EFFECTS OF *BACILLUS CEREUS* AND ITS TOXINS ON MICROORGANISMS FROM USED MOUTH-GUARDS

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CHAPTER I

INTRODUCTION

In October 2001, anthrax was sent in powder form through the United States mail; the intent being murder. This attack resulted in 22 cases of anthrax infection (11 inhalation, 11 cutaneous) (Todar, 2005) and 5 deaths. Anthrax is now considered at the top of the list for weaponization by terrorists because of the ease with which terrorists can quickly obtain and distribute it to masses of people in an aerosol form, thus causing severe illness and death.

Anthrax is a naturally occurring disease of domesticated animals such as cattle, goats, and sheep. This disease is caused by the bacteria *Bacillus anthracis*, which is a gram-positive, spore-forming rod that is 3-5μm long. The cells have characteristically squared ends and the ellipsoidal spores are located centrally in the sporangium (Todar, 2005). The ability to form spores allows *B. anthracis* to survive harsh environments for long periods of time. However, its virulence is caused by two other factors: an anti-phagocytic poly-D-glutamyl capsule and a multicomponent anthrax toxin (Burgess & Horwood, 2006).

The poly-D-glutamyl capsule is formed by all virulent strains of *B. anthracis* and is coded by the pX02 plasmid. Transduction of this plasmid into nonencapsulated *B. anthracis* produces the encapsulated phenotype. Though the poly-D-glutamyl capsule is

non-toxic itself, it aides in establishing the anthrax infection by resisting phagocytic engulfment of the bacteria. The anthrax exotoxin is encoded on the pX01 plasmid and is comprised of three factors: edema factor, protective antigen, and lethal factor. Together the toxin complex mediates the toxigenic stage of the disease, which impairs phagocytic activity and destroys leukocytes, eventually causing death (Todar, 2005).

B. anthracis was the first bacteria shown to be the cause of a disease (Todar, 2005). Humans become infected with anthrax when they come in contact with the flesh, bones, hides, hair, or excrement of diseased animals. The transmission of infection in this manner was first noted in the mid 1800's and became known as "Woolsorter's Disease" (Bell, 2002). It was soon thereafter that a vaccine was developed for the cutaneous form of the disease and as of 1998, the U.S. government has required the immunization of all U.S. military personnel against anthrax (Sidel, Cohen, & Gould, 2002). The anthrax vaccine is a preparation of the protective antigen that is produced by a nonencapsulated strain of *B. anthracis*. The immunization treatment involves multiple subcutaneous injections given over an 18-month period to establish immunity and requires annual boosters to maintain a protective level against anthrax (Todar, 2005).

In order for infection to occur, *B. anthracis* must be transmitted into the body through the gastrointestinal tract, skin, or by inhalation, thereby causing gastrointestinal, cutaneous, and pulmonary/inhalational anthrax, respectively. Gastrointestinal anthrax results from ingesting poorly cooked meat of infected animals. The bacteria invade through the mucosa and into the lymphatic system. Once inside the body, the spores germinate. The vegetative cells grow and manufacture toxins producing edema at the site of infection. Cutaneous anthrax is analogous to gastrointestinal anthrax, except for the

location of infection. Anthrax spores enter through the skin, usually from an abrasion on exposed areas of the body. Small, itchy bumps resembling insect bites develop within 12 to 36 hours of infection. These change rapidly into vesicles, pustules, and necrotic ulcers that give rise to septicemia (Todar, 2005). Inhalational anthrax occurs when spores are inhaled. It usually occurs when people handle infected animal hair or hides. The bacteria germinate in the lungs, causing systemic hemorrhagic pathology (Agency for Healthcare Research and Quality [AHRQ], 2001; Todar, 2005).

Although the cutaneous form is the most common presentation (Centers for Disease Control and Prevention [CDC], 2004), inhalation is the most lethal route for anthrax infection. Thus, the most likely mechanism for the weaponization of anthrax is an aerosol release of spores, although it is possible for food to be contaminated with the bacteria as well (Center for Infectious Disease Research and Policy [CIDRAP], 2004). As few as 10-50 *B. anthracis* spores are considered a fatal dose (AHRQ, 2001) and between 80% and 90% of the people who become infected will die despite treatment (Orange County Health Care Agency [OCHCA], 2004). Review of the literature to date reveals no reported cases of person-to-person anthrax transmission (CDC, 2004; OCHCA, 2004; AHRQ, 2001).

Because of its ability to form protective spores, *B. anthracis* can withstand harsh conditions both during and after aerosol dispersal. Studies in England have shown that *B. anthracis* spores can survive an explosion due to bomb dispersal and still infect people effectively (Public Health Foundation [PHF], 1999). These spores have even been known to stay viable in soil for decades.

B. anthracis is easily found in soil samples where there has been a previous anthrax endemic. In the United States, areas of incidence have been noted in North Dakota, South Dakota, Oklahoma, Nebraska, Arkansas, Texas, Louisiana, Mississippi, and California (American Veterinary Medical Association, 2001), though small areas of infection exist in other states. However, the incidence of naturally-acquired anthrax is quite rare in the United States, with only one to two cases of cutaneous disease reported per year. The incidence of anthrax worldwide is not known due to unreliable reporting (Todar, 2005).

Anthrax bacteria are more commonly found in developing countries and areas where there are no veterinary public health programs, therefore allowing *B. anthracis* to thrive among local livestock. Certain regions of the world, including the Middle East, report more cases of anthrax in animals than others (Discovery Health Channel, 2004). Consequently, terrorists in the Middle East may find it relatively simple to obtain the *B. anthracis* bacteria. In addition, countries including Iraq, Iran, Syria, Libya, and North Korea that are known to support terrorism (Franck, n.d.), also have the knowledge to weaponize anthrax. They are known to have actively recruited experts who previously worked in the biological weapons program for the former Soviet Union (CIDRAP, 2004). These countries have since been suspected of developing offensive bioweapon programs. Iraq has even admitted to producing and weaponizing anthrax after the Persian Gulf War (PHF, 1999).

B. cereus is very similar phenotypically and genotypically to *B. anthracis*. Both are gram-positive rods from the genus *Bacillus* and both produce spores that do not distend the sporangia (Drobniewski, 1993). Some experts even believe these two bacteria

are varieties of the same species after results from DNA hybridization analysis revealed chromosomal similarities. When sequencing the 16S rRNA between *B. anthracis* and an emetic strain of *B. cereus*, results revealed a continuous stretch of 1,446 identical bases and differences in only four to nine nucleotides (Ash et. al, 1991). Variable-number tandem repeat (VNTR) analysis cannot even distinguish these two *Bacillus* species (Helgason et. al, 2004).

Researchers have found similarities between the toxins of *B. anthracis* and *B. cereus* as well. The complete sequence of the cereulide synthetase gene cluster in the *B. cereus* genome, which is the multi-enzyme complex that produces the emetic toxin cereulide, shows substantial (90%) homology to the toxin-encoding pX01 plasmid sequence found in *B. anthracis* (Ehling-Schulz et. al, 2006).

One particular strain of *B. cereus* isolated from the sputum and blood of a patient with life-threatening pneumonia has been found to harbor a plasmid so similar to the pX01 plasmid of *B. anthracis* that an inhalation anthrax-like illness ensued (Hoffmaster et. al, 2004). The clinical features and laboratory findings from this patient resemble the medical reports of ten people with inhalational anthrax contracted from terrorist attacks in 2001. Shotgun sequencing of these homologous plasmids found 99.6% similarity in their genetic sequences and the presence of all three anthrax toxin genes in this *B. cereus* genome (Hoffmaster et. al, 2004). This is notable since the virulence of anthrax is based on two plasmids: pX01 and pX02, which code for the toxin complex and the poly-D glutamyl acid capsule respectively.

The evidence not only suggests that *B. cereus* contains the genetic codes for anthrax toxins, but *B. anthracis* also harbors a homologous gene to the *B. cereus* non-

hemolytic enterotoxin (Nhe) AB gene. Along with the hemolysin BL (HBL) toxin, Nhe causes the diarrheal form of *B. cereus* illness. Translated NheAB sequences exhibit 98% similarity to the suspected *B. anthracis* counterpart proteins. *B. anthracis* even produces and secretes the NheA subunit of the *B. cereus* Nhe complex in vitro (Mendelson et. al, 2004).

The differences between these bacteria are that *B. cereus* is motile, hemolytic on blood agar, penicillin resistant, and resistant to bacteriophage gamma; *B. anthracis* is not (Drobniewski, 1993). However, due to the overwhelming similarities between these two bacteria and transmission issues, researchers have often used *B. cereus* to study *B. anthracis* in the laboratory.

B. cereus can be identified by its specific growth patterns on agar. On blood agar, *B. cereus* colonies grow and produce discontinuous hemolysis, which is an area of non-hemolysis surrounded by a zone of complete hemolysis. On Mannitol-egg yolk-polymyxin (MYP) agar, *B. cereus* colonies produce a precipitate of lecithinase from the hydrolysis of the egg yolk in the media. Generation time for *B. cereus* is less than 30 minutes and gram-positive spores form with heat shock (Todar, 2005).

B. cereus is ubiquitous in the soil but food products such as rice, meat, and milk also harbor the bacteria. The known toxins of *B. cereus* include hemolysins, phospholipase C's, enterotoxins, and the emetic toxin, cereulide. The ability to produce enterotoxins varies between different strains of *B. cereus* and some strains can produce both enterotoxins and emetic toxins (Kotiranta, Lounatmaa, & Haapasalo, 2000). However, not all *B. cereus* toxins cause sickness in humans.

The two types of food-borne illness caused by the toxins of *B. cereus* are emetic and diarrheal. The emetic disease has an incubation period of one to six hours and is characterized by nausea, vomiting, and abdominal cramps (U.S. Food and Drug Administration [FDA], 1992). It is caused by the emetic toxin that is produced by *B. cereus* cells growing in contaminated food (Kramer & Gilbert, 1989). The emesis inducing dose for humans is not known, however depending on the strain, *B. cereus* can release more than 5μg/mL of this toxin. Unlike other *B. cereus* toxins, cereulide is not inactivated by heat and therefore can be found in heat-treated foods. It causes damage by inhibiting the cytotoxicity of natural killer cells, swelling their mitochondria, and eventually inducing apoptosis (Paananen et. al, 2002).

The diarrheal illness manifests as abdominal cramps and diarrhea. The incubation period is eight to sixteen hours and is mediated by heat-labile enterotoxins produced by the vegetative form of *B. cereus* in the small intestine. These toxins activate intestinal adenylate cyclase and cause the secretion of intestinal fluids (FDA, 1992). Two enterotoxins produce the diarrheal illness of *B. cereus*: enterotoxin HBL and enterotoxin NHE. Enterotoxin HBL causes vascular permeability (Beecher, Schoeni, & Wong, 1995) and produces the characteristic hemolysis of *B. cereus* on blood agar (Beecher & Wong, 1994). Enterotoxin NHE is non-hemolytic (Beecher & Wong, 2002), but is highly toxic to Vero cells (Lund & Granum, 1997). Both the emetic and diarrheal illnesses typically last less than 24 hours after the onset of symptoms (Todar, 2005).

The toxins of *B. cereus* have been shown to induce deleterious effects on humans, however the bacteria has probiotic properties as well. It has shown measurable

antimicrobial activity against *B. pumilus, B. clausii,* and *B. sphaericus* bacteria (Duc et. al, 2004).

Though *B. cereus* is mainly found in the soil, the bacteria can be transmitted to a variety of surfaces. This is primarily due to its ability to produce spores, which are resistant to many harsh environmental conditions including extreme heat, cold, drying, irradiation, and exposure to disinfectants. A previous study recovered *B. cereus* spores from infected coffee, toothbrushes, and doorknobs (Miller, 2005), revealing the ease at which this bacteria, and therefore most likely *B. anthracis*, can be distributed for infective purposes.

B. cereus spores from environmental sources can also adhere to the used mouth-guards (MG) of football players. This has the potential of causing illness if the preformed toxins of *B. cereus* are ingested into the body. MG not only provide a niche for some bacteria, but can also alter "normal" oral microbial ecology (Bullard, 2004).

MG are primarily worn during participation in athletic sports. However, they are also used as a protective measure against grinding teeth during sleep. They function as a buffer between the teeth and soft tissues, help prevent tooth fractures, prevent contact between opposing teeth, give mandibular support, act as a shock absorber for the jaw thus reducing the likelihood of concussions, and help prevent neck injuries during athletic events (Vastardis, 2005).

There are three main types of MG: stock, boil and bite, and custom. Stock MG are the least expensive, yet the least effective and least preferred. They do not come in many sizes and do not fit nor function well. The boil and bite variety is the most popular since it is reasonably priced and works reasonably well. The custom MG, which are

custom made by dentists to ensure a better fit to the teeth with the required thickness, are the most expensive. These MG tend to last longer than the boil and bite variety, as they do not lose their shape as quickly.

The Everlast® brand of athletic MG is synonymous with boxing, but these MG are commonly used for a variety of athletic sports. They are currently manufactured in China, but were first developed and sold in New York. Today, these MG are readily available in all states.

MG are constructed of either single- or multiple-layer ethylene vinyl acetate (EVA) that is laminated around a mold of the subject's mouth (Vastardis, 2005). By first softening the MG material in hot water, the subject can form the boil and bite style MG to the mouth by using pressure from the lips, tongue, cheeks, fingers, and the act of biting. This gives a better fit than a stock MG, however errors in forming can result in a MG that is not of the proper thickness for protection.

Most MG, regardless of the type, have been made of EVA for the last 30 years. It has minimal moisture absorption and is non-toxic, elastic, and easy to manufacture (Weserman, Stringfellow, & Eccleston, 2002). However, porosities readily develop in the EVA due to the process of polymerization. These pores in the MG provide selective niches for bacteria and other microorganisms that naturally inhabit the oral cavity (Paster et. al, 2001). The surfaces of the MG are also susceptible to adhesion by microbes, including *B. cereus* (Banerjee et. al, 1988). Figure 1 shows a microscopic image of the irregular surface of a MG.

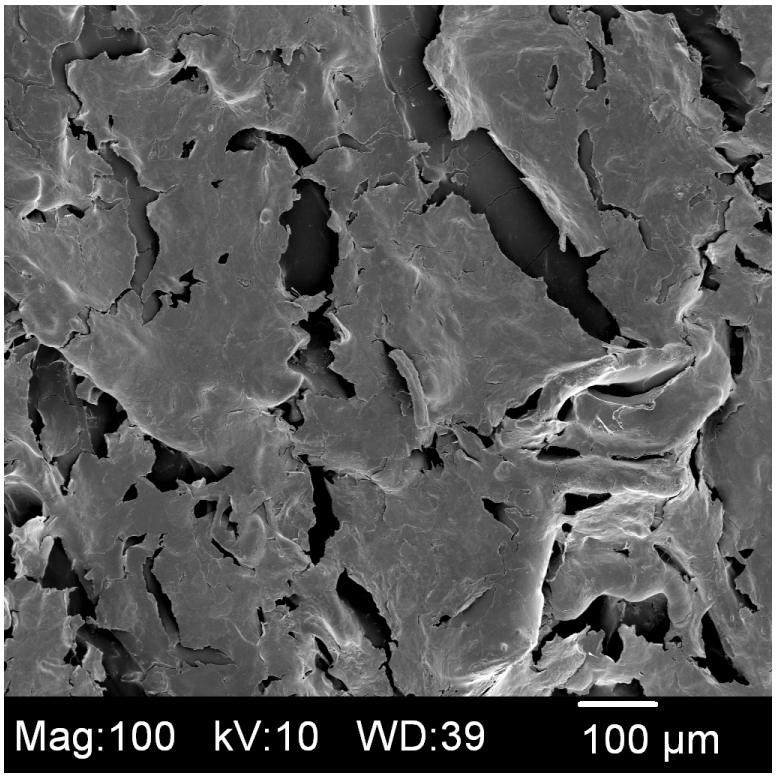

Figure 1. Magnification (100x) of the Surface of a Mouth-Guard.

A recent study found that when the number of air inclusions within a MG was increased, the MG was better at reducing opposing forces and was therefore more protective (Weserman, Stringfellow, & Eccleston, 2002). However, more microorganisms may be able to inhabit a MG if there are more air spaces within. Moreover, MG can not only be contaminated by one's "normal" oral microorganisms, but also foreign bacteria, yeasts, and molds from the surrounding environment (Glass, Bullard, & Conrad, 2006).

It is not unlikely that intentional contamination of a MG by pathogenic organisms could occur. MG are used by many people in the United States and their intimate contact with the human body makes them susceptible for abuse as a mode of transmission for harmful microorganisms. Given the conditions of the MG support the adherence and

growth of the pathogens, it would not be long before these organisms are inhaled or swallowed, thus introducing them into the body for infection.

In order to evaluate the presence and effects of contaminated MG, the following research questions are posed:

1. Can mouth-guards be purposefully contaminated with *B. cereus* bacteria?
2. What organisms are found in the worn mouth-guards of students?
3. What inhibitory effects do the *B. cereus* bacteria have on the growth of microorganism populations on worn mouth-guards?
4. What inhibitory effects do the *B. cereus* toxins have on the growth of microorganism populations on worn mouth-guards?

CHAPTER II

MATERIALS AND METHODS

Bacillus cereus Treatments and Controls

Two *B. cereus* strains, Whimpy and Arnold, were used in this research. The Whimpy strain was collected from a soccer field at Oklahoma State University (Bullard, 2004) and the Arnold strain was collected from a soil sample along Oklahoma I-35 (Miller, 2005). As described by Bullard (2004), the Whimpy strain does not produce discontinuous hemolysis, clear hemolysis immediately surrounding the growth, lecithinase on a minimal media, enterotoxin, or verotoxin when grown on a minimal medium. There were no toxin analyses performed on the Arnold strain by Miller (2005).

There were four *B. cereus* treatments (labeled A, B, C, and D) and one control (labeled E) that worn MG sections and microorganisms from them were subject to in this study. Treatment A introduced a $2x10^6$ CFU/mL concentration of the Whimpy bacteria in a brain heart infusion broth (BHI) to the MG organisms. Treatment B introduced a $2x10^6$ CFU/mL concentration of the Arnold bacteria in BHI to the MG organisms. Treatment C introduced 10mL of the Whimpy culture filtrate stock solution (CF) to the MG organisms. Treatment D introduced 10mL of the Arnold CF to the MG organisms. The preparation of the *B. cereus* CF is described in the next section, "*Bacillus cereus*

Toxin Collection". The control conditions had no *B. cereus* bacteria or CF added to the MG organisms, only an equal amount of BHI.

The $2x10^6$ CFU/mL concentrations of the Whimpy and Arnold strains were obtained by first inoculating 10mL BHI with each *B. cereus* strain and allowing the bacteria to grow in a 37°C incubator for 24 hours. The *B.* cereus inoculated BHI was added to clean BHI by a sterile dropper until the absorbance was the same as a McFarland standard 1. A micropipette was then used to transfer 0.333mL of the Whimpy and Arnold McFarland standards into 50mL of BHI in treatment flasks A and B respectively for the experiments.

B. cereus growth curve controls were also created by first making a McFarland standard 1 of both the Whimpy and Arnold strains in BHI. Two Nephelo culture flasks were each filled with 50mL of BHI and into each was added 0.333mL of either the Whimpy or Arnold strain McFarland standard. The optical density of these solutions was recorded every 30 to 60 minutes to create a growth curve for the Whimpy and Arnold bacteria over 24 hours.

Bacillus cereus **Toxin Collection**

The stock solutions of the Whimpy and Arnold CF were prepared by first inoculating two 500mL flasks of BHI with *B. cereus*, one with the Whimpy strain and one with the Arnold strain, and allowing them to grow for 24 hours in a 37°C incubator. These suspensions were centrifuged and filtered through a 0.22 micron filter. The filtered CF of each was collected and frozen at $^{-}20$°C for future use.

The presence of specific toxins produced by the Whimpy and Arnold strains was determined by adding 20µL of Whimpy or Arnold CF into four 4mm diameter wells that were punched in TSA-II and MYP. These were then placed in a 37°C incubator for 24 hours. The diameter of the hemolysis, lecithinase, and lipase activity was recorded.

A toxin production timeline was also made for both *B. cereus* strains. Two Nephelo culture flasks were filled with 50mL of a $2x10^6$ CFU/mL concentration of either the Whimpy or Arnold strain in BHI. At one to two hour intervals for 25 hours, the optical densities of the Whimpy and Arnold flasks were recorded and 2mL of liquid was removed from each. These liquids were centrifuged, sterile filtered through a 0.22 micron filter, and 20µL of each was placed into 4mm wells that were punched around Trypticase™ Soy Agar plates with 5% sheep blood (TSA-II) and Mannitol-egg yolk-polymyxin plates (MYP). These plates were placed in an incubator at 37°C for 25 hours, after which the diameter of hemolysis, lecithinase, and lipase activity was recorded.

Contamination of Mouth-Guards

Each of four new Everlast® MG was divided in half using flame sterilized razor blades. The surfaces and depths of each MG half were touched three times to TSA-II and incubated at 37°C for 24 hours to see if there were any intrinsic microorganisms on the MG. Each MG half was then placed into 10mL of a $2x10^6$ CFU/mL concentration of either the Whimpy or Arnold strain in BHI and incubated at 37°C for 24 hours. The MG halves were removed from the *B. cereus* cultures and allowed to dry for three days in a sterile covered Petri dish.

The eight MG halves were cut 5mm from the previously cut edge with flame sterilized razor blades to expose new depths, which along with the surfaces were touched three times to TSA-II. These plates were incubated at 37°C for 24 hours. The MG halves were boiled for 12 seconds in 100mL sterile water and placed in 50mL cold sterile water for two seconds as directed by Everlast®. While in the boiling water for 12 seconds, a TSA-II was held 1cm above the 250mL beaker in the steam. After being in cold water for two seconds, the MG halves were again cut with flame sterilized razor blades 5mm from the previously cut edges to expose new depths, which along with the surfaces were touched three times to TSA-II that were then incubated at 37°C for 24 hours. This procedure was replicated with two MG and the PA01 strain of *Pseudomonas* bacteria.

Collection and Preparation of Worn Mouth-Guards

After obtaining informed consent (Appendix A), new Everlast® boil and bite MG were given to eleven students of the Oklahoma State University Center for Health Sciences (OSU-CHS) to wear intermittently for 12-24 hours. These were collected in an aseptic manner by expectoration of the MG directly into a sterile Petri dish in the lab. The total number of hours each student wore the MG was recorded.

Upon collection, each MG was divided into sections using flame sterilized razor blades and instruments. The sections were cut as illustrated in Figure 2 below. The unlabeled section (bottom left) was frozen for future use. Sections A, B, C, D, and E were frozen until needed for the *in vivo* growth curves. Sections 1, 2, and 3 were used in

the *in vitro* growth curves and the identification of microorganisms from MG as detailed below.

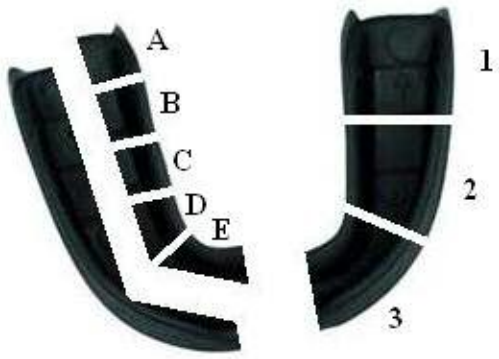

Figure 2. Sectioned Mouth-Guard. Sections 1, 2, and 3 were used for the *in vitro* growth curves. Sections A, B, C, D, and E were used for the *in vivo* growth curves.

Identification of Microorganisms from Worn Mouth-Guards

Pre-treatment cultures were made by touching the surfaces and depths of MG sections 1, 2, and 3 three times to TSA-II. Microorganisms from these cultures were streaked for isolation and Analytical Profile Index (API) identification procedures were performed for identification on these pure cultures. All isolates were stored in the freezer at $^{-}65^{o}$C.

Interaction of *Bacillus cereus* and Individual Mouth-Guard Microorganisms

Sixty-eight isolates were randomly chosen from the 181 that were collected from the OSU-CHS student MG. For each isolate, a sterile inoculating loop was used to collect the frozen bacteria and streak it for isolation on TSA-II. Of those that grew, one

isolated colony was collected and added to 1mL sterile water. A cotton swab was dipped into this solution and used on TSA-II to streak for lawn growth.

Five wells with 4mm diameters were equally spaced around the streaked plate and labeled A, B, C, D, and E. For wells A and B, 20µL of a $2x10^6$ CFU/mL concentration of the Whimpy and Arnold strains was added respectively. For wells C and D, 20µL of the Whimpy and Arnold CF was added respectively. A total of 20µL BHI was added into well E as a control. The TSA-II were incubated at 37°C for 24 hours. The diameters of the *B. cereus* growth and inhibition of the lawn growth were measured as seen in Figure 3 and recorded.

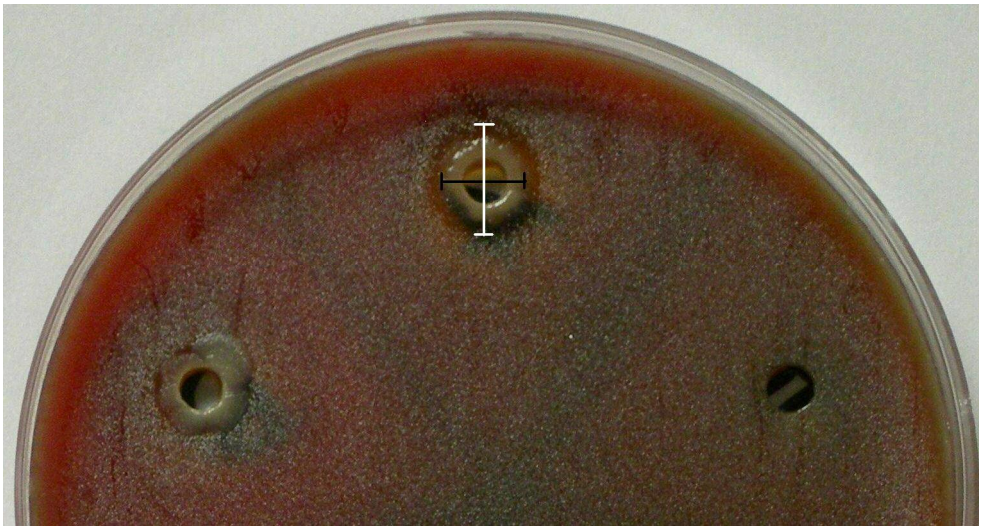

Figure 3. Measuring *B. cereus* Growth and Lawn Inhibition. Measurements of the diameter of *B. cereus* growth (black line) and lawn inhibition (white line).

Gram stains of the Whimpy, Arnold, and lawn bacteria were made on microscopic slides. A sterile inoculating loop was used to collect the Whimpy and Arnold growth from the top edge of their respective wells. The lawn bacteria were collected from the

middle of the plates by a sterile inoculating loop. The morphologies of Whimpy, Arnold, and the lawn bacteria were observed with a light microscope and recorded.

Growth Curves (*in vitro*)

Pre-treatment cultures were made on TSA-II for all eleven OSU-CHS student MG as outlined in the "Identification of Microorganisms from Mouth-Guards" section above. For each MG, sections 1, 2, and 3 were macerated together, weighed, and added to 10mL sterile water. This solution was vortexed for five minutes and serial dilutions were made to determine the concentration of microorganisms per gram of MG. The vortexed solution was poured into a flask with 50mL BHI and incubated at 37^{o}C for 24 hours to create a stock solution of the MG microorganisms.

For each MG, a 2x10^{6} CFU/mL concentration of the MG microorganisms was prepared. This was done by making a McFarland standard 1 of the MG stock solution and adding 0.333mL of this to 40mL BHI in each of five Nephelo culture flasks labeled A, B, C, D, and E. For treatment A, 0.333mL of a 2x10^{6} CFU/mL concentration of the Whimpy strain was added to the prepared Nephelo culture flask. For treatment B, 0.333mL of a 2x10^{6} CFU/mL concentration of the Arnold strain was added to the prepared Nephelo culture flask. These two flasks were then brought up to a final volume of 50mL by the addition of BHI. For treatment C, 10mL of the Whimpy CF was added to the prepared Nephelo culture flask for a final volume of 50mL. For treatment D, 10mL of the Arnold CF was added to the prepared Nephelo culture flask for a final volume of 50mL. For the control, E, 10mL BHI was added to the prepared flask to bring the final volume to 50mL. The five flasks were then placed in a 37^{o}C incubated shaker and the

optical densities of each were recorded every 30-60 minutes for 24 hours. Post-treatment serial dilutions were prepared on TSA-II from the suspensions in each flask.

Growth Curves (*in vivo*)

The frozen MG sections A, B, C, D, and E were thawed at room temperature for 30 minutes and utilized in this phase. Pre-treatment cultures were made for the five MG sections by touching the surface and depth of each to a TSA-II three times and observing any growth on the plates after incubation for 24 hours at 37^{o}C.

For treatment A, 0.333mL of a 2x10^{6} CFU/mL concentration of the Whimpy strain was added to 50mL of BHI in a Nephelo culture flask and the optical density was recorded just after the addition of MG section A. For treatment B, 0.333mL of a 2x10^{6} CFU/mL concentration of the Arnold strain was added to 50mL of BHI in a Nephelo culture flask and the optical density was recorded just after the addition of MG section B. For treatment C, 10mL of the Whimpy CF was added to 40mL of BHI in a Nephelo culture flask and the optical density was recorded just after the addition of MG section C. For treatment D, 10mL of the Arnold CF was added to 40mL of BHI in a Nephelo culture flask and the optical density was recorded just after the addition of MG section D. Control E involved the addition of 50mL of BHI to a Nephelo culture flask and recording the optical density just after the addition of MG section E. The flasks were placed in an incubated shaker at 37^{o}C and the optical densities of each were recorded every 30-60 minutes for 24 hours.

After this time, MG sections A, B, C, D, and E were removed from the flasks and new depths were created by cutting 5mm from the previously cut edge with flame

sterilized razor blades. Post-treatment cultures were made by touching the surface and depth of each MG section three times to TSA-II and incubating the plates for 24 hours at 37^{o}C.

Data Collection and Statistics

API strips were used to identify the bacteria from the OSU-CHS students' worn MG. Different API strips were used depending on the gram stain results of the isolates. Gram positive cocci that were catalase positive were identified using the API STAPH strip. Gram positive cocci that were catalase negative were identified using the API 20 STREP strip. Gram negative cocci were identified by using the API NH strip. Gram positive rods were identified by using the API CORYNE strip. Gram negative rods were identified using the API 20 NE strip. The API strips were obtained from bioMerieux Industry.

The optical densities of the treatment solutions in the *in vivo* and *in vitro* experiments were measured using a spectrophotometer set at a wavelength of 640nm. Growth curves were plotted and analyzed using GraphPad's Prism software. The differences between the growth curves of the treatment conditions and controls reflected the effects that microorganisms in the worn MG and *B. cereus* have on each other. Statistical analyses determining the significance of the difference between the bacterial growth curves were performed by the analysis of the regression line, with emphasis on the endpoint absorbance, midpoint, and slope of the growth curve.

The amount of *B. cereus* growth on the streaked lawns and the measures of inhibition of the lawn were quantified by a ruler with mm increments and digital calipers.

An ANOVA test was used to analyze the significance of these measurements. A Nikon digital camera was used to take pictures of all TSA-II.

CHAPTER III

RESULTS

Bacillus cereus Controls and Toxin Production

The Whimpy and Arnold strains were cultured in BHI and allowed to grow in a 37^{o}C incubated shaker for 24 hours. After this time, the CF was collected for both *B. cereus* strains and put into 4mm wells in TSA-II and MYP to assess the types and relative amounts of toxins being produced. The measurements of the diameters of the hemolysis, lecithinase, and lipase activity from these plates are detailed in Table 1.

Table 1. Diameters (in mm) of the Toxin Activity of *B. cereus*. The diameters of hemolysis, lecithinase, and lipase activity were recorded for the Whimpy and Arnold *B. cereus* strains after 24 hours of growth.

Hemolysis		Lecithinase		Lipase	
Whimpy	Arnold	Whimpy	Arnold	Whimpy	Arnold
10	6	20	15	0	0
11	6	19	15	0	0
10	7	19	15	0	0
10	6	19	15	0	0

For both Whimpy and Arnold, the presence of hemolysis on TSA-II and lecithinase on MYP showed that these *B. cereus* strains have hemolysin and phospholipase C toxins respectively. The CF from the Whimpy strain produced a significantly larger diameter of hemolysis ($p=0.0011$) and lecithinase ($p=0.0002$) than the

CF from the Arnold strain. There was no lipase activity observed from the CF of either Whimpy or Arnold. Differences in the hemolysis patterns on TSA-II were also noted between the two *B. cereus* strains. Around the Whimpy strain there was a zone of complete hemolysis only. The Arnold strain exhibited a zone of complete hemolysis that was surrounded by a second zone of incomplete hemolysis.

While the *B. cereus* strains were being incubated in the shaker for 25 hours, the optical density of each solution was being recorded every 30-60 minutes. From this data, growth curves for both *B. cereus* strains were made (Figure 4). The Whimpy and Arnold growth curves were not significantly different (p=0.2281). Both had a lag phase of about three hours, a log phase for three to four hours with very similar midpoints (p=0.1205) and growth rates (p=0.7764), as well as very similar endpoint absorbances (p=0.8184) during stationary phase.

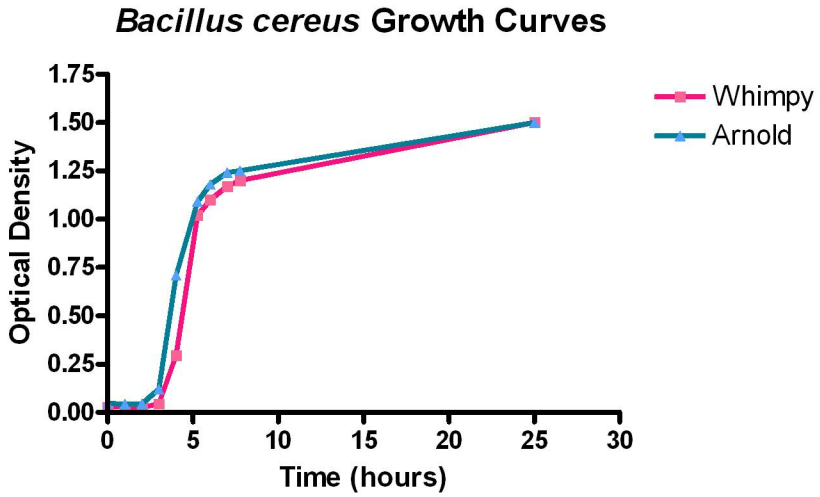

Figure 4. Growth Curves of *B. cereus* Strains Whimpy and Arnold.

A toxin production timeline was plotted for both the Whimpy and Arnold strains (Figure 5). Since the toxins of *B. cereus* are proteins that are excreted outside of the

bacterial cells, the CF collected from the Whimpy and Arnold strains every hour was analyzed for the presence of the hemolysin and phospholipase C toxins. Wells were punched in TSA-II and MYP agar plates into which 20µL of either the Whimpy or Arnold CF was placed. The diameters of the hemolysis and lecithinase activity were recorded after 25 hours of incubation.

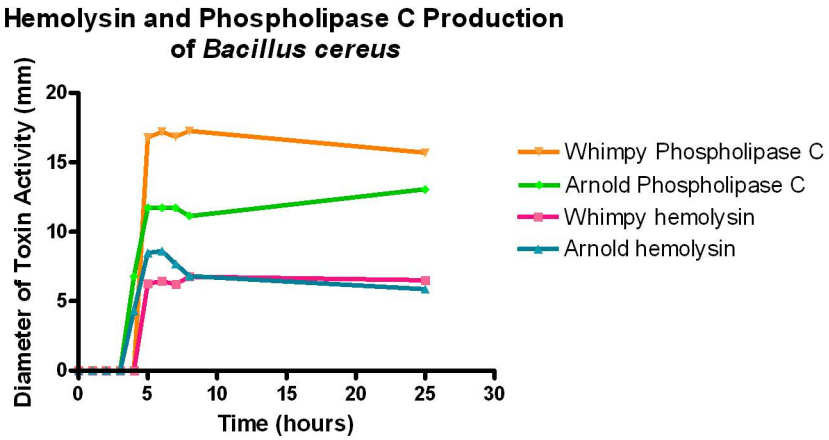

Figure 5. Production of Individual *B. cereus* Toxins over Time. The production of hemolysin and phospholipase C toxins per hour as measured by the diameter of hemolysis and lecithinase activity respectively.

There was no hemolysis or lecithinase activity for the CF of either *B. cereus* strain after three hours. At hour four there was an increase in activity for both Arnold toxins while there was no evidence of the presence of either toxin in the Whimpy CF; at five hours, hemolysis and lecithinase activity was observed from the Whimpy CF as well. After five hours, there was no significant difference in the amount of hemolysin produced by either the Whimpy strain ($p=0.6722$) or the Arnold strain ($p=0.0636$). However, from hours five to twenty-five there was a significant drop in phospholipase C activity from the Whimpy CF ($p=0.0336$) and a significant increase in this toxin from the Arnold CF

(p=0.0440). Until hour 25, the Arnold CF produced a larger diameter of hemolysis than the Whimpy CF at every hour tested. With the exception of the fourth hour when no lecithinase activity was reported from the Whimpy CF, the Arnold CF produced a smaller diameter of lecithinase for every hour tested. At 25 hours, the Whimpy CF produced a larger diameter of hemolysis and lecithinase than the Arnold CF.

Contamination of Mouth-Guards

Four new MG were purposefully contaminated with *B. cereus* strains Arnold and Whimpy and then tested to see if the bacteria would survive the MG boiling procedure as documented by the Everlast® MG directions. As a control, two new MG were also contaminated with the PA01 strain of *Pseudomonas*, which unlike *B. cereus* does not produce spores.

Pre-treatment plates were prepared by touching the surfaces and depths of each newly opened MG to TSA-II. There was no bacterial growth on these plates after 24 hours of incubation at 37°C (Figure 6a). This figure is representative of all pre-treatment plates from this experiment. After the MG sat in the *B. cereus* or PA01 inoculated BHI for 24 hours and were allowed to dry, they were touched to TSA-II. Substantial *B. cereus* growth occurred where the surfaces and depths of the Whimpy and Arnold contaminated MG had touched TSA-II for all four MG. Likewise, the PA01 plates had growth of *Pseudomonas* where the surfaces and depths of the *Pseudomonas* contaminated MG were touched.

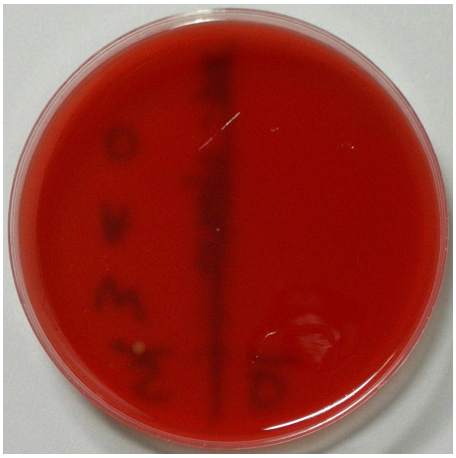

Figure 6a. Pre-Treatment Touch Plate. There is no growth from where the surfaces (left) or depths (right) were touched.

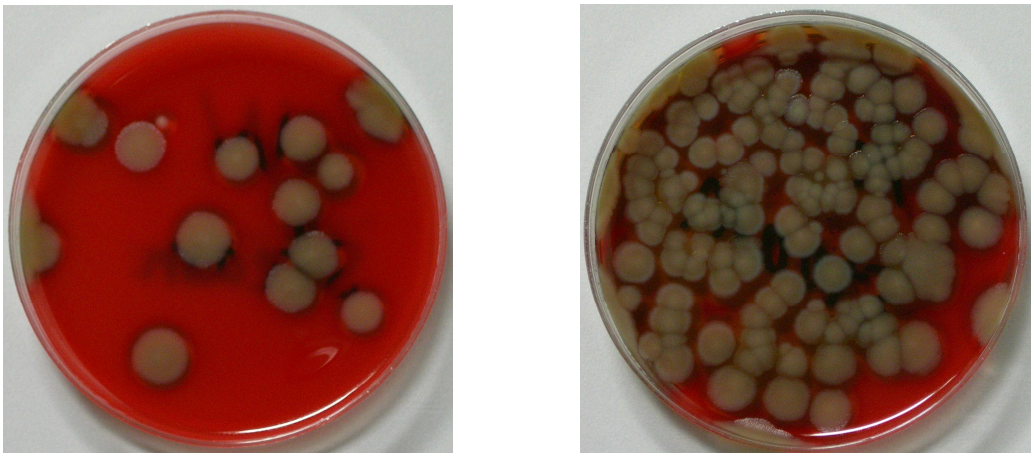

Figure 6b. While-Boiling *B. cereus* Plates of Whimpy and Arnold Respectively. These TSA-II were held above boiling *B. cereus* contaminated MG.

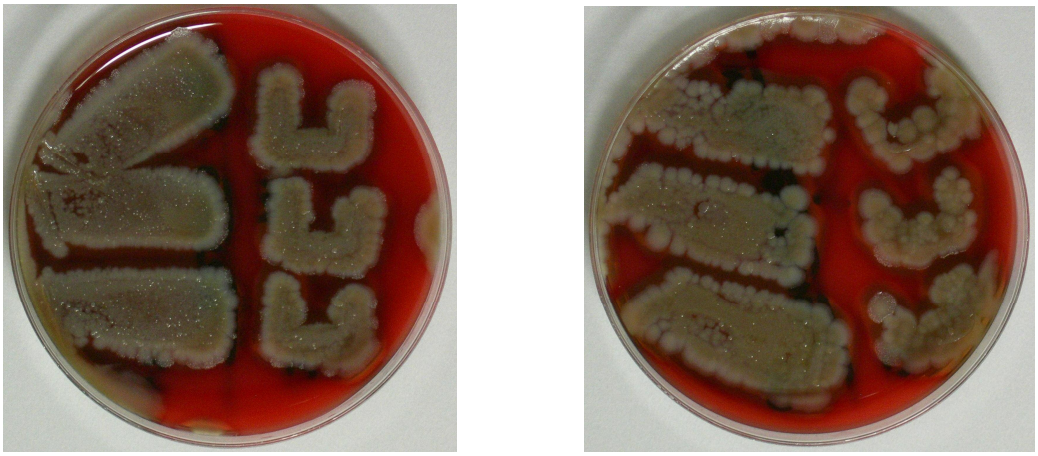

Figure 6c. Post-Boiling *B. cereus* Plates of Whimpy and Arnold Respectively. Surfaces were touched three times on the left side and depths were touched three times on the right side of each plate.

Each contaminated MG half was placed into boiling water and a TSA-II plate was held above the beaker in the steam. For one of the four *B. cereus* contaminated MG tested, the TSA-II for both the Whimpy and Arnold strain grew colonies on these plates as seen in Figure 6b. The TSA-II held above the boiling MG contaminated with the Arnold strain had more colonial growth than that held above the boiling MG contaminated with the Whimpy strain. None of the TSA-II plates held above the boiling MG contaminated with *Pseudomonas* produced any colony growth.

After the contaminated MG had been boiled for 12 seconds and placed in cool water for two seconds, surfaces and depths were again touched to TSA-II. All of the Arnold and Whimpy post-boiling plates had *B. cereus* growth where the surfaces and depths had been touched (Figure 6c), which is representative of all *B. cereus* post-boiling plates for this experiment. Both of the PA01 post-boiling plates had *Pseudomonas* growth where the surfaces and depths were touched to TSA-II.

Collection of Worn Mouth-Guards

The number of hours each OSU-CHS student wore his or her MG was recorded when the MG was received in the laboratory. Pre-treatment serial dilutions were performed on weighed MG pieces to determine the concentration of microorganisms that inhabited the MG during the time worn (Table 2).

The number of CFU/g/hour of MG wear ranged from 5.50×10^4 to 1.58×10^7. There was no statistical significance between the number of hours a MG was worn and the number of colony forming units with which it was inhabited when worn for 12 to 24 hours ($p=0.1414$).

Table 2. The Rate of Colonization of the Mouth-Guards

Hours Worn	Mouth-Guard	CFU/g/hour
12	MG-06	$8.47x10^5$
12	MG-10	$1.15x10^6$
12.5	MG-09	$8.06x10^5$
13	MG-04	$2.53x10^5$
14	MG-08	$6.80x10^4$
15	MG-01	$1.58x10^7$
16	MG-03	$5.50x10^4$
20	MG-07	$9.77x10^5$
24	MG-02	$8.84x10^6$
24	MG-05	$1.07x10^6$

Note: no serial dilutions were performed for MG-11

Identification of Microorganisms from Worn Mouth-Guards

A total of 181 isolates were collected from the pre-treatment touch plates of the 11 MG worn by the OSU-CHS students. From these, six isolates could not be recultured for identification and eight isolates had API profiles that were unidentifiable. Identification of the other 167 isolates yielded a total of 42 distinct microbial species as detailed in Table 3.

On average, 15 isolates were collected for identification from each MG and each MG harbored an average of eight different microbial species. Over seventy percent of the isolates collected and identified came from four main genera: *Arcanobacterium, Neisseria, Staphylococcus,* and *Streptococcus*. Each of these four common genera was present on at least seven MG. There was no *B. cereus* cultured from the OSU-CHS student MG.

Table 3. Species Identified from Worn Mouth-Guards. A list of species isolated from the 11 MG worn by OSU-CHS students.

Gram Positive Cocci	Gram Positive Rods
1. *Aerococcus viridans* (2) 2. *Gemella morbillorum* (1) 3. *Kocuria kristinae* (1) 4. *Lactococcus lactis* (2) 5. *Leuconostoc spp* (4) 6. *Micrococcus spp* (2) 7. *Staphylococcus spp* (5) 8. *Staphylococcus aureus* (5) 9. *Staphylococcus auricularis* (1) 10. *Staphylococcus conhii* (1) 11. *Staphylococcus epidermidis* (4) 12. *Staphylococcus hominis* (1) 13. *Staphylococcus xylosus* (2) 14. *Streptococcus spp* (5) 15. *Streptococcus bovis II* (3) 16. *Streptococcus pneumoniae* (1) 17. *Streptococcus salivarius* (1) 18. *Streptococcus sanguis* (1) 19. *Streptococcus uberis* (1)	1. *Actinomyces spp* (1) 2. *Actinomyces turicensis* (1) 3. *Arcanobacterium haemolyticum* (9) 4. *Brevibacterium spp* (3) 5. *Cellulomonas/Microbacterium spp* (2) 6. *Corynebacterium spp* (3) 7. *Corynebacterium aquaticum* (1) 8. *Corynebacerium diphtheriae* (1) 9. *Corynebacterium pseudodiphtheriticum* (1) 10. *Corynebacterium striatum/amycolatum* (1) 11. *Rothia dentocariosa* (2)
Gram Negative Cocci	Gram Negative Rods
1. *Branhamella catarrhalis* (1) 2. *Neisseria spp* (6) 3. *Neisseria cinerea* (6) 4. *Neisseria gonorrhoeae* (1) 5. *Neisseria meningitidis* (3) 6. *Neisseria polysaccharea* (1) 7. *Neisseria sicca* (2) 8. *Neisseria subflava* (1)	1. *Chryseobacterium indologenes* (1) 2. *Chryseomonas luteola* (1) 3. *Moraxella spp* (1) 4. *Pasteurella haemolytica* (1)

Note: Numbers in parentheses are the number of MG on which the species were found.

Interactions of *Bacillus cereus* and Individual Mouth-Guard Microorganisms

B. cereus strains Whimpy and Arnold were grown in wells punched in TSA-II labeled A and B respectively. These *B. cereus*-filled wells were surrounded by streaked lawns of individual bacterial species from the MG in order to test the inhibitory effects *B. cereus* has on specific microorganisms. The amount of inhibition of the lawn and *B. cereus* growth onto the streaked TSA-II was measured in mm with a ruler and recorded.

Sixty-eight isolates were randomly selected to be streaked as lawns on TSA-II and subjected to the Whimpy and Arnold treatments. Of these chosen, five isolates could not be recultured and one had an API profile that was unidentifiable. The remaining 62 isolates were comprised of 28 different bacterial species as outlined in Table 4.

Table 4. Species Streaked as Lawns on TSA-II.

Gram Positive Cocci	Gram Positive Rods
1. *Aerococcus viridans* (1) 2. *Gemella morbillorum* (1) 3. *Kocuria kristinae* (1) 4. *Lactococcus lactis* (1) 5. *Leuconostoc spp* (3) 6. *Micrococcus spp* (1) 7. *Staphylococcus spp* (2) 8. *Staphylococcus aureus* (3) 9. *Staphylococcus epidermidis* (2) 10. *Staphylococcus xylosus* (1) 11. *Streptococcus spp* (4) 12. *Streptococcus bovis II* (1) 13. *Streptococcus salivarius* (2) 14. *Streptococcus uberis* (1)	1. *Actinomyces turicensis* (1) 2. *Arcanobacterium haemolyticum* (11) 3. *Brevibacterium spp* (2) 4. *Cellulomonas/Microbacterium spp* (1) 5. *Corynebacterium spp* (2) 6. *Corynebacterium striatum/amycolatum* (1) 7. *Rothia dentocariosa* (2)
Gram Negative Cocci	**Gram Negative Rods**
1. *Neisseria spp* (5) 2. *Neisseria cinerea* (6) 3. *Neisseria meningitidis* (1) 4. *Neisseria sicca* (3)	1. *Chryseobacterium indologenes* (1) 2. *Chryseomonas luteola* (1) 3. *Pasteurella haemolytica* (1)

Note: The numbers in parentheses are the numbers of isolates of that species which were chosen to be streaked as lawns.

Complete Inhibition of the Lawn Bacteria

Of the 62 lawns made, nine had measurable complete inhibition around the *B. cereus* strain wells (Table 5). The complete inhibition of the lawn was defined as a measurable area around any treatment well where no lawn colonies were observed to grow.

Table 5. Complete Inhibition of the Lawn Bacteria by *B. cereus*. The diameter (in mm) of the zone of complete inhibition (seen as no lawn growth) around the Whimpy and Arnold wells A and B.

Lawn Species (MG it was from)	Whimpy	Arnold
Arcanobacterium haemolyticum (MG-02)	0	19
Arcanobacterium haemolyticum (MG-03)	0	19
Arcanobacterium haemolyticum (MG-04)	0	14
Streptococcus spp (MG-02)	0	21
Aerococcus viridans (MG-02)	0	21
Brevibacterium spp (MG-03)	0	22
Corynebacterium spp (MG-001)	0	22
Leuconostoc spp (MG-01)	0	18
Rothia dentocariosa (MG-02)	0	11

Though seven different species showed inhibitory effects against the Arnold strain, not every lawn of these species had a measurable zone of complete inhibition around *B. cereus*. However, all isolates that were noticeably inhibited did so around the Arnold strain of *B. cereus* with an average of about 18mm in diameter. No complete inhibition was noted around the Whimpy strain for any lawn.

Partial Inhibition of the Lawn Bacteria

For some isolates there was a measurable area around wells A and B where the lawn colonies grew noticeably smaller in size (partial inhibition). The measure of this partial inhibition of the lawn growth around Whimpy and Arnold is detailed in Table 6.

Eleven lawns comprised of eight different species had measurable zones of partial inhibition. Though these isolated species demonstrated inhibitory effects against the Arnold strain, not every lawn of these species had a measurable zone of partial inhibition around *B. cereus*. However, there were no zones of partial inhibition around the wells of any Whimpy strains. The average diameter of the partial inhibition was 15mm.

Table 6. Partial Inhibition of the Lawn Bacteria by *B. cereus*. The diameter (in mm) of the zone of partial inhibition (seen as smaller colonies of lawn growth) around the Whimpy and Arnold strain wells.

Lawn Species (MG it was from)	Whimpy	Arnold
Arcanobacterium haemolyticum (MG-03)	0	21
Arcanobacterium haemolyticum (MG-04)	0	16
Arcanobacterium haemolyticum (MG-06)	0	12
Streptococcus spp (MG-02)	0	23
Aerococcus viridans (MG-02)	0	23
Brevibacterium spp (MG-04)	0	16
Lactococcus lactis (MG-03)	0	18
Leuconostoc spp (MG-01)	0	11
Leuconostoc spp (MG-05)	0	11
Pasteurella haemolytica (MG-03)	0	17
Rothia dentocariosa (MG-02)	0	13

The bacteria *Aerococcus viridans, Rothia dentocariosa, and Streptococcus spp* on MG-02, *Arcanobacterium haemolyticum* on MG-03, and *Arcanobacterium haemolyticum* on MG-04 exhibited a zone of complete inhibition around the growth of Arnold and a zone of partial inhibition outside of that. The other lawns from Tables 5 and 6 only had the complete or partial inhibition, not both, around the Arnold strain growth.

Inhibition of the Lawn Bacteria within the Growth of Bacillus cereus

For all 62 lawns tested, slides were prepared of both *B. cereus* strains and the lawn bacteria. This was done to visualize whether the lawn bacteria that were streaked on the plates before the addition of *B. cereus* into its treatment wells was still present after *B. cereus* grew onto the surface of the agar plate or whether growth of the lawn was inhibited here as well. The Whimpy and Arnold bacteria were collected via an inoculating loop touched to the top edge of the wells in which they grew. The lawn bacteria were collected via an inoculating loop touched to the center of the plate. The slides of the bacterial cultures were observed with a microscope and the presence or

absence of the lawn bacteria within the samples taken from the *B. cereus* growth was recorded. From these slides, it was apparent that some lawn species did not grow in the presence of *B. cereus* while others were present up to the well edge regardless of the growth of the Whimpy or Arnold strain (Table 7).

Table 7. Inhibition of the Lawn Bacteria within *B. cereus* Growth. The percentage of the lawns from the specified genus that were inhibited in the presence of the Whimpy and Arnold bacteria.

Genus (# of isolates)	Whimpy	Arnold
Arcanobacterium (11)	5 (45%)	9 (82%)
Neisseria (14)	3 (21%)	7 (50%)
Staphylococcus (7)	0 (0%)	0 (0%)
Streptococcus (7)	0 (0%)	3 (43%)
All identifiable lawn isolates (62)	15 (24%)	30 (48%)

Of the four main genera represented by the OSU-CHS student MG, the Arnold strain had a stronger inhibitory effect on the growth of the lawn up to the *B. cereus* well edge for *Arcanobacterium*, *Neisseria,* and *Streptococcus*. This effect was significant for *Arcanobacterium* ($p=0.0190$) and *Streptococcus* ($p=0.0398$). All *Staphylococcus spp* made into lawns were not inhibited by either *B. cereus* strain. This was evident from the lack of complete or partial inhibition around the *B. cereus* growth as well as *Staphylococcus spp* being present within all *B. cereus* growth on the plates. Likewise, though some *Neisseria spp* were present in the *B. cereus* cultures from the well edges, none of these isolates produced measurable zones of complete or partial inhibition around the Whimpy or Arnold strain wells.

For the other species, there were too few replicates per genus (three or less) to make any notable observations concerning the inhibition by the *B. cereus* growth.

However, when all of the species are analyzed collectively, the Arnold strain is more likely to inhibit the growth of the lawn up to the *B. cereus* well edge on the TSA-II more often than the Whimpy strain ($p<0.0001$). Of the 62 lawns made and tested, 48% were inhibited by the growth of the Arnold strain while only 22% were inhibited by the Whimpy strain.

It should be noted that all isolates with measurable zones of complete inhibition around the growth of the Arnold strains were not present within the Arnold cultures taken from the edge of the TSA-II wells. Of the isolates with measurable incomplete hemolysis around the Arnold strain growth, almost half (46%) were absent in the cultures of the Arnold strain taken from the edge of the TSA-II wells.

Inhibition of the Lawn Bacteria by Bacillus cereus Culture Filtrates

The CF of *B. cereus* also produced some inhibition among the lawn bacteria, however the effects were much less frequent and less pronounced than those produced from the Arnold and Whimpy bacteria themselves. A table of the complete and partial inhibition of the lawn species is detailed in Table 8.

Only five isolates had measurable zones of inhibition around the Whimpy and Arnold treatment wells C and D respectively. No other isolates of these species that were tested showed any measurable inhibition to the *B. cereus* CF. Of these five isolates, *Rothia dentocariosa* from MG-02 was the only one affected by the Whimpy CF and did not show any effect in response to the Arnold CF. It had a measurable zone of complete inhibition of 9mm in diameter around the Whimpy CF well. Three other isolates (*Corynebacterium spp, Kocuria kristinae,* and *Corynebacterium striatum/amycolatum*)

exhibited complete inhibition to the Arnold CF. The inhibitory effect of the Arnold CF on the lawn bacteria was greater than that achieved by the Whimpy CF as seen by the larger diameter of the complete inhibition zones. One isolate of *Neisseria spp* demonstrated a zone of partial inhibition around the Arnold CF well only. There was no partial inhibition observed around any Whimpy CF wells for any lawns.

Table 8. Complete and Partial Inhibition of the Lawn Bacteria by *B. cereus* Culture Filtrates. The measure (in mm) of the diameters of complete and partial inhibition of the lawn by the Whimpy and Arnold CF.

Lawn Species	Complete Inhibition by Whimpy CF (mm)	Complete Inhibition by Arnold CF (mm)	Partial Inhibition by Whimpy CF (mm)	Partial Inhibition by Arnold CF (mm)
Corynebacterium spp (MG-001)	0	14	0	0
Neisseria spp (MG-01)	0	0	0	8
Rothia dentocariosa (MG-02)	9	0	0	0
Kocuria kristinae (MG-07)	0	11	0	0
Corynebacterium striatum/amycolatum (MG-08)	0	13	0	0

Inhibition of Bacillus cereus by the Lawn Bacteria

Measurements of the diameter of *B. cereus* growth around treatment wells A and B on the streaked TSA-II were also recorded. The average diameter of the *B. cereus* growth on lawns of each species is listed in Table 9. Out of the 28 species studied, the Arnold strain did exhibit a smaller diameter of growth for more species (14) than the Whimpy strain (10). Only for lawns of the *Neisseria* genus was the diameter of the Arnold strain growth significantly smaller than the diameter of growth for the Whimpy

strain (p=0.0069). Generally though, there was no difference between the diameter of growth of the Whimpy and Arnold strains on the lawns (p=0.2414).

Table 9. The Average Diameter (in mm) of *B. cereus* Growth on the Lawns.

	Whimpy	Arnold
*Actinomyces turicensis**	9	11
*Aerococcus viridans**	15	13
Arcanobacterium haemolyticum	11	11
Brevibacterium spp	13	13
*Cellulomonas spp/Microbacterium spp**	14	10
*Chryseobacterium indologenes**	6	6
*Chryseomonas luteola**	6	5
Corynebacterium spp	9	11
*Corynebacterium striatum/amycolatum**	12	11
*Gemella morbillorum**	11	12
*Kocuria kristinae**	8	9
*Lactococcus lactis**	13	14
Leuconostoc spp	11	9
*Micrococcus spp**	13	16
Neisseria cinerea	11	8
*Neisseria meningitidis**	9	7
Neisseria sicca	9	7
Neisseria spp	13	7
*Pasteurella haemolytica**	9	13
Rothia dentocariosa	9	10
Staphylococcus aureus	8	6
Staphylococcus epidermidis	8	8
Staphylococcus spp	8	10
*Staphylococcus xylosus**	9	7
*Streptococcus bovis II**	9	10
Streptococcus salivarius	11	10
Streptococcus spp	13	11
*Streptococcus uberis**	10	9

* Only one isolate of the species was made into a lawn, and therefore the average values depicted are the actual measurements from that one lawn.

Other Changes in Bacillus cereus Growth

The lawn bacteria also altered the morphology of the *B. cereus* growth on the agar. For 9 of the 63 lawns, the *B. cereus* growth was very diffuse and opaque in appearance (Figure 7). All nine of these lawns had abnormal *B. cereus* growth for the Whimpy strain, while only two had an effect on the Arnold strain as well. Six of the nine lawns causing this opaque growth were streaked with a *Neisseria* spp. The other three lawns were of two *Staphylococcus* strains and one *Streptococcus* strain. Gram stains performed on the *B. cereus* with this abnormal growth revealed no morphological difference from other *B. cereus* isolates.

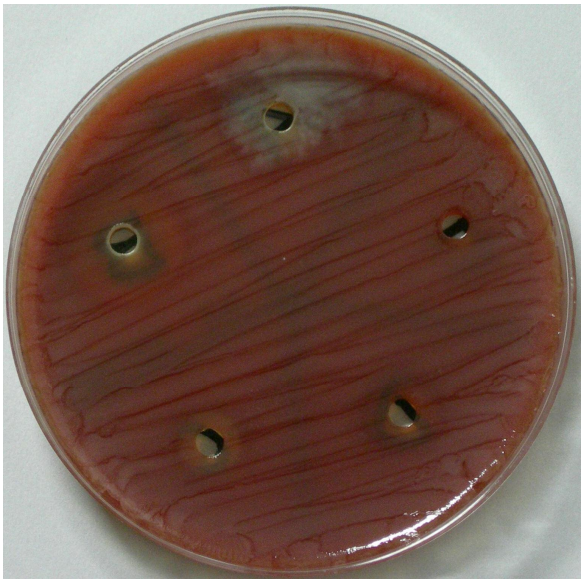

Figure 7. An Example of Opaque Growth of *B. cereus* on a Lawn. Opaque growth is seen at the noon position (Whimpy strain well A); normal *B. cereus* growth is at 10 o'clock (Arnold strain well B).

Growth Curves

The collection of microorganisms from each OSU-CHS student MG was grown for 24 hours in suspensions of *B. cereus* strains and CF. The absorbance of these suspensions was recorded at regular intervals to produce growth curves for each *B. cereus* treatment. Growth curve data for MG-01, MG-02, and MG-11 were not recorded and therefore are not represented in these results.

The growth curves from the MG microorganisms inoculated with the *B. cereus* strains (treatments A and B) were compared to the respective *B. cereus* growth curve and the growth curve of the MG microorganisms by themselves as controls. Data from these analyses could not be meaningfully interpreted since it was not possible to separate the *B. cereus* growth from the MG bacteria growth when the cultures were combined.

However, data from the inoculation of MG bacteria with the *B. cereus* CF (treatments C and D) during growth curves could be analyzed. For the *in vitro* growth curves, bacteria from each MG were cultured in BHI and subjected to the *B. cereus* CF treatments. For the *in vivo* growth curves, the actual worn MG pieces were introduced to the *B. cereus* CF treatments in BHI. Growth curves (Appendix B) from these solutions were compared to a control to assess the significance that the *B. cereus* toxins have on the growth of MG microorganisms.

Significant differences between the CF treatment treatments and MG controls were observed for three different parameters: the endpoint absorbance (E), the midpoint (M), and the slope (S) of the growth curve. The endpoint absorbance is the last optical density value recorded and represents the cell density at stationary phase after 24 hours of growth. The midpoint is the time at which the optical density of bacterial growth is

halfway between the starting point (zero) and the endpoint absorbance. The slope of the growth curve is representative of the growth rate. A chart showing the types of significant differences observed for all MG when compared to controls is listed in Table 10.

Table 10. The Effects of *B. cereus* Culture Filtrates on the Growth of Mouth-Guard Bacteria *in vitro* and *in vivo*.

MG	*In vitro*		*In vivo*	
	Whimpy CF	Arnold CF	Whimpy CF	Arnold CF
03	E M S	E M S	E	-
p-value	< 0.0001	< 0.0001	= 0.0405	-
04	E S	E	E M	E M S
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
05	E M	E M	-	E
p-value	< 0.0001	< 0.0001	-	= 0.0036
06	M	-	E M S	E M S
p-value	= 0.0202	-	< 0.0001	< 0.0001
07	S	E	M S	M S
p-value	= 0.0077	< 0.0001	< 0.0001	< 0.0001
08	E M	E M S	M S	M
p-value	< 0.0001	< 0.0001	< 0.0001	< 0.0001
09	M	M S	E M	M
p-value	= 0.0490	= 0.0065	< 0.0001	< 0.0001
10	E	E M	E	E M
p-value	= 0.0228	= 0.0053	< 0.0001	< 0.0001

- = no significant difference was seen between the treatment and control
E = endpoint absorbance was significantly different than the control
M = midpoint was significantly different than the control
S = slope was significantly different than the control

The *B. cereus* CF caused significant changes for at least three of the four growth curves that were recorded for each MG. However, there were three instances overall where there were no significant differences between the growth curves of the treatment conditions and the control. One was in the Arnold *in vitro* CF treatment for MG-06; one

was in the Arnold *in vivo* CF treatment for MG-03; and one was the Whimpy *in vivo* CF treatment for MG-05.

The CF of *B. cereus* caused similar significant changes among the growth curves of each individual MG, consistently affecting the endpoint absorbance, midpoint, and/or the slope of the curves. This effect was regardless of the *in vitro* or *in vivo* condition or which *B. cereus* CF treatment was used. However, both the *B. cereus* CF had more of an effect on the endpoint absorbance and midpoint than the slope of the MG bacteria growth curves. In general, the *B. cereus* CF tended to reduce the endpoint absorbance (70% of the time) and increase the midpoint (62% of the time) of the MG growth curves. Although the CF did significantly alter the slope of the growth curves, they did not consistently increase or decrease this value. There was no substantial difference between the effects that the Whimpy CF had on the MG growth curves and those caused by the Arnold CF.

Among the MG controls, the MG bacteria tended to grow to a higher capacity *in vivo* than *in vitro*; only for MG-09 was the endpoint absorbance value higher *in vitro*. All other MG controls had a significantly higher *in vivo* endpoint absorbance than *in vitro* ($p=0.0064$). Thus, the bacteria from each MG were at a higher concentration at stationary phase when the worn MG was present than when just the bacteria from the MG were grown in BHI.

For the most part, the MG growth curves also had a lower endpoint absorbance than the individual bacterial species that made them up when grown separately. An example of this can be seen with MG-04 (Figure 8), which is typical of other MG growth curves. As noted before, the *in vivo* growth curve for MG-04 had a higher endpoint

absorbance than the *in vitro* growth curve. The endpoint absorbances for *S. conhii*, *Streptococcus spp*, *N. meningitidis*, and *Neisseria spp* were all higher than the endpoint absorbance of both the *in vitro* and *in vivo* MG-04 growth curves.

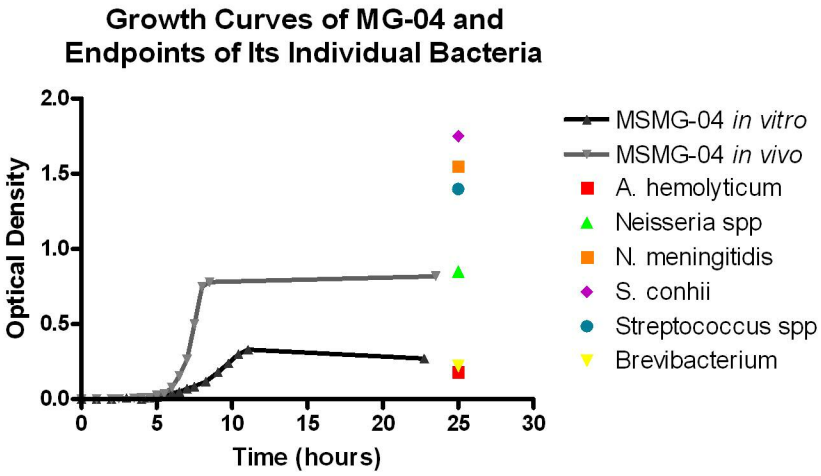

Figure 8. Growth Curves of a Mouth-Guard and Endpoints of Its Individual Bacteria. The *in vivo* and *in vitro* growth curves of MG-04 with the endpoint absorbances of its individual bacterial species.

Biofilms formed on MG pieces and in the flasks during incubation for the growth curves (Figure 9). This precipitation in BHI was noted in the *in vitro* MG-07 flasks C, D, and E (Whimpy CF treatment, Arnold CF treatment, and MG control respectively) and the *in vivo* flasks C, D, and E for MG-03, MG-05, MG-06, MG-07, and MG-09. MG-04 only had this precipitous growth in flasks C and D, while for MG-08 and MG-10, it was only present in flask D. The biofilm formation on the MG pieces was only present from the flasks with the bacterial precipitation.

Figure 9. Examples of the Precipitation of Mouth-Guard Bacteria. It was present in liquid media (left) and on the MG itself (right).

The pre- and post-treatment serial dilutions performed for the *in vitro* growth curves as well as the *in vivo* pre- and post-treatment surface and depth touch plates helped shed light on the effects that the *B. cereus* treatments were having on the MG microorganisms. Appendix C documents the serial dilutions and touch plate results from MG-07, however these pictures are typical of what was found in the other MG as well.

Figure C-1 (Appendix C) is a pre-treatment serial dilution showing the bacteria present in the MG after being worn in the mouth for 12-24 hours. The different colonial morphologies are indicative of different bacterial species. Figure C-2 (Appendix C) shows a serial dilution of the same MG after being incubated at 37^{o}C in BHI for 24 hours. This was a post-treatment control. The concentration of bacteria in the MG was higher post-treatment ($3.5x10^{8}$) than pre-treatment ($9.8x10^{5}$). There was also a difference in the types of bacteria present between these two plates, as noted by the difference in the sizes and colors of the bacteria growing on the agar.

Figures C-3 and C-4 (Appendix C) show post-treatment serial dilutions after the MG bacteria were inoculated with the Whimpy and Arnold strains respectively and

allowed to grow for 24 hours. There were no observable MG bacteria present on these post-treatment plates, however there was abundant *B. cereus* growth.

Figures C-5 and C-6 (Appendix C) show post-treatment serial dilutions after the MG bacteria were introduced to the Whimpy and Arnold CF respectively. The bacterial growth from the Whimpy CF treatment was visually similar to the post-treatment control in that the colors and sizes of the bacteria growing were comparable. However, the concentration of these bacteria was about ten times less for the Whimpy CF treatment ($3.8x10^7$) than the control ($3.5x10^8$).

The concentration of the MG bacteria exposed to the Arnold CF was similar to that of the control ($5.0x10^8$), however there was a difference in the types of bacteria present. The control plate showed an abundance of small, grey, α hemolytic colonies and a few medium white colonies. The plates of the Arnold CF treatment showed comparatively less of the small, grey, α hemolytic colonies and more of the medium white colonies. In addition, the presence of larger grey colonies was noted in the Arnold CF post-treatment serial dilutions and was not seen in the plates from the post-treatment Whimpy CF or the control.

The surface and depth touch plates from the *in vivo* experiment also documented the effects of the different *B. cereus* treatments. Figure C-7 (Appendix C) shows the bacteria present on the surface and in the depths of MG-07 pre-treatment. There was no observable difference between the bacteria present where the surface of the MG was touched versus where the depth of the MG was touched. On visual inspection, these bacteria were similar to the ones on the pre-treatment serial dilution plate.

Figure C-8 (Appendix C) depicts the bacteria present on the surfaces and depths of the MG pieces after being subjected to their respective treatments. The surfaces were touched on the left side of the agar plates, the depths were touched on the right. The five touches per side are from MG sections A, B, C, D, and E and are represented as such from top to bottom.

Therefore, the top two touches were from the Whimpy and Arnold bacterial treatments A and B respectively (Figure C-8). There were no MG bacteria observed here, however there was abundant *B. cereus* growth for the surfaces and depths of both strains.

The growth from the surface and depth touches of the MG piece subjected to treatment C had fewer of the medium, white colonies and none of the larger, yellow colonies seen in the control (E), which is the bottom surface and depth touch on the plate (Figure C-8). As in the serial dilutions, there was also less confluence of bacterial growth in the Whimpy CF treatment than the control.

Like the serial dilutions, larger white colonies were seen growing from surface and depth touches from the MG piece subjected to treatment D that were not present in the control (Figure C-8). There was also some *B. cereus* contamination on the plate where the surface of this MG piece was touched.

CHAPTER IV

DISCUSSION

Bacillus cereus Controls and Toxin Production

Growth curves of *B. cereus* were made and compared to the toxin production of both strains. The growth curves of Whimpy and Arnold were not significantly different; from hours zero to three was the lag phase, from hours three to six was the log phase, and from hours six to 24 was stationary phase.

Results from hemolysis and phospholipase C production from the *B. cereus* CF demonstrated that these toxins are present in both strains. Both *B. cereus* strains produced hemolysin and phospholipase C on TSA and MYP respectively after about five hours of growth as demonstrated from the appearance of hemolysis and lecithinase from CF collected at this time. This time frame suggests that the majority of toxins were being produced either at the end of the growth phase or beginning of the lag phase for both strains. This is consistent with other studies looking at the production of diarrheal toxins by *B. cereus* (Granum & Lund, 1997; Fermanian et al., 1995).

Contamination of Mouth-Guards

When new MG were opened and touched to TSA-II, there were no bacteria, yeasts, or molds present after 24 hours of incubation. However, this is not to say that MG

are aseptic before they are worn. Contamination can easily occur before the MG is packaged and distributed (Figure 10).

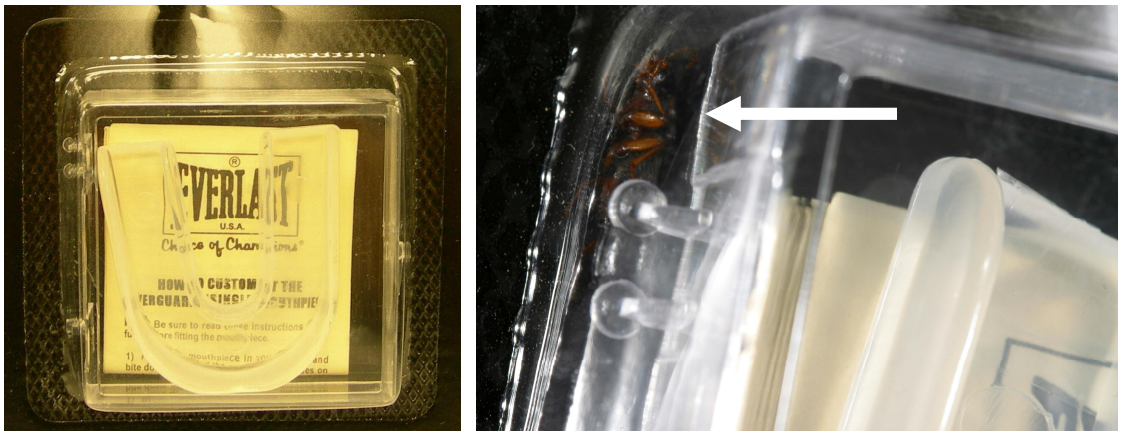

Figure 10. Pictures of Mouth-Guard Contamination. The photograph on the left is an overall view of a packaged MG. The photograph on the right is a close-up of a roach inside the packaging (white arrow).

With this in mind, it is not improbable that someone could intentionally contaminate pre-packaged MG with a bioweapon such as *B. anthracis*. Though *B. cereus* has been found in the used MG of athletes (Glass, Bullard, & Conrad, 2006) there has been no research to date that has looked at the ability of MG to be purposefully contaminated with a bioagent. The present study shows that when newly opened MG are suspended in *B. cereus* inoculated liquid media, they can be contaminated with *B. cereus*. This contamination occurred not only three days after the MG had been removed from the Whimpy and Arnold solutions, but also after being submerged in boiling water for 12 seconds. Thus, the act of molding such a contaminated MG to the mouth could transmit the microorganisms to the oral cavity, allowing for ingestion and/or inhalation of the bacteria.

The TSA-II plates of the *Pseudomonas* contaminated MG also had growth after the MG were boiled, however the number of colonies present was less than that from

either the Whimpy or Arnold strain. This is not surprising since although *B. cereus* vegetative cells are readily killed by heat (Byrne, B. Dunne, G., & Bolton, D.J., 2006), the spores are relatively heat resistant. In distilled water, *B. cereus* spores can withstand temperatures of 95^{o}C from 1.5 to 36.2 minutes depending on the strain (FDA, 2000). Thus, the reduced number of *Pseudomonas* colonies may be attributed to the absence of spores, and therefore only the few surviving vegetative cells were present on the agar.

The resistance of the *B. cereus* spores to heat may also explain why *B. cereus* was cultured from the steam of the Arnold and Whimpy contaminated MG while the *Pseudomonas* steam plates were free of bacterial growth. Though both *B. cereus* and *Pseudomonas* were present on the post-treatment touch plates, the ability for *B. cereus* to form spores may have aided in its transportation and/or survival in the steam. This finding raises new cautions about the ability of *B. cereus*, and possibly *B. anthracis*, to be spread to the masses.

Collection and Identification of Bacteria from Worn Mouth-Guards

Eleven MG were worn and collected from students of the Oklahoma State University Center for Health Sciences. An average of eight different bacterial species was cultured from each MG. The number of hours a MG was worn was not significantly related to the concentration of bacteria per gram of MG material regardless of whether the MG was worn for 12 or 24 hours. This indicates that it takes less than 12 hours of MG wear for it to be colonized to capacity by the specific microorganisms *in vivo*.

The wide range seen in the concentration of bacteria in the MG may be attributed to the differences in the "normal" oral flora of the student volunteers, how well the

individual bacterial species can flourish in the MG material, or the order in which the different species colonize the MG. Variations in the "normal" oral flora of the OSU-CHS student participants were reflected in the differences of bacterial profiles between MG. Since individual bacterial species within a MG were shown to have a wide range of stationary phase concentrations (Figure 8), the differences in the CFU/g between MG may be attributed to the differences in bacterial species that make up each profile. Thus, some MG may have more bacteria with lower stationary phase concentrations while other MG may have more bacterial species that have higher stationary phase concentrations. Likewise, some bacteria may be better able to proliferate in the MG matrix than others. Thus, how well the bacteria flourish in the MG can alter the CFU/g of each MG depending on the bacterial profile of each student.

The order in which the different bacterial species colonize a MG could also contribute to the differences seen in concentrations among MG. *Streptococcus spp* have been found to bind to salivary proteins and thus may be pioneer bacteria that are the first to adhere to the MG. Other early colonizers include species of *Actinomyces* and *Neisseria*. After this initial community is setup, protein-protein interactions and metabolic associations between bacterial species continue to attract other bacterial species (Jenkinson & Lamont, 2005). Conversely, differences in the presence of certain species can attract or repel other bacteria to the biofilm. For example, the adherence of *Streptococcus sanguinis* to newly erupted baby teeth delays the colonization of *Streptococcus mutans*, which is known to cause tooth decay (Caufield et al., 2000). Thus, the types of bacteria present in the mouth and the order in which they inhabit the MG may affect the CFU/g and cause the differences in profiles observed.

Of the 181 isolates that were taken from the worn MG, 42 different species were identified. Unlike football player MG, but like the oral swabs of first-year medical students (Glass, Bullard, & Conrad, 2006), the OSU-CHS student MG did not contain any strains of *B. cereus*. Rather, the species identified from the OSU-CHS student MG consisted of nineteen gram positive cocci, eight gram negative cocci, eleven gram positive rods, and four gram negative rods. Although oral swabs from first-year medical students did not yield any gram negative cocci (Glass, Bullard, & Conrad, 2006), the bacteria identified from the OSU-CHS student MG are comparable to those normally found in the mouth.

There were four predominating genera of bacteria represented on at least seven different MG: *Arcanobacterium, Neisseria, Staphylococcus,* and *Streptococcus*. While most MG had species from all four of these genera, no two MG had the same bacterial profile.

Interactions of *Bacillus cereus* and Individual Mouth-Guard Microorganisms

Of the 62 isolates studied, only the *B. cereus* Arnold strain completely or partially inhibited the growth of the individual MG species on TSA-II. When looking for the presence of the lawn bacteria within the growth of the *B. cereus* strains on TSA-II, the Arnold strain inhibited the lawn growth in this location significantly more often than the Whimpy strain. Furthermore, the Arnold CF produced inhibitory effects of the lawns four times more often than the Whimpy CF.

The lawn bacteria were observed to be completely or partially inhibited where there was no *B. cereus* colony growth. This occurred either beyond the growth of the

Whimpy and Arnold strains in their wells or around the wells where the CF were housed. Thus, these inhibitory effects were most likely from the toxin production of the bacteria.

The experiments with the individual lawn bacteria suggested that there is dilution and/or resistance to the *B. cereus* toxins at some point as indicated by the presence of complete and partial inhibition of the lawns. The Arnold and Whimpy strains and CF only caused measurable amounts of complete and partial inhibition of the lawns away from the *B. cereus* growth for a few isolates. However, almost half of the isolates tested were not present at the well edge where the *B. cereus* actually grew onto the agar. This increase in the number of isolates that are inhibited as one gets closer to the *B. cereus* growth is consistent with the dilution of and/or resistance to the toxins being produced. The different species making up the lawns most likely contribute to the differences seen in the measured amount of inhibition, the type of inhibition, or the complete lack thereof. However, the lawns were inhibited more often and in larger diameters by the Arnold strain than by the Arnold CF alone. This suggests that either there was a higher concentration of bactericidal toxins produced by the Arnold strain placed in the well than the stock CF placed in the well, or that the presence of *B. cereus* coupled with its toxin production produced a greater inhibitory effect. Competitive inhibition is also a relevant factor when *B. cereus* is present with the individual lawn species, as both bacteria would vie for the limited amount of nutrients available in the agar.

Not only does *B. cereus* effect the growth of the lawns, but the lawn species also appear to affect the size and morphology of the growth of *B. cereus*. When all lawn species were grown together, there was no significant difference between the diameters of growth of the *B. cereus* strains on the lawns. However, the Arnold strain had a smaller

growth diameter than the Whimpy strain for more lawn isolates. This suggests that the Arnold strain is responding to the MG bacteria by producing more toxins and thus producing the larger inhibitory effect observed.

Atypical *B. cereus* growth was also noted when grown on selective lawns. Nine lawns consisting of six *Neisseria spp*, two *Staphylococcus spp*, and one *Streptococcus spp* produced a more diffuse, opaque growth of the Whimpy strain onto the agar surface. The Arnold strain produced this abnormal growth for only two of the nine lawns: one *Neisseria spp* and one *Staphylococcus spp*. The susceptibility of the Whimpy strain to be altered by the lawn bacteria may be related to its reduced ability to inhibit the lawns when compared to the Arnold strain. It appears that when a *B. cereus* strain is not strong enough to effectively inhibit a certain bacterial species, that species may be strong enough to have some inhibitory effect on *B. cereus*. For example, the *Neisseria spp* and *Staphylococcus spp* in particular are resistant to *B. cereus* strains Whimpy and Arnold. Species of both genera do not exhibit complete nor partial inhibition to either *B. cereus* strain or their CF on TSA-II. All isolates of the *Staphylococcus spp* were also present at the edge of the agar wells where both the Whimpy and Arnold strains were growing, indicating that they can grow even in the presence of *B. cereus*. Also, only for lawns streaked with species of the *Neisseria* genus was the diameter of the Arnold strain growth significantly smaller than Whimpy. This implies that the growth of the Arnold strain is being inhibited by *Neisseria spp*.

Results such as these that provide evidence against the deleterious effects of *B. cereus* are noteworthy when the similarities to *B. anthracis* are taken into consideration. If certain species of bacteria are less susceptible to the toxins of *B. cereus*, it is possible

that these same species or others very similar are also less susceptible to the toxins of *B. anthracis*.

Growth Curves

The growth curves were not suitable for studying the effects that the *B. cereus* bacteria had on the growth of the MG bacteria. However, growth curves for MG strains cultured in medium containing *B. cereus* CF could be produced and analyzed.

The growth curves of each MG were similarly affected by the CF of *B. cereus*, regardless of the *in vitro* or *in vivo* condition. The growth curves also demonstrated the consistency of the MG bacteria to react similarly to the *B. cereus* CF regardless of which strain's CF was used. This is unlike data from the lawn experiment, where the Arnold CF produced inhibition more often than the Whimpy CF. The reason for this is most likely the difference in the number of MG bacteria being tested in each experiment. For the lawn experiment, isolated MG species were tested for inhibition to the *B. cereus* CF. For the growth curves, it was the collection of all bacteria from a MG that was subjected to the *B. cereus* CF. In addition, the different inhibitory effects between the *B. cereus* CF were only noted for five out of sixty-two isolates that were streaked as lawns; most single MG species were not noticeably affected by the *B. cereus* CF alone. Therefore, while the Arnold CF had more of an inhibitory effect than the Whimpy CF for the few isolated MG species that were inhibited, these effects were not noticeable when multiple MG bacteria were present.

When the growth curves of all MG were examined together, there were no differences observed between the effects of the Whimpy and Arnold CF. Both the

Whimpy and Arnold CF significantly decreased the endpoint absorbances and increased the midpoints of the MG growth curves more often, as would be expected. The decrease in the endpoint absorbance was indicative of a lower bacterial cell density at stationary phase. Therefore, the *B. cereus* toxins were inhibiting the amount of bacteria that could grow or were eliminating certain strains from the population. Likewise, the larger midpoint value signified either a lag in the start time of the MG bacterial growth or coincided with a slower growth rate. Either way, the *B. cereus* toxins tended to have an adverse effect on the growth of the MG bacteria collectively. These results are consistent with other research that documents *B. cereus* probiotic activities against harmful intestinal bacteria (Duc et al., 2004). However, the disease-producing toxins of *B. cereus* would likely hamper its use for humans as an antimicrobial agent.

Among the MG growth curve controls, endpoint absorbances measured during the *in vivo* growth curves were significantly higher than those from the *in vitro* growth curves for all but MG-09. It is likely that the concentration of bacteria in the MG pieces was higher than the initial concentration of MG bacteria added for the *in vitro* experiment. The presence of the MG piece may also have allowed some bacteria a sheltered niche in which to survive, and therefore alter the concentration of bacteria at the end of 24 hours of growth. Likewise, the lower endpoint absorbances observed for the MG bacteria collectively versus the individual species could be due to competitive inhibition among the bacteria.

There were a few MG whose bacterial growth curves were not always significantly different from the control in either the *in vitro* or *in vivo* growth curve experiments: MG-03, MG-05, and MG-06. These three MG all had *Neisseria cinerea*

present. There were no *N. cinerea* bacteria identified from any of the other MG studied. This is consistent with the data from the lawn experiments, as both illustrate a very strong resistance to the effects of *B. cereus* by *Neisseria spp*.

Biofilm formation was present in most of the flasks and on most of the MG pieces that were subjected to the Whimpy and Arnold CF treatments during data collection for growth curves. This precipitation was also noted for most of the MG controls. There was no precipitous growth observed in any flask or on any MG piece that was inoculated with either *B. cereus* strain. Therefore, the addition of *B. cereus* not only affected concentration of the MG bacterial growth as seen in the pre- and post-treatment serial dilutions and touch plates, but also the interactions between the MG microorganisms with the resultant biofilm formation.

In the pre- and post-treatment serial dilutions and touch plates from the *in vitro* and *in vivo* experiments respectively, more specific effects of the *B. cereus* CF on the MG bacteria could be seen. All post-treatment plates for the Whimpy and Arnold strain treatments A and B exhibited an abundance of *B. cereus* and no observable MG bacteria when compared to the control. However, whether or not the *B. cereus* treatments completely prevented the growth of individual MG species can not be determined from these results. Treatments C and D demonstrated differences in the concentrations and types of bacteria present when compared to the controls. Generally, the concentration of bacteria from the *B. cereus* CF treatments was less than that of the control. Differences in the colonies observed between the pre- and post-treatment plates do infer that the *B. cereus* strains and toxins produce noticeable changes in the MG bacterial communities.

CHAPTER V

CONCLUSION

The results of this study demonstrated that *B. cereus*, a human pathogen, also has adverse effects against other bacteria. Its toxin production seems to be its main mode of pathogenesis, however the effects are greater when the bacteria are present as well. It is able to contaminate a MG after only 24 hours of inoculation. Of the 42 different species identified from worn MG of students, only half of these species will grow in the presence of *B. cereus*.

Different strains of *B. cereus* were studied and found to have different effects on the growth of the MG bacteria. The Arnold strain inhibited the growth of individual MG species, whereas the Whimpy strain did not. The difference in inhibition of individual bacterial species was attributed to the probable differences in toxin profiles of these two strains.

The *B. cereus* toxins typically caused lags in the start of growth and decreases in the stationary phase concentrations of the MG bacteria. In addition, the bacterial profiles of the MG were observably different after being subjected to the *B. cereus* toxin treatments when compared to a control. This demonstrated that *B. cereus* toxins disrupt the normal environment of these bacteria, inhibiting some species and possibly allowing

others to flourish. This raises concerns since *B. cereus* toxin activity may allow for the proliferation of other pathogenic microorganisms.

Interestingly, a few MG species demonstrated resistance to the effects of *B. cereus*. This was especially observed from bacteria of the *Staphylococcus* and *Neisseria* genera. These results not only demonstrate how *B. cereus* and its toxins adversely effect the growth of individual species and bacterial communities, but also the effects that some species may have on *B. cereus* and therefore possibly *B. anthracis*.

In summary, the answers to the research questions are as follows:

1. Mouth-guards can be intentionally contaminated with different strains of *B. cereus*. The *B. cereus* can also survive 12 seconds in boiling water and can travel in the steam.
2. Forty-two species of bacteria were identified from the worn mouth-guards of non-athlete OSU-CHS students. The microorganisms found include 19 gram positive cocci, 8 gram negative cocci, 11 gram positive rods, and 4 gram negative rods. No yeasts, molds, or *B. cereus* were found.
3. The *B. cereus* Arnold strain inhibited the growth of individual bacterial species when grown on TSA-II.
4. The *B. cereus* toxins caused a decrease in the concentration at stationary phase and a lag in the start of log phase growth of the mouth-guard bacteria.

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APPENDIX

APPENDIX A – IRB Approval Pages

OSU-CHS
IRB

JAN 06 2006

APPROVED

Subject Number _____

Oklahoma State University Center for Health Sciences

Informed Consent

Title of Project: Amendment #4 to Protocol #2002003 — Evaluation of the Microbiota in Athletic Mouth-guards

Investigators: **Richard Thomas Glass, D.D.S., Ph.D., Co-Principal Investigator**
Professor of Pathology
918-561-8240

Robert S. Conrad, Ph.D., Co-Principal Investigator
Professor of Microbiology
Chair, Department of Biochemistry and Microbiology
918-561-8263

This is to certify that I, _____, hereby agree to participate as a volunteer in a scientific investigation as part of an authorized research program of OSU-CHS under the supervision of Dr. R. Tom Glass, Co-Principal Investigator and Dr. Robert S. Conrad, Co-Principal Investigator.

The purpose of this research is to determine whether protective athletic mouth-guards become contaminated with harmful microorganisms or germs when they are used. For this study, you are being asked to participate by:

1. **initially having your teeth and gingival tissues (gums) swabbed with a sterile swab by Dr. Glass.**
2. **then wearing a new boil-and-bite mouth-guard that is made just for this study for cumulative minimum of 6 hours to a cumulative maximum of 12 hours (except when eating). Boil-and-bite mouth-guards are commercially available and are used in many contact sports. A mouth-guard can easily be adapted to your mouth by placing it in boiling water for 12 seconds followed by placing it into cool water for 2 seconds and inserting it into your mouth for biting and molding (Manufacturer's instructions). Drs. Glass and Conrad will assist you in custom fitting your mouth-guard for comfortable wear prior to your participation in the study.**
3. **surrendering the mouth-guard to Drs. Glass and Conrad immediately after the designated time period of wear (6-12 hours).**

Initials_____

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There will be no physical or mental discomfort associated with this research project. The mouth-guard that you wear will be completely destroyed as part of the research project and, therefore, will never be available for you to wear again.

There is no known benefit from this study for you. You will receive a one-time compensation of fifty dollars ($50.00) for your time and effort.

By agreeing to participate in this research and signing this form, you do not waive any of your legal rights.

Your identity will be kept in strictest confidence and your mouth-guard will be identified only by your Subject Number (above). This identification link will be destroyed at the end of the study.

You are free to refuse to participate in this study without penalty.

If you have any questions about your rights as a study participant, you may contact Dr. Stephen Eddy at Oklahoma State University-Center for Health Sciences (918) 561-8287. If you have any questions about the study, you may contact Dr. R. Tom Glass (918) 561-8240 or Dr. Robert S. Conrad (918) 561-8263

Subject's Signature _____ Date _____

I certify that I have explained all elements of this consent form to the subject or legal representative and answered all questions asked before requesting they sign it.

Witness _____ Date _____

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JAN 06 2006
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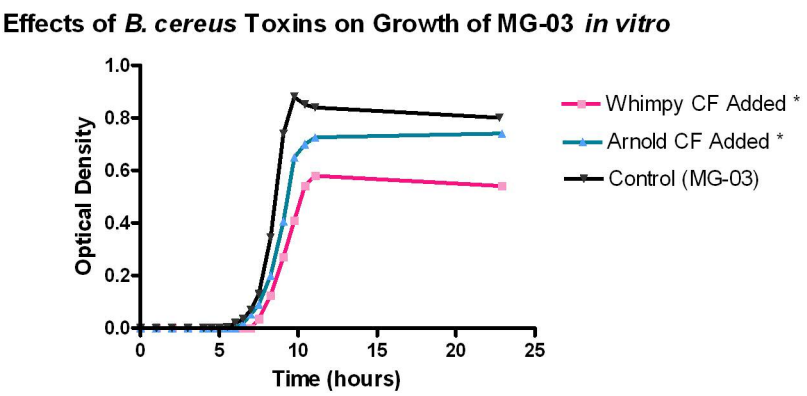
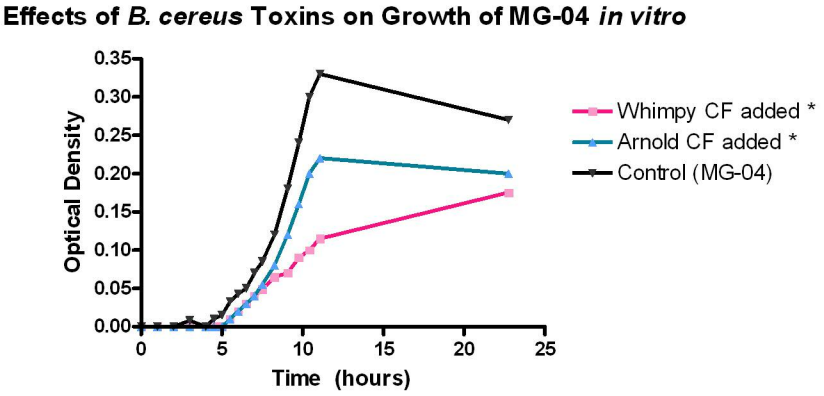
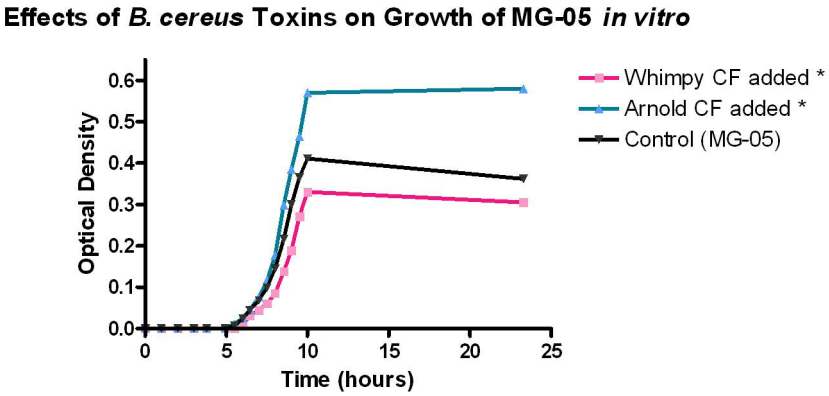
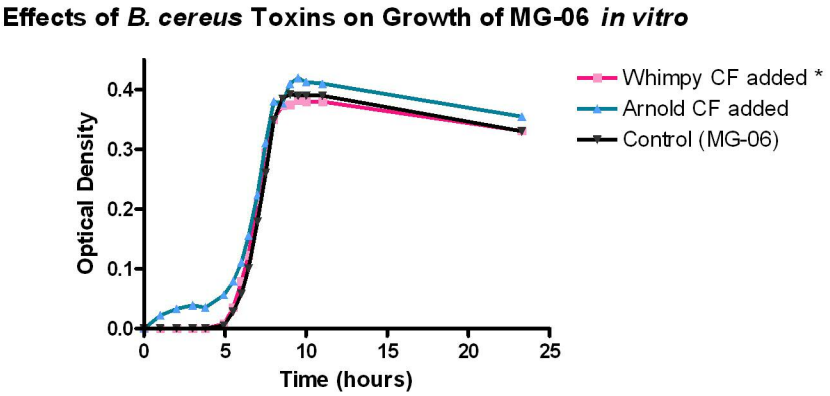
APPENDIX B – Growth Curves of Mouth-Guard Treatments C and D



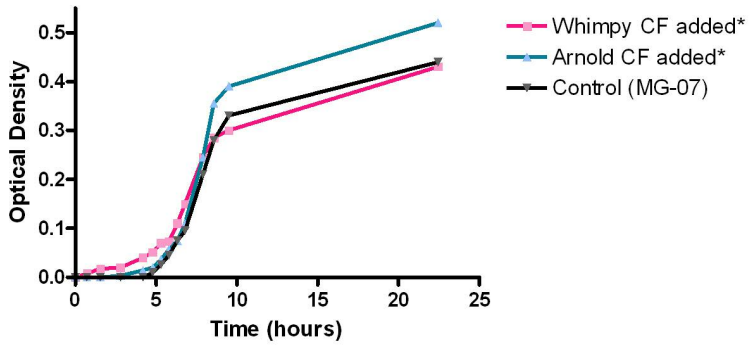




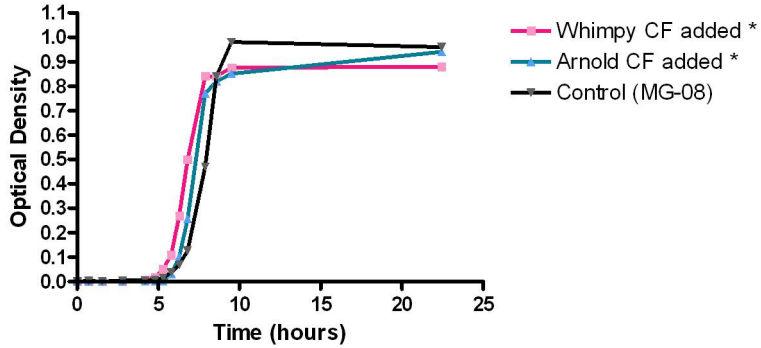


* Growth curve is significant

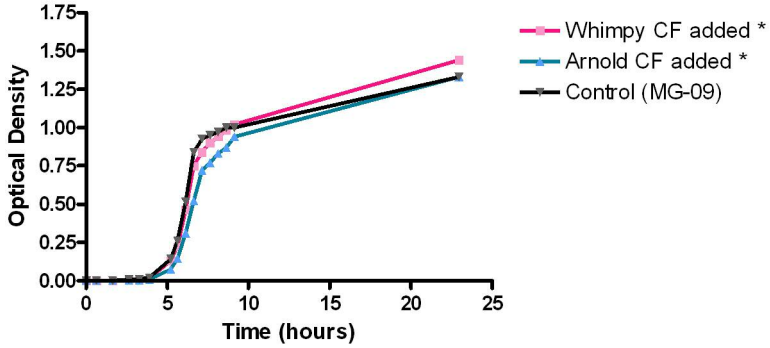
Effects of *B. cereus* Toxins on Growth of MG-07 *in vitro*



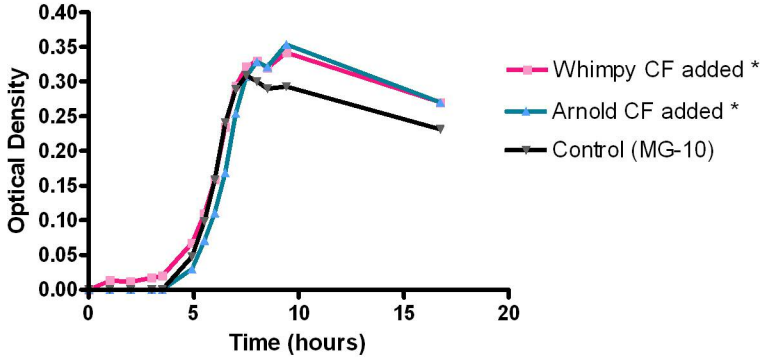
Effects of *B. cereus* Toxins on Growth of MG-08 *in vitro*



Effects of *B. cereus* Toxins on Growth of MG-09 *in vitro*

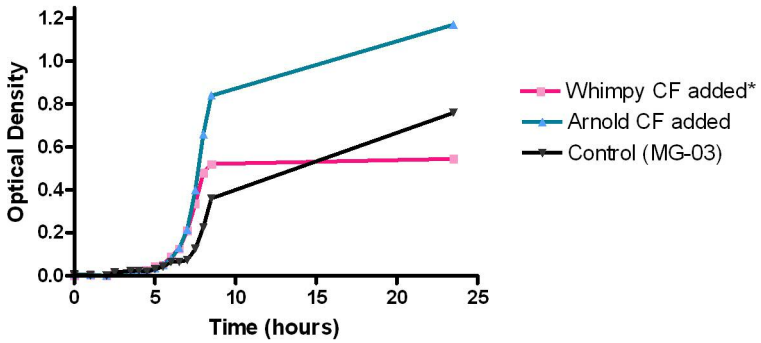


Effects of *B. cereus* Toxins on Growth of MG-10 *in vitro*

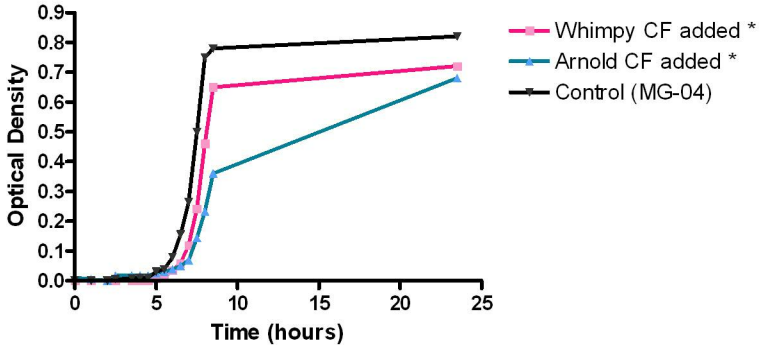


* Growth curve is significant

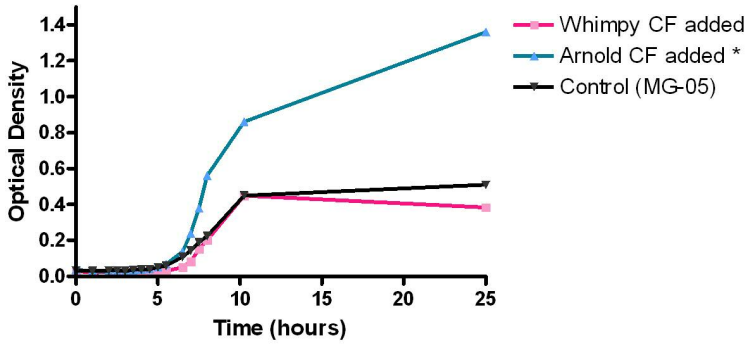
Effects of *B. cereus* Toxins on Growth of MG-03 *in vivo*



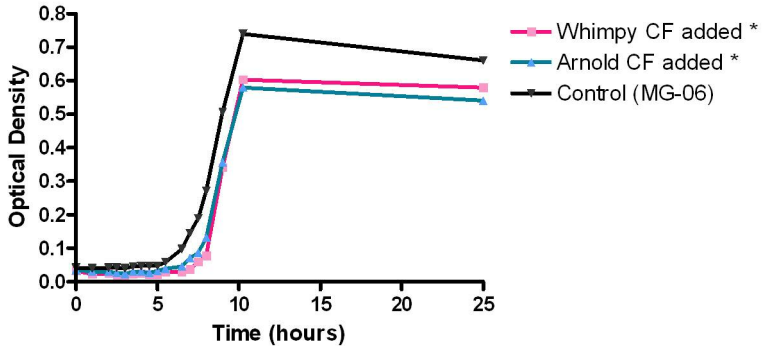
Effects of *B. cereus* Toxins on Growth of MG-04 *in vivo*



Effects of *B. cereus* Toxins on Growth of MG-05 *in vivo*

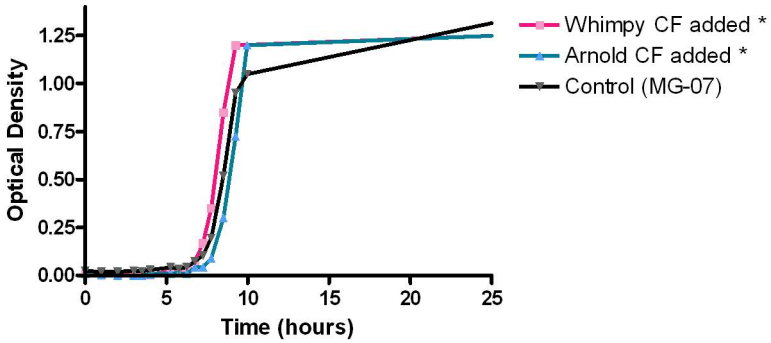


Effects of *B. cereus* Toxins on Growth of MG-06 *in vivo*

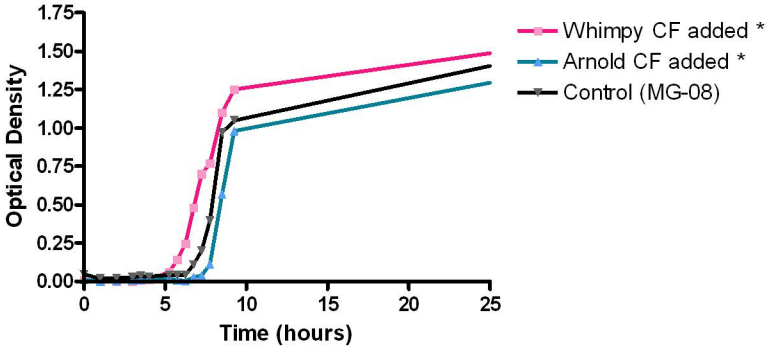


* Growth curve is significant

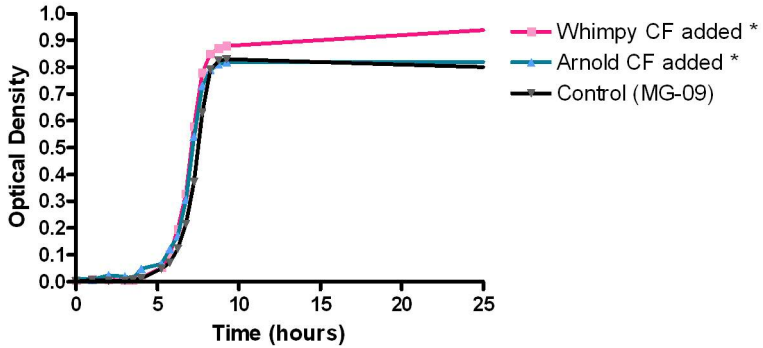
Effects of *B. cereus* Toxins on Growth of MG-07 *in vivo*



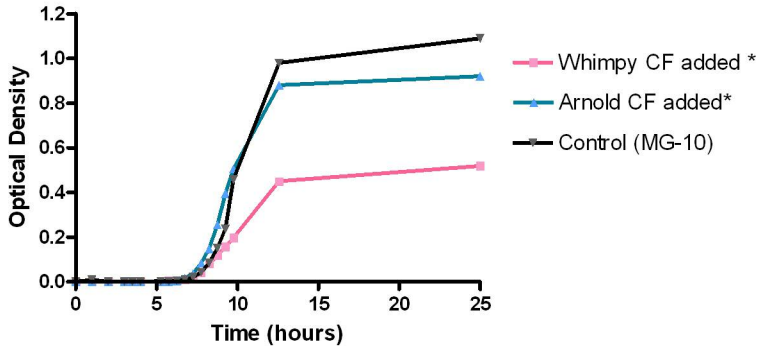
Effects of *B. cereus* Toxins on Growth of MG-08 *in vivo*



Effects of *B. cereus* Toxins on Growth of MG-09 *in vivo*



Effects of *B. cereus* Toxins on Growth of MG-10 *in vivo*



* Growth curve is significant

APPENDIX C – Comparisons of Pre- and Post-Treatment Bacterial Growth

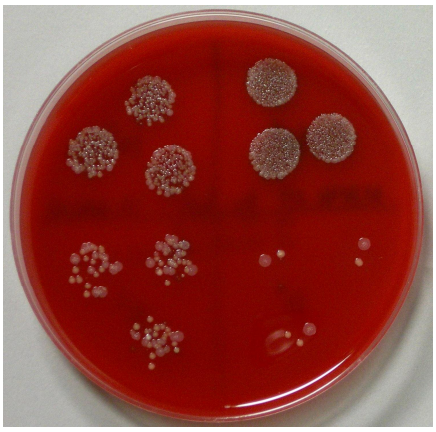


Figure C-1. Pre-treatment serial dilution.

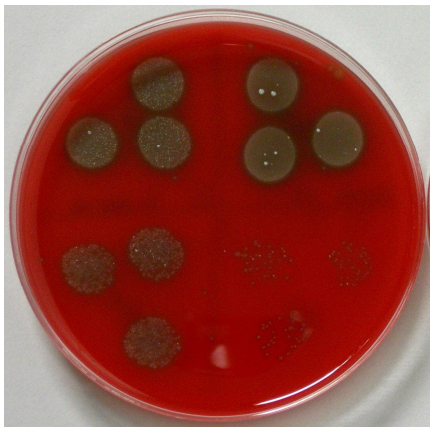


Figure C-2. Post-treatment serial dilution of treatment E (MG alone).

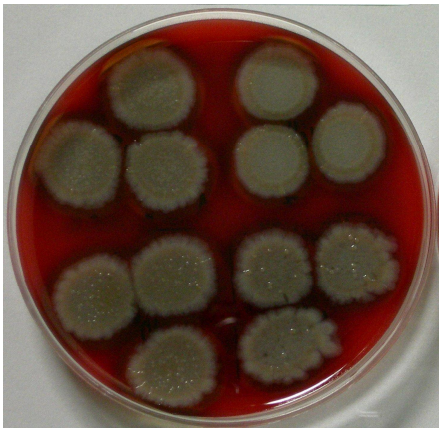


Figure C-3. Post-treatment serial dilution of treatment A (Whimpy strain).

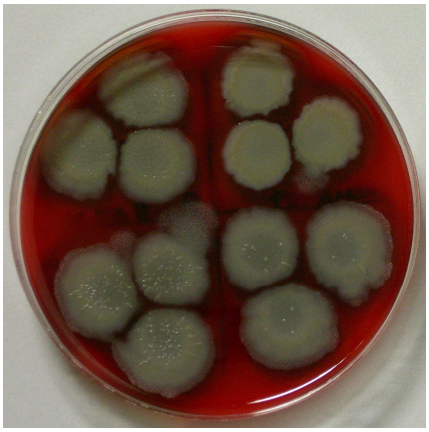


Figure C-4. Post-treatment serial dilution of treatment B (Arnold strain).

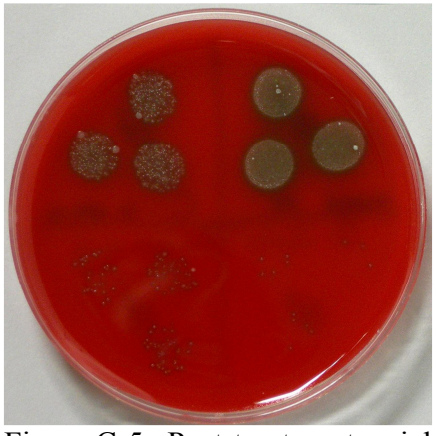


Figure C-5. Post-treatment serial dilution of treatment C (Whimpy CF).

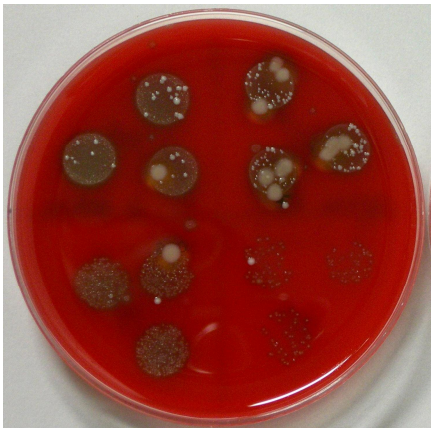


Figure C-6. Post-treatment serial dilution of treatment D (Arnold CF).

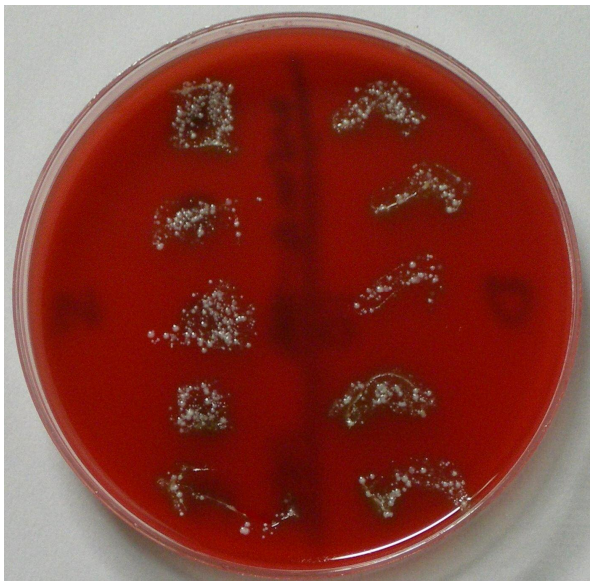


Figure C-7. Pre-treatment touch plate of the surfaces (left) and depths (right) of a MG. The five touches per side are from MG sections A, B, C, D, and E before being applied to their respective treatments and are represented in descending order on the plate from top to bottom.

Figure C-8. Post-treatment touch plate of the surfaces (left) and depths (right) of a MG. Treatment conditions A, B, C, D, and E are represented in descending order from top to bottom.

VITA

Erin Kathryn Doyle

Candidate for the Degree of

Master of Science

Thesis: EFFECTS OF *BACILLUS CEREUS* AND ITS TOXINS ON MICROORGANISMS FROM USED MOUTH-GUARDS

Major Field: Forensic Science

Biographical:

Personal Data: Born in Bryn Mawr, Pennsylvania, on June 16, 1982, the daughter of Robert and Kathryn Doyle.

Education: Graduated from Upper Merion Area High School in King of Prussia, Pennsylvania in June, 2000.

Received a Bachelor of Arts degree with a major in Biology and minor in Neuropsychology from Bucknell University, Lewisburg, Pennsylvania, in May 2004.

Completed the requirements for the Master of Science degree at Oklahoma State University Center for Health Sciences with a concentration in Forensic Pathology in December, 2006.

Name: Erin Kathryn Doyle Date of Degree: December, 2006

Institution: Oklahoma State University-CHS Location: Tulsa, Oklahoma

Title of Study: EFFECTS OF *BACILLUS CEREUS* AND ITS TOXINS ON MICROORGANISMS FROM USED MOUTH-GUARDS

Pages in Study: 68 Candidate for the Degree of Master of Science

Major Field: Forensic Pathology

Scope and Method of Study: The purpose of this study was to evaluate the inhibitory effects that *B. cereus* bacteria and toxins have on the growth of oral microorganisms as well as look at the possibility that oral appliances, such as mouth-guards, can be contaminated with this pathogen. *B. cereus* was used as a surrogate for *B. anthracis*, which causes anthrax. Two strains (Whimpy and Arnold) with different toxin profiles were employed. New mouth-guards were placed in media inoculated with these strains and boiled according to the directions in the mouth-guard packaging to assess the ability of *B. cereus* to contaminate the mouth-guards. Oral bacteria were collected, isolated, and identified from worn mouth-guards of students. These mouth-guard bacteria were cultured with the *B. cereus* bacteria and culture filtrates and the resultant growth curves were analyzed. Individual mouth-guard species were streaked as lawns on agar and inhibition to the *B. cereus* bacteria and culture filtrates was recorded.

Findings and Conclusions: Mouth-guards placed in *B. cereus* inoculated media were found to possess *B. cereus* on the surfaces and in the depths of the mouth-guards after boiling. *B. cereus* was also cultured from the steam from the boiling, contaminated mouth-guards, an occurrence that may be aided by the ability of *B. cereus* to produce spores. Results from the growth curve experiments demonstrated that the culture filtrates of both Whimpy and Arnold tended to decrease the growth rate and increase the midpoint of the mouth-guard growth curves. This suggests that the *B. cereus* toxins cause a lag in and decrease the concentration at stationary phase of oral bacteria. The inhibitory effect of *B. cereus* culture filtrates on individual mouth-guard bacteria was less noticeable, and most likely due to the number of bacteria being tested. However, the growth of certain isolates of mouth-guard bacteria on agar was inhibited by the presence of the Arnold strain only. These results demonstrate that *B. cereus* not only contaminates mouth-guard material, but also inhibits the growth of oral bacteria. Different strains of *B.cereus* can have a stronger inhibitory effect than others and inhibition is increased when both bacteria and toxins are present.

ADVISOR'S APPROVAL: Dr. R. Thomas Glass