An Investigation of the Relationship Between Black Soldier Fly Larvae Pathogen Suppression and Growth Substrate in Relation to Maine Agricultural Industries

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AN INVESTIGATION OF THE RELATIONSHIP BETWEEN BLACK SOLDIER FLY LARVAE PATHOGEN SUPPRESSION AND GROWTH SUBSTRATE IN RELATION TO MAINE AGRICULTURAL INDUSTRIES

by

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ABSTRACT

Insects are promoted as cost-effective and sustainable protein sources for animal feed. Their utilization may help to avoid a predicted global protein shortage. Black soldier fly (*Hermetia illucens*) larvae (BSFL) grow on organic wastes, converting these wastes into larval biomass which can fulfill this purpose. Potential benefits of using BSFL to remediate organic wastes include reduction of waste mass and bacterial load, along with the sale of larvae as a protein supplement. BSFL suppress the growth of some Gram-positive and Gram-negative human pathogens in these substrates; though suppression of *Bacillus cereus*, a spore-forming bacterium that causes food-poisoning, has not been documented. This project focused on *Bacillus cereus* suppression by BSFL on byproducts (used as growth substrates) of 2 Maine agricultural industries: potatoes and blueberries. Colony counts on *B. cereus* selective media were higher for larvae fed on potatoes spiked with pathogen than pathogen alone on potatoes after 2 days. After 4 days, an opposite effect was observed, with lower colony counts observed for larvae fed on potato with *B. cereus* than pathogen alone on potato. PCR analysis of samples from the potato confirmed the detection of *B. cereus*. Blueberry substrate was not capable of supporting *B. cereus* as colony counts for all treatments were below the detection threshold after 2 days of larval feeding. While 100% viability was observed for BSFL reared on blueberries, larval weight decreased by an average of 82% with pathogen compared to a 32% decrease without pathogen, indicating that blueberries are not a suitable growth substrate.
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LITERATURE REVIEW

The Black Soldier Fly (*Hermetia illucens*)

The black soldier fly (*Hermetia illucens*) is a large, wasp-like fly that is prominent in most tropical, subtropical, and temperate regions. Black soldier flies can be found in most of the United States, South America, and Europe, as well as parts of Asia, Africa, and Australia. These insects have grown in popularity over recent years due to their ability to process a wide variety of organic wastes. They convert these wastes into larval biomass, which can be used for animal feed. Additionally, they have demonstrated antimicrobial abilities against several known human pathogens.

Black soldier flies are holometabolous insects, meaning they undergo metamorphosis through 4 life stages: egg, larva, pupa, and adulthood (Figure 1). Adult females lay their eggs around the edges of a larval food source, in dry crevices within two days after mating (Tomberlin & Sheppard, 2002). Each female lays between 500 to 900 eggs at a time (De Smet, et. al, 2018). From the time the eggs are laid until the larvae hatch takes about 4 days. Larvae represent an immature, mobile phase where nutrients are accumulated as they feed for approximately two weeks before entering the prepupae stage. Pupae represent an immobile, non-feeding stage that concludes when the adult insect emerges from the pupal case after about 2 weeks. After emerging from the pupal case, adults mate within 2 to 4 days (Tomberlin & Sheppard, 2002).

Adult black soldier flies do not eat during their 5 to 8 day lifespan, so they must obtain fat in their larval stage to survive as adults (Nguyen, et. al, 2013). Therefore, the larvae must consume a large amount of food, such as various decomposing organic
wastes. These include food wastes and agricultural byproducts. Larvae are even capable of growing on human fecal matter (Lalander, et. al, 2013), cow manure (Myers, et. al, 2008), and chicken manure (Sheppard, et. al, 1994).

Specific abiotic factors such as temperature and diet have been shown to significantly impact larval growth. Eggs remain viable at temperatures between 15°C and 40°C but temperatures lower than 15°C and greater than 40°C are fatal (Chia, et. al, 2018). Variations in diet composition affect the development time and mortality. Some diets lead to a faster development into the prepupal stage (Meneguz, et. al, 2018b), as well as observed changes in the length and weight of larvae grown on various rearing substrates (Harnden & Tomberlin, 2016). One example of a dietary factor that affects mortality is using fish as a rearing substrate. Even though fish has a high fat content,
black soldier fly larvae exhibited 100% mortality when fed exclusively on fish (Nguyen, et. al, 2013). This may be attributed to the accumulation of toxic metals, like mercury, in the fish. Larvae reared on pig liver also exhibited a high mortality rate (Nguyen et. al, 2013). High mortality of larvae reared on liver is likely due to the accumulation of various toxins in the liver. In addition to affecting the growth and mortality of the larvae, the composition of the food substrate impacts the nutrient profile of the larvae. For example, if the growth substrate contains high amounts of metals, these can accumulate within the larvae. It was also shown that larvae fed on a high protein diet had a higher protein content (Tschirner & Simon, 2015). Organic wastes can vary significantly in their composition and since the BSFL can grow on most organic wastes, they are highly malleable. When grown on different organic wastes, BSFL demonstrate nutrient profiles that correlate with that of their growth substrate (Spranghers, et. al, 2016). Therefore, using a growth substrate with high protein content to rear the BSFL can make them suitable for use as a high protein supplement in animal feed.

**Black Soldier Fly Larvae in Organic Waste Processing**

Around the globe, large amounts of wastes are generated daily. One study projected that by the year 2025, over 6 million tons of waste will be generated globally per day. This waste, when not dealt with properly, can have negative impacts on public health and the environment (Hoornweg & Bhada-Tata, 2012). These wastes can harbor human pathogens which can contaminate food and water sources leading to disease outbreaks. Additionally, poor management of organic wastes leaves behind nutrients and energy that could be utilized. An emerging method to combat these issues is to allow fly
larvae to grow on the waste. Fly larvae reduce the mass of the waste and extract the nutrients in the form of larval biomass that can be used for other purposes, such as animal feed. One of the most studied insects for this purpose is the black soldier fly (Čičková, et. al, 2015).

The black soldier fly larvae (BSFL) are capable of feeding and growing on almost all organic wastes. BSFL have been shown to reduce the mass of the organic waste by up to 74% depending on the composition (Gold, et. al, 2018). To maximize the conversion to larval biomass, substrates with easily available carbon sources and high protein are recommended, such as fecal matter and food waste (Lalander, et. al, 2019). BSFL have also been shown to decrease the time for waste processing when compared to regular aerobic composting (Cai, et. al, 2018).

The conversion of organic waste to larval biomass is a major benefit of using BSFL. After rearing on organic wastes, larvae can be processed and utilized in a variety of ways. One of the most popular uses is as a protein additive in animal feeds. In the European Union the black soldier fly is one of only 7 insects approved for and used in animal feeds (European Commission, 2017). In the United States, BSFL are already being used as feeds in the poultry and aquaculture industries (McDougal, 2018; Simke, 2019). BSFL are high in protein and fat content by weight, making them an excellent additive to various animal feeds (Wang & Shelomi, 2017). Additionally, BSFL are much cheaper to produce than other alternative sources of protein additives for animal feed. Another use for the larvae is that they can be processed and used as a source of lipids for biodiesel production (Li, et. al, 2011). By producing marketable products from the larvae,
users are able to profit from the waste system. In this way, BSFL represent a financially viable way to process organic wastes.

Black soldier flies leave behind a residue after processing that has similar properties to immature compost (Gold, et. al, 2018). This leftover residue can be utilized. For example, the residual product of BSFL processing increases the yield of produce when used as an additive for soilless growing systems (Setti, et. al, 2019). In Switzerland a fertilizer was designed using the residue left behind by BSFL reared on plant material. This fertilizer increased produce yield from nutrient poor soil systems at a rate similar to currently used mineral-based fertilizers (Kebli & Sinaj, 2017). Additionally, the composting system using BSFL was found to degrade the antibiotic tetracycline 1.6 times faster than the aerobic composting system (Cai, et. al, 2018). This is important because residual antibiotics in agricultural waste can pollute the environment and contribute to the development of resistant microbial populations. Overall, the versatility of BSFL in reducing organic waste mass and as feed additives has made them an increasingly popular choice for use in sustainable agriculture systems.

Pathogen Suppression by Black Soldier Fly Larvae

In addition to reducing the mass of organic wastes and use as a feed additive, BSFL have demonstrated suppressive effects against various pathogens in growth substrates. This ability to reduce the pathogenic bacterial load in organic wastes makes them even more valuable to sustainable agriculture systems. BSFL have been shown to reduce the amount of *Escherichia coli* present in cow manure (Liu, et. al, 2008). As manure amount increased, suppression of *E. coli* by the larvae increased. This
suppression varied with temperature, with complete growth inhibition occurring at 35°C. Additionally, 72 hours of larval feeding were necessary for suppression to develop (Liu, et. al, 2008).

BSFL reduced E. coli O157:H7 populations in chicken manure to undetectable levels after 3 days of larval feeding at 27°C and 32°C (Erickson, et. al, 2004). In the same study by Erickson, et. al (2004), BSFL reared on hog manure actually exacerbated the growth of E. coli O157:H7. Variation in the pH of the manures may play a role in the ability of the larvae to suppress E. coli. The BSFL exhibited increased suppression on alkaline chicken manure as opposed to acidic hog manure. In addition to suppression of E. coli, BSFL suppressed the growth of Salmonella in the chicken manure (Erickson, et. al, 2004). BSFL also significantly reduce the amount of total bacteria present in finfish waste after both 7 and 14 days of larval feeding (Bernard, et. al, 2020). In particular, the gram-negative human pathogen Shigella sonnei in finfish waste accounted for less of the bacterial population in waste treated with BSFL (Bernard, et. al, 2020).

BSFL suppress the growth of pathogenic bacteria in human wastes, as well. After 8 days of larval feeding, a 6 log\textsubscript{10} reduction in the amount of Salmonella species was observed in the human fecal waste, compared to only a 2 log\textsubscript{10} reduction when larvae weren’t present (Lalander, et. al, 2013). A slight reduction in the amount of Enterococcus species was also noted in the same study, with less than 1 log\textsubscript{10} reduction in BSFL treated human waste compared to 1 log\textsubscript{10} increase when BSFL were not present. No significant reduction in the amount of the bacteriophage \Phi X174 in the presence of BSFL compared to the control was found (Lalander, et. al, 2013). Though pathogen suppression is often observed, additional steps to further sanitize waste before it is utilized as a fertilizer may
be necessary when pathogenic bacterial load is high. Further understanding of the immunological aspects that enable pathogen suppression by the BSFL may lead to optimization of pathogen suppression and the avoidance of these additional sanitation steps.

**Immunology of Black Soldier Fly Larvae**

Organic wastes typically contain a high amount of bacteria, including both human and insect pathogens. The black soldier fly larvae’s ability to survive in these environments laden with bacterial pathogens suggests that they possess a wide variety of antimicrobial properties to protect themselves. One defense mechanism that is an ubiquitous part of the innate immune response in almost all organisms is the production of low-molecular weight, cationic antimicrobial peptides (CAMPs). In insects, antimicrobial peptides serve as the primary innate immune response. These antimicrobial peptides are synthesized in their body fat and released into the hemolymph (Bulet, et. al, 1999).

When isolated, the larval hemolymph exerts antimicrobial effects on various pathogens (Dang, et. al, 2006). BSFL extracts, consisting of homogenized larvae with buffer, were shown to inhibit the growth of several Gram-negative bacterial pathogens including *Neisseria gonorrhoeae, Klebsiella pneumoniae, and Shigella sonnei* (Choi, et. al, 2012). Both aqueous and methanol-based BSFL extracts exhibited antimicrobial effects against both Gram-negative and Gram-positive human pathogens (Park, et. al, 2014). The Gram-positive pathogens against which the BSFL extracts showed antimicrobial activity included methicillin-resistant *Staphylococcus aureus* and the Gram-
positive environmental bacterium *Bacillus subtilis* (Park, et al, 2014). Since the effects could be observed using aqueous fractions, the antimicrobial activity could be due to the production of soluble factors such as CAMPs.

Analysis of the BSFL transcriptome revealed 53 putative antimicrobial peptides including 26 suspected defensins, the largest number found in any animal (Vogel, et al, 2017). Only one of these putative antimicrobial peptides has been isolated and was 75% similar to a defensin found in the sand fly *Phlebotomus duboseqi*. This peptide is referred to as defensin-like peptide 4 and had potent antimicrobial effects against the Gram-positive pathogen *Staphylococcus aureus*, regardless of methicillin-sensitivity (Park, et al, 2015). With 52 other putative antimicrobial peptides left to be isolated and characterized, BSFL provide a promising source for novel antimicrobials.

BSFL present an interesting case for the emerging field of nutritional immunology. Nutritional immunology explores the relationship between nutrition and the function of the immune system. Changes in diet composition for the BSFL significantly increase the expression of putative antimicrobial peptides and other genes, including lysozymes and pattern-recognition receptors, involved in the immune response (Vogel, et al, 2017). These diet-dependent changes in expression produced different antimicrobial profiles from both methanol and aqueous extracts from the larvae. A diet containing bacteria lead to a higher expression of antimicrobial peptides (Vogel, et al, 2017). These changes in the expression of various immunity-related genes suggest that the BSFL are able to tailor their immune response to their environment. However, more research is needed to fully understand how diet impacts the antimicrobial capabilities of the BSFL. In particular, *in vivo* research is required to confirm the effects seen by the
larval extracts against Gram-positive pathogens and to further elucidate the effects of growth substrate on larval suppression. This *in vivo* research is important because in an industrial waste processing setting whole larvae will likely be used, rather than larval extracts.

**Bacteriology of Bacillus cereus**

*Bacillus cereus* is an aerobic Gram-positive, spore-forming, rod-shaped, soil bacterium. It is mesophilic, with optimal growth occurring at temperatures between 28°C and 35°C, and motile via peritrichous flagella (Vilas-Bôas, 2007). *B. cereus* displays hemolytic activity when grown on blood agar, visible as beta-hemolysis of red blood cells. Most strains of *B. cereus* are also ampicillin resistant. Another defining characteristic of *B. cereus* is the presence of lecinthinase, demonstrated by a distinct appearance on agar containing egg yolk (Vilas-Bôas, 2007).

Despite these characteristics, *B. cereus* can be hard to identify. It belongs to the larger *Bacillus cereus* group, consisting of six closely related organisms: *B. cereus*, *Bacillus anthracis*, *Bacillus thuringiensis*, *Bacillus mycoides*, *Bacillus pseudomycoides*, and *Bacillus weihenstephanensis* (Vilas-Bôas, 2007). More recent additions to the group include *Bacillus cytotoxicus* and *Bacillus toyonensis*. All members of this group are very closely related aerobic, Gram-positive, spore-forming rods (Liu, 2015). Interestingly, *B. cytotoxicus* was found to be a common microorganism in finfish waste, a rearing substrate for BSFL (Bernard, et. al, 2020).

Within the group, *B. anthracis*, *B. thuringiensis*, and *B. cereus* show the most genetic overlap, and all have pathogenic tendencies (Vilas-Bôas, 2007). They have only
about 5% genetic divergence in seven main chromosomal genes (Helgason, et. al, 2000). These three species are often distinguished by probing housekeeping genes \textit{pcyA} and \textit{ccpA} (Liu, 2015). \textit{B. anthracis} is the causative agent of anthrax in mammals, \textit{B. thuringiensis} forms a crystalline structure outside of its spore that can be toxic to certain insect larvae, and \textit{B. cereus} is can cause food poisoning. Strains of \textit{B. anthracis} are easily distinguished from \textit{B. cereus} and \textit{B. thuringiensis} by capsular production, lack of motility, absence of hemolytic activity, and sensitivity to penicillin. The main physical characteristic that distinguishes \textit{B. thuringiensis} from \textit{B. cereus} is the production of the parasporal crystal by \textit{B. thuringiensis} (Vilas-Bôas, 2007). The insecticidal toxin genes in \textit{B. thuringiensis} are carried on a plasmid (Helgason, et. al, 2000).

\textit{B. cereus} contains strains capable of being human pathogens. Pathogenic strains of \textit{B. cereus} produce a wide variety of toxins that induce illness, including enterotoxins, an emetic toxin, and two hemolysins (Majed, et. al, 2016). The natural reservoirs of \textit{B. cereus} include soil, fresh as well as marine water, vegetables and other inert surfaces, as well as in the intestines of some invertebrates (Jensen, 2003). It is capable of colonizing both inert and living surfaces (Majed, et. al, 2016) \textit{B. cereus} is able to survive in many environments due in part to the presence of several two-component signal transduction systems (De Been, et. al, 2006).

Other environments where \textit{B. cereus} can grow include various surfaces in the food processing industry. Contamination of food products by \textit{B. cereus} is often hard to eliminate due to their ability to form spores and biofilms. These include both immersed and floating biofilms, though floating biofilms are more common (Majed, et. al, 2016). Free iron significantly impacts the ability of \textit{B. cereus} to form biofilms, as surfaces with
available iron are much more likely to host biofilms. This may be one reason why they are more likely to colonize stainless steel surfaces than plastics in industrial settings. Biofilm cells can switch back to the planktonic state to spread and form new biofilms (Hayrapetyan, et. al, 2015). A protein, ComER, serves as a positive regulator for both sporulation and biofilm formation related genes (Huang, et. al, 2020). This suggests that sporulation and biofilm formation are closely related. Sporulation rates within the biofilms can reach up to 90% when nutrients are depleted and with an aerobic interface (Majed, et. al, 2016). The presence of B. cereus biofilms poses a public health risk when formation occurs in industrial food processing settings. This pre- or post-processing contamination leads to food spoilage or foodborne illness in produce, poultry, dairy, and red meat processing facilities (Rajkovic, et. al, 2008).

B. cereus is also a common contaminant of potatoes. There have been 10 separate studies identifying B. cereus as a contaminant in various potato products including raw potatoes and dehydrated potato flakes (Doan & Davidson, 2000). Consumption of potato products that are contaminated with B. cereus can cause outbreaks of food poisoning. There have been several outbreaks of food poisoning around the world that have been linked specifically to mashed potatoes contaminated with B. cereus (Doan & Davidson, 2000). One notable outbreak occurred in South Dakota where 450 school children contracted food poisoning linked to B. cereus contaminated mashed potatoes (MaEnteffer, 1978).
Bacillus cereus as a Pathogen

There are approximately 9.4 million outbreaks of foodborne illness with a known etiologic agent in the United States each year. Of these 14% are caused by three pathogens: *B. cereus*, *Clostridium perfringens*, and *Staphylococcus aureus* (Scallen, et al, 2011). Due to its ability to form spores, contamination by *B. cereus* can persist in prepared foods and various agricultural products, causing food-borne illness. Food contaminated with *B. cereus* can cause both emetic and diarrheal disease syndromes shortly after ingestion. The form of the disease produced depends on the production of the 3 enteric toxins and the emetic toxin, cereulide (McDowell, et al, 2020). Toxin production is both strain- and environment-dependent (Jeßberger, et al, 2015).

The emetic toxin, commonly called cereulide, is a small, ring-shaped peptide (Ehling–Schulz, et al, 2015). Cereulide is produced via non-ribosomal peptide synthesis, a large multienzyme complex in which each module attaches another monomer to the growing peptide. The *ces* gene cluster encodes both the toxin monomer and the non-ribosomal peptide synthesis complex. This gene cluster is found on a plasmid, named the pCER270. Also encoded on pCER270 are approximately ten regulator proteins which are tightly linked to the metabolism of the host bacterium (Ehling–Schulz, et al, 2015).

Illness is caused by the ingestion of pre-formed cereulide in contaminated food (Ehling–Schulz, et al, 2004) and requires about 8 to 10 micrograms of cereulide per kilogram of body weight in animal models for symptom development (Stenfors Arnesen, et al, 2008).

The enteric toxins are produced via a classical ribosomal synthesis pathway (Ehling–Schulz, et al, 2015) and include hemolysin BL (HBL), nonhemolytic enteric toxin, and cytotoxin K (Huang, et al, 2020). All three of the enterotoxins are pore-
forming toxins (Bottone, 2010). The hemolysin BL consists of three protein components, one binding component, B, and two lytic components, L₁ and L₂ (Lund & Granum, 1997). In addition to its hemolytic capabilities, the HBL enterotoxin can cause tissue necrosis and vascular permeability, leading to fluid accumulation in the intestinal lumen. The non-hemolytic enterotoxin is also consists of three components, but it is not hemolytic (Lund & Granum, 1997). Cytotoxin K is strongly cytotoxic with both necrotic and hemolytic capabilities, leading to lysis of epithelial cells in the intestinal lumen and diarrhea (Lund, et. al, 2000). The enterotoxins are produced in the body when a person ingests food contaminated with *B. cereus* spores. These spores germinate in the small intestine, leading to local growth of vegetative cells and the production of the enterotoxins, triggering the diarrheal symptomology (Ceuppens, et. al, 2012). An infectious dose of 10⁵ to 10⁸ colony forming units is required to be ingested for the diarrheal illness to develop but is lower for spores (Clavel, et. al, 2004).

The emetic and diarrheal forms of *B. cereus*-implicated food poisoning have differing incubation periods and durations. The emetic form has an incubation period of thirty minutes to six hours, with the duration of the illness ranging from six hours to one day long (Stenfors Arnesen, et. al, 2008). The diarrheal form has a longer incubation period of twelve hours to one day. This longer incubation is coupled with a longer duration of the illness, usually twelve hours to one day, but sometimes symptoms can last up to several days. Foods linked to the two forms of *B. cereus* illness are also different. Protein-rich foods, such as meat and dairy products, are most linked to the diarrheal form. Foods rich in starches, such as pastas and rice, are most often linked to the emetic form.
Lethal cases of both forms are rare but have been observed (Stenfors Arnesen, et. al, 2008).

Though food poisoning is the most common presentation of *B. cereus* infection, it has been identified as the etiological agent in extraintestinal infections of various anatomical sites in both immunocompetent and immunocompromised individuals. These include infections of the central nervous system, respiratory system, eyes, bones, skin, and muscles. Severe *B. cereus* infections of muscles produce a myonecrotic symptomology similar to the gas gangrene caused by *Clostridium perfringens* (Bottone, 2010). In addition, strains of *B. cereus* have been isolated that produce an Anthrax like illness in mammals (Baldwin, 2020).
INTRODUCTION

Maine agricultural industries generate a lot of waste products. In 2019 Maine produced over 54 million pounds of blueberries and over 1.6 billion pounds of potatoes (USDA, 2019). This amount of agricultural productivity results in the production of a lot of waste, for example products (potatoes or blueberries) that are not fit to sell. Culled potatoes can be sources of disease for other crops grown nearby if they are not properly maintained. This is such a large problem that the state of Maine has even established strict regulations for the maintenance of cull potato piles. Improperly maintained cull potato piles are considered a public nuisance and are punishable by up to a $1,000 fine (Animals and Agriculture, 2007). Additionally, run off from contaminated agricultural wastes can pollute water and food sources leading to the spread of human disease.

Black soldier fly larvae (BSFL) present an opportunity to reduce the mass of agricultural wastes while generating sustainable feed sources for Maine livestock and aquaculture. The potential benefits are three-fold: biowaste processing, reduction of pathogenic bacteria, and sale of larvae to Maine industries as feed. A prime example of this is the dairy cow industry in Maine. BSFL can be reared on cow manure and have been shown to reduce the amount of Escherichia coli present in the manure (Liu, et. al, 2008). BSFL convert the manure into larval biomass that can then be used as a protein supplement in animal feed. The female black soldier fly can lay 500 – 900 eggs at one time and with their short developmental period, they would be an economical and sustainable food source for agriculture and aquaculture industries. BSFL have already received FDA approval to be used in poultry feed (McDougal, 2018). By reducing the number of bacteria present in cow manure, BSFL have the potential to reduce
contamination of water sources (from manure pile runoff) and food crops that are fertilized with this manure.

Other Maine agricultural systems that could benefit from the use of BSFL include the blueberry and potato farming industries. For example, potato cull piles could be composted using BSFL. This would reduce the mass and bacterial load of the cull pile while also generating marketable larvae for use in animal feed. Previous research shows cull potatoes are a suitable growth substrate for BSFL, since BSFL demonstrated a significant increase in mass when reared on this diet (Alyokhin, et. al, 2018). As for blueberries, there are no previously published data about BSFL rearing on this substrate. Blueberries have their own antimicrobial properties, so their use in composting could also help to reduce bacterial contamination. However, it is important to understand the interaction between BSFL and blueberries as a growth substrate before they are implemented in composting systems.

Literature on the suppression of Gram-positive human pathogens by BSFL is sparser than Gram-negative human pathogens. Some studies have demonstrated that BSFL lack the capability to suppress Gram-positive pathogens in their substrate (Lalander, et. al, 2013; Choi, et. al, 2012). However, both aqueous- and methanol-based larval extracts have demonstrated antimicrobial abilities against Gram-positive human pathogens (De Smet, et. al, 2018), including methicillin-resistant *Staphylococcus aureus* (Park, et. al, 2014). The interactions between BSFL *in vivo* and Gram-positive human pathogens needs to be more fully understood since whole larvae are utilized in composting. Understanding the interaction between Gram-positive pathogens more fully will allow for the BSFL to be better utilized.
*Bacillus cereus* is a public health concern since it is a major etiologic agents of food borne illness (Scallen, et. al, 2011). *B. cereus* is a Gram-positive bacterium that is commonly found in soil, so it has the potential to contaminate many agricultural crops. Its ability to form both spores and biofilms makes contamination by *B. cereus* hard to eliminate. Unpublished preliminary data from the Bernard lab on the University of Maine campus suggested that crushed BSFL might exhibit antimicrobial properties against *B. cereus*. Additionally, Park et. al (2014) demonstrated that both aqueous and methanol BSFL extracts exhibited antimicrobial capabilities against *Bacillus subtilis* which belongs to the same genus.

This project aimed to narrow a knowledge gap about the relationship between pathogen suppression and growth substrate in black soldier fly larvae, focusing on Maine agricultural industries. We hypothesized that the BSFL may reduce populations of *B. cereus* on potato and blueberry substrates. To characterize the effects of BSFL on *B. cereus* populations in these substrates, we conducted dilution plating and molecular assays to detect and quantify *B. cereus*. Additionally, BSFL growth on blueberries needed to be measured since there was no existing data. This was accomplished by assessing larval weight before and after the trial.
METHODS AND MATERIALS

*B. cereus* Growth Conditions and Counting

To prepare inoculum for growth substrates, *B. cereus* was grown on trypticase soy agar (TSA) for 24 hours at 37°C and used to inoculate a flask containing 50 mL of sterile trypticase soy broth (TSB). This flask was incubated for 24 hours at 37°C. After this incubatory period, the culture was diluted with TSB to an absorbance of 0.3 at 600 nm (A\textsubscript{600}). The A\textsubscript{600} was measured using a ThermoFisher spectrophotometer and 1 mL plastic cuvettes. To correct for the base absorbance of the growth medium, 1 mL of sterile TSB was used to calibrate the spectrophotometer. Five 1 mL samples of this culture were serially diluted to 10\textsuperscript{-7} and the 10\textsuperscript{-6} and 10\textsuperscript{-7} dilutions were plated onto Oxoid *Bacillus cereus* agar to enumerate bacterial colonies (see below). These plates were incubated overnight at 37°C and colonies consistent with a *B. cereus* colony morphology (color/lecithinase activity) were counted. From these data, the A\textsubscript{600} of 0.3 corresponded to an average of 4 \(\cdot\) 10\textsuperscript{7} CFU per mL.

Oxoid *Bacillus cereus* agar is a selective and differential medium used for isolating *B. cereus* from food samples. This medium selects for Gram-positive organisms through the inclusion of Polymixin B. Polymixin B is a cationic peptide that interacts with the negatively charged Lipid A portion of the lipopolysaccharide layer on Gram-negative organisms (Avedissian, et. al, 2019). *B. cereus* can be differentiated from other Gram-positive organisms on this media based on colony morphology. Colonies of *B. cereus* are visually identified by a precipitate in the medium (cloudy appearance) and a peacock blue color. The precipitate results from lecithinase activity that hydrolyzes
lecithin in egg yolk. The peacock blue color results from failure to ferment mannitol.

These characteristics are sufficient to distinguish *B. cereus* from all other species except *Bacillus thuringiensis*.

**Experimental Design Overview**

Two experiments were designed to examine the suppression of *B. cereus* by BSFL on different substrates. For the first trial, the growth substrate was autoclaved organic potatoes, while in the second trial the growth substrate was autoclaved frozen blueberries. In each trial there were four treatments. (Figure 2, Table 1): sterile substrate with TSB added (Treatment 1), substrate with larvae and TSB added (Treatment 2), substrate with larvae and *B. cereus* culture at an $A_{600}$ of 0.3 (Treatment 3), and substrate with *B. cereus* culture at an $A_{600}$ of 0.3 (Treatment 4). The trials were conducted in 12-oz. Rainforce glass jars sterilized by autoclaving. On Day 0, 165 grams of the substrate (either potato or blueberry) was placed into each jar. The potatoes used were Marketside organic gold potatoes obtained from Walmart. These were autoclaved prior to the start of the trial. The blueberries were frozen Wyman’s brand obtained from Hannaford. Blueberries were autoclaved while frozen prior to the start of the trial. Based on previously published literature (Liu, et. al, 2008), either *B. cereus* culture or sterile TSB was added at a ratio of 0.01 mL per gram of substrate to each jar. Therefore, treatments 1 and 2 received 1.65 mL of sterile TSB, while treatments 3 and 4 received 1.65 mL of the culture standardized to an OD$_{600}$ of 0.3. Treatments 2 and 3 received 50 BSFL in each jar. A double layer of sterile cheesecloth and a sterile jar lid with holes were secured on top. The jars were placed at random into a fume hood with the fan deactivated and the sash lowered. As shown in Figure 1, each treatment was conducted in triplicate.
Figure 2. Graphic representation of experimental trial set up.

Table 1. Treatment names and codes. The treatment numbers correspond to those shown in Figure 1 for reference.

<table>
<thead>
<tr>
<th>Treatment Number</th>
<th>Treatment Name</th>
<th>Treatment Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Substrate</td>
<td>S</td>
</tr>
<tr>
<td>2</td>
<td>Larvae</td>
<td>L</td>
</tr>
<tr>
<td>3</td>
<td>Larvae and Pathogen</td>
<td>LP</td>
</tr>
<tr>
<td>4</td>
<td>Pathogen</td>
<td>P</td>
</tr>
</tbody>
</table>

Plating Assay

On day 0 of the potato trial, 3 randomly selected jars were swabbed on TSA plates before the addition of the pathogen to ensure starting sterility of the substrate. During the blueberry trial this number was increased to 6 randomly selected jars. In both trials after 2 days and 4 days of larval feeding, 5-gram samples were removed from each jar and placed into Erlenmeyer flasks containing 45 mL of sterile water in triplicate. The contents of the flasks were serially diluted to $10^{-6}$ and 0.1 mL of the $10^{-5}$ and $10^{-6}$ dilutions were
plated onto Oxoid *Bacillus cereus* agar (Figure 3). The plates were incubated for approximately 24 hours before the colonies were counted. There were three types of counts taken: total colony count, colonies consistent with *B. cereus* morphology, and colonies not consistent with *B. cereus* morphology (the total less the number consistent with *B. cereus* morphology).

During the blueberry trial, no growth was observed on the Oxoid *Bacillus cereus* agar after 2 days of larval feeding. Since there was nothing observed on this selective media, plating for day 4 was conducted on TSA. TSA is a non-selective media that will grow both Gram-positive and Gram-negative organisms. There are no differential components to the TSA, so colony counts could not be separated into *B. cereus* and non-*B. cereus* morphologies. Due to this change, only one 5-gram sample was taken from each jar and plated. The dilution series were conducted in the same format, with the $10^{-5}$ and $10^{-6}$ dilutions plated (Figure 3). Additionally, to capture the level of the bacterial population in the blueberry substrate, for one larvae and pathogen treatment jar 0.1 mL of each dilution was plated.
Figure 3. Graphic representation of the plating procedure conducted on Days 2 and 4 of each trial. Each flask contained 45 mL of sterile water while each tube contained 9 mL of sterile water.

Figure 4. *B. cereus* colony on the Oxoid Bacillus cereus selective agar. The cloudy appearance is due to the precipitate generated by lecithinase activity while the blue color indicates an inability to ferment mannitol.
Larval Weight Analysis

During the blueberry trial, larval weight was measured by jar before and after the trial. The larvae were weighed prior to the start of the trial then weighed again after 4 days of feeding on the blueberry substrate. The larval percent weight change was calculated for each sample. Jars for each treatment were then averaged together to obtain average percent weight change per treatment. A t-test was then conducted to determine the significance of any differences in the data.

DNA Extraction

On day 0 of both trials six random jars were sampled for DNA extraction, 3 inoculated with pathogen and 3 without the pathogen. An additional 0.75-gram sample was taken from every jar on days 2 and 4. All these samples were placed in a –20°C freezer for one week before the DNA was extracted using the MO BIO Laboratories, Inc., DNeasy PowerFood Microbial Kit (Qiagen, Germantown, MD, USA). This kit is designed for microbial DNA extraction from food samples. Extraction protocols followed the manufacturer’s specifications for extracting DNA directly from food without prior enrichment. Genomic DNA from each extraction was then assessed on 0.8% agarose Tris-acetate-EDTA (TAE) gels. Electrophoresis conditions for genomic DNA were 35 volts for approximately four hours.

In addition to the samples from both the potato and blueberry trials, the MO BIO Laboratories, Inc., DNeasy PowerFood Microbial Kit (Qiagen, Germantown, MD, USA) was used to extract DNA from a dilution series of 24-hour old B. cereus culture. An extraction was taken from undiluted culture as well as from a series of ten-fold dilutions
performed on culture standardized to an OD\textsubscript{600} of 0.35. The culture diluted with sterile TSB to an OD\textsubscript{600} of 0.35 was found to have approximately $10^8$ colony forming units (CFU) per mL.

**Diagnostic PCR**

Extracted DNA samples from the potato trial were used to conduct diagnostic PCR for detection of *B. cereus*. Primers were chosen from previously published literature (Table 2) targeting two genes from *B. cereus*: hemolysin (*hblD*) and cereulide synthase (*ces*). Additionally, a primer targeting the transcriptional regulator of the insecticidal toxin (XRE) found only in *B. thuringiensis* was used in an attempt verify that colony counts reflected mostly *B. cereus* and not *B. thuringiensis*, since the two are visibly indistinguishable on that growth medium. The chosen primers (Table 2) were used to probe the DNA extraction from undiluted culture to verify the identity of *B. cereus* and the efficacy of the primer sets. Each primer set had specific reaction conditions listed in Table 3. The culture dilution series was then reacted with the *hblD* primer set to establish a detection threshold. All DNA extractions from the potato trial samples were probed with the *hblD* primers and the XRE primer sets. PCR products were visualized by gel electrophoresis using 2% agarose TAE gels. Electrophoresis conditions were 50 volts for about 2 hours. Gels were stained prior to imaging using a 1 ng per mL solution of ethidium bromide.
Table 2. Primer information for diagnostic PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer Sequence (5’→3’)</th>
<th>Function</th>
<th>Amplicon Length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hblD</td>
<td>GTTAGATACAGCGAAGCCAC</td>
<td>Forward Primer</td>
<td>465 bp</td>
<td>Zhang, et. al, 2016</td>
</tr>
<tr>
<td></td>
<td>CCGCCAGTTACAACAATA</td>
<td>Reverse Primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ces</td>
<td>CGCCGAAAGTGATTATACCAA</td>
<td>Forward Primer</td>
<td>103 bp</td>
<td>Fricker, et. al, 2007</td>
</tr>
<tr>
<td></td>
<td>TATGCCCGTTCTCAAACTG</td>
<td>Reverse Primer</td>
<td></td>
<td></td>
</tr>
<tr>
<td>XRE</td>
<td>AAGATATTGCAAGCGGTAAGAT</td>
<td>Forward Primer</td>
<td>270 bp</td>
<td>Wei, et. al, 2019</td>
</tr>
<tr>
<td></td>
<td>GTTTGTTCAGATCCGTACGTA</td>
<td>Reverse Primer</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 3. PCR reaction conditions for each of the chosen Primer sets.

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Melting Temp</th>
<th>Annealing Temp</th>
<th>Elongation Temp</th>
<th>Primer Concentration</th>
<th>Cycle number</th>
</tr>
</thead>
<tbody>
<tr>
<td>hblD</td>
<td>95°C Initial = 10min Cycle = 30s</td>
<td>52.2°C Cycle=30s</td>
<td>72°C Cycle=30s Final=10min</td>
<td>0.2µM</td>
<td>35</td>
</tr>
<tr>
<td>ces</td>
<td>95°C Initial = 2min Cycle = 30s</td>
<td>60°C Cycle=60s</td>
<td>72°C cycle=30s Final=5min</td>
<td>0.3µM</td>
<td>35</td>
</tr>
<tr>
<td>XRE</td>
<td>95°C Initial = 5 min Cycle = 30s</td>
<td>49°C Cycle=30s</td>
<td>72°C Cycle= 30s Final = 5min</td>
<td>0.3µM</td>
<td>35</td>
</tr>
</tbody>
</table>

Statistical Analyses

Plate count data was analyzed using SAS University Edition Software. Standard deviation and standard error were calculated for each treatment. Normality of the data was assessed using the Shapiro-Wilk test. Treatment, jar, and treatment by jar effects were analyzed using a generalized linear model. For plating data, mean separation was carried out using Tukey’s honestly significant difference (HSD) test ($P<0.05$). A t-test was conducted to determine the significance of any differences in larval weight data.
RESULTS

Potato Trial Plating Results

The Oxoid Bacillus cereus selective agar plates swabbed on day 0 exhibited no growth of any colonies. After 2 days, the larvae and pathogen treatment had significantly higher colony counts than the substrate, larvae, and pathogen treatments in terms of total colony counts (Figure 5) with an average colony count of $1.702 \pm 0.472 \cdot 10^8$ CFU per gram of substrate. Total colony counts of the substrate, larvae, and pathogen treatments were statistically indistinguishable from one another. On day 4, however, the larvae treatment was significantly higher than the larvae, substrate, and larvae and pathogen treatments. On day 4 the larvae treatment had an average of $2.03 \pm 0.176 \cdot 10^9$ CFU per gram of substrate.

On both days, average colony counts not consistent with B. cereus (Figure 6) were highest in treatments that had not been spiked with B. cereus. On day 2, the substrate treatment had significantly more colonies not consistent with B. cereus than the larvae, pathogen, and larvae and pathogen. The larvae, larvae and pathogen, and pathogen treatments were statistically indistinguishable from one another. This changed on day 4, when the larvae treatment exhibited a significantly higher number of colonies not consistent with B. cereus morphology when compared to the substrate, larvae and pathogen, and pathogen treatments. The other treatments were statistically indistinguishable from one another.

Average colony counts consistent with B. cereus (Figure 7) were highest in treatments spiked with B. cereus at the start of the trial. On day 2, the larvae and pathogen treatment had the most B. cereus-consistent colonies with an average of $4.28 \pm$
$0.736 \cdot 10^8$ CFU per gram of substrate. The amount of \textit{B. cereus}-consistent colonies in the larvae and pathogen treatment was significantly higher than in the substrate, larvae, and pathogen treatments. However, on day 4, the treatment that exhibited the highest burden of \textit{B. cereus} colonies was the pathogen treatment. The pathogen treatment was considered statistically higher compared to the larvae and pathogen, substrate, and larvae treatments. Additionally, the larvae and pathogen treatment was significantly higher when compared to the substrate and larvae treatments. The substrate treatment and the larvae treatment were indistinguishable from each other.
Figure 5. Average total colony counts on Oxoid Bacillus cereus selective agar of the potato trial. Bars topped with the same letter are statistically indistinguishable. Error bars represent standard error for each treatment. (A) Total colony counts for day 2 are shown separated by treatment, the y-axis represents CFU per gram of substrate on the order of $10^5$. (B) Total colony counts for day 4 are shown separated by treatment, the y-axis represents CFU per gram of substrate on the order of $10^5$. 
Figure 6. Average colony counts not consistent with *B. cereus* morphology on the Oxoid *Bacillus cereus* selective agar of the potato trial. Bars topped with the same letter are statistically indistinguishable. Error bars represent standard error for each treatment. (A) Average of colonies not consistent with *B. cereus* morphology for day 2 are shown separated by treatment, the y-axis represents CFU per gram of substrate on the order of $10^5$. (B) Average of colonies not consistent with *B. cereus* for day 4 are shown separated by treatment the y-axis represents CFU per gram of substrate on the order of $10^6$. 
Figure 7. Average colony counts consistent with B. cereus morphology on Oxoid Bacillus cereus selective agar during the potato trial. Bars topped with the same letter are statistically indistinguishable. Error bars represent standard error for each treatment. (A) Average count of colonies consistent with B. cereus morphology for day 2 are shown separated by treatment, the y-axis represents CFU per gram of substrate on the order of $10^5$. (B) Average count of colonies consistent with B. cereus for day 4 are shown separated by treatment, the y-axis represents CFU per gram of substrate on the order of $10^5$. 
Genomic DNA Extractions

Gel electrophoresis of genomic DNA from the day 0 samples of the potato trial did not show DNA in any of the lanes. This could be because the substrate was sterile and only a small amount of culture was added. DNA from day 2 potato trial extractions was only observed for one jar in the pathogen treatment but in all of the larvae and pathogen treatment samples. No DNA was detected in any of the day 2 samples from the substrate or larvae treatments. Day 4 potato trial extraction samples showed DNA in one of the pathogen treatment jars. DNA was detected in all of the day 4 samples from the larvae and pathogen treatment. No DNA was detected in any of the day 4 samples from the substrate or larvae treatments. These results are summarized in Table 4.

DNA extracted from the *B. cereus* culture dilution series was detected by gel electrophoresis for undiluted culture and the $10^8$ to $10^4$ CFU per mL dilutions. The visual intensity of the genomic DNA decreased from the undiluted culture lane to the $10^4$ CFU per mL lane and became undetectable below that threshold.

Table 4. Summary of genomic DNA gel electrophoresis results for samples from the potato Trial. A (+) indicates that DNA was detected in the lane for that sample while a (–) indicates that no DNA was detected in the lane for that sample.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Jar</th>
<th>Day 2</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>A</td>
<td>–</td>
<td>–</td>
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<tr>
<td></td>
<td>B</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Larvae</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>–</td>
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<tr>
<td></td>
<td>C</td>
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<td>–</td>
</tr>
<tr>
<td>Larvae and Pathogen</td>
<td>A</td>
<td>+</td>
<td>+</td>
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<tr>
<td></td>
<td>B</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Pathogen</td>
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<td></td>
<td>C</td>
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<td>–</td>
</tr>
</tbody>
</table>
PCR Analysis of Culture Extractions

The chosen primer sets (Table 2) were used to probe an undiluted culture of *B. cereus*. The *hblD* and *ces* primers were specific to *B. cereus* while the *xre* primer set was used to rule out *B. thuringiensis*. When the undiluted culture was probed, only the *hblD* primer reaction produced a band of the expected size (Figure 8). This band was about 500 bp which is the appropriate size for the fragment targeted by these primers (Table 1). The *ces* and *XRE* primers failed to produce detectable bands on the undiluted culture reaction (Figure 8), likely indicating that these genes were not present. This is expected for a non-emetic strain of *B. cereus*, which would lack both the *ces* and *XRE* genes. Bands of the appropriate size for *hblD* were observed in PCR reactions from the $10^8$ CFU per mL, $10^7$ CFU per mL, and $10^6$ CFU per mL culture dilutions (Figure 9). This indicates that threshold of detection is $10^6$ CFU per mL in the assay.
Figure 8. Gel electrophoresis of PCR reactions on undiluted *B. cereus* culture. The (-) indicates a negative control which was nuclease free water and loading dye. The Ld. abbreviation represents the ladder which was NE BioLabs 1 kilobase (kb) ladder. The length of the fragments on the ladder is listed to the left of each band in the number of bp. The only band shown was produced in the *hblD* reaction lane and was slightly smaller than 0.5 kb or 500 base pairs (bp).

Figure 9. Gel electrophoresis of *hblD* PCR reactions on culture standards. The (+) indicates the positive control which was an *hblD* PCR reaction on undiluted *B. cereus* culture. The (-) indicates a negative control which was nuclease free water and loading dye. The Ld. abbreviation represents the ladder which was NE BioLabs 100 base pair (bp) ladder. The length of the fragments on the ladder is listed to the left of each band in the number of bp. All of the bands were produced were 500 bp or slightly smaller.
PCR Analysis of Potato Trial Samples

The \textit{hblD} and \textit{XRE} primer sets were to probe extracted DNA from each jar in the potato trial. None of the PCR reactions using the \textit{XRE} primer sets produced a band (Table 5). This indicates that the insecticidal toxin of \textit{B. thuringiensis} was undetectable and, since all reactions for \textit{XRE} were negative, a positive PCR reaction for \textit{hblD} likely indicates the presence of \textit{B. cereus} (above the detection threshold of $10^6$ CFU per mL).

The detection of \textit{B. cereus} using the \textit{hblD} primer sets had variable results (Table 6). All the larvae treatment samples came back negative for \textit{hblD}. This means that the larvae did not introduce a significant amount ($>10^6$ CFU per mL) of \textit{B. cereus} naturally. In contrast, the pathogen treatment samples from both day 2 and day 4 were all positive for \textit{hblD}, verifying that \textit{B. cereus} did persist on the potato substrate. The substrate treatment tested negative for the presence of \textit{hblD} in two jars while one was positive. This indicates potential contamination with \textit{B. cereus}, since no bacteria were deliberately introduced into these jars. All the larvae and pathogen treatment jars were positive for \textit{hblD} on day 2 but on day 4 one jar was negative.
Table 5. Summary of XRE PCR reactions on the potato trial samples from days 2 and 4. A (+) would indicate the presence of a band consistent with the expected fragment size generated by the XRE primers while a (–) indicates that no band of comparable size was observed on the gels.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Jar</th>
<th>Day 2</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>A</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>–</td>
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<td></td>
<td>C</td>
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<tr>
<td>Larvae</td>
<td>A</td>
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<td></td>
<td>B</td>
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<td>Larvae and Pathogen</td>
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<td>C</td>
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</tbody>
</table>

Table 6. Summary of hblD PCR reactions on the potato trial samples from days 2 and 4. A (+) indicates that a band consistent with the hblD positive control was seen while a (–) indicates that no band consistent with the hblD positive control was observed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Jar</th>
<th>Day 2</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
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</table>

**Blueberry Trial Results**

The six random jars of substrate that were swabbed on day 0 prior to the addition of pathogen or larvae to any of the treatments showed no growth on TSA. This suggested that the substrate was sterile prior to the addition of the larvae or pathogen. Plating of day 2 blueberry samples also exhibited no growth on the Oxoid *Bacillus cereus* selective agar. This indicated that any pathogen or Gram-positive bacterial presence within the
samples was below the plating threshold of 10^6 CFU per gram of substrate. Since nothing was observed on the day 2 selective agar plates, day 4 plating was completed on TSA. There was no growth on the TSA for the substrate, pathogen, or larvae and pathogen treatments. The larvae treatment TSA plates exhibited growth with an average of 2.67 ± 0.577 • 10^6 CFU per gram of substrate. Thus, the larvae treatment was the only treatment with bacteria present above the plating threshold of 10^6 CFU per gram of substrate. On day 4, Jar B of the larvae and pathogen treatment had each dilution plated individually on TSA. This one full set of dilution TSA plates revealed that jar B of the larvae and pathogen treatment had a bacterial load of 1.15 ± 0.2203 • 10^3 CFU per gram of substrate. This is well below the plating threshold (10^6 CFU per gram of substrate) that was used for the other jars.

The weight analysis revealed that when larvae were fed on blueberries for 4 days they lost weight. Significantly more weight was lost in the presence of *B. cereus* than when *B. cereus* was not present (Table 7). The average weight loss was -29.2% ± 5.839 when *B. cereus* was not present and -75.1% ± 12.053. These were determined to be significantly different by performing a T-test (p = 0.004, T = -5.94).

Table 7. Larval Weight Analysis from Blueberry Trial. The P-Value was 0.0040 and t-value was -5.94 indicating that the two treatments were significantly different from one another in terms of percent change in weight.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Average Weight Before Trial</th>
<th>Average Weight After Trial</th>
<th>Average Percent Change in Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>No <em>B. cereus</em></td>
<td>0.983 ± 0.095</td>
<td>0.697 ± 0.106</td>
<td>-29.2% ± 5.839</td>
</tr>
<tr>
<td>In the Presence of <em>B. cereus</em></td>
<td>0.914 ± 0.105</td>
<td>0.228 ± 0.110</td>
<td>-75.1% ± 12.053</td>
</tr>
</tbody>
</table>
DISCUSSION & FUTURE DIRECTIONS

BSFL represent an excellent opportunity for sustainable agriculture systems in Maine. They provide a method for composting various organic wastes while serving as a source of income when used as a feed additive. These benefits are enhanced by the potential for BSFL to reduce the burden of bacteria in their growth substrates. Previously published literature indicates BSFL are capable of reducing the burden of certain pathogenic Gram-negative bacteria in their growth substrate (Erickson, et. al, 2004; Lalander, et. al, 2013; Liu, et. al, 2008). Homogenized larval extracts also exhibit antimicrobial effects on both Gram-negative and Gram-positive bacteria (Park, et. al, 2014; Park, et. al, 2015; Vogel, et. al, 2018). In order to fully harness their antimicrobial capabilities, there is a need to better understand the suppression of Gram-positive pathogens in vivo. Results from this study suggest a delayed suppressive effect on the Gram-positive pathogen B. cereus when BSFL are reared on potatoes. On the other hand, blueberries were not a suitable growth substrate.

When reared on potatoes, BSFL appeared to exacerbate the growth of B. cereus early in the feeding process (Figure 7). This is consistent with some previously published data. A study by Erickson, et. al (2004) showed that when BSFL were present, the amount of E. coli within hog manure increased. This increase could have been due to the acidic nature of the substrate. Most digestive enzymes of BSFL exhibit optimal activity under basic conditions (Kim, et. al, 2011). The initial increase in B. cereus burden on potatoes in the presence of BSFL has been corroborated by the Bernard laboratory on the University of Maine campus (Bernard, unpublished data).
Despite the initial increase of \textit{B. cereus}, on day 4 there was a significant decrease (Figure 7). This may indicate a delayed suppressive effect by the BSFL on \textit{B. cereus} in the potato substrate. BSFL increase the pH of their substrate over time, with the most significant change in pH occurring between days 2 and 3 (Meneguz, et. al, 2018a). The modification of the growth substrate within the time frame could explain the emergence of suppression in samples taken after 4 days of larval feeding.

Gel electrophoresis of PCR reactions using the \textit{XRE} primer set (Table 1) confirmed that \textit{B. thuringiensis} was undetectable in the potato trial (Table 5). The published detection threshold for \textit{B. thuringiensis} from spiked foods was $10^3$ CFU per gram (Wei, et. al, 2019). This detection threshold could be assay-specific or vary based on the substrate. For this reason, further testing should be undertaken to confirm the detection threshold of the \textit{XRE} primer set when using potatoes as a substrate. The primer set has a published 97.3\% success rate in differentiating \textit{B. thuringiensis} strains from other \textit{B. cereus} group pathogens, with only \textit{B. thuringiensis} strains appearing positive for \textit{XRE} PCR reactions (Wei, et. al, 2019). The absence of detection of the \textit{XRE} gene in the indicates that \textit{B. thuringiensis} was likely not introduced to the growth substrate by the larvae.

Gel electrophoresis of PCR reactions using the \textit{hblD} primer set (Table 1) on the potato trial DNA extractions confirmed the presence of \textit{B. cereus} in both the pathogen and the larvae and pathogen treatments (Table 6). Based on the initial assay, a positive \textit{hblD} reaction indicates that the presence of \textit{B. cereus} was above the detection threshold of the primer set ($10^6$ CFU per mL). One jar of the larvae and pathogen treatment did appear negative for \textit{hblD} on Day 4 (Table 6). This jar had \textit{B. cereus} above the detection
threshold on day 2, indicating that between days 2 and 4 the population of *B. cereus* decreased to less than $10^6$ CFU per mL. Since this was not observed in any of the pathogen treatment jars, this decrease in *B. cereus* could be due to the presence of larvae. This coupled with the decrease of *B. cereus* seen in the plating data on day 4 in the larvae and pathogen treatment strengthens the idea that the BSFL exhibit a delayed suppressive effect on *B. cereus*.

The *hblD* gene was detected in one of the substrate treatment jars (Table 6), indicating the presence of *B. cereus* in an amount above the detection threshold. Even though *B. cereus* is a soil-dwelling bacterium and could naturally be present on the potatoes, this contamination likely occurred during the plating procedure or the experimental setup as the potatoes were autoclaved prior to the start of the trial. Autoclaves have been shown to eliminate spores of bacteria within the *Bacillus* genus (Lin, et. al, 2018). Additionally, the 3 random jars swabbed onto TSA showed no growth indicating that substrate was initially sterile. Though there was contamination of this treatment, plating data (Figure 7) still showed that the amount of *B. cereus* present in the substrate sample was significantly less than the pathogen and larvae treatment and the pathogen treatment. The larvae treatment exhibited no signs of contamination with *B. cereus*. The substrate treatment was considered statistically indistinguishable from the larvae treatment (Figure 7).

Even though there is potential suppression of *B. cereus* by BSFL reared on potato substrate, it is not complete. Therefore, the larvae and/or substrate may require additional sanitation steps before use in a commercial setting. This is reinforced since suppression of *E. coli* in both dairy manure (Liu, et. al, 2008) and chicken manure (Erickson, et. al,
2004) was not complete. Remaining pathogens could be a potential public health concern if the waste is used as fertilizer or if the larvae are used a protein supplement. Additional sanitation steps, such as drying the larvae, could help to mediate concerns related to incomplete suppression of pathogens (De Smet, et. al, 2018).

In the blueberry trial, the BSFL lost significant amounts of weight (Table 4). This indicates that blueberries would not be a sole good substrate for rearing larvae. For industrial use, larvae should gain weight when growing on a substrate in order to maximize their biomass. The observed weight loss could be due to the acidic nature of blueberries. Fresh high bush blueberries exhibit a pH between 3.15 and 3.2 (Almenar, et. al, 2008), which we can expect to have a similar pH to the low bush blueberries. Ma, et. al (2018) demonstrated that BSFL gained the most mass when reared on substrates with an initial pH of 6.0 or greater. The poor growth of BSFL on blueberries could be due to their digestive enzymes exhibiting peak function under basic conditions (Kim, et. al, 2011). If their digestive enzymes are not functioning optimally, the BSFL are not able to obtain nutrients from their substrate as efficiently. This could lead to the weight loss observed when larvae were reared on blueberries. It was also observed that the BSFL lost significantly more weight in the presence of *B. cereus* (Table 4). This suggests that *B. cereus* may somehow interfere with larval growth or development.

The blueberry substrate appeared to exhibit some antimicrobial properties on its own demonstrated by the fact that pathogen presence was below the plating threshold on day 2. Lowbush blueberry extracts have demonstrated inhibitory effects against both Gram-negative and Gram-positive food borne pathogens (Lacombe, et. al, 2012). Their antimicrobial capabilities are due to the presence of high amounts of phenolics,
anthocyanins, and proanthocyanidins (Lacombe, et. al, 2012). These compounds are all partially hydrophobic which enables them to bind to and disrupt the outer membrane structure of bacteria (Kwon, et. al, 2007). Extracts from lowbush blueberries demonstrated inhibitory capabilities against *Listeria monocytogenes*, a Gram-positive intestinal pathogen (Lacombe, et. al, 2012). Due to their antimicrobial abilities, blueberries should be explored as a compost additive to help regulate pathogenic burden in organic wastes. Blueberries could be added to waste when rearing BSFL to help reduce pathogen contamination. This has potential to cut down on extra sanitation steps required for the larvae and remaining substrate to be used safely. However, further experiments are necessary to determine what ratio of blueberries to organic waste would be optimal to maximize pathogen suppression without deterring larval weight gain.

Future research should explore the potential delayed suppressive effect on *B. cereus* exhibited by BSFL grown on potatoes. To fully elucidate the effects, data should be collected for longer than 4 days to see if the suppressive trend continues beyond this point. Additionally, the early exacerbation of *B. cereus* by larvae reared on potatoes should be further investigated. This could be achieved by examining the interaction between BSFL and *B. cereus* on different substrates at multiple time points between 0 and 4 days. Another avenue to investigate this would be to employ quantitative molecular methods. The DNA extractions and primer sets from this project could be used to conduct quantitative PCR (qPCR). The qPCR would allow for quantitative comparison of *B. cereus* amongst the different treatments. This may also allow for detection of *B. cereus* below the $10^6$ threshold. Extracted DNA could be used to conduct community sequencing
analysis in order to get a fuller picture of the microbial community in the various treatments.

The blueberry trial outlined in this project should be repeated using a lower plating threshold to establish the burden of *B. cereus* in the growth substrate. This would allow one to better understand any effects in the population of *B. cereus* on blueberries and the presence of BSFL. DNA was extracted from samples of each jar on days 2 and 4 of this trial. This DNA can be used to conduct a diagnostic PCR process analogous to the one for the potato samples in this project. This would allow for a molecular assessment of the pathogen within the samples. The DNA and primers could be used to conduct qPCR on the samples and the extracted DNA can also be used to conduct community sequencing analysis. There is a possibility that due to the antioxidant properties of blueberries they may have an overall lower microbial presence than potato samples.

Further research is also necessary to understand the reason behind larval weight loss on the blueberry substrate. There is a possibility that pH levels may affect larval growth and pathogen suppression (Erickson, et. al, 2004). Using substrates with different pH values to compare pathogen suppression and larval growth would answer this question. Using a pathogen that BSFL have already reliably exhibited suppressive effects (e.g. *E. coli*) against would also be best to compare the effects of substrate. The effect of *B. cereus* on larval weight should also be assessed using more replicates to allow for a more robust statistical analysis. A substrate better suited for rearing the larvae, such as potatoes, would be best to analyze the effects of *B. cereus* on larval weight gain.

In summary, this trial has revealed several interesting ideas about the interaction between BSFL, their growth substrate, and suppression of *B. cereus*. The BSFL may
initially exacerbate the growth of *B. cereus* in potato substrates. Despite this initial trend, a delayed suppressive effect by the BSFL on *B. cereus* was observed after 4 days of larval feeding. Further research is needed to fully understand this suppression pattern.

When grown on blueberries the BSFL lost significantly more weight in the presence of *B. cereus*. This could suggest that *B. cereus* has an impact on larval growth. Additionally, blueberries will not make a good substrate for commercial rearing of BSFL since the larvae lost weight. However, the blueberries may be useful compost additives as they have their own antimicrobial capabilities. A better understanding of the interaction between BSFL and blueberries could help harness the full potential of both the BSFL and blueberries in composting systems.
REFERENCES


Haley Lynn Morrill was born on October 21, 1998 in Farmington, Maine. Raised in Rangeley, Maine, she graduated from Rangeley Lakes Regional School in 2017. She began her undergraduate career at the University of Maine in the fall of 2017 where she pursued a bachelor’s of science in microbiology. Haley is a member of the Phi Beta Kappa, Phi Kappa Phi, and Alpha Lambda Delta honors societies. She was the 2019 recipient of the Edith McVay King scholarship, recognizing outstanding students in microbiology at the University of Maine. She also received the E. Reeve Hitchner memorial scholarship in 2021. This award recognizes an outstanding senior majoring in microbiology at the University of Maine.

Upon graduation from the University of Maine in May of 2021, Haley will be matriculating to the University of New England. At the University of New England she plans to obtain her doctorate of pharmacy and a graduate certificate in public health.