. cereus* and other Gram-positive bacteria, induces the inflammatory response in the vitreous of the eye by initiating production of cytokines and other inflammatory cells. The main cytokines involved in the ocular inflammatory response are TNF- α, IFN-γ, IL-1, IL-2, IL-6, and IL-8 (29). In addition to cytokines, toll-like receptor (TLR) cells also initiate inflammation during an innate immune response (30).

Tumor necrosis factor-alpha (TNF- α) is a pro-inflammatory cytokine often found during a systemic inflammatory response, but it can also be produced by astrocytes in the eye (30). TNF- α stimulates apoptosis and cell proliferation along with inflammation (31). During infection with endophthalmitis, TNF- α is up-regulated in the eye and recruits polymorphonuclear leukocytes (PMNs) to the site of infection (23). A study by Ramadan *et al*. illustrated the importance of this pro-inflammatory cytokine by observing that TNF- α knockout mice infected with *B. cereus* had significantly lower levels of polymorphonuclear leukocytes within the eye (32). TNF-α also induces inflammation by activating the transcriptional regulator NF-κB, which regulates additional chemokines and cell adhesion molecules (4). Ramadan *et al*. found that TNF-α is present in the eye as early as 2 hours after initial infection, but a sharp increase can be seen after 10 hours of infection (33).

Interferon-gamma (IFN-γ) is a pro-inflammatory cytokine produced by a wide variety of immune cells, such as CD4+ helper T cells, CD8+ cytotoxic T cells, NK cells, B cells, NKT cells, and antigen-presenting cells (34). IFN-γ is induced by additional cytokines, specifically IL-12 and IL-18, and transmits signals *via* the Jak-Stat pathway. In the absence of IFN-γ, fewer PMNs are recruited to the site of infection, and NK cell activity suffers as well. IFN-γ is highly expressed in the eye 24 hours after initial infection (35).

Interleukin-1 (IL-1) is a cytokine primarily involved in the acute-phase response (4). This pro-inflammatory cytokine is produced by Müller cells, ciliary body cells, and corneal epithelial cells in response to ocular infection. However, IL-1 can be produced by macrophages, monocytes, and dendritic cells during systemic infections (30). IL-1 acts in a cell-signaling pathway to activate additional components of the inflammatory response, such as prostaglandins, phospholipase A_2, collagenases, and pro-inflammatory cytokines IL-6 and TNF-α (4). Intraocular injection with IL-1β leads to blood-retinal barrier degradation and aggregation of leukocytes within the eye.

Interleukin-2 (IL-2) is a cytokine involved in antigen removal in conjunction with activated lymphocytes (29) such as CD4+ T cells, CD8+ T cells, NKT cells, and NK cells (36). IL-2 plays an important role in the differentiation of CD4+ T cells into immunosuppressive regulatory T cells. This cytokine also helps differentiate CD8+ T cells into either temporary effector T cells or more permanent memory T cells. Activated dendritic cells can also produce IL-2. In a study by Granucci *et al*., mice deprived of dendritic cells had lower levels of IL-2, which in turn decreased the activation of NK cells and their subsequent production of IFN- γ (37). This same study revealed that initially after bacterial infection, the main

source of IL-2 came from activated dendritic cells rather than T cells or B cells. Overall, IL-2 is an important cytokine for both protective and regulatory immune responses.

Interleukin-6 (IL-6) is another cytokine involved in the acute-phase response, and it also plays a role in the differentiation of B cells and T cells (4). This pro-inflammatory cytokine is induced by IFN-γ, TNF-α, and IL-1. IL-6 is produced by retinal pigment epithelial cells within the eye, but this cytokine can also be produced by a wide variety of immune cells during a systemic infection (30). IL-6 also has anti-inflammatory qualities that help regulate the immune response due to its negative feedback relationship with TNF-α and IL-1. IL-6 is important to both the inflammatory immune response and the regulatory immune response during bacterial infection.

Interleukin-8 (IL-8) is a potent pro-inflammatory cytokine that works by recruiting PMNs to the site of infection (38). This cytokine is produced by activated macrophages and monocytes, as well as vascular endothelial cells. IL-8 expression is induced by cytokines such as IFN-γ, TNF-α, IL-1, and IL-6 but suppressed by IL-10. IL-8 may play a role in early retinal detachment because under stress, retinal pigment epithelial cells express this cytokine. IL-8 is particularly unique because it remains at the site of infection long after other cytokines are gone.

Toll-like receptor (TLR)-expressing host cells activate cell signaling pathways that eventually induce an inflammatory response after binding to pathogen-associated molecular patterns (PAMPs) (30). Retinal pigment epithelial cells host TLR-2, TLR-4, and TLR-9. These TLR cells are involved in an inflammatory immune response in the posterior of the eye, where endophthalmitis infections occur.

Although an intraocular immune response to invading bacteria is necessary in order to clear an endophthalmitis infection at its source, the immune response can often do more harm than good due to excessive inflammation (30). Infiltration of PMNs into the eye is a characteristic immune response to endophthalmitis infections, and it is very damaging to intraocular tissue. Although PMNs kill bacterial cells through the process of phagocytosis, they also release metalloproteases and oxyradicals that are very toxic to the retina. In endopthalmitis cases with high levels of these immune cells, a vitrectomy would be most effective.

Carvacrol

Although antibiotics such as vancomycin are effective at killing endophthalmitis-causing bacteria, they do not retain anti-inflammatory qualities that would be helpful to prevent excess ocular damage caused by host immune cells. Although the use of corticosteroids such as dexamethasone has been investigated, no significant anti-inflammatory benefits have been found (21). For this reason, essential oils from plants have been investigated for antimicrobial and anti-inflammatory qualities. These essential oils typically reduce inflammation by scavenging oxygen and nitrogen free radicals generated by various immune cells (39). These oxygen and nitrogen free radicals initiate inflammatory responses by acting as cell signaling molecules and inducing expression of transcription factors such as NF-κB, which plays an important role in the activation of various cytokines. By quenching these dangerous free radicals, essential oils are able to prevent the activation of an unwanted inflammatory response.

Carvacrol is the main component in oregano oil with antimicrobial, antioxidant, and anti-inflammatory qualities (39). Carvacrol is a hydrophobic phenol synthesized by the

oxidation of γ-terpinene to ρ-cymene (40). Carvacrol is created when a hydroxyl group is added to ρ-cymene. However, ρ-cymene is not nearly as effective as carvacrol on bacteria such as *B. cereus*, although the chemical mechanism for this has yet to be elucidated (41). This difference in antimicrobial activity suggests that the hydroxyl group of carvacrol is of great importance. Another important quality of carvacrol is its possession of a system of delocalized electrons. The only difference between carvacrol and menthol is that menthol does not possess a system of delocalized electrons. However, this difference prevented menthol from inhibiting *B. cereus* growth even at concentrations 10 times higher than those required by carvacrol.

Carvacrol is lethal to *B. cereus* at concentrations above 1 mM (42). A study by Ultee *et al*. revealed that carvacrol administered at 0.9 mg/ml or above caused a rapid decrease in *B. cereus* toxin production *in vitro* (43). Carvacrol causes damage by disturbing the cytoplasmic membrane of *B. cereus* and subsequently decreasing intracellular ATP levels. This disruption is illustrated by an increase in swelling within the membrane (41). Carvacrol also reduces ATP levels by disrupting the K^+ ion gradient within the cell membrane of *B. cereus* (42). Disruption of the K^+ ion gradient results in structural damage to the bacterial cell membrane. When carvacrol disrupts the *B. cereus* cytoplasmic membrane, it also decreases the membrane potential, which in turn reduces ATP synthesis.

Carvacrol also appears to inhibit the expression of various pro-inflammatory cytokines and transcription factors (44). In a study by Aristatile *et al*., carvacrol significantly reduced mRNA and protein expression of TNF-α, IL-6, iNOS, COX-2, and NF-κB. This is an important ability, because TNF-α and IL-6 are especially important in the ocular inflammatory response during endopthalmitis. Carvacrol is an ideal substitute for

antibiotics during treatment for *B. cereus* endophthalmitis because not only does it kill *B. cereus*, but it also inhibits some of the main cell signaling pathways used to initiate a harmful inflammatory response in the eye.

Effects of Sub-Inhibitory Concentrations of Carvacrol

A study by Ultee *et al*. confirmed that *B. cereus* is able to grow in the presence of carvacrol at concentrations below the lethal limit (45). This same study found that carvacrol concentrations of 0.4 mM or below can inhibit *B. cereus* growth, but do not kill the bacteria. When exposed to a subinhibitory concentration (SIC) of carvacrol, *B. cereus* alters the fatty acid composition within its cell membrane in an attempt to protect intracellular ATP and K^+ from leaving the bacterial cell.

Previous work in our lab has determined that the minimum inhibitory concentration (MIC) of carvacrol is 2 mM and the SIC of carvacrol is 1 mM (Nimmer, unpublished). We have also discovered that in response to the SIC of carvacrol, *B. cereus* HblC toxin expression increases by approximately 50%, and NheA toxin expression increases by approximately 46.8%. Additional work with *Caenorhabditis elegans* provided an *in vivo* model illustrating the effects of the SIC of carvacrol on *B. cereus* virulence. *C. elegans* treated with *B. cereus* stressed with the SIC of carvacrol had a 62% increase in mortality compared to *C. elegans* treated with the bacteria alone. In the current study, the effects of subinhibitory carvacrol concentrations on *B. cereus* virulence were assessed during infection with endophthalmitis in an *in vivo* mouse model. We also assessed the systemic effects of this eye infection by quantifying pro-inflammatory cytokines TNF-α and IL-6 in mouse spleen tissue. Histological analysis was performed on enucleated eyes by staining with fluorescent DAPI and Rose Bengal dyes. DAPI stains the nuclei of normal cells

blue, and functioned in this study to stain healthy eye tissue. Rose Bengal stains dead or damaged eye tissue red, and functioned in this study to stain eye tissue damaged by *B. cereus* and the SIC of carvacrol (46).

Hypothesis: Growth of *B. cereus* ATCC14579 in subinhibitory carvacrol concentrations will significantly increase the ability of the bacteria to elicit eye tissue damage in a mouse ocular infection model but will not progress into a systemic infection.

Objective 1: To determine if *B. cereus* stressed with the SIC of carvacrol increases virulence during endophthalmitis and progresses into a systemic infection.

Objective 2: To determine if *B. cereus* stressed with the SIC of carvacrol causes significantly more tissue damage in the eye compared to the bacteria or carvacrol alone.

II. Manuscript

INTRODUCTION

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming bacterium that, in addition to being well-known in foodborne illness (47), is capable of causing endophthalmitis (1, 4). *B. cereus* produces a variety of damaging toxins that play an important role in its 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) (3). *B. cereus* is the second most common source of posttraumatic endophthalmitis, next to *Staphylococcus aureus* (4).

Endophthalmitis is an inflammatory infection that occurs after introduction of bacteria into the posterior segment of they eye (4). 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.

Because it is difficult for health professionals to immediately identify the bacteria responsible for endophthalmitis, broad-spectrum antibiotics are commonly administered (4). 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 is toxic to sensitive retinal cells, ceftazidime is often preferred. However, *B. cereus* is not always sensitive to this drug. A vitrectomy is also a fast and effective way to drain the eye of fluid containing the causative bacteria, toxins, and host inflammatory cells (10).

While intravitreal antibiotics can effectively control *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 (29). TNF-α and IL-6 are two pro-inflammatory cytokines produced in the eye during endophthalmitis (4). TNF-α signals polymorphonuclear leukocytes to rapidly migrate to the site of infection within the eye, causing extensive inflammatory damage along the way (23). IL-6 is stimulated by TNF- α, and plays a role in the differentiation of T- B-lymphocytes (4) 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 (21). 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 (39). 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 (41) while releasing intracellular ATP (43). Carvacrol also disrupts the K^+ ion gradient within the cell membrane and is lethal to *B. cereus* at concentrations exceeding 1 mM (42). However, it is the anti-inflammatory qualities of carvacrol that render it most useful as a possible treatment for endophthalmitis. A study by Aristatile *et al.* determined

that carvacrol inhibits TNF- α and IL-6 expression at both transcriptional and translational levels, along with other pro-inflammatory cytokines *in vitro* (44). Carvacrol may be an ideal 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. In a study by Ultee *et al*., *B. cereus* was able to adjust to carvacrol at concentrations of 0.4 mM or below (45). *B. cereus* had different levels of fatty acids within its cell membrane after exposure to sub-inhibitory concentrations (SIC) of carvacrol, most likely in an attempt to protect its intracellular ATP and K^+ ions. Studies performed in this lab 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 nonstressed cultures.

In this study, we dropwise infected the eyes of mice with *B. cereus* exposed to the SIC of carvacrol. 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 that of eyes infected with *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. The intraocular reaction of *B. cereus* to the SIC of carvacrol is of great importance because the ability of carvacrol to penetrate the vitreous has not yet been studied, and it is possible that only sublethal levels of this chemical could persist into the eye. It is important to study both

the benefits and potentially harmful effects of using carvacrol to treat endophthalmitis caused by *B. cereus*.

MATERIALS AND METHODS

Bacteria Preparation

Bacillus cereus ATTC14579 (VWR, West Chester, PA) was cultured in Tryptic Soy Broth (TSB) (Weber Scientific, Hamilton, NJ) 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 (W224502, SAFC Supply Solutions, St. Louis, MO). The bacteria were sub-cultured twice before being administered to mice at $5x10^7$ colony forming units (cfu) in sterile saline.

Infection with *Bacillus cereus* and the 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. Three BALB/c mice were randomly assigned to five different treatment groups for this experiment. All mice were approximately 10 weeks of age, and cages were separated by gender throughout the experiment. 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 included a control group receiving only saline, a group receiving only the SIC of carvacrol without bacteria, a group receiving $5x10^7$ cfu of *B. cereus* in saline, a group receiving $5x10^7$ cfu of *B. cereus* cultured with the SIC of carvacrol, and a group receiving an intraperitoneal injection of $5x10^7$ cfu of *B. cereus* alone. Mice were infected with the same

volume of eye drops (15µl) in each eye by micropipet. The mice were closely monitored throughout the infection period of 14 days, after which they were sacrificed.

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 x 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 (28908, Thermo Scientific, Rockford, IL) containing 20µl 10 N NaOH added dropwise (Sigma-Aldrich, St. Louis, MO)). After removing the lens, the eyes were stored at -20°C for future histological analysis.

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 performed using the Mouse TNF-α ELISA Ready-SET-Go!® (88-7324-88, eBioscience, San Diego, CA) and Mouse IL-6 ELISA Ready-SET-Go!® (88-7064-22, eBioscience) kits and following the manufacturer's instructions. A TNF-α protein standard (39-8321-60, eBioscience) and IL-6 protein standard (39-8061-60, 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 (P1037, Sigma-Aldrich), 0.25% glutaraldehyde (P8648, Eastman Kodak Company,

Rochester, NY) and $5x10^7$ cfu *B. cereus*. 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.

Histological Analysis of Eye Tissue

Fixed mouse eyes were sectioned at 12 μm thickness using the MICROM HM 505 N cryostat. Each eye was stored in 30% sucrose (15503-014, Life Technologies, Carlsbad, CA) cryoprotectant at least 24 hours prior to sectioning. For each eye, five slides were prepared using TRUBOND 380 Microscope Slides (0380W, Tru Scientific, Japan). Each slide contained three slices of eye tissue.

Eye tissue slides were then stained with 1% DAPI (D1306, Life Technologies) for four minutes and 0.001% Rose Bengal (R3877, 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 1X PBS, the slides were imaged using a fluorescence microscope and stored at -20°C. The images acquired at 100X magnification were analyzed using Image-Pro Express 6.0 software. Data for each slide were averaged together, resulting in a single mean intensity value for red spectrum fluorescence and blue spectrum fluorescence in each eye.

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

TNF-α pro-inflammatory cytokine expression during *B. cereus* ocular infection. Mice infected with SIC carvacrol-treated *B. cereus* had significantly higher ($p < 0.001$) detectable TNF-α levels in the spleen than all other treatment groups except for mice treated with *B. cereus* alone (Fig. 3, pg. 73). Mice injected intraperitoneally with *B. cereus* displayed lower TNF-α levels, but not significantly different 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 ($p < 0.001$) detectable IL-6 levels in the spleen than those of all other treatment groups except for mice injected intraperitoneally with *B. cereus* (Fig. 4, pg. 74). 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 *B. cereus* ocular infection. Mice infected with SIC carvacrol-treated *B. cereus* had significantly higher ($p < 0.001$) detectable IgG levels than mice infected with *B. cereus* alone (Fig. 5, pg. 75). No significant difference was observed between mice infected with SIC carvacrol-treated *B. cereus* and mice injected intraperitoneally with *B. cereus* however.

Histological analysis of tissue damage during *B. cereus* ocular infection. Mouse eyes infected with SIC carvacrol-treated *B. cereus* and fluorescently stained with DAPI and Rose Bengal had significantly higher red saturation intensity values, and therefore more damaged tissue than eyes infected with *B. cereus* alone ($p = 0.023$) and eyes treated with saline ($p = 0.048$) (Fig. 6, pg. 76). However, eyes infected with SIC carvacrol-treated *B. cereus* and eyes treated with SIC carvacrol alone were not significantly different.

DISCUSSION

Although our hypothesis predicted that a systemic infection would not result from the administration of *B. cereus* treated with the SIC of carvacrol into the eyes of mice, our findings, specifically for the ELISA-based detection of TNF-α and IL-6 pro-inflammatory cytokines, supported the notion that at least a modest systemic bacterial infection did occur. The goal of this 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 in the eye 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. Levels of IL-6 in spleen lysates were significantly higher in mice infected with stressed *B. cereus* ($P = 0.012$). Anti-*B. cereus* IgG levels were also significantly higher in mice infected with the bacteria exposed to the SIC of carvacrol compared to those of *B. cereus* alone ($P < 0.0001$). Histological analysis confirmed that *B. cereus* stressed with the SIC of carvacrol caused more damage to the eye than the bacteria alone ($P = 0.023$). 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. This conclusion suggests that carvacrol alone was irritating to the eye and seemed to cause some damage in the absence of bacteria. However,

mice treated with carvacrol alone did not display any external signs of irritation during the 14d immune challenge. Although no conclusive difference was noted in ocular damage between these two treatment groups, the increased levels of pro-inflammatory cytokines and anti-*B. cereus* IgG suggest that the SIC of carvacrol did increase the virulence of *B. cereus* during endophthalmitis. Previous *in vitro* work in this lab supports this overall conclusion. However, another possibility is that the SIC of carvacrol caused damage to the blood-retinal barrier and allowed *B. cereus* to escape the eye more readily. The third possibility is that the effects seen here are a combination of increased bacterial virulence from sublethal stress, and carvacrol effects on retinal tissue. Future studies will examine the effects of carvacrol on RPE cells, which make up the outer layer of the blood-retinal barrier (22). These studies will investigate whether or not the SIC of carvacrol is toxic or in any way damaging to retinal tissue in the eye. Future studies will also assess the expression of tight junction proteins that connect RPE cells to determine whether or not the blood-retinal barrier is compromised in response to the SIC of carvacrol.

Although there is no universal method for fixing or storing ocular tissue prior to cryosectioning, the results of these experiments reveal at least some degree of tissue damage by allowing the ocular tissue to temporarily freeze in fixative solution. Although histological methods vary in their length of time for fixation, as well as whether or not the tissue should be stored frozen prior to sectioning, it is possible that the ocular tissue used in this experiment suffered additional damage due to either over-fixation or the formation of ice crystals during the freeze. Although the ocular tissue was moved to a cryoprotectant solution 24h before sectioning, damage may have occurred prior to this protective treatment. For this reason, it is possible that some of the tissue damage observed may be

due to the methods employed prior to sectioning rather than the treatment alone. The data from this experiment suggest that *B. cereus* stressed with the SIC of carvacrol caused significantly more damage to eyes than the bacteria caused by itself, but this conclusion can be made stronger by performing future studies with better quality eye tissue sections from tissue that was not frozen during the preparation phase. In future experiments, the ocular tissue would not be stored in fixative, but in a cryoprotectant solution.

Another potential limitation of this study was the lack of ocular tissue specificity. The interest of this experiment focused on overall ocular tissue damage. Because specific ocular structures were not quantified individually, the possibility that Rose Bengal stained specific tissues differently among eye sections remains. Rose Bengal was chosen as a specific stain for this experiment because it is commonly used to stain ocular tissue damage specifically, especially in the form of eye drops *in vivo* (48). Prior to this experiment, it was not known how Rose Bengal would stain specific intraocular structures, and in fact the focus of this experiment was not on these specific intraocular structures, but rather damage to the eye overall. However, innovative use of Rose Bengal to stain intraocular tissue presented a challenge when normalizing data from one fluorescent image to another. Images were normalized to the eyes treated with saline, but these eye sections did not always maintain the same level of tissue integrity. For example, eyes treated with the SIC of carvacrol alone maintained their tissue integrity better than other treatment groups, and this preserved group of tissue also stained more readily with Rose Bengal. A tissue type suspected to be the choroid also stained readily with Rose Bengal throughout the experiment. However, this tissue type was not present in all treatment groups. As stated previously, future experiments will work to achieve higher quality ocular tissue sections,

allowing for a more standard method of normalization against background fluorescence. One possible method would be to normalize replicate stained images of presumptive damaged tissue against the background fluorescence elicited by the intraocular structure(s) confirmed to be the choroid, for example. Additional methods of normalization could also be used, such as comparing an ocular tissue section stained with both Rose Bengal and DAPI to an ocular tissue section from the same treatment group stained with Rose Bengal alone or DAPI alone, the latter stain of which would show distinct intact nuclei. Overall, this study took an important step in examining ocular tissue damage using Rose Bengal and DAPI stains in sectioned tissue samples.

It is important to understand the response of *B. cereus* to the SIC of carvacrol in the ocular environment because carvacrol is a potential alternative treatment for endophthalmitis caused by *B. cereus,* due to its antimicrobial and anti-inflammatory qualities (40). However, it is important to understand both 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 lab, 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 could have a more virulent *B. cereus* eye infection, resulting in more eye tissue damage than the bacteria would have caused by itself.

Future studies will be performed using RPE cells *in vitro* to assess damage done to retinal cells by the SIC of carvacrol alone. We will also assess tight junction protein expression in RPE cells infected with *B. cereus* and 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 change how doctors treat endophthalmitis caused by these bacteria For example, if toxins are not actively produced inside the eye, then doctors may focus on controlling the bacteria with antibiotics rather than flushing the eye with saline in a vitrectomy. However, if *B. cereus* actively produces toxins within the eye, then clinicians would benefit from knowing the specific circumstances prior to administering treatment.

III. Proposed Future Research

ABSTRACT

B. cereus is a pathogenic bacterium capable of causing an infection in the posterior of the eye called endophthalmitis. *B. cereus* can cause irreversible damage to the eye as early as nine hours after initial infection by using its unique bacterial toxins. Studies have shown that *B. cereus* causes damage to retinal pigment epithelial (RPE) cells and increases permeability of the blood-retinal barrier. One characteristic of endophthalmitis caused by *B. cereus* is the rapid inflammatory response mounted against it. This inflammatory response also causes damage to the eye. Carvacrol is an extract from oregano oil that may be able to treat *B. cereus* endophthalmitis due to its inhibition of bacterial growth and anti-inflammatory qualities. However, sub-inhibitory levels of carvacrol increase *B. cereus* virulence. To the best of our knowledge, the retinal tissue response to carvacrol is currently unknown. This study will investigate the cytotoxicity of carvacrol to RPE cells. We will also determine if exposure to the subinhibitory concentration (SIC) of carvacrol causes *B. cereus* to increase its production of toxins within the eye. This study will also assess the effects of the SIC of carvacrol on the ability of *B. cereus* to damage the blood-retinal barrier and progress into a systemic infection. We hypothesize that *B. cereus* will damage the blood-retinal barrier during endophthalmitis and progress into a systemic infection by increasing its production of virulence factors when treated with subinhibitory levels of carvacrol. Understanding the behavior of *B. cereus* in the eye when it is stressed with the SIC of carvacrol is important when deciding if carvacrol could be used as an alternative treatment for endophthalmitis because it is possible that patients may receive treatment levels below the minimum inhibitory concentration (MIC) inside the eye.

BACKGROUND AND SIGNIFICANCE

Bacillus cereus is a Gram-positive, rod-shaped, spore-forming bacterium capable of causing endophthalmitis (1, 4). *B. cereus* produces a variety of damaging toxins that play an important role in its virulence during infection with endophthalmitis. Many of these toxins are under control of the global transcriptional regulator PlcR, including hemolysin BL (Hbl), nonhemolytic enterotoxin (Nhe), and cytotoxin K (CytK) (3). Endophthalmitis is an inflammatory infection that occurs after the introduction of bacteria into the posterior segment of they eye (4). Because of its motile nature, *B. cereus* can rapidly migrate from the vitreous of the eye to other intraocular structures and cause damage as early as nine hours after initial infection (4). Although damage to the blood-retinal barrier is often a consequence of *B. cereus* endophthalmitis, the progression of a systemic infection is rare.

The blood-retinal barrier is a stringent regulatory system in the posterior of the eye that defines an immunoprivileged region protected from potentially damaging systemic molecules found in the bloodstream (22, 23). The blood-retinal barrier consists of an inner and outer layer. The inner blood-retinal barrier is composed of retinal endothelial cells connected by tight junctions, and the outer blood-retinal barrier is composed of retinal pigment epithelial (RPE) cells joined together by tight junctions (22).

Tight junctions consist of over 40 proteins responsible for forming a tight seal between retinal cells and preventing gaps between these cells that might allow harmful systemic molecules to flow through (23). When tight junctions become damaged, the connection between retinal cells is lost, and therefore permeability of the blood-retinal barrier increases (23). During inflammation, ocular infiltration of leukocytes causes damage to the blood-retinal barrier and the tight junction proteins that hold it together.

The conventional treatment for *B. cereus* endophthalmitis is the direct intraocular injection of antibiotics, most commonly vancomycin, in addition to an aminoglycoside or cephalosporin (10). However, the use of antibiotics can also cause damage by inducing intraocular inflammation while trying to eliminate the bacteria (4). Photoreceptor cells are extremely sensitive to both the invading microbe and the host response mounted against it. In response to ocular infection with *B. cereus*, a variety of pro-inflammatory cytokines are produced (29), such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) (4). TNF- α signals polymorphonuclear leukocytes to rapidly migrate to the site of infection within the eye (23). IL-6 is stimulated by TNF- α, and plays a role in the differentiation of T-B-lymphcytes (4) 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 (21). 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.

Carvacrol is a phenolic chemical compound found in oregano oil that displays anti-microbial, antioxidant, and anti-inflammatory qualities (39). Carvacrol causes damage to *B. cereus* by disrupting the cell membrane and causing it to swell (41) while releasing intracellular ATP (43). A study by Aristatile *et al.* determined that carvacrol inhibits TNF- α and IL-6 expression along with other pro-inflammatory cytokines *in vitro* (44). Although

carvacrol can effectively inhibit *B. cereus* growth and suppress inflammation, it is very important to administer this chemical compound at the correct concentration. Studies performed in this lab have determined that the sub-inhibitory concentration (SIC) of carvacrol against *B. cereus* ATCC14579 is 1 mM, and early experiments indicated that *in vitro* toxin production increased compared to that in cultured bacteria alone.

Although studies have illustrated the importance of *B. cereus* toxins to the virulence of posttraumatic endophthalmitis (17, 18), to our knowledge no studies have been performed to determine if *B. cereus* actively produces toxins in the ocular environment. Understanding the behavior of *B. cereus* within the unique ocular environment is an important step in learning how to treat posttraumatic endophthalmitis more effectively.

Preliminary studies in this lab have investigated the effects of carvacrol treatment in the eye and the consequences of administering the SIC. Although ocular tissue damage was observed, we cannot confirm if the source of the damage was due to increased virulence of *B. cereus*, or cytotoxicity of carvacrol itself. The effects of carvacrol on ocular tissue are currently unknown.

The proposed study will investigate the intraocular behavior of *B. cereus* by observing if this bacterium actively produces toxins in the eye and causes damage to the blood-retinal barrier. This study will also determine whether or not carvacrol is cytotoxic to RPE cells in order to better understand the source of increased tissue damage in eyes treated with *B. cereus* and the SIC of carvacrol. Finally, this study will examine the damage done to the blood-retinal barrier by *B. cereus* treated with the SIC of carvacrol and quantify the systemic bacteria present in the spleen.

HYPOTHESIS: *Bacillus cereus* damages the blood-retinal barrier during endophthalmitis and progresses into a systemic infection by increasing virulence factor production when treated with sub-inhibitory levels of carvacrol.

PRELIMINARY DATA:

Identification of the sub-inhibitory concentration (SIC) of carvacrol. The SIC of carvacrol is the concentration capable of injuring *B. cereus* ATCC14579 and inhibiting its growth without killing all of the bacteria (45). A 1999 study by Ultee *et al.* found that carvacrol did not effectively kill *B. cereus* at a concentration of 1 mM, but bacterial cell death resulted after exposure to 1.25 and 1.5 mM concentrations (42). After serially diluting carvacrol in Mueller-Hinton broth, we found that the SIC was indeed 1 mM, and the minimum inhibitory concentration (MIC) was 2 mM, which supports the conclusions made by Ultee *et al* (Pierre Nimmer, unpublished). We also determined that the minimum bactericidal concentration (MBC) of carvacrol was 11 mM. Determining the SIC of carvacrol allowed us to treat *B. cereus* with the appropriate dosage in order to injure the bacteria without killing it for use in subsequent studies.

***B. cereus* exposed to the SIC of carvacrol decreased *hblc* transcript abundance and increased *nheA* transcript abundance.** Bacteria often undergo changes in gene expression under stress in order to ensure their survival (49). For this reason, we used RT-PCR to analyze mRNA transcript abundance levels for *B. cereus* toxins *hblC* and *nheA*, which are both positively regulated by the global transcription regulator *plcR* (9). The SIC of carvacrol caused *B. cereus* to down-regulate transcription of *hblC* by 16.25% and up-regulate transcription of *nheA* by 14.1% (Pierre Nimmer, unpublished). The positive

control, *gyrB*, was down-regulated by 23.9%. These results suggest that *B. cereus* undergoes transcriptional changes when exposed to the SIC of carvacrol.

***B. cereus* increases HblC and NheA toxin production when stressed with the SIC of carvacrol.** Many bacteria produce toxins as a response to stress in order to protect themselves (49). After running an enzyme-linked immunosorbant assay (ELISA) on *B. cereus* stressed with the SIC of carvacrol, we determined that the bacteria increases its production of the HblC toxin by 50% and the NheA toxin by 46.8% (Pierre Nimmer, unpublished). However, *B. cereus* treated with the MIC of carvacrol did not produce a detectable level of these toxins. These results conclude that *B. cereus* increases its production of toxins in response to treatment with the SIC of carvacrol.

***Caenorhabditis elegans* had a higher mortality rate when exposed to *B. cereus* stressed with the SIC of carvacrol.** Although we found that the SIC of carvacrol caused *B. cereus* to increase its toxin production, the virulence of this stressed bacterium had not been assessed *in vivo*. For this reason, we fed *B. cereus* stressed with the SIC of carvacrol to *Caenorhabditis elegans* in order to determine the effects of increased virulence on the nematode. We observed a 95.63% mortality rate in *C. elegans* given *B. cereus* with the SIC of carvacrol, but only a 59.26% mortality rate in *C. elegans* given *B. cereus* with the MIC of carvacrol ($P < 0.05$) (Jill Bange, unpublished). This study suggests that the SIC of carvacrol increases the virulence of *B. cereus* in our *C. elegans in vivo* model.

Mice infected intraocularly with *B. cereus* stressed with SIC levels of carvacrol have higher systemic levels of pro-inflammatory cytokines IL-6 and TNF-α. Studies have shown that endophthalmitis caused by *B. cereus* often involves degradation to the blood-retinal barrier (4, 50), although the progression of a systemic infection is rare. However,

the systemic effects of *B. cereus* stressed with the SIC of carvacrol had not been analyzed. In this study, pro-inflammatory cytokines were quantified from the spleens of mice infected with *B. cereus* and the SIC of carvacrol to determine if a systemic immune response was present. We found higher concentrations of IL-6 ($P = 0.012$) in mice intraocularly infected with *B. cereus* stressed with the SIC of carvacrol than in mice who received the bacteria alone (Fig. 4, pg. 74). There was also a slight but insignificant increase in TNF-α concentrations in the spleen (Fig. 3, pg. 73). Mice intraocularly infected with *B. cereus* and the SIC of carvacrol had higher levels of anti-*B. cereus* IgG in the spleen than in mice infected with the bacteria alone ($P < 0.001$) (Fig. 5, pg. 75). The results of this study suggest that the virulence of *B. cereus* treated with the SIC of carvacrol induces a systemic response greater than that of the bacteria alone. The proposed study will further investigate this systemic response by looking for additional pro-inflammatory cytokines in mouse spleen tissue, as well as quantifying the cfu of *B. cereus* in the spleen.

***B. cereus* treated with the SIC of carvacrol increases damage to the eye.** *B. cereus* causes extensive ocular tissue damage during infection with endophthalmitis (4, 50). However, the tissue damage caused by *B. cereus* when it is stressed with the SIC of carvacrol had not yet been assessed. We performed histological analysis on infected mouse eyes to determine if *B. cereus* stressed with the SIC of carvacrol caused more damage to ocular tissue than that of the bacteria alone. To quantify the tissue damage, we stained sections of eyes with DAPI and Rose Bengal dyes. DAPI stains the nuclei of healthy cells blue, so fluorescent blue tissue were considered unaffected by the bacterial infection. Rose Bengal dye stains damaged or dead tissue red (48). We found that *B. cereus* treated with the SIC of carvacrol caused more damage to the eye than the bacteria by itself ($P = 0.023$)

(Fig. 6, pg. 76). However, we also saw tissue damage in eyes treated with the SIC of carvacrol alone. Although *B. cereus* treated with SIC carvacrol had more tissue damage than the bacteria alone, we cannot yet determine whether the source of damage was due to increased bacterial virulence or cytotoxicity from SIC carvacrol treatment. The proposed study will investigate whether or not carvacrol is cytotoxic to ocular tissue.

Specific Aim 1: To determine if exposure to the SIC of carvacrol causes *B. cereus* to increase its production of PlcR-regulated toxins *in vivo*.

Rationale for Specific Aim 1

Although it is known that *Bacillus cereus* causes ocular tissue damage using its unique toxins during infection with endophthalmitis (4), it is not known if toxins are actively produced in the microenvironment of the eye. Studies performed by Beecher *et al*. have analyzed the effects of *B. cereus* toxins without the bacteria present in ocular tissue both *in vitro* and *in vivo* to determine the role of specific toxins in virulence (17, 18). These studies concluded that the toxins alone caused ocular damage similar to that seen in tissue infected with *B. cereus*. It is possible that pre-existing *B. cereus* toxins are responsible for the rapid rate of vision deterioration, with permanent damage observed as early as 12 hours after the initial infection (10). However, it is also possible that *B. cereus* immediately begins producing toxins upon entering the eye, causing the rapid damage observed. A study performed by Moyer *et al*. found that after only 8 hours of infection, 86-89% of retinal epithelial (RPE) cells were necrotic (16). In order to gain a better understanding of the intraocular behavior of *B. cereus*, it is important to determine if this bacterium is capable of producing toxins within the eye. Knowing whether or not toxins are actively produced can change the way the patient is treated. For example, if toxins are not produced but rather

introduced along with *B. cereus*, then treatment should focus on first neutralizing these toxins to prevent damage. However, if *B. cereus* is capable of producing toxins in the ocular environment, it would be beneficial to know the time point when toxins are expressed most highly. Knowing when toxin production is at its highest would give doctors a window of time in which they can treat the eye with antibiotics and kill the bacteria before they produce an overwhelming amount of toxins. Most toxins produced by *B. cereus* are under the control of PlcR, a transcriptional regulator unique to this bacterium (51). For this reason, this experiment will assess protein expression levels of PlcR by performing an immunoblot on protein extracted from the vitreous of infected mouse eyes.

Experimental Procedures for Specific Aim 1

Study 1: Does *B. cereus* exposed to the SIC of carvacrol increase its distribution of PlcR in mice infected ocularly?

Methods for Study 1

Bacteria Preparation. *Bacillus cereus* ATTC14579 (VWR, West Chester, PA) will be cultured in Tryptic Soy Broth (TSB) (3089-00, Weber Scientific, Hamilton, NJ) and incubated at 30°C under aeration at 150 rpm. *B. cereus* will also be cultured in the same way with the addition of the 1 mM subinhibitory concentration (SIC) of carvacrol (W224502, SAFC Supply Solutions, St. Louis, MO). *B. cereus* will also be treated with the 2 mM minimum inhibitory concentration (MIC) of carvacrol for comparison. A *plcR*-GFP construct made previously in this lab will be transformed into *B. cereus* prior to ocular infection in mice. The bacteria will be sub-cultured twice and washed with sterile saline before being administered to mice at $5x10^7$ cfu in sterile saline.

Infection with *Bacillus cereus* and the SIC of Carvacrol. All experiments will be performed following approval from the Ball State University Institutional Biosafety Committee (IBC), as well as the Institutional Animal Care and Use Committee (IACUC). BALB/c mice will be randomly assigned to seven different treatment groups for this study. All mice will be approximately 10 weeks of age, and mice will be separated according to gender throughout the experiment. Six treatment groups will have six mice while one positive control group will only require three mice. Mice will be 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 to facilitate bacterial inoculation by eye drops. The seven treatment groups are listed in Table 1. Mice will be infected with the same bacterial or carvacrol concentration in each eye by micropipet. Three of the six mice will be closely monitored throughout the infection period of 24 h, after which they will be sacrificed via CO_2 chamber. The three remaining mice will be monitored over a 14-day infection period and subsequently sacrificed for use in Specific Aim 3.

Table 1. Seven treatment groups of BALB/c mice will be required for this experiment.

Treatment	Number of Mice
Sterile saline negative control	6
1 mM SIC of carvacrol alone	6
2 mM MIC of carvacrol alone	6
$5x10^7$ cfu *B. cereus*	6
$5x10^7$ cfu *B. cereus* with the SIC of carvacrol	6
$5x10^7$ cfu *B. cereus* with the MIC of carvacrol	6
*IP injection of $5x10^7$ cfu *B. cereus*	3

*Only three mice are required for the group receiving an IP injection of *B. cereus*. This group will not be used in this experiment, but rather for experiments described in Specific Aim 3.

Biophotonic Imaging. To detect PlcR-expressing *B. cereus,* three mice from each treatment group will be anesthetized with an intraperitoneal injection of ketamine (50 mg/kg body weight) and xylazine (5 mg/kg body weight) (Henry Schein) prior to imaging. The IVIS® Lumina Series III pre-clinical in vivo imaging platform (PerkinElmer Inc., Waltham, Massachusetts) will be used to image mice every 2 hours for 12 hours, along with a final image after 24 hours. This experiment will analyze the migration of the GFP-PlcR protein expressed by *B. cereus* over the course of a 24h infection. Three images will be taken of each mouse at the specified time intervals. Images will be analyzed using Living Image® In Vivo Imaging software (PerkinElmer Inc.).

Statistical Analysis. Minitab® 17 software (Minitab, State College, PA) will be used to perform a one-way ANOVA on mean fluorescence intensity values obtained at each time interval to observe differences in PlcR protein distribution in response to different treatments throughout the course of infection.

Study 2: Does exposure to the SIC of carvacrol cause *B. cereus* to increase protein expression of PlcR in the eye?

Methods for Study 2

Bacteria Preparation. *B. cereus* will be cultured and treated with carvacrol as described in Study 1.

Infection with *Bacillus cereus* and the SIC of Carvacrol. Mice will be infected in the same was as described in Study 1. The same mice imaged with the biophotonic imager will be sacrificed after 24 hours for PlcR protein analysis. The remaining three mice in each group will be used for Specific Aim 3.

Dot Blot of PlcR Protein Expression. After 24 hours of infection, mouse eyes will be harvested and protein lysates will be made according to methods used by Moyer *et al.* (50). Whole cell lysates from two eyes will be pooled together for each mouse to increase the protein content. Protein concentrations will be measured using the BCA Protein Assay Kit (23227, Thermo Scientific, Rockford, IL). Three different protein concentrations will be blotted onto an Immun-Blot PVDF Membrane (162-0175, Bio-Rad Laboratories Inc., Hercules, California) in addition to a blot containing 100 µg/ml primary antibody by itself according to a protocol provided by R&D Systems (Minneapolis, MN). After drying for one hour at room temperature, the blots will be incubated for one hour with a blocking solution consisting of 5% dry milk (P4739-10PAK, Sigma-Aldrich, St. Louis, MO) and TTBS (50 mM Tris (T1503-25G, Sigma-Aldrich), 0.5 M NaCl (71393-1L, Sigma-Aldrich), Tween-20 (P1379-25ML, Sigma-Aldrich) at a pH of 7.4). After one hour, the membrane will be incubated with the primary antibody, rabbit anti-PlcR (1:3000; generously provided by Pomerantsev *et al.*) (52) in TTBS for another hour at room temperature. After this incubation, the membrane will be washed in TTBS three times for 10 minutes before incubating with the secondary antibody, goat anti-mouse IgG DyLight 680 conjugate (1:5,000-20,000; 35518, Life Technologies, Carlsbad, CA) for one hour in TTBS. After washing the membrane three times for 10 minutes in TTBS, the Odyssey CLx scanner (9140-01, LI-COR, Lincoln, NE) will be used for imaging, and images will be analyzed using Image Studio software.

Positive Expected Outcomes

It is expected that mice infected ocularly with *B. cereus* stressed with the SIC of carvacrol will have an increased distribution of the GFP-PlcR protein throughout the eye

compared to mice infected with the bacteria alone. It is also expected that *B. cereus* stressed with the SIC of carvacrol will have higher PlcR protein expression levels over time compared to eyes infected with *B. cereus* alone and eyes treated with carvacrol alone. We also hypothesize that *B. cereus* treated with the MIC of carvacrol will have lower levels of PlcR protein expression when compared to the bacteria alone. Fluorescence observed by the IVIS® Lumina Series III will signify the distribution of the PlcR-GFP protein throughout the eye. The intensity of fluorescence will be quantified by Living Image® software.

It is also expected that the PlcR protein will be more highly expressed in eyes infected with *B. cereus* stressed with the SIC of carvacrol when compared to eyes infected with the bacteria alone. We hypothesize that eyes treated with *B. cereus* and the MIC of carvacrol will have lower levels of PlcR protein expression than that of *B. cereus* alone. The dot blot experiment will be considered a success if dots are present only in treatment groups receiving *B. cereus* after imaging due to successful binding of the primary antibody. This outcome will determine that PlcR is being expressed after *B. cereus* infection of the eye. Because PlcR is being expressed, it can be suggested that the toxins it activates are also being expressed. Future experiments will address the expression of specific toxins regulated by PlcR.

Contingency Plans for Specific Aim 1

Biophotonic imaging would be the most desirable method for assessing GFP-PlcR protein distribution because it allows one mouse to be imaged multiple times to see how PlcR protein distribution changes during the course of infection without having to kill mice at each measuring time point. However, it is possible that this technology may not quantify the PlcR-GFP protein above background fluorescence, or fluorescence could not be

detected at all. GFP has an emission spectrum within the range of the IVIS® Lumina Series III according to PerkinElmer Inc., but it is possible that construct could be damaged as *B. cereus* enters the eye and is met with the host immune response. For this reason, it is important to have a different way of quantifying PlcR protein expression. Although an immunoblot would not quantify the distribution of the PlcR protein throughout the eye, the protein content could still be effectively quantified. This method is implemented in Study 2.

Although a dot blot would be an efficient way to compare PlcR protein concentrations within different treatment groups of eyes, this test does not provide information regarding the molecular weights of the proteins because the different proteins are not separated out by electrophoresis prior to blotting (53). The dot blot is beneficial for this study to compare differences in PlcR antibody binding and therefore protein expression levels among different treatment groups. However, because we will be administering the whole cell lysates directly to the membrane without first separating the different proteins, it is possible non-specific binding may occur and result in false-positive results. If a dot appears in the location that received the primary antibody by itself, then non-specific binding would be a problem. Another possible problem would be that the antibody dilution is not the best option for the whole cell lysates and would respond better to the protein once it is separated out. An example of this situation would be if no dots were present after treatment with the proper antibodies. If non-specific binding or problems with the primary antibody occur, an alternative way to perform this experiment is to run a Western blot. This would require an additional step in which proteins from the whole cell lysate are separated according to molecular weight on an Optiblot SDS Gel 10% (ab119202, Abcam, Cambridge, MA) and then transferred over to the membrane. The

subsequent steps would remain the same as those performed during the dot blot. This separation of proteins may make it easier for the primary antibody to bind with more specificity.

Another problem that may occur in this experiment is if PlcR expression is not observed in any of the treatment groups in a dot blot or a Western blot. If this situation occurs, the first plan of action would be to buy all new antibodies and make sure they are optimized for use in the Western blot. However, if changing the antibodies does not resolve the issue, then it is possible that the protein used in the Western blot is too degraded to be recognized by the antibody. In this situation, the experiment would have to be repeated with fresh whole cell lysates, and a stronger protease inhibitor may be added to protect the protein from degrading. If all of these optimizations fail, these results would conclude that PlcR is not expressed by *B. cereus* in the ocular environment, particularly if these data are consistent with the mRNA work.

Specific Aim 2: To determine if *B. cereus* stressed with the SIC of carvacrol causes more damage to the blood-retinal barrier than the bacteria or carvacrol alone.

Rationale for Specific Aim 2

B. cereus toxins play an important role in the virulence of endophthalmitis and breakdown of the blood-retinal barrier (16, 18). Although carvacrol may serve as an alternative treatment for endophthalmitis due to its antimicrobial and anti-inflammatory qualities (39), studies in this lab have shown that *B. cereus* increases its production of Hbl and Nhe toxins in response to the SIC of carvacrol *in vitro*. We have also found that mice infected in the eyes with *B. cereus* and the SIC of carvacrol have higher levels of systemic pro-inflammatory cytokines and anti-*B. cereus* IgG levels *in vivo*. Taken together, these

results suggest that the SIC of carvacrol increases the virulence of *B. cereus* during endophthalmitis. However, histological analysis showed that the SIC of carvacrol was able to cause damage to the eye by itself. This observation suggests that the SIC of carvacrol is irritating to the eye and may be causing ocular damage that is not due to an increase in *B. cereus* virulence. It is possible that carvacrol is cytotoxic to retinal cells, which are known to be very sensitive to microbes, antibiotics, and even their own host inflammatory response (4). However, the eye's reaction to carvacrol is not currently known, and therefore the source of damage observed in *B. cereus* stressed with the SIC of carvacrol cannot yet be confirmed. For this reason, this study will investigate the effects of carvacrol on the blood-retinal barrier, specifically RPE cells. We will quantify tight junction protein expression in RPE cells treated with *B. cereus* and the SIC of carvacrol, as well as the SIC of carvacrol alone. Because the MIC of carvacrol is higher than that of SIC and possibly more damaging to retinal tissue, we will also assess the difference in tissue damage between these groups. This study is important because it will investigate whether the observed ocular damage is due to the cytotoxicity of carvacrol by itself or the increased virulence of *B. cereus* due to stress caused by the SIC of carvacrol treatment.

Experimental Procedures for Specific Aim 2

Study 1: Do RPE cells treated with *B. cereus* and the SIC or MIC of carvacrol have lower levels of tight junction protein expression when compared to cells treated with *B. cereus* or carvacrol alone?

Methods for Study 1

Bacteria Preparation. *B. cereus* will be cultured and treated with carvacrol as described in Study 1 of Specific Aim 1 without the addition of the *plcR*-GFP construct.

Cell Culture Methods. Human RPE cells will be examined *in vitro* using the ARPE-19 cell line (CRL-2302™, ATTC®, Manassas, VA) and treated according to procedures published by Moyer *et al.* (16). Cells will be grown in 100x20 mm cell culture dishes (83.3902, Sarstedt Inc., Newton, NC) using Dulbecco modified Eagle medium (DMEM)/F12 (11320-033, Life Technologies) along with 10% fetal bovine serum (FBS), certified One Shot™ (16000-077, Life Technologies). According to Life Technologies, this brand of FBS has the lowest endotoxin content and is the ideal choice for sensitive cells, such as RPE cells. Using this brand of FBS will help eliminate variables that could possibly cause damage to RPE cells. This growth medium will also be supplemented with 1% GlutaMAX™ (35050-061, Life Technologies). After growing to 100% confluence, ARPE-19 cells will be seeded onto a 24-well tissue culture plate (83.3922, Sarstedt) 48 hours before being used for cytotoxicity experiments.

RPE Cell Polarization. To mimic the polarized environment of the blood-retinal barrier required for immunocytochemistry experiments, ARPE-19 cells will be seeded in 6.5mm Transwell® inserts with a 0.4 µm membrane pore size within a 24-well tissue culture plate (3470, Corning, Tewksbury, MA). A protocol written by Sonoda *et al.* will be followed during cell polarization methods (54) with modifications taken from methods from Moyer *et al.* (16). Transwells will be coated with a solution containing DMEM/F-12 with 5% FBS, 1% GlutaMAX™, 102 µg/ml bovine serum albumin (BSA) (BP675-1, Thermo Fisher Scientific Inc., Waltham, MA), 30 µg/ml bovine type I collagen (OB1200-02, Thermo Fisher Scientific Inc.), and 10 µg/ml human fibronectin (CB-40008, Thermo Fisher Scientific Inc.). These Transwells will be allowed to dry overnight under a laminar flow hood. After coating, Transwells will be washed twice with 1X phosphate-buffered saline (PBS) (10010023, Life

Technologies). ARPE-19 cells will then be seeded on Transwells at $2x10^5$ cells/ml in growth medium containing DMEM/F-12 with 5% FBS and 1% GlutaMAX™ according to the manufacturer's instructions.

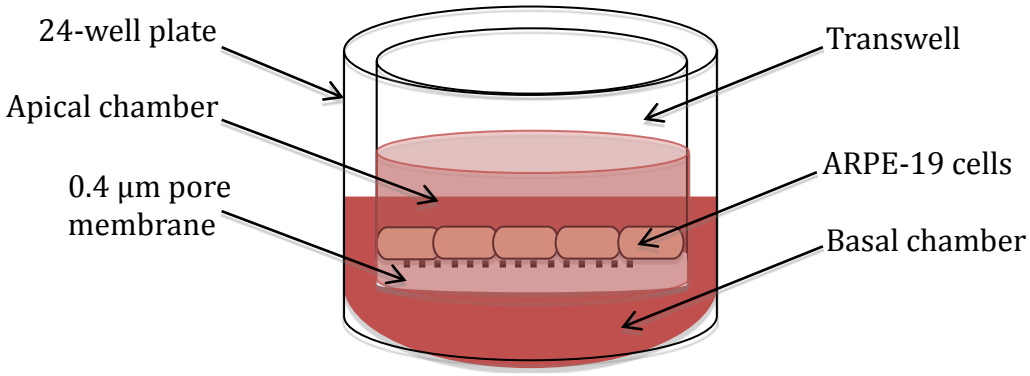

Figure 7. A schematic illustrating the seeding of ARPE-19 cells on a Transwell in the 24-well plate to facilitate polarization similar to that observed in the blood-retinal barrier *in vivo*.

After growing for approximately 48 to 72 hours, medium will be aspirated from the apical chamber (Fig. 7) to allow cells to induce polarization. However, the basal chamber (Fig. 7) will continue to receive growth medium. ARPE-19 cells will be left undisturbed for 15 days before infection to allow polarization to occur. Successful polarization will be confirmed using immunocytochemistry.

Infection with *B. cereus* and the SIC or MIC of Carvacrol. ARPE-19 cells will be infected with *B. cereus* by diluting the bacterium in DMEM/10% FBS/1% GlutaMAX™ and adding this infected medium to three wells of cells at 10^5 cfu/ml in each well. The same method will be used for wells receiving *B. cereus* treated with either the SIC or MIC of carvacrol. A negative control group will consist of sterile saline and growth medium. Additional wells will receive the appropriate concentrations of carvacrol (1 mM or 2 mM) in addition to growth medium. Each treatment or control group will include three replicate wells, and all

wells will receive 1 ml total volume. The infection period will last 4 hours. This infection period was set based on results obtained by Moyer *et al.*, which observed that ZO-1 protein signals were below the detectable limit after 8 hours of infection, and occludin protein signals were almost undetectable after 4 hours (16). However, the infection period may be decreased if tight junction protein degradation is hindering image analysis after 4 hours. ZO-1 and occludin proteins will be used as markers for the formation of tight junctions within RPE cells of the blood-retinal barrier.

Immunocytochemistry. Polarized ARPE-19 cells will be fixed in ice-cold 2% paraformaldehyde (28908, Thermo Scientific) and permeabilized in Triton X-100 (85111, Life Technologies) for 30 minutes at room temperature. The Transwells will then be washed twice with 1X PBS/0.1% Tween 20. ARPE-19 cells will then be blocked using a serum-free protein block (X0909, Dako North America Inc., Carpinteria, CA) for one hour at room temperature. After blocking non-specific binding, cells will be washed with 1X PBS/0.1% Tween 20 three times for five minutes each. ARPE-19 cells will be treated with the same primary antibodies used by Moyer *et al.*, including mouse anti-human ZO-1 Alexa Fluor® 488 conjugate (1:100; 339188, Life Technologies), mouse anti-human occludin Alexa Fluor® 488 conjugate (1:200; 331588, Life Technologies), and mouse anti-human Na^+/K^+ ATPase Alexa Fluor® 488 conjugate (1:100; 16-243, EMD Millipore, Darmstadt, Germany) (16). All antibodies will be diluted in 0.05 M Tris-HCl (15506-017, Life Technologies), 1% BSA, and 50 mM NaN_3 (S2002-5G, Sigma-Aldrich). ARPE-19 cells will be incubated with primary antibodies for 30 minutes at room temperature. After washing with PBS/0.1% Tween 20 three times, ARPE-19 cells will be incubated with the secondary antibody, goat anti-mouse IgG Alexa Fluor® 488 conjugate (1:200; A-11001, Life

Technologies) for 30 minutes in the dark at room temperature. After washing the cells three more times in PBS/0.1% Tween 20, the 0.4 μm pore membranes will be removed from the Transwells using a sterile razor. These membranes will be placed onto glass microscope slides (10144633B, Thermo Scientific) with the ARPE-19 cells facing up. Slides will be mounted using ProLong® Gold Antifade Mountant with DAPI (P-36931, Life Technologies) and coverslips (12-545-88, Thermo Fisher Scientific). The slides will be imaged using a fluorescence microscope with the 488 nm filter. Five images from five different fields of view will be acquired for each well, and mean intensity values will be quantified using ImagePro Express 6.0 software. Data from each treatment group will be normalized against background fluorescence by quantifying intensity levels from images of RPE cells that did not receive treatment with antibodies.

Statistical Analysis. Minitab® 17 software will be used to perform a one-way ANOVA on mean fluorescence intensity values to quantify changes in tight junction protein signals after infection with *B. cereus* and the SIC of carvacrol.

Study 2: Is carvacrol cytotoxic to RPE cells?

Methods for Study 2

Bacteria Preparation. *B. cereus* will be cultured and treated with carvacrol as described in Study 1 of Specific Aim 1 without the addition of the *plcR*-GFP construct.

Cell Culture Methods. ARPE-19 cells will be cultured using the same techniques described in Study 1.

RPE Cell Polarization. Cell polarization will be induced using the same methods described in Study 1.

Carvacrol Treatment. ARPE-19 cells will be treated with either the SIC or MIC of carvacrol to determine if this treatment is cytotoxic to retinal tissue. Carvacrol will be diluted to the appropriate concentration (SIC or MIC) in growth medium containing DMEM/10% FBS/1% GlutaMAX™ and administered to three wells of cells. Three wells will also receive growth media alone as a comparative control. Treatment with carvacrol will last 4 hours.

Lactate Dehydrogenase (LDH) Assay. Supernatants will be harvested from each well of ARPE-cells after 4 hours of exposure to carvacrol or media alone and transferred to a 96-well plate (36985, Qiagen, Venlo, Limburg). The CytoTox-ONE™ Homogenous Membrane Integrity Assay (G7890, Promega, Madison, WI) will be used to test APRE-19 cell supernatants for the presence of LDH in response to cell membrane damage according to the manufacturer's instructions. LDH will be quantified based on the intensity of the fluorescence signal read by a fluorometer at 560 nm for excitation and 590 nm for emission. The treatment groups are listed in Table 3 below.

Table 3. The following treatment and control groups will be used for the CytoTox-ONE™ Assay to measure fluorescence signals due to LDH release. Triton X-100 will be used as a positive control in which LDH release and cytotoxicity are expected to occur.

LDH Assay Treatment Groups
Media alone
ARPE-19 cells, media alone
No cells, SIC of carvacrol
ARPE-19 cells, SIC of carvacrol
No cells, MIC of carvacrol
ARPE-19 cells, MIC of carvacrol
ARPE-19 cells, Triton X-100 (positive control)

Statistical Analysis. Fluorescence values will first be normalized against the values obtained from the wells without cells or carvacrol. Minitab® 17 software will be used to perform a Student's T test on normalized mean fluorescence values of untreated ARPE-19

cells and ARPE-19 cells treated with the SIC of carvacrol. This test will determine whether or not carvacrol is cytotoxic to RPE cells. A one-way ANOVA will be performed on normalized mean fluorescence values of untreated ARPE-19 cells, cells treated with the SIC of carvacrol, and cells treated with the MIC of carvacrol. This statistical test will determine if increased levels of carvacrol increases cytotoxicity to retinal cells.

Positive Expected Outcomes

It is expected that ARPE-19 cells treated with *B. cereus* and the SIC of carvacrol will have lower tight junction protein expression levels and higher levels of LDH release when compared to cells infected with *B. cereus* alone. We also expect to see some damage to RPE cells due to carvacrol alone based on results obtained from previous experiments. If carvacrol proves to be cytotoxic to RPE cells, we will expect to see lower tight junction protein expression levels and higher LDH release in cells treated with MIC of carvacrol than that of the SIC of carvacrol.

If ARPE-19 cell monolayers are grown successfully, we will expect to see hexagonal RPE cells surrounded by fluorescent tight junction proteins that link these cells together during immunocytochemistry imaging. This will be determined by a +/- scoring system in which 200 cells are assessed on each slide for the presence of an intact hexagonal shape. For example, cells with an irregular or circular shape will receive a – score, as well as cells with an interrupted hexagonal shape. Only intact hexagonal cells will be given a + score.

If RPE cell polarization is successful, we will expect to observe Na^+/K^+ ATPase protein expression as well (16). Expression of Na^+/K^+ ATPase would show that tight junction proteins are not only present, but functional, because an ion gradient was established between the basal chamber of the well and the apical chamber of the Transwell.

We will also expect to see blue fluorescent nuclei within the RPE cells if staining with DAPI was successful. Growing polarized ARPE-19 cells successfully is important in order to mimic the environment of the blood-retinal barrier *in vitro.*

Although we are unsure of the expected outcomes for carvacrol cytotoxicity, the LDH assay will be considered successful if the negative and positive controls behave as expected. We expect RPE cells receiving no treatment to release very low levels of LDH, and we expect the cells treated with Triton X-100 to have very high levels of LDH release. Based on previous studies in this lab, we expect RPE cells treated with the SIC of carvacrol to release LDH, but we are not sure how much will be released. We also expect RPE cells treated with the higher MIC of carvacrol to release more LDH than cells treated with the SIC.

Contingency Plans for Specific Aim 2

Although cell polarization is a unique quality of the blood-retinal barrier, it is a difficult environment to mimic *in vitro*. Although Moyer *et al.* found that RPE cell polarization was successful after just 15 days by measuring Na^+/K^+ ATPase expression (16), a study by Sonoda *et al.* determined that Transwells require at least one month of culturing before Na^+/K^+ ATPase was expressed (54). The results of these two studies suggest that the amount of culturing time required for Na^+/K^+ ATPase expression and cell polarization varies between experiments. If our experiment does not detect Na^+/K^+ ATPase expression, we will treat RPE cells with an antibody against RPE65. Polarized RPE cells express high levels of the RPE65 protein within a few weeks of culturing.

While immunocytochemistry will reveal tight junction protein structural changes caused by treatment with *B. cereus* and carvacrol, it may be difficult to quantify changes in

protein expression, especially for individual tight junction proteins. It is also possible that we could be detecting tight junction protein expression for cells that are dead. Another method we could use to quantify expression of individual tight junction proteins is by performing a dot blot using the same techniques discussed in Specific Aim 1. RPE cell lysates would be blotted on a membrane, and the same antibodies would be used from immunocytochemistry with the addition of mouse anti-human Glyceraldehyde 3 phosphate dehydrogenase (GAPDH) with a biotin conjugate (MA5-15738-BTIN, Life Technologies). GAPDH is a protein expressed only in living cells (16). By normalizing tight junction protein expression levels to GAPDH, we could ensure that we are only quantifying changes in protein expression due to tight junction disruption and increased permeability of the blood-retinal barrier *in vitro* rather than overall cytotoxicity. However, if the antibodies have trouble binding to the whole cell lysates on the dot blot, a Western blot could also be performed. The only additional step required for a Western blot is the separation of proteins from the whole cell lysates according to molecular weight by electrophoresis. According to Moyer *et al.* and manufacturers' websites, we would expect to a band for Na^+/K^+ ATPase at approximately 111 kDa, a band for ZO-1 at approximate 225 kDa, and a band for occludin at approximately 65 kDa. We would also expect to see a band for GAPDH at approximately 35 kDa according to the manufacturer's website. These alternative methods to immunocytochemistry would quantify changes in tight junction protein expression in response to different treatments with *B. cereus* and carvacrol.

Although the LDH assay is commonly used for measuring cytotoxicity, it is possible that the experiment could not function as expected. For example, it is possible that may see higher levels of LDH expression than expected in ARPE-cells without treatment. One

possible reason for this could be non-specific binding of the LDH reagent to metabolites released by the cells. In this situation, we would not be able to determine if LDH release was higher due to treatment with carvacrol, or extracellular components of the whole cell lysates. For this reason, it is important to have additional methods to assess cytotoxicity. One possible method would be making an ARPE-19 cell suspension, staining with trypan blue, and counting viable cells that did not stain blue using a hemocytometer. Flow cytometry could also be used to quantify necrotic cells compared to apoptotic cells. Flow cytometry would be able assess the status of untreated ARPE-19 cells and determine if LDH was observed due to cytotoxicity or non-specific binding of the LDH reagent. The trypan blue exclusion assay and flow cytometry are alternative methods to the LDH assay that could be used to determine if carvacrol is cytotoxic to RPE cells.

Specific Aim 3: To determine if *B. cereus* treated with the SIC of carvacrol escapes the eye and progresses systemically more readily than the bacteria alone.

Rationale for Specific Aim 3

Although it is a rare occurrence for *B. cereus* endophthalmitis to progress into a systemic infection (4), increased virulence coupled with the weakening of the blood-retinal barrier may provide a potential way for the bacteria to escape the eye and enter the bloodstream. A systemic response was observed in a 1987 clinical study that reported a fever and high white blood cell count after infection with posttraumatic endophthalmitis caused by *B. cereus* (14). Although studies have illustrated possible mechanisms for *B. cereus* to escape the eye (16, 17, 50), little is known about whether or not *B. cereus* actually uses these methods and progresses from the eye into the bloodstream. Endogenous endophthalmitis occurs when a systemic bacterial infection invades the eye (10). *B. cereus*

is often the causative bacterium for endogenous endophthalmitis in intravenous drug users. Because *B. cereus* is capable of crossing the blood-retinal barrier and entering the eye during endogenous endophthalmitis, we hypothesize that this bacterium is also capable of progressing from the eye into the bloodstream after enough damage is caused by its toxins and the host inflammatory response. Although carvacrol may be a possible alternate treatment for *B. cereus* endophthalmitis due to its antimicrobial and anti-inflammatory qualities (39), previous studies performed in this lab have shown that the SIC of carvacrol increases the virulence of *B. cereus in vitro.* We have also demonstrated that mice infected ocularly with *B. cereus* and the SIC of carvacrol have higher levels of TNF-α and IL-6 in the spleen than mice infected with bacteria alone. For this reason, this study will quantify additional pro-inflammatory cytokines in mouse spleen tissue, such as IL-1β and IFN-γ, after ocular infection with *B. cereus* and the SIC of carvacrol. We will also directly quantify *B. cereus* in the spleen tissue to determine if the bacteria were able to progress from the initial infection site to other parts of the body. The goal of this study is to determine if the increased virulence observed in *B. cereus* stressed with the SIC of carvacrol causes the bacteria to leave the eye and progress systemically.

Experimental Procedures for Specific Aim 3

Study 1: Does an ocular infection with *B. cereus* and the SIC of carvacrol result in increased levels of pro-inflammatory cytokines IL-1β and IFN-γ compared to mice infected with the bacteria alone?

Methods for Study 1

Bacteria Preparation. *B. cereus* will be cultured and treated with carvacrol as described in Study 1 of Specific Aim 1 without the addition of the *plcR*-GFP construct.

Infection with *Bacillus cereus* and the SIC of Carvacrol. Mice will be infected in the same was as described in Study 1. All mice from each treatment group will be monitored for 14 days after initial infection to allow time for an immune response to develop.

Spleen Tissue Preparation. After a 14 day immune challenge, mice will be sacrificed *via* CO^2 chamber, and spleens will immediately be harvested from each mouse and stored in ice-cold lysis buffer containing 150 mM NaCl (0241, Amresco, Solon, OH), 50 mM Tris pH 7.4, and 1 mM EDTA (0105, Amresco). The spleen tissue will be homogenized and centrifuged at 2K x G for three minutes at 4°C. After centrifugation, the supernatant will be collected and stored at -20°C for future use in ELISA experiments.

ELISA-based Detection of IL-1β and IFN-γ Pro-inflammatory Cytokines. Spleen lysates will be transferred to pre-coated 96-well plates provided by ELISA kits and treated with either the IL-1β Mouse ELISA Kit (KMC0011, Life Technologies) or the IFN-γ Mouse ELISA Kit (KMC4021, Life Technologies) according to the manufacturer's instructions. IL-1β and IFN-γ standards are also provided by the ELISA kits for use in constructing a standard curve and determining protein concentrations. After processing the plates, absorbance values will be measured by the Bio-Rad Model 680 Microplate Reader at 450 nm. Absorbance values will be normalized to the mean absorbance value of wells that did not receive spleen lysates to account for background absorbance.

Statistical Analysis. Minitab® 17 software will be used to perform a one-way ANOVA on mean protein concentrations calculated for each treatment group.

Study 2: Does *B. cereus* treated with the SIC of carvacrol escape the eye more readily than the bacteria alone?

Bacteria Preparation. *B. cereus* will be cultured and treated with carvacrol as described in Study 1 of Specific Aim 1 without the addition of the *plcR*-GFP construct.

Infection with *Bacillus cereus* and the SIC of Carvacrol. Mice will be infected in the same was as described in Study 1. Three mice from each treatment group will be monitored for 14 days after initial infection to allow time for an immune response to develop.

Spleen Tissue Preparation. After a 14-day infection period, mice will be sacrificed by a CO^2 chamber for use in RT-PCR. Spleen tissue will be harvested using the same methods as Study 1, except spleen lysates will be homogenized in Trizol® Reagent (15596-026, Life Technologies) instead of the lysis buffer described previously.

RNA Isolation. The Trizol method will be used for the RNA extraction. Spleen supernatants will be centrifuged at 4°C at 11,000 x G for three minutes. The supernatant will be discarded, and the resulting pellet will be resuspended in Trizol® Reagent on ice. After allowing this solution to incubate on ice for 15 minutes, 0.2 ml of chloroform (C7559, Sigma-Aldrich) will be added to the tube for every 1 ml of Trizol® Reagent. This solution will be mixed thoroughly by inversion and will incubate on ice for 2-3 minutes. After this incubation step, the solution will be centrifuged at 4°C at 12,000 x G for 15 minutes. Because RNA is found in the resulting top aqueous phase after centrifugation, only this top layer will be transferred to a fresh microcentrifuge tube. RNA will be precipitated by adding cold isopropanol (W292907, Sigma-Aldrich) and will incubate for 10 minutes on ice. After this incubation step, the solution will be centrifuged at 12,000 x G for 20 minutes at 4°C. The supernatant will be discarded, and the resulting pellet will be allowed to dry completely. A DNase digestion will be performed on the RNA pellet for 45 minutes at 37°C to eliminate contaminating DNA. To prevent DNase I from degrading cDNA synthesized

during PCR, the RNA sample will be incubated at 65°C for 2 minutes to inactivate the enzyme. A_{260}/A_{280} ratios will be generated by a spectrophotometer to determine RNA content and quality, and RNA samples will be equated to 0.5 µg for use in PCR.

RT-PCR. The MasterAmp™ RT-PCR Kit for High Sensitivity (RT712100, Ecogen, Barcelona, Spain) will be used for cDNA synthesis and subsequent PCR reactions. A negative control group will not receive any RNA, but will receive all reagents. This negative control group will detect if PCR reagents are contaminated. A Reverse Transcriptase (RT) control group will receive template RNA, primers, and DNA polymerase alone instead of the RT enzyme. The RT control group will determine if amplification is due to cDNA alone, or contaminating gDNA, because any amplfication observed in this group has to be due to gDNA. An internal control group will receive all reagents and template RNA, but primers will be designed for *gyrB*, a gene that is constitutively expressed in *B. cereus* (55). Treatment groups will receive all reagents and primers designed for *plcR* and a positive control group will receive the *plcR* primers along with RNA extracted from cultured *B. cereus* to ensure that the primers are fully functional. Primer sequences are listed in Table 4, and PCR cycling parameters are listed in Table 5. RNA will also be serially diluted (1:2) from a starting concentration of 10 µg/µl to create standard curves for each primer set and quantify mRNA expression using the resulting standard curve equations.

Table 4. Forward and reverse primers designed for *plcR* for use in RT-PCR (Integrated DNA Technologies, Grand Island, NY)

	Primer Sequence	**Reference #**
plcR Forward	5’ ACTAGGATCCATGCAAGCAGAGAAATTAG 3’	91897266
plcR Reverse	5’ ACTAAGGTCCTTATCTGCTGATTTTATTTAC 3’	91897267
gyrB Forward	5’ GCGTTAGAAGTTTCAAGTTTAC 3’	AF090330
gyrB Reverse	5’ CCGATTGCCGTAATAATTGTAC 3’	AF090330

Table 5. PCR cycling parameters will include 40 cycles of Denaturation, Annealing, and Extension. cDNA will first by synthesized for 30 minutes, followed by inactivation of the RT enzyme.

RT-PCR Cycling Parameters

	Temperature	Time	Cycles
cDNA Synthesis	42°C	30 min	
RT Inactivation	94°C	30 s	
Denaturation	94°C	30 s	40
Annealing	52°C	30 s	40
Extension*	72°C	40 s	40

*A Melt Curve reading was taken at the end of each Extension phase.

Statistical Analysis. Minitab® 17 software will be used to perform a one-way ANOVA on mean C_T values and mean mRNA concentrations obtained for each treatment group to compare the amount of viable *B. cereus* cells in mouse spleen tissue after ocular infection.

Positive Expected Outcomes

Based on previous experiments in this lab with TNF-α and IL-6 pro-inflammatory cytokines, we expect to see significantly higher levels of IL-1β and IFN-γ in the spleens of mice infected in the eye with *B. cereus* + the SIC of carvacrol compared to those observed for mice infected with the bacteria alone. Because both IL-1β and IFN-γ are involved in the systemic inflammatory response (29, 34), it is expected that mice undergoing a systemic infection will have significantly higher levels of these two cytokines in the spleen. This result would conclude that the SIC of carvacrol increased *B. cereus* virulence and resulted in a more prominent inflammatory response by the host. The presence of systemic pro-inflammatory cytokines would also suggest that *B. cereus* was able to migrate from the eye into the bloodstream.

Although previous experiments quantified anti-*B. cereus* IgG levels in the spleen tissue, the current study will directly quantify the *B. cereus* antigens found in the spleen. Because we observed higher levels of anti-*B. cereus* IgG in mice infected with *B. cereus* and

the SIC of carvacrol, we expect to also see higher levels of *plcR* mRNA expression in mice infected in the eye with *B. cereus* and the SIC of carvacrol in the current study.

Contingency Plans for Specific Aim 3

Alternative methods can be used to quantify the presence of pro-inflammatory cytokines as well as the antigen itself in mouse spleen tissue if the methods we have chosen become problematic. One example of a challenge we could face is unequal binding of antibodies to their corresponding cytokines within the spleen lysates. ELISAs are dependent on the ability of the primary antibody to successfully bind to the antigen. Although this method is considered to be very sensitive, ELISAs are often cross-reactive, meaning the secondary antibody can sometimes interact with components of the antigen rather than just the primary antibody. ELISAs are also limited by their need for the antigen or antibody to bind well to the surface of a 96-well plate. If problems arise during ELISA analysis, one alternate method would the Western blot. This method would detect IL-1β and IFN-γ protein expression using antibodies against the two cytokines after separating the proteins according to molecular weight. If the Western blot were unable to quantify IL-1β and IFN-γ proteins in the spleen tissue, another option would be to analyze a different systemic source of pro-inflammatory cytokines. We could perform the same experiments on mouse blood serum to quantify systemic IL-1β and IFN-γ pro-inflammatory cytokines.

An alternative method to RT-PCR for quantifying *B. cereus* directly in the spleen would be homogenizing the spleen tissue and plating Tryptic Soy Agar (TSA) plates with spleen lysates at different dilutions. After culturing the plates, the bacteria could be quantified and compared between different treatment groups. Although this method is less sensitive than PCR, it is also less complicated and inexpensive. Culturing the bacteria from

spleen tissue would be another way to quantify *B. cereus* in the spleen and determine if the bacteria were able to migrate from the eye into the bloodstream.

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V. References

1. **Bottone EJ.** 2010. Bacillus cereus, a volatile human pathogen. Clin Microbiol Rev **23:**382-398.
2. **Drobniewski FA.** 1993. Bacillus cereus and related species. Clin Microbiol Rev **6:**324-338.
3. **Arnesen LPS, Fagerlund A, Granum PE.** 2008. From soil to gut: Bacillus cereus and its food poisoning toxins. Fems Microbiology Reviews **32:**579-606.
4. **Callegan MC, Engelbert M, Parke DW, Jett BD, Gilmore MS.** 2002. Bacterial endophthalmitis: Epidemiology, therapeutics, and bacterium-host interactions. Clinical Microbiology Reviews **15:**111.
5. **Ngow HA, Wan Khairina WM.** 2013. Bacillus cereus endocarditis in native aortic valve. J Infect Chemother **19:**154-157.
6. **Gaur AH, Patrick CC, McCullers JA, Flynn PM, Pearson TA, Razzouk BI, Thompson SJ, Shenep JL.** 2001. Bacillus cereus bacteremia and meningitis in immunocompromised children. Clin Infect Dis **32:**1456-1462.
7. **Kotiranta A, Lounatmaa K, Haapasalo M.** 2000. Epidemiology and pathogenesis of Bacillus cereus infections. Microbes Infect **2:**189-198.
8. **Sasahara T, Hayashi S, Morisawa Y, Sakihama T, Yoshimura A, Hirai Y.** 2011. Bacillus cereus bacteremia outbreak due to contaminated hospital linens. Eur J Clin Microbiol Infect Dis **30:**219-226.

9. **Gohar M, Okstad OA, Gilois N, Sanchis V, Kolsto AB, Lereclus D.** 2002. Two-dimensional electrophoresis analysis of the extracellular proteome of Bacillus cereus reveals the importance of the PlcR regulon. Proteomics **2:**784-791.
10. **Durand ML.** 2013. Endophthalmitis. Clin Microbiol Infect **19:**227-234.
11. **Seal D, Reischl U, Behr A, Ferrer C, Alio J, Koerner RJ, Barry P, Group EES.** 2008. Laboratory diagnosis of endophthalmitis: comparison of microbiology and molecular methods in the European Society of Cataract & Refractive Surgeons multicenter study and susceptibility testing. J Cataract Refract Surg **34:**1439-1450.
12. **Callegan MC, Cochran DC, Kane ST, Ramadan RT, Chodosh J, McLean C, Stroman DW.** 2006. Virulence factor profiles and antimicrobial susceptibilities of ocular bacillus isolates. Curr Eye Res **31:**693-702.
13. **Miller JJ, Scott IU, Flynn HW, Jr., Smiddy WE, Murray TG, Berrocal A, Miller D.** 2008. Endophthalmitis caused by Bacillus species. Am J Ophthalmol **145:**883-888.
14. **Davey RT, Jr., Tauber WB.** 1987. Posttraumatic endophthalmitis: the emerging role of Bacillus cereus infection. Rev Infect Dis **9:**110-123.
15. **Vanbijsterveld OP, Richards RD.** 1965. Bacillus Infections of the Cornea. Arch Ophthalmol **74:**91-95.
16. **Moyer AL, Ramadan RT, Thurman J, Burroughs A, Callegan MC.** 2008. Bacillus cereus induces permeability of an in vitro blood-retina barrier. Infect Immun **76:**1358-1367.
17. **Beecher DJ, Olsen TW, Somers EB, Wong AC.** 2000. Evidence for contribution of tripartite hemolysin BL, phosphatidylcholine-preferring phospholipase C, and collagenase to virulence of Bacillus cereus endophthalmitis. Infect Immun **68:**5269-5276.
18. **Beecher DJ, Pulido JS, Barney NP, Wong ACL.** 1995. Extracellular Virulence Factors in Bacillus-Cereus Endophthalmitis - Methods and Implication of Involvement of Hemolysin Bl. Infection and Immunity **63:**632-639.
19. **Chan FK, Moriwaki K, De Rosa MJ.** 2013. Detection of necrosis by release of lactate dehydrogenase activity. Methods Mol Biol **979:**65-70.
20. **Ferencz JR, Assia EI, Diamantstein L, Rubinstein E.** 1999. Vancomycin concentration in the vitreous after intravenous and intravitreal administration for postoperative endophthalmitis. Arch Ophthalmol **117:**1023-1027.
21. **Wiskur BJ, Robinson ML, Farrand AJ, Novosad BD, Callegan MC.** 2008. Toward improving therapeutic regimens for Bacillus endophthalmitis. Invest Ophthalmol Vis Sci **49:**1480-1487.
22. **Cunha-Vaz J.** 2010. Blood-Retinal Barrier, p 293-299. *In* Dartt D (ed), Encyclopedia of the Eye, Four-Volume Set, ed 1, vol 1. Elsevier Ltd.,
23. **Vinores SA.** 2010. Breakdown of the Blood-Retinal Barrier, p 300-306. *In* Dartt D (ed), Encyclopedia of the Eye, Four-Volume Set, ed 1, vol 1. Elsevier Ltd.,
24. **Simo R, Villarroel M, Corraliza L, Hernandez C, Garcia-Ramirez M.** 2010. The retinal pigment epithelium: something more than a constituent of the blood-retinal barrier--implications for the pathogenesis of diabetic retinopathy. J Biomed Biotechnol **2010:**190724.
25. **Erickson KK, Sundstrom JM, Antonetti DA.** 2007. Vascular permeability in ocular disease and the role of tight junctions. Angiogenesis **10:**103-117.

26. **Rajasekaran SA, Palmer LG, Moon SY, Peralta Soler A, Apodaca GL, Harper JF, Zheng Y, Rajasekaran AK.** 2001. Na,K-ATPase activity is required for formation of tight junctions, desmosomes, and induction of polarity in epithelial cells. Mol Biol Cell **12:**3717-3732.
27. **Taylor AW.** 2010. Immunosuppressive and Anti-Inflammatory Molecules that Maintain Immune Privilege of the Eye, p 44-49. *In* Dartt D (ed), Encyclopedia of the Eye, Four-Volume Set, ed 1, vol 1. Elsevier Ltd.,
28. **Streilein JW.** 2003. Ocular immune privilege: the eye takes a dim but practical view of immunity and inflammation. J Leukoc Biol **74:**179-185.
29. **Vallejo-Garcia JL, Asencio-Duran M, Pastora-Salvador N, Vinciguerra P, Romano MR.** 2012. Role of inflammation in endophthalmitis. Mediators Inflamm **2012:**196094.
30. **Suzuki T, Gilmore MS.** 2010. Pathogenesis and Immunology of Bacterial Endophthalmitis, p 165-171. *In* Dartt D (ed), Encyclopedia of the Eye, Four-Volume Set, ed 1, vol 1. Elsevier Ltd.,
31. **Agarwal R, Agarwal P.** 2012. Glaucomatous neurodegeneration: an eye on tumor necrosis factor-alpha. Indian J Ophthalmol **60:**255-261.
32. **Ramadan RT, Moyer AL, Callegan MC.** 2008. A role for tumor necrosis factor-alpha in experimental Bacillus cereus endophthalmitis pathogenesis. Invest Ophthalmol Vis Sci **49:**4482-4489.
33. **Ramadan RT, Ramirez R, Novosad BD, Callegan MC.** 2006. Acute inflammation and loss of retinal architecture and function during experimental Bacillus endophthalmitis. Curr Eye Res **31:**955-965.
34. **Schroder K, Hertzog PJ, Ravasi T, Hume DA.** 2004. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol **75:**163-189.
35. **Callegan MC, Gilmore MS, Gregory M, Ramadan RT, Wiskur BJ, Moyer AL, Hunt JJ, Novosad BD.** 2007. Bacterial endophthalmitis: therapeutic challenges and host-pathogen interactions. Prog Retin Eye Res **26:**189-203.
36. **Boyman O, Sprent J.** 2012. The role of interleukin-2 during homeostasis and activation of the immune system. Nat Rev Immunol **12:**180-190.
37. **Granucci F, Zanoni I, Pavelka N, Van Dommelen SL, Andoniou CE, Belardelli F, Degli Esposti MA, Ricciardi-Castagnoli P.** 2004. A contribution of mouse dendritic cell-derived IL-2 for NK cell activation. J Exp Med **200:**287-295.
38. **Ghasemi H, Ghazanfari T, Yaraee R, Faghihzadeh S, Hassan ZM.** 2011. Roles of IL-8 in ocular inflammations: a review. Ocul Immunol Inflamm **19:**401-412.
39. **Miguel MG.** 2010. Antioxidant and anti-inflammatory activities of essential oils: a short review. Molecules **15:**9252-9287.
40. **Russo M, Galletti GC, Bocchini P, Carnacini A.** 1998. Essential Oil Chemical Composition of Wild Populations of Italian Oregano Spice (*Origanum vulgare* ssp. *hirtum* (Link) Ietswaart): A Preliminary Evaluation of Their Use in Chemotaxonomy by Cluster Analysis. 1. Inflorescences. J Agric Food Chem **46:**3741-3746.
41. **Ultee A, Bennik MH, Moezelaar R.** 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl Environ Microbiol **68:**1561-1568.
42. **Ultee A, Kets EP, Smid EJ.** 1999. Mechanisms of action of carvacrol on the food-borne pathogen Bacillus cereus. Appl Environ Microbiol **65:**4606-4610.

43. **Ultee A, Smid EJ.** 2001. Influence of carvacrol on growth and toxin production by Bacillus cereus. International Journal of Food Microbiology **64:**373-378.
44. **Aristatile B, Al-Assaf AH, Pugalendi KV.** 2013. Carvacrol suppresses the expression of inflammatory marker genes in D-galactosamine-hepatotoxic rats. Asian Pac J Trop Med **6:**205-211.
45. **Ultee A, Kets EP, Alberda M, Hoekstra FA, Smid EJ.** 2000. Adaptation of the food-borne pathogen Bacillus cereus to carvacrol. Arch Microbiol **174:**233-238.
46. **Lima MD, Quintans LJ, de Santana WA, Kaneto CM, Soares MBP, Villarreal CF.** 2013. Anti-inflammatory effects of carvacrol: Evidence for a key role of interleukin-10. European Journal of Pharmacology **699:**112-117.
47. **Nimmer PS, Beer MR, McKillip JL.** 2014. *Bacillus cereus*: a bacterial species of environmental and clinical significance. Journal of Liberal Arts and Sciences **18:**21-32.
48. **Khan-Lim D, Berry M.** 2004. Still confused about rose bengal? Curr Eye Res **29:**311-317.
49. **Yamaguchi Y, Park JH, Inouye M.** 2011. Toxin-antitoxin systems in bacteria and archaea. Annu Rev Genet **45:**61-79.
50. **Moyer AL, Ramadan RT, Novosad BD, Astley R, Callegan MC.** 2009. Bacillus cereus-Induced Permeability of the Blood-Ocular Barrier during Experimental Endophthalmitis. Investigative Ophthalmology & Visual Science **50:**3783-3793.
51. **Agaisse H, Gominet M, Okstad OA, Kolsto AB, Lereclus D.** 1999. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in Bacillus thuringiensis. Mol Microbiol **32:**1043-1053.
52. **Pomerantsev AP, Pomerantseva OM, Leppla SH.** 2004. A spontaneous translational fusion of Bacillus cereus PlcR and PapR activates transcription of PlcR-dependent genes in Bacillus anthracis via binding with a specific palindromic sequence. Infect Immun **72:**5814-5823.
53. **Fawcett PT, Rose CD, Gibney KM, Doughty RA.** 1998. Comparison of immunodot and western blot assays for diagnosing Lyme borreliosis. Clin Diagn Lab Immunol **5:**503-506.
54. **Sonoda S, Spee C, Barron E, Ryan SJ, Kannan R, Hinton DR.** 2009. A protocol for the culture and differentiation of highly polarized human retinal pigment epithelial cells. Nat Protoc **4:**662-673.
55. **Bavykin SG, Lysov YP, Zakhariev V, Kelly JJ, Jackman J, Stahl DA, Cherni A.** 2004. Use of 16S rRNA, 23S rRNA, and gyrB gene sequence analysis to determine phylogenetic relationships of Bacillus cereus group microorganisms. J Clin Microbiol **42:**3711-3730.

VI. Manuscript Results Figures

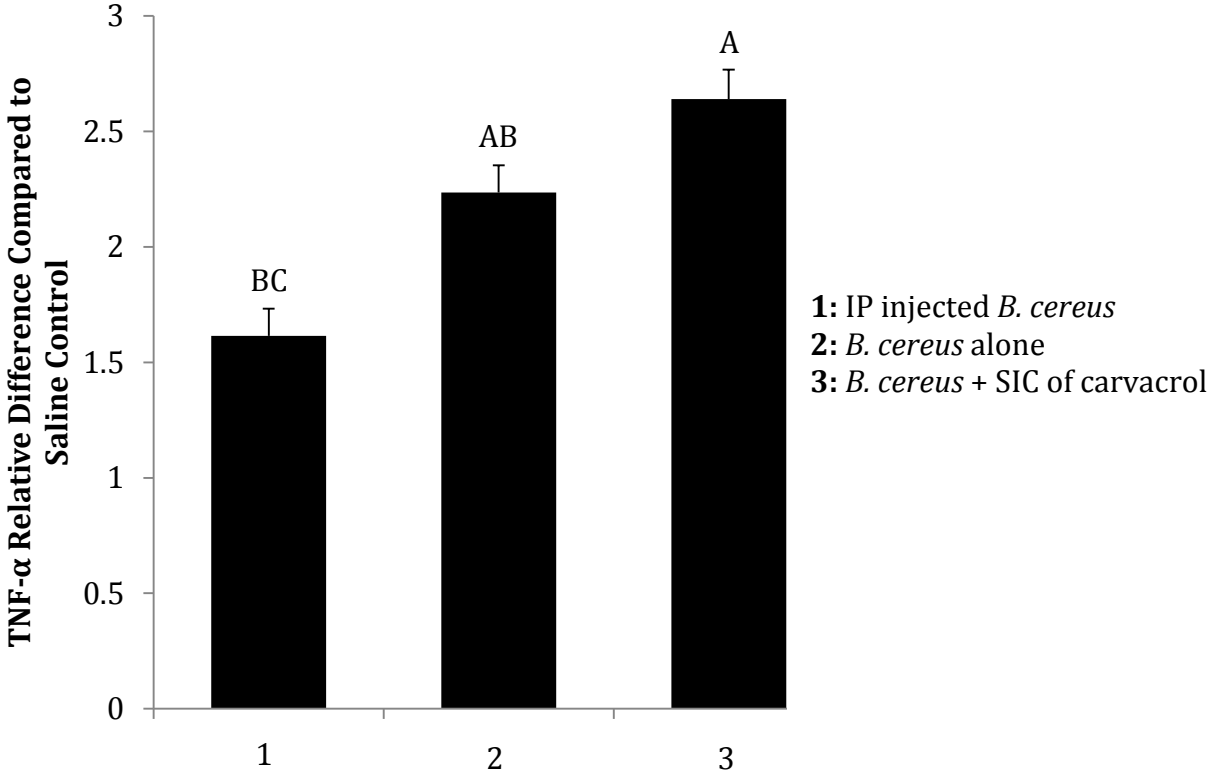

Figure 3. Relative difference of mean TNF-α levels in mice following ocular infection with *Bacillus cereus* ATCC14579 as indicated by treatment group on the x-axis. Mean log TNF-α protein concentrations were obtained from ELISA analysis of spleen lysates for three mice within each treatment group and compared using a one-way ANOVA with Tukey's Pairwise Comparisons Test (Mean log protein concentration ± SEM). Treatment groups with different letters are significantly different ($P < 0.05$).

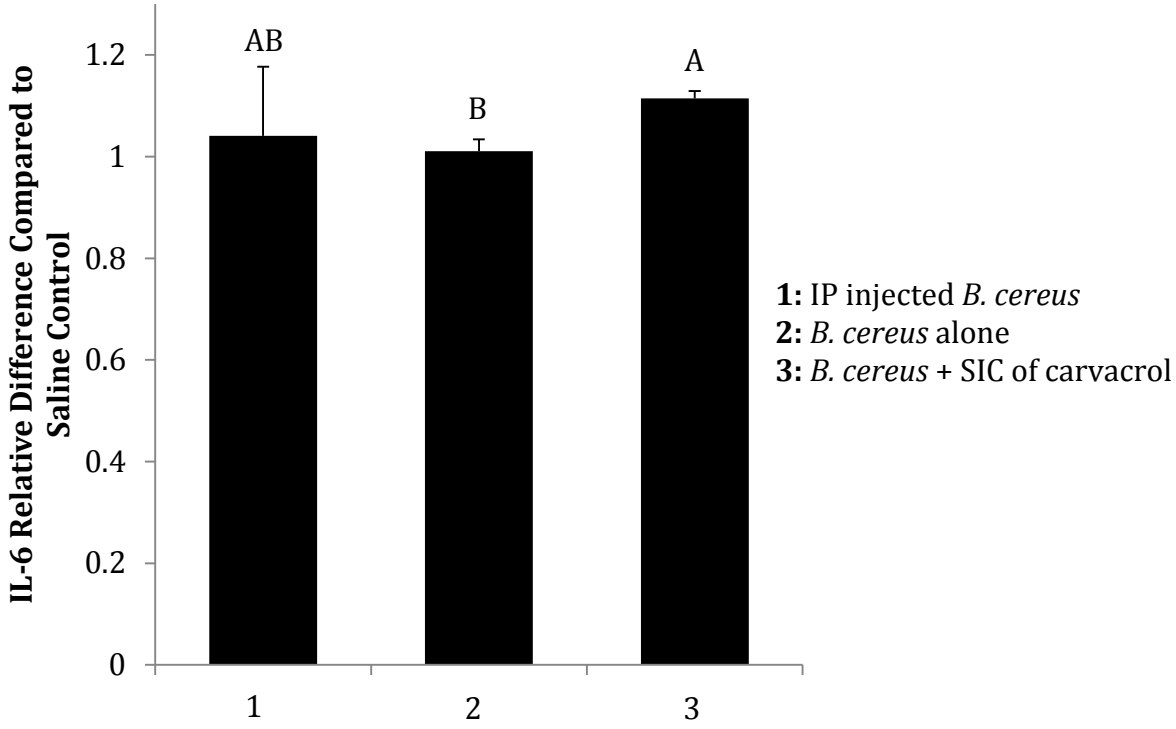

Figure 4. Relative difference of mean IL-6 levels in mice following ocular infection with *Bacillus cereus* ATCC14579 as indicated by treatment group on the x-axis. Mean log IL-6 protein concentrations were obtained from ELISA analysis of spleen lysates for three mice within each treatment group and compared using a one-way ANOVA with Tukey's Pairwise Comparisons Test (Mean log protein concentration ± SEM). Treatment groups with different letters are significantly different ($P < 0.05$).

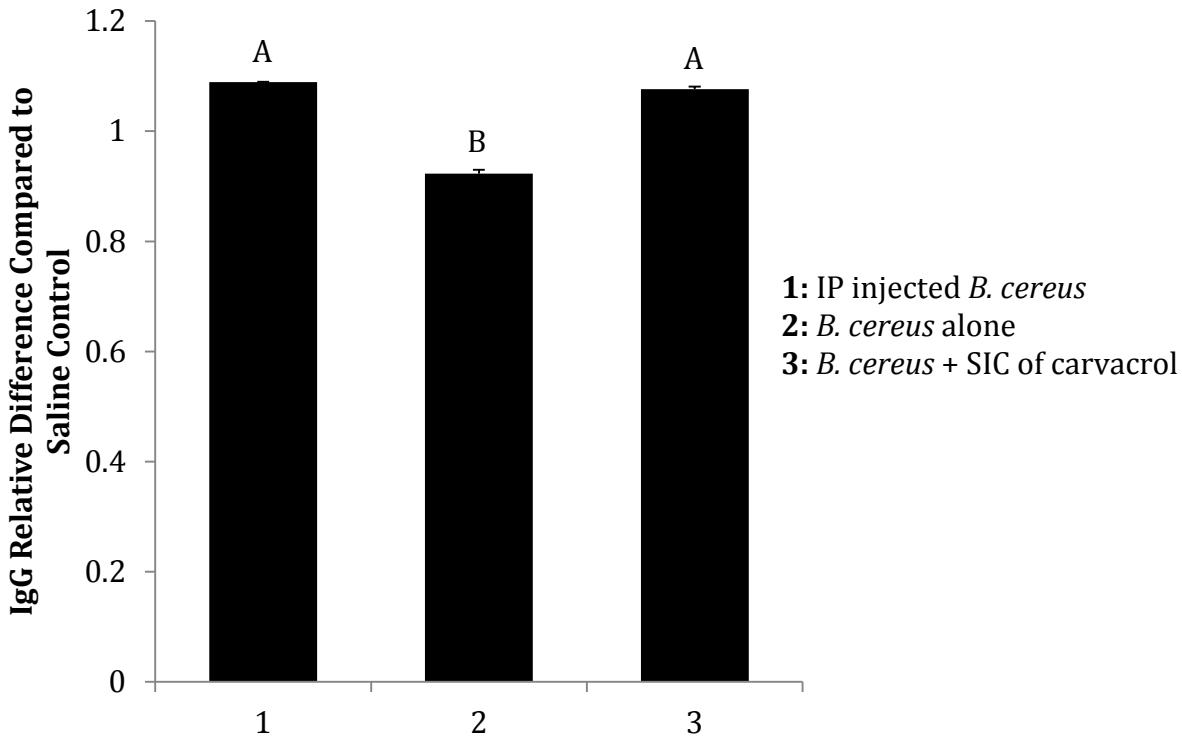

Figure 5. Mean A_{450} values were obtained using indirect ELISA of spleen lysates for three mice within each treatment group to assess relative levels of anti-*B. cereus* IgG. Mean A_{450} values were compared using one-way ANOVA with a Tukey's Pairwise Comparisons Test (Mean A_{450} value ± SEM). Treatment groups with different letters are significantly different ($P < 0.05$).

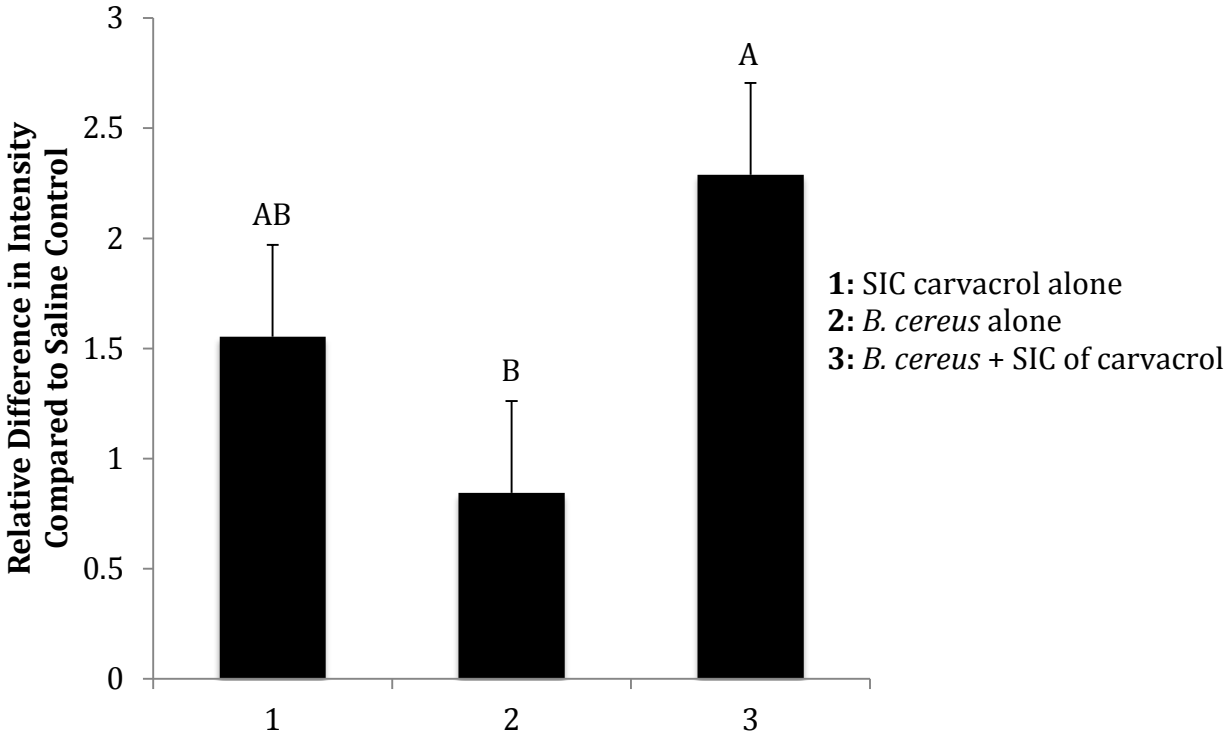

Figure 6. Relative difference of mean fluorescent red intensity values in mouse eyes (n = 6), representing ocular tissue damage after infection with *B. cereus*. Mean intensity values were obtained from mouse eye cryosections stained with DAPI and Rose Bengal using Image Pro Express 6.0 software. Mean intensity values were transformed to the fourth power and compared using a one-way ANOVA with Tukey's Pairwise Comparisons Test (Mean intensity value^4 ± SEM). Treatment groups with different letters are significantly different ($P < 0.05$).